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The impacts of endothelial cell senescence on ageing-related blood-brain barrier dysfunction and the potential therapeutic strategies

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Abstract

Background: Disruption of the blood-brain barrier (BBB) contributes to various age-related neurological disorders. Brain microvascular endothelial cells (BMECs) are the most critical components of the BBB, forming a tightly sealed yet selectively permeable monolayer to maintain central nervous system (CNS) homeostasis. The accumulation of senescent cells in the CNS is thought to play a significant role in age-related BBB dysfunction. A large number of recent pre-clinical and clinical studies have focused on the development of senotherapeutic interventions to prevent or delay BBB ageing and extend health span. However, the understanding of BMEC senescence and age-related BBB dysfunction remains limited, and studies illustrating the effects of potential interventions are urgently needed.

Methods: Replicative senescence (RS) in human brain microvascular endothelial cells (HBMECs) was induced by repetitive passage and confirmed to exist by a panel of senescent markers, including senescence-associated-β galactosidase staining (SA-β-gal), γH2AX staining, telomere length measurement, WST-1 proliferation assay, and cyclin-dependent kinase inhibitor p16 expression. BMECs were treated with a cocktail of senolytics (dasatinib and quercetin, D+Q), or senomorphics targeting p38MAPK (BIRB796) or NF-κB (QNZ) signalling pathways from passage 16 until passage 20 to explore the effects of senotherapeutics on cellular senescence. Premature senescence was induced by exposing HBMECs (passages 6-8) to 400 μM H_2O_2 for 48 hours, followed by 12 days of incubation under normal complete media for the development of senescence. BIRB796 or QNZ were added to the culture media of HBMECs along with H_2O_2 to inhibit the activation of p38MAPK or NF-κB, respectively. D+Q was added to the culture media of HBMECs on day 10 after the H_2O_2 exposure for 24 hours to eliminate senescent cells. Similar phenotypic assays as the replicative senescence

experiments were conducted to evaluate the senescence-associated changes evoked by oxidative stress. Using an *in vitro* model of BBB, composed of BMECs, astrocytes and pericytes, this study explored the correlation between BMEC senescence and BBB dysfunction. Crucial factors of BBB function were analysed in young, RS and SIPS HBMECs in the presence or absence of senotherapeutics to explore the mechanism of age-related BBB dysfunction, including expression and localisation of tight junction proteins, levels and activity of MMPs, and the expressions of angiogenic factors and pro-inflammatory cytokines.

Results: Replicative senescence was deemed present at passage ≥ 19, where HBMECs displayed enlarged morphology, shortened telomere length, reduced proliferative capacity and increased SA-β-gal activity, γH2AX staining, actin stress fibers and cyclin-dependent kinase inhibitor p16 expression. The angiogenesis capacity and migration capacity revealed a significant decline in RS BMECs, evidenced by diminished tubulogenic potentials and wound repair rate. Significant impairments observed in the integrity and function of BBB established with RS BMECs, ascertained successively by decreases in transendothelial electrical resistance and increases in paracellular flux, revealed a close correlation between BMEC senescence and BBB dysfunction. Disruptions in the localisation and decreased expression of tight junction proteins, zonula occluden-1 (ZO-1), occludin, and claudin-5, and increased activity of matrix metalloproteinase 2 (MMP2) in RS BMECs may elucidate the underlying mechanisms of the BBB dysfunction. Stress-induced premature senescence (SIPS) HBMECs exhibited a set of similar phenotypic changes and functional impairment to those induced by RS. However, in contrast to the 70% senescent rate induced by RS, oxidative stress induced approximately 30% of SA-β-gal and γH2AX positively stained cells, yet it still caused significant disruption of the

BBB. In addition, RS HBMECs exhibited markedly reduced overall expressions of tight junction proteins, including ZO-1, occludin, and claudin-5. Meanwhile, ZO-1 and occludin levels remained stable, whereas claudin-5 levels showed further elevation in SIPS HBMECs. Selectively elimination of senescent cells by D+Q, as well as the inhibition of the p38MAPK signalling pathway, attenuated the effects of both replicative stress and oxidative stress on senescent markers in HBMECs and preserved BBB function by restoring subcellular localisation of ZO-1 and inhibiting the activation of MMP2. NF-κB inhibitor (QNZ) delayed SIPS and protected BBB from oxidative stress, yet accelerating cellular senescence and induced cell death with RS HBMECs. Furthermore, inhibition of the p38MAPK/NF-κB pathway significantly suppressed the oxidative stress-evoked expression of senescence-associated secretory phenotypes (SASPs), namely interleukin-8, monocyte chemoattractant protein-1 and intercellular adhesion molecule-1.

Conclusion: This study supports the concept that BMECs enter senescence under replicative stress and enhanced oxidative stress during the ageing process, which ultimately damages the integrity of the BBB. The selective elimination of senescent cells by senolytics and the inhibition of p38MAPK effectively mitigate the accumulation of senescent BMECs in the cerebrovasculature and preserve BBB functions.

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List of publications and conference

Publications

1. **Ya, J.,** Bayraktutan, U. (2024). Senolytics and Senomorphics Targeting p38MAPK/NF-κB Pathway Protect Endothelial Cells from Oxidative Stress-Mediated Premature Senescence. Cells, 13(15), 1292. DOI: 10.3390/cells13151292
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4. **Ya, J.,** Bayraktutan, U. (2023). Vascular ageing: mechanisms, risk factors, and treatment strategies. International Journal of Molecular Sciences, 24(14), 11538. DOI: 10.3390/ijms241411538
5. **Ya, J.,** Kadir, R. R. A., Bayraktutan, U. (2023). Delay of endothelial cell senescence protects cerebral barrier against age-related dysfunction: Role of senolytics and senomorphics. Tissue Barriers, 11(3), 2103353. DOI: 10.1080/21688370.2022.2103353
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Conference

Ya, J., Bayraktutan, U. Delay of endothelial cell senescence protects cerebral barrier against age-related dysfunction: role of senolytics and senomorphics. 6th UK Pre-clinical Stroke Symposium. (Poster presentation)

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List of abbreviations

ARDs	Age-related diseases
ATM	Ataxia-telangiectasia mutated protein
ATR	ATM and rad3-related
ATRIP	ATR-interacting protein
BBB	Blood-brain barrier
BRCA1	Breast cancer type 1
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
CHK	Cycle checkpoint kinase
CNS	Central nervous system
CXCL-1	CXC motif chemokine ligand 1
D+Q	Dasatinib combined with quercetin
DAPI	4,6-diamidino-2-phenylindole
DCE-MRI	Dynamic contrast-enhanced magnetic resonance imaging
DDR	DNA damage response
EBA	Evan's blue labelled albumin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	EGF receptor
eNOS	Endothelial NO synthase
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor

FOXO4	Forkhead Box protein O4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GUK	Guanylate kinase
HA	Human astrocyte
HB-EGF	Heparin-binding EGF-like growth factor
HBMEC	Human brain microvascular endothelial cell
HBSS	Hank’s Balanced Salt Solution
HP	Human pericyte
HSP	Heat shock protein
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
JAK	Janus kinase
JAM	Junctional adhesion molecules
LDH	Lactate dehydrogenase
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MDM	Mouse double minute
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MRN	MRE11-RAD50-NBS1
NaF	Sodium fluorescein
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NVU	Neurovascular unit
OIS	Oncogene-induced senescence

Ox-LDL	Oxidized low-density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
P38MAPK	P38mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase
PLGF	Placental growth factor
POT1	Protection of Telomeres 1
pRB	Phosphorylated retinoblastoma protein
qPCR	Quantitative polymerase chain reaction
RAP1	Repressor/activator protein 1
RNF	Ring finger containing nuclear factor
ROS	Reactive oxygen species
RPA	Replication protein A
RS	Replicative senescence
SA-β-gal	Senescence-associated β galactosidase
SASP	Senescence-associated secretory phenotype
SEM	Standard error of the mean
SH3	Src homology 3
SIPS	Stress-induced premature senescence
SIRT	Sirtuins
STAT	Signal transducer and activator of transcription protein
TEER	Transendothelial electrical resistance
TGF-β	Transforming growth factor-β
TIMP-1	Tissue inhibitor metalloproteinase-1
TIN	Telomeric repeat binding factor 1-interacting nuclear factor
TJ	Tight junction

TopBP1	DNA Topoisomerase II binding protein 1
TPP1	POT1-interacting protein 1
TRF	Telomeric repeat binding factor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
ZO	Zonula occluden

Chapter 1: General Introduction

1. Blood-brain barrier

1.1. Structure and function

The blood-brain barrier (BBB) is a mechanical barrier between the cerebral vessels and the central nervous system that regulates the selective passage of a variety of cells, ions and molecules between the bloodstream and brain tissue to maintain homeostasis, filter toxic compounds and pathogens, transport essential nutrients, dispose of waste and modulate immunity (Wu et al., 2023). Brain microvascular endothelial cells (BMECs) and their cell-to-cell junction complex are the fundamental elements of the BBB, closely encircled by the basement membrane, pericytes, and astrocytic endfeet (Ballabh et al., 2004) (Figure 1). Different from the systemic capillaries, the BBB exhibits tightly sealed endothelium that strictly restricts the entry of most of the blood-borne molecules and cells into the brain and controls the transport of nutrients, metabolites and other essential components via substrate-specific carriers or receptors (Sweeney et al., 2018).

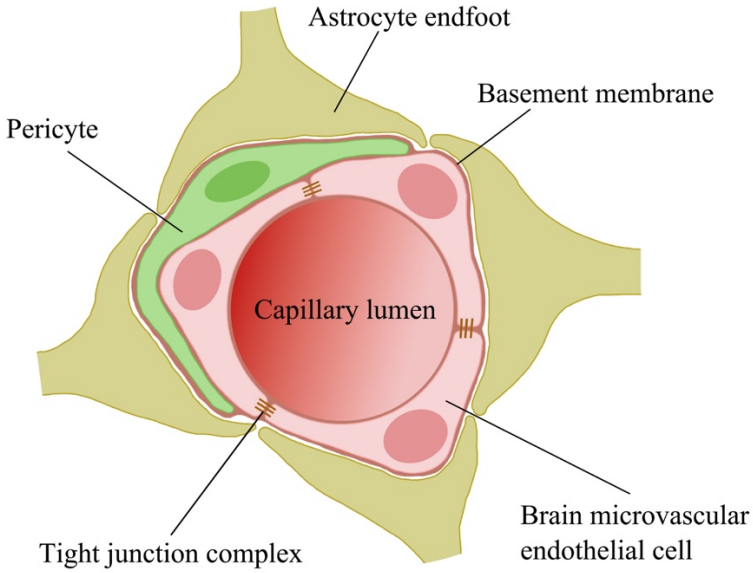


Figure 1. Structure of the blood-brain barrier.
BBB consists of brain microvascular endothelial cells, pericytes, astrocytes end-foot and the basement membrane, which work together to maintain its integrity and function.

Increased permeability and dysfunction of BBB have been associated with the progression of several neurological diseases, such as stroke, vascular dementia, Alzheimer's disease, and glaucoma (Archie et al., 2021).

1.2. Brain microvascular endothelial cells

Endothelial cells form the endothelium which covers the entire inner surface of all blood vessels. Apart from functioning as a selective mechanical barrier, endothelial cells also express and release various bioactive components to regulate vascular tone and inflammation, modulate the recruitment of platelets and immunocytes, and maintain the procoagulant/anticoagulant balance (Pober and Sessa, 2007). Compared to other components of the vascular system, the endothelial cells in the CNS have more extensive tight junctions (TJs), no fenestrations, lower expression of the adhesion molecules, and lower level of vesicular transport, which together limit the paracellular diffusion and the specifically selective transcellular diffusion (Galea, 2021). The main proteins for the BMEC junction complex include occludin, claudins, junctional adhesion molecules (JAMs) and zona occludens proteins (ZOs). Most claudins exhibit tissue-specific expression, and claudin-5 is the predominant claudin in BMECs. (Ohtsuki et al., 2008). Claudin-5 consists of four transmembrane domains, two extracellular loops, one intracellular loop, and the NH_2-terminal and COOH-terminal located in the cytoplasm, which together restrict the paracellular permeability and interact with scaffolding proteins such as ZO-1. Apart from the larger size of the extracellular loops and intracellular terminals, occludin shares a similar structure with claudin-5 (Zheng et al., 2023). ZO proteins are a family of key scaffolding proteins composed of three PDZ domains, one Src homology 3 (SH3) domain and one

guanylate kinase (GUK)-like domain. These domains provide multiple binding sites to regulate the tight junction complex by linking the transmembrane proteins, the actin cytoskeleton and intracellular signalling pathways (Tornavaca et al., 2015).

BMECs release a wide range of molecules that serve important functions in the BBB. The balance between the production of nitric oxide and endothelin-1 in BMECs participates in the regulation of vascular constriction and dilation to maintain normal cerebral blood flow (Zhu et al., 2016). In addition, BMECs secrete a range of inflammatory cytokines, growth factors and metalloproteinases (MMPs) that contribute to angiogenesis, tissue repair and immune responses under both physiological and pathological conditions (Verma et al., 2006, Kadir et al., 2023, Wang et al., 2006).

1.3. Astrocytes

Astrocytes are characteristic star-shaped glial cells that constitute the majority of cells in the CNS. Apart from functioning as a supportive component in the brain tissue, astrocytes also play crucial roles in neuronal activity, synaptic transmission and BBB composition (Sofroniew and Vinters, 2010). Astrocytic end-feet covers most of the vessel surface and closely interacts with endothelial cells to maintain the integrity of the BBB (Manu et al., 2023). Besides mechanical barrier formation, astrocytes-derived secretory factors, such as sonic hedgehog, angiopoietin-1, retinoic acid and Wnt growth factors, have been reported to promote BBB formation and enhance BBB integrity by promoting the expression of junction proteins in ECs, downregulating the expression of adhesion molecule and suppressing the levels of pro-inflammatory cytokines (Alvarez et al., 2011, Lippmann et al., 2014, Guerit et al., 2021, Siddiqui et

al., 2015). The presence of astrocytes in the *in vitro* model of BBB significantly enhanced the barrier integrity and increased the levels of junction proteins, thereby supporting the indispensable role of astrocytes in BBB function (Watanabe et al., 2021, Shao, 2014).

1.4. Pericytes

Pericytes are cells located in the basement membrane as part of the neurovascular unit (NVU) and closely interact with BMECs through paracrine effects and gap junctions (Benarroch, 2023). Pericytes secrete various cytokines to interact with endothelial cells to maintain the normal function of the BBB. For example, pericytes produce angiopoietin-1 to enhance the formation of endothelial tight junction complex (Hori et al., 2004), and vascular endothelial growth factor (VEGF) to promote the proliferation, survival and migration of ECs (Zechariah et al., 2013). Pericytes also play a crucial role in the stability of the extracellular matrix by secreting the essential ECM proteins such as laminin and collagen, as well as releasing the matrix metalloproteinases (MMP)-2 and MMP-9 to degrade the ECM during angiogenesis (Gautam et al., 2016, Benarroch, 2023). Brain pericytes wrap around the vessels in the microvasculature and express relatively high levels of α-smooth muscle actin (α-SMA) compared to endothelial cells, which enable the cells to regulate capillary blood flow in response to neuronal activity and vascular homeostasis (Hall et al., 2014). Smooth muscle cells located in large vessels express higher levels of α-SMA to regulate the active vasoconstriction and dilation (Brozovich et al., 2016).

2. Cellular senescence

Cellular senescence is a state characterised by a permanent cell cycle arrest, associated with differences in cell morphology and function compared to proliferative cells (Di Micco et al., 2021). This physiological phenomenon was first identified by Hayflick, who observed the irreversible cell cycle arrest after limited numbers of subcultivation of various types of human diploid cells in 1961 (Hayflick and Moorhead, 1961). Cellular senescence occurs in response to many triggers, including telomere attrition, oxidative stress, ionising radiation, chemical exposure or oncogene activation (Ya and Bayraktutan, 2023). Senescent cells can be detected in all organs at all ages, as they participate in multiple physiological processes, including embryogenesis, inflammatory modulation, tumour suppression and tissue repair by preventing the proliferation of cells with critical DNA damage and secreting essential cytokines (Di Micco et al., 2021, Huang et al., 2022). The exact percentage of senescent cells at different ages is difficult to define, as it varies by tissue, organism, health conditions, genomic variation and markers used to assess senescence. However, a substantially higher percentage of senescent cells has been observed in elderly or age-related diseases (ARDs) affected individuals (Di Micco et al., 2021, Baker et al., 2011), which contributes to age-related dysfunction across multiple tissues and organs. Accumulation of senescent cells represents the fundamental physiological process of organismal ageing and promotes the progression of ARDs, evidenced by the wide detection of senescent cells at sites of age-related pathologies, such as atherosclerotic plaques, brain cortical grey matter with Alzheimer's disease or premalignant lesions of certain cancers (Minamino et al., 2002, Schmitt et al., 2022, Fancy et al., 2024).

2.1. Replicative senescence

The telomere attrition theory is regarded as one of the more widely accepted theories of cellular senescence. This theory was first proposed by Olovnikov in 1973, which suggested that senescence culminates from a critical shortening of telomere length arising from the progressive loss of telomeric DNA with every cell division (Olovnikov, 1973). Telomeres are repeated nucleotide sequences at each end of the chromosomes that protect the DNA strands for genome stability (Lu and Liu, 2024). At each cell division, the telomeres shorten owing to the incomplete replication of the linear DNA molecules by the conventional DNA polymerases (Makarov et al., 1997). Although the shelterin complex, a group of proteins that exists at the end of chromosomes, recruits a reverse transcriptase called telomerase to add telomere repeat sequences to the 3' end of telomeres, telomeres undergo a gradual shortening as a result of continuous cell division. Critically shortened telomeres trigger DNA damage response (DDR), signalling the cells to undergo senescence, known as replicative senescence (Childs et al., 2014) (Figure 2). Length-independent telomere dysfunction also plays a significant role in cellular senescence. DNA damage accumulation with telomeric DNA has been observed in senescent cells independently of telomere attrition (Fumagalli et al., 2012).

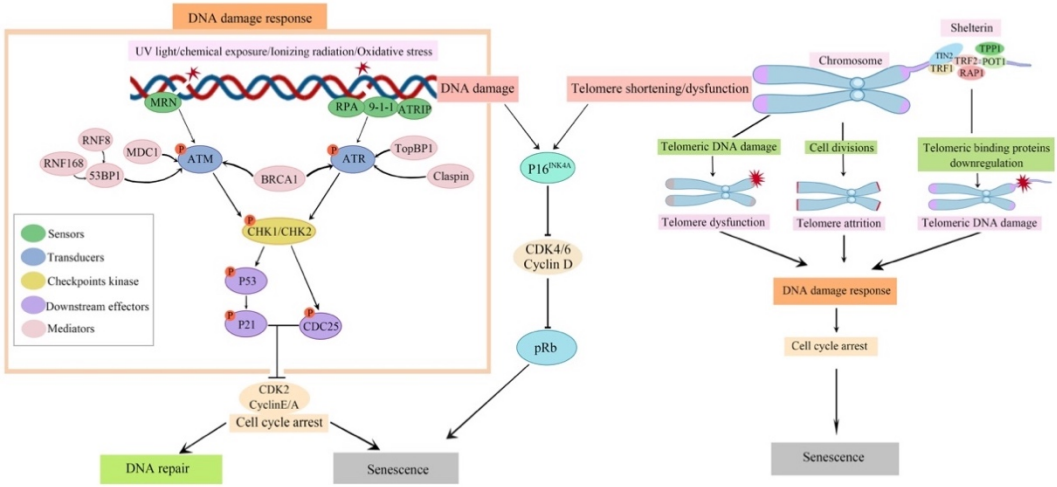


Figure 2. Triggers and general signalling pathways of cellular senescence.
Telomere attrition, telomeric DNA damage, shelterin protein downregulation, and damage accumulated on non-telomeric DNA trigger DNA damage response (DDR) and p16/pRb pathway, resulting in either temporary cell cycle arrest for the DNA repair process or permanent cell cycle arrest, known as cellular senescence. Figure adopted from (Ya and Bayraktutan, 2023).

2.2. Stress-induced premature senescence

Exposure to different sub-lethal stresses, such as oxidative stress, genotoxic agents or radiation, leads to cellular senescence within a relatively short period of time, with or without telomere shortening, termed stress-induced premature senescence (SIPS) (Toussaint et al., 2002). Stress-induced DNA damage activates DDR, which temporarily arrests the cell cycle for the damaged DNA to repair and remodel (Figure 2). However, the cell cycle will be permanently arrested if the DNA damage is beyond repair (Zhao et al., 2020).

Oxidative stress, characterised by the excessive availability of reactive oxygen species (ROS), is one of the leading causes of physiological ageing and ARDs, which create DNA damage lesions and subsequently accelerate cellular senescence, and senescent

cells in turn, have mitochondrial dysfunction and increased reactive oxygen species (ROS) production (Passos et al., 2010). The vicious cycle between oxidative stress and cellular senescence accelerates the ageing process and the development of ARDs (Yang et al., 2024).

2.3. Oncogene-induced senescence

Oncogenic signalling activation, resulting from an activating mutation of oncogene or inactivation of tumour-suppressor gene, leads to DDR activation, inflammatory transcriptome alteration, and hyperproliferation-related DNA replication stress, which collectively cause a permanent cell cycle arrest, regarded as oncogene-induced senescence (OIS) (Kuilman et al., 2008). OIS is considered a tumour suppression mechanism, but a recent study has found that OIS promotes the initiation and development of certain cancers via the secretion of tumourigenesis factors, such as MMPs, interleukin-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Liu et al., 2018, Ya and Bayraktutan, 2023).

2.4. General pathways involved in cellular senescence

Despite differences in the nature of stimuli, cell cycle arrest in senescence is generally mediated by the activation of the p53/p21WAF1 or p16INK4A/retinoblastoma protein (RB) pathways or both (Korotchkina et al., 2010, Baker et al., 2011). Severe DNA damages, dysfunctional telomeres or oncogene activation are known to trigger p53-mediated DDR, which in turn activates its downstream effector, cyclin-dependent kinase inhibitor (CDKI), aka p21, which promotes cell cycle arrest. However, the elevation of p53 expression is not observed in all senescent cells. For instance, the

level of p53 expression appears to be relatively lower in senescent human keratinocytes or renal epithelial cells than in their non-senescent counterparts. Here, the downregulation of p53 is regarded as a mechanism to resist apoptosis (Kim et al., 2015). The 16/RB pathway can be triggered by telomere attrition, DNA damage, genotoxic stress, oxidative stress and oncogene activation. In the process of senescence, p16 keeps the tight bond between unphosphorylated pRB tumour suppressor protein and E2F transcription factor, consequently preventing them from stimulating DNA replication and blocking the cells from entering the S phase of the cell cycle (Mooi and Peeper, 2006, Sulli et al., 2012) (Figure 2). A previous study has demonstrated that cell cycle arrest in cells with low level p16 expression can be reversed by the inactivation of p53, yet senescent cells with high levels of p16 expression cannot re-enter the cell cycle by inactivation of either p53 or RB (Beausejour et al., 2003).

2.5. Oxidative stress in cellular senescence

Endogenous ROS, the byproducts of aerobic mitochondrial respiration, evoke a variety of DNA damage, including accelerated telomere shortening, oxidase DNA bases, abasic sites, and DNA strand breaks, and as a consequence potentiate DDR (Barnes et al., 2019, Kowalska et al., 2020). ROS overproduction from NADPH oxidase overactivity and mitochondrial dysfunction during the ageing process activates a series of mechanisms leading to cellular senescence via activation of cell cycle checkpoints and mitochondrial homeostasis disruption, which reciprocally produce more ROS and maintain this vicious cycle in an active state (Passos et al., 2010). Ageing not only aggravates oxidative stress but also impairs cellular resistance to oxidative stress in that attenuation of antioxidant capacity in the elderly through

suppression of Nrf2, a ubiquitous transcription factor that responds to oxidative stress and promotes the genes encoding antioxidant enzymes (Ungvari et al., 2019). The exaggerated availability of ROS compromises endothelial function and results in vascular tone disturbance, pro-inflammatory cytokines generation and BBB integrity impairment (Chung et al., 2022, Faraci, 2006). Among the mechanisms involved in oxidative stress-mediated cellular senescence, ataxia-telangiectasia-mutated protein (ATM), p38mitogen-activated protein kinase (p38MAPK) and nuclear factor-κB (NF-κB) play pivotal roles (Salminen et al., 2012, Hongo et al., 2017).

Antioxidant vitamin C has been shown to increase lifespan in premature aged mice induced by the $Wrn^{\triangle/hel/\triangle hel}$ gene mutant but not in wild-type mice, even treatments with vitamin C successfully reduced the ROS level and oxidative DNA damage in both ageing models (Massip et al., 2010). Transgenic mice expressing dominant-negative IκBα selectively in endothelial cells exhibited lower levels of oxidative stress marker, delayed the progression of vascular ageing and increased longevity compared to wild-type counterparts (Hasegawa et al., 2012).

Many clinical conditions can lead to the generation of oxidative stress and possibly accelerate the accumulation of senescent cells. Smoking is one of the widely regarded risk factors of ageing and ARDs (Linli et al., 2022). It has been observed that endothelial cells isolated from patients who smoke have significantly higher levels of oxidative stress and premature senescent rate, compared to those isolated from non-smoking counterparts (Farhat et al., 2008). Similar to cigarette smoking, exposure to cigarette smoke extract also induces endothelial cell senescence, apoptosis, and dysfunction via ROS overproduction and mitochondrial dysfunction, which are repressed by ubiquinol and menaquinone-7 supplements due largely to their

antioxidant effects (Cirilli et al., 2020). Excessive production of ROS and activation of the Notch 1 pathway are among the major mechanisms in hypercholesterolemia-induced vascular dysfunction (Tie et al., 2014).

3. Age-related blood-brain barrier dysfunction

Age-dependent loss of BBB integrity has been observed with a linear increase in the BBB K_{trans} permeability in the human hippocampus area, quantified by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (Montagne et al., 2015). In elderly individuals and aged animal models, an increased burden of senescent markers with multiple cell types was detected in the CNS, including glial cells and endothelial cells (Idda et al., 2020, Novo et al., 2024). Endothelial cell senescence contributes to BBB dysfunction and age-related neurological diseases, including cerebral vascular disease, vascular dementia and Alzheimer's disease (Bomboi et al., 2010, Ouvrier et al., 2024, Kuhlmann et al., 2009).

3.1. Endothelial cell senescence

In the central nervous system (CNS), the endothelial cells form a mechanical barrier between the blood and the brain and regulate the selective passage of a variety of cells, molecules, and metabolic waste between the two compartments (Kadry et al., 2020). Brain microvascular endothelial cells (BMECs) express a higher abundance of tight junction proteins occludin, claudins and zonula occludens compared to ECs located in other organs (Galea, 2021), which enables the formation of a tightly sealed and highly selected BBB. Compared to young ECs, senescent ECs express occludin, claudins and zonula occludens at considerably lower levels, and form disrupted monolayer (Cong and Kong, 2020, Krouwer et al., 2012). As demonstrated with increased permeability,

contact of senescent ECs with young ones also compromises the overall barrier integrity (Kadir et al., 2022). The loss of endothelium integrity impacts the selective permeability of the vascular wall and promotes the accumulation of oxidized low-density lipoprotein (ox-LDL) cholesterol, inflammatory cytokines and ROS in the subendothelial space, which subsequently aggravates atherosclerosis (Fledderus et al., 2021). Increased secretion of pro-inflammatory SASP factors, such as MMPs, IL-6 and IL-8, also contribute to age-related BBB breakdown, along with diminished availability of LDL receptor-related protein-1, they diminish the ability of restriction and clearance of deleterious components from the CNS (Storck et al., 2016, Khan et al., 2017).

ECs also regulate vascular homeostasis and brain perfusion by synthesizing and releasing a wide range of vasoconstrictors, vasodilators, inflammatory mediators, as well as pro- and anti-thrombogenic factors (Ashby and Mack, 2021). Nitric oxide (NO) constitutes the most important endothelium-derived agent that regulates vascular relaxation, coagulation, oxidative stress and inflammatory response (Cyr et al., 2020). Due to the decreased endothelial NO synthase (eNOS) activity, senescent ECs generate limited amounts of NO and, therefore, elicit significant impairments in all the aforementioned crucial functions (Ungvari et al., 2018). The decreased response of senescent ECs to vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) as effectively as their younger counterparts adversely affects angiogenesis and adds to the extent of vascular damage (Berenjabad et al., 2022). Dysfunctional brain endothelial cells with decreased NO production and impaired responses to vasoactive factors perturb normal blood flow and result in chronic cerebral hypoperfusion (Graves and Baker, 2020, Yamazaki and Kanekiyo, 2017). BBB breakdown and chronic hypoperfusion contribute to the initiation and

progression of vascular cognitive loss and Alzheimer's disease (Rajeev et al., 2022, Wu et al., 2021). Enhanced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) on senescent EC plasma membrane consequently leads to the increased adhesive and prothrombotic propensity of the endothelium (Salmon et al., 2020).

Taken together these studies indicate that the progressive accumulation of senescent ECs in the CNS vasculature may be a driving force for atherogenesis, inflammation, thrombosis, cognitive loss and impaired angiogenesis.

3.2. Role of MMPs in blood-brain barrier functions

MMPs belong to a family of zinc-dependent endopeptidases, which are initially synthesized as inactive proenzymes (pro-MMPs) and can be activated by proteolytic cleavage, inflammatory cytokines, oxidative stress, growth factors and tissue injury (Rempe et al., 2016). MMPs exert both beneficial and detrimental effects on BBB function. Under physiological conditions, MMPs participate in the maintenance of vascular homeostasis, cell migration, tissue remodelling, angiogenesis and wound healing by degrading extracellular matrix proteins such as collagen, elastin and fibronectin (Van Doren, 2015). However, under pathological conditions, such as stroke or neuroinflammation, the upregulation and activation of MMPs were determined to contribute to the disruption of BBB by degrading the basement membrane and tight junction proteins (Abdullah and Bayraktutan, 2016, Yang and Rosenberg, 2011). During ageing process, MMP synthesis was enhanced, which results in age-related extracellular matrix (ECM) remodelling (Freitas-Rodriguez et

al., 2017). It has been reported that senescent vascular smooth muscle cells (VSMCs) release active MMPs that may accelerate atherosclerosis (Gardner et al., 2015).

3.3. Senescence-associated secretory phenotypes

Despite their non-proliferation status, senescent cells remain metabolically active and secrete a large number of soluble and insoluble cytokines, chemokines, growth factors, and MMPs that collectively constitute the SASP (Chen et al., 2015). SASPs have both beneficial and deleterious effects. While the former effects include wound healing and tumour suppression, the latter effects include the promotion of certain cancers, atherosclerosis, insulin resistance, endothelial dysfunction, chronic inflammatory reactions, and the induction and reinforcement of cellular senescence by paracrine activity (Ritschka et al., 2017). SAPS has been coupled to the pathophysiological process of several ARDs, including cardiovascular diseases, type 2 diabetes, osteoarthritis, some cancers, heart failure, fibrosis and some chronic inflammatory diseases (Shakeri et al., 2018, Birch and Gil, 2020). Persistent DDR initiates the formation of SASP, whereas autocrine and paracrine effects amplify the signal and reinforce the senescent state (Davalos et al., 2010). Multiple signalling pathways participate in the complex regulation of SASP. Ataxia-telangiectasia mutated (ATM) kinase, a core component of DDR signalling, activates nuclear factor-κB (NF-κB) in response to genotoxic and oxidative stress via post-translational modifications. NF-κB, an important transcription factor, in turn regulates the expression of many genes responsible for both the innate and adaptive immune responses, including those of several pro-inflammatory factors, including IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (GM-CSF), that make up SASP (Cuollo et al., 2020). P38MAPK activation has been observed in response to various stimuli, including

DNA damage, oxidative stress or oncogene activation (Zarubin and Han, 2005). Inhibition of p38MAPK suppressed the secretion of IL-6, IL-8, GM-CSF, MCP-1 and CXCL-1, determining the crucial role of the p38MPAK pathway in SASP production (Alimbetov et al., 2016, Freund et al., 2011, Ya and Bayraktutan, 2024). The suppression of the sirtuins (SIRT), a family of signalling proteins involved in metabolic regulation, also contributes to the formation and modulation of SASP. Moreover, differences in cellular origin and stimuli play pivotal roles in the expression of factors that make up SASP (Gorgoulis et al., 2019).

3.4. Biomarkers of cellular senescence

To date, several elements, relying mostly on differences between young and senescent cell morphology, telomere attrition, enzymatic activity, genomic instability and epigenetic alterations, have been proposed as biomarkers to detect senescence in vitro and in vivo (Hernandez-Segura et al., 2018, Kudlova et al., 2022). Since the abundance and positivity of most biomarkers differ from physiopathological stimuli and from one cell type to another, it is essential to determine and evaluate the cellular senescence using multiple biomarkers from different aspects (Wallis et al., 2022). Senescent cells tend to manifest enlarged, flattened and incongruous morphology compared to their young counterparts (Aan et al., 2013). Detectable β-galactosidase (β-gal), aka senescence-associated β-gal activity (SA-β–gal), is the most commonly used biomarker to distinguish senescent cells by cleaving X-gal and staining the cells blue (Itahana et al., 2013). However, as serum starvation or cellular confluence-induced quiescence may also increase β-gal activity (Yang and Hu, 2005), it cannot be used as the sole parameter to determine cellular senescence. Another category of senescence markers is based on the accumulation of DNA damage and the activation of DDR,

such as γH2AX phosphorylated on Ser-139, a double-stranded DNA breaks marker, or activated ataxia-telangiectasia mutated (ATM) or ATM and Rad3-related protein kinase (Feringa et al., 2018). Telomere attrition serves as a convincing marker of cellular senescence (Bernadotte et al., 2016). However, length-independent telomere dysfunction and persistent DNA damage can also trigger permanent cell cycle arrest, especially with premature senescence (Anderson et al., 2019, Ksiazek et al., 2007). Cyclin-dependent kinases (CDKs) are a group of protein-serine/threonine enzymes that regulate the progression of the cell cycle (Ding et al., 2020). Inhibitors of the CDKs can be activated by various stimuli and consequently arrest the cell cycle, which provides reliable senescence markers, such as p53/p21, and p16/pRB (Althubiti et al., 2014). This mechanism participates in tumour suppression, DNA repair and cellular senescence (Lukasik et al., 2021). Other markers, including decreased proliferation capacity, ROS overproduction, mitochondrial dysfunction, and increased release of SASP, are all significant parameters to evaluate senescence status, but not to determine the presence or absence of cellular senescence because these changes can also be observed with non-senescent cells under certain conditions (Kudlova et al., 2022).

4. Anti-ageing strategies

The world is gradually entering an era of ageing. Data from World Health Organization indicate that the number of individuals aged ≥60 years reached 1.4 billion in 2020 and may exceed 2.1 billion by 2050 (Organisation, 2022). Age-related diseases (ARDs) already constitute a public health and economic issue for most countries globally and are likely to become an even bigger issue in the future. Determination of the relative mechanisms of ageing and ARDs, as well as the development of anti-ageing strategies, are crucial to the improvement of global health

and the limitation of healthcare-related costs. In this regard, increasing studies are focused on exploring various anti-ageing interventions, including modification of lifestyle, adoption of special dietary patterns, use of synthetic or natural therapeutic agents and cell-based therapeutics (Guo et al., 2022, Liu, 2022, Duan et al., 2022, Martel et al., 2024).

Anti-ageing strategies are highly diverse for different individuals. To maximise the beneficial effects and minimize the potential side effects, individualized plans to delay ageing and prevent ARDs need to be provided according to the actual age, health status, risk factors, and ageing-related pathological burden (Ya and Bayraktutan, 2023).

Given the crucial role of senescent cell accumulation in the pathogenesis of various ARDs such as vascular diseases, neurodegeneration diseases, cancer and metabolic disorders, therapeutic strategies targeting senescent cells have recently gained considerable importance (Calcinotto et al., 2019, Hekmatimoghaddam et al., 2017). Since cellular senescence, characterized by permanent cell cycle arrest, cannot be reversed by any existing clinical interventions, and may further induce secondary senescence of the neighbouring cells through paracrine effects. Present senotherapeutics focused either on the delay of senescence progression, removal of senescent cells or modulation of their secretory factors.

4.1. Senolytics

Senolytics are a group of drugs that selectively obliterate senescent cells and are divided into seven categories as per their mechanism of action; tyrosine kinase inhibitors, heat shock protein 90 (HSP90) inhibitors, B-cell lymphoma 2 (BCL-2) family inhibitors, mouse double minute 2 (MDM2) inhibitors, Forkhead Box protein

O4 (FOXO4) inhibitors, glutaminase inhibitors and histone deacetylase inhibitors (Ge et al., 2021). Dasatinib, a tyrosine kinase inhibitor, combined with quercetin, a natural flavonoid with multiple targets, has attracted increasing attention as a potential anti-ageing strategy. Significant increases in NO bioavailability and reduction in senescent burden and aortic calcification in aged mice and hypercholesterolemic mice, prove the anti-vascular ageing effect of D+Q (Roos et al., 2016). An open-labelled, phase 1 clinical trial consolidates the potential anti-ageing effect of D+Q by showing that 3 days of oral administration can significantly decrease senescent markers, including the numbers of p16INK4A+ cells, p21CIP1+ cells and circulation SASP factors in 55-79 years old patients with diabetic kidney disease without noticeable adverse effects (Hickson et al., 2019). In patients (age 76±5 years old) with early-stage symptomatic Alzheimer's disease, 3 months of intermittent oral administration of D+Q significantly decreased the levels of SASP in plasma compared to baseline, and the safety parameters indicated good tolerability of the treatment (Gonzales et al., 2023). Another clinical trial has shown marked improvements in physical functions in 55-84 years old patients with idiopathic pulmonary fibrosis receiving D+Q treatment, though it has also reported some adverse side effects, including coughing, gastrointestinal discomfort, heartburn and headache in these patients (Justice et al., 2019).

4.2. Senomorphics

The mechanism by which senomorphics function is to delay the senescent progression in senescing cells or suppress the release of SASP. Senomorphics are generally divided into nine categories; telomerase activators, sirtuin activators, mTOR inhibitors, antioxidants, NF-κB inhibitors, ATM inhibitors, Janus kinase (JAK) inhibitors, signal transducer and activator of transcription proteins (STAT) inhibitors, and p38MAPK

inhibitors (Knoppert et al., 2019). Considering that senescent cells mediate both autocrine and paracrine effects through SASP, modulation of SASP is considered an important therapeutic strategy. In this regard, p38MAPK, a major transducer of stress stimuli that is activated in response to diverse cellular and environmental stresses such as DNA damage, oxidative stress and pro-inflammatory cytokines, attracts much of the attention. P38MAPK mediates various events including cell proliferation, cell cycle progression and apoptosis (Martinez-Limon et al., 2020). Inhibition of p38MAPK suppresses the secretion of many SASP factors such as IL-1α, IL-1β, IL-6, IL-8 and GM-CSF and delays senescence in different cell lines e.g. fibroblasts and bone primary cells subjected to repetitive passaging, lipopolysaccharides or oncogenes (Alimbetov et al., 2016, Aquino-Martinez et al., 2021). The transcription factor NF-κB is a key regulator of a group of SASP factors. NF-κB inhibition has been proven to downregulate the secretion of MMP-2, MMP-3 and MMP-13, which results in the degradation of collagen II and IV, two crucial structure proteins that participate in the formation of cartilage, basal lamina and capillary, also pro-inflammatory factors including TNF-α, IL-6 and IL-8 (Zang et al., 2017, Zhu et al., 2020). CCAAT/enhancer-binding protein β (C/EBPβ), another key transcription factor that cooperates with NF-κB, also plays a crucial role in regulating SASP, especially with oncogene-induced senescence (Acosta et al., 2008).

5. Thesis aims

This thesis aimed to investigate the impact of endothelial cell senescence on BBB function, focusing on the mechanisms and potential treatment strategies. The first part of the experiments aimed to determine the effect of replicative senescence and stress-induced premature senescence on the morphological and functional characteristics of

brain microvascular endothelial cells (BMECs). The following chapter then explored how BMEC senescence leads to BBB disruption, focusing on the alteration of tight junction proteins and extracellular matrix and the effect of senotherapeutics. Finally, the last chapter focused on the role of the p38MAPK/NF-κB pathway in oxidative stress-induced BMEC senescence and the ensuing BBB disruption.

Chapter 2: General Methods

1. Cell preparation

1.1. General cell culture

Human brain microvascular endothelial cells (HBMECs), human brain microvascular pericytes (HPs), and human brain astrocytes (HAs) were purchased at passage 3 from Neuromics (Minneapolis, MN, USA). All cells were cultured in their respective media (Sciencell Research Laboratories, San Diego, USA) in a humidified atmosphere of 75% N2, 20% O2 and 5% CO2 at 37℃. The endothelial cell media (ECM, SC-1001, Sciencell) contained 5% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, and 1% endothelial growth supplement. Astrocyte media (AM, SC-1801) and pericyte media (PM, SC-1201) contain 2.5% FBS, and same concentration of antibiotics and astrocyte/pericyte growth supplements.

1.2. Cell count and cell viability

To minimize the inter-experimental bias, before seeding, freezing or passaging, the cell numbers and viability were calculated. 20µl of the cell suspension were mixed with 0.1% trypan blue at a ratio of 1:1 and incubated at room temperature for 3 minutes before counting on the haemocytometer under the light microscope (Figure 3). Cells situated on the four grids were counted. Viable cells appear clear and refractile and dead cells are stained with blue colour (Figure 3A). The number and viability of the cells were calculated using the following formulas.

The number of cells.

Cells per mL= average number of cells per grid × 2 ×10^4

Cell viability

Cell viability=Number of viable cells/Total cell count ×100%

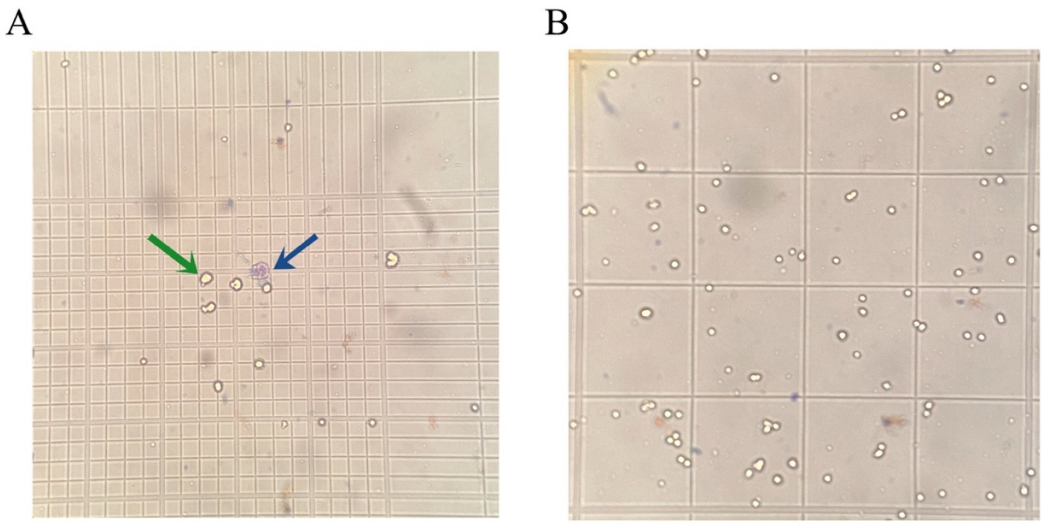


Figure 3. Cell counting on haemocytometer.

Viable cells are identified by their clear appearance (green arrow), which is different from the trypan blue stained dead cells (blue arrow) (A). The image of a single grid under light microscope displays 4 × 4 large corner squares (B).

2. Experimental conditions

2.1. Induction of replicative senescence

HBMECs before passage 8 were defined as young cells. Replicative senescence was induced through repetitive culture of young cells till no observation of cell proliferation with fresh complete culture media. Senescence was deemed to exist when about 70% of the cultured cells were stained positive for SA-β-gal and γ-H2AX, which appeared around passage 19.

2.2. Induction of stress-induced premature senescence

Premature senescence was induced by exposing HBMECs (passage 6-8) to media containing H_2O_2. To determine the optimal condition that yields high levels of premature senescence without compromising overall viability, HBMECs were cultured in complete medium containing different concentrations of H_2O_2 (100, 400,

1000 μM) for 24, 48 or 72 hours. A further 1, 6 or 12 days of incubation under normal experimental conditions followed these treatments. SA-β-gal staining (detailed methodology in section 2.6.1.) was used to assess the senescence rate of the cells and LDH cytotoxicity assay (detailed methodology in section 2.5.3.) was used to evaluate the level of cellular toxicity induced by H_2O_2. Cells subjected to 400 μM H_2O_2 for 48 hours followed by further culture in normal conditions for 12 days displayed the optimal percentage of SA-β-gal positive staining and 1.13% cytotoxicity.

2.3. Treatment of cells with senotherapeutics

A variety of senolytics and senomorphics were chosen after a comprehensive review of literature. Before their application in experimental settings, the dose-response experiments were carried out for each agent, namely dasatinib, quercetin, p38MAPK inhibitor (BIRB796) and NF-κB inhibitor (QNZ). WST-1 assay was performed to assess cell viability rates. HBMECs (passage 7) were cultured in different concentrations of the abovementioned agents for 24 hours and then incubated with fresh media containing WST-1 assay reagent for 2 hours (detailed methodology in section 2.5.4.). Results indicated low concentrations of dasatinib (1nM and 20nM) and quercetin (1 μM, 5 μM, and 10 μM) significantly increased HBMEC viability compared to the control cells. In contrast, 50nM dasatinib decreased cell viability compared to 20nM group, while 100nM reduced viability compared to controls (Figure 4). Low concentrations of quercetin (1, 2.5 and 5 μM) were reported to promote cell proliferation, whereas higher concentrations of quercetin (7.5 and 10 μM) suppress it (Zhang et al., 2020). BIRB796 (0.1, 1 and 5 μM) and QNZ (10, 100 nM, 1 μM) did not display any negative impact on cell viability at low concentrations (Figure

4). Based on the evidence, the optimal dose of each senotherapeutic was employed in the following experiments.

The modulatory impact of senolytics (20 nM dasatinib+5 μM quercetin) and senomorphics targeting p38MAPK (5 μM BIRB796), or NF-κB (1 μM QNZ) on senescent markers and endothelial function were assessed by treating passage 16 endothelial cells till passage 20 in replicative senescence experiments. Passage 16 cells were identified as the initial therapeutic target because they exhibited signs of senescence but had not entered irreversible cell cycle arrest. The same doses of the abovementioned agents were also applied in the following premature senescence experiments, except for QNZ. As QNZ dramatically decreased the viability of passage 16 cells at 1 μM, the dose of 100 nM was utilised instead in the SIPS experiments. BIRB796 or QNZ were added to the culture media of HBMECs along with H_2O_2 to inhibit the p38MAPK or NF-κB, respectively. D+Q was added to the culture media of HBMECs on day 10 after the H_2O_2 exposure for 24 hours to eliminate senescent cells.

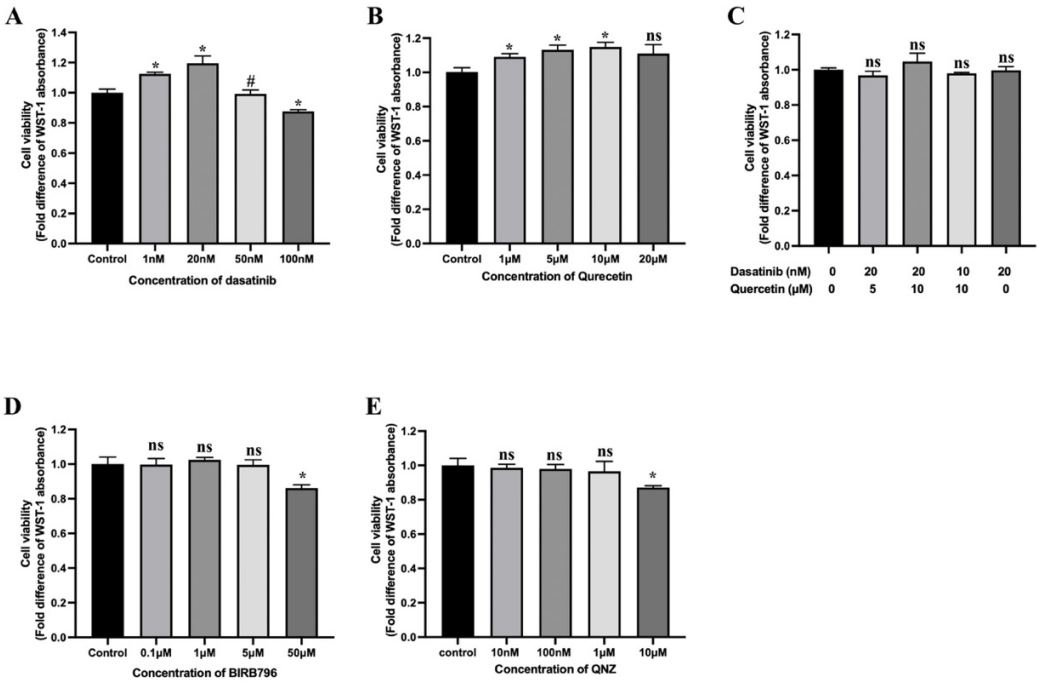


Figure 4. Impact on cell viability of the therapeutic agents.

High concentrations of dasatinib (100nM), BIRB796 (50 μM) and QNZ (10 μM) reduced the cell viability (A-E). The combination of dasatinib and quercetin at low concentrations did not affect cell viability (C). *p< 0.05 compared to control group, #p< 0.05 compared to 20nM dasatinib group, ns= not significant compared to control.

3. Assessment of BBB function

3.1. Establishment of an in vitro triple-cell culture model of human BBB

In a previous study, Bayraktutan's group has shown that the in vitro model of human BBB established with HBMECs, astrocytes and pericytes display better barrier integrity and function compared to those established with HBMECs and astrocytes or pericytes or to HHBMECs monolayers (Shao, 2014) (Figure 5). HBMECs, HAs and HPs were cultured with their respective media to ~90% confluence prior to the following procedures. Approximately 1×10^5 HAs were seeded on the basolateral side of the transwell insert (polyester membrane, 12 mm diameter, 0.4 μm pore size, Corning Costar, UK) and incubated for 4-6 hours. Once the cells adhered to the membrane, the inserts were inverted to the original orientation and cultured in sterile 12-well plates containing fresh astrocyte media to ~90% confluence. HBMECs (5×10^4) subjected to different experimental conditions were then seeded onto the apical side of the inserts containing HAs and cultured to full confluence. For Replicative senescent HBMECs, to ensure the full confluency of the endothelial cell monolayer, 1×10^5 cells were seeded onto the apical side of the inserts containing HAs.

1 ml of daily changed astrocyte complete media was added into the abluminal chamber and 750 μl of daily changed endothelial cells complete media was added into the luminal chamber to nourish the cells till they reached 100% confluence. In the meantime, HPs (150,000 cells) were seeded into a new 12-well plate and cultured in pericyte complete medium till 90% confluence. The inserts containing fully confluent

HBMECs and HAs were transferred to the well plate containing confluent HPs, to set the triple culture model of human BBB. 750µl of pericyte media and 750µl astrocyte media was added in the abluminal chamber, and 750µl of endothelial media was added in the luminal chamber at a daily base for at least another 24 hours.

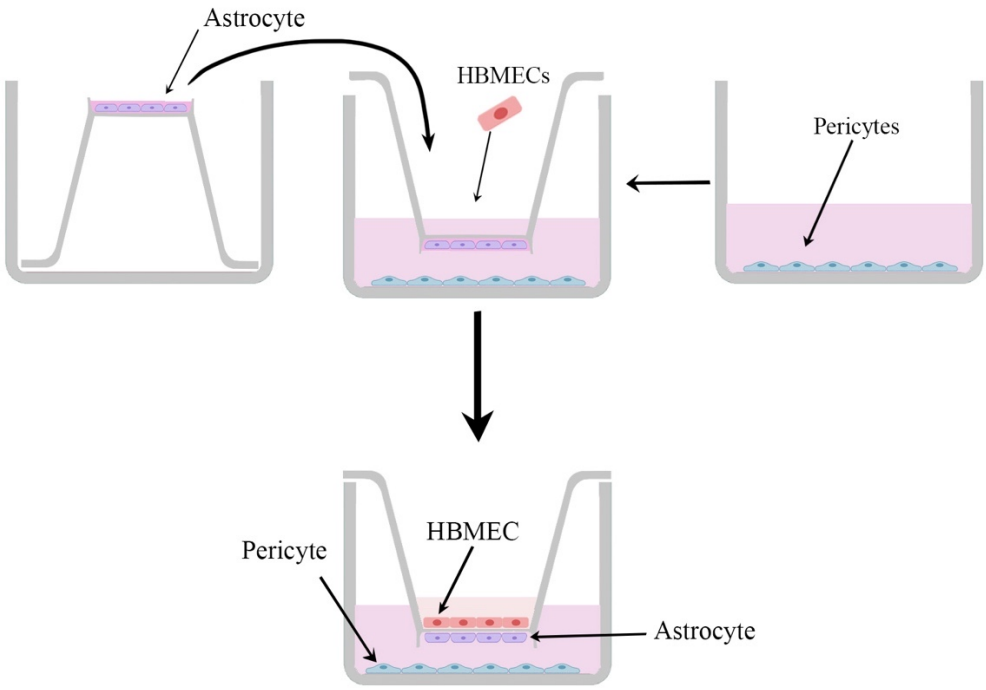


Figure 5. Setup of the in vitro tri-culture model of human BBB.
To set up the tri-culture model of BBB, astrocytes were seeded onto the abluminal side of the transwell inserts and cultured to ~90% confluence. The inserts with astrocytes were flipped, HBMECs were seeded onto the luminal chamber, and pericytes were seeded on the bottom of another 12 well plates before combining with the inserts to create the BBB model.

3.2. Assessment of the BBB integrity and function

The transendothelial electrical resistance (TEER) is an extensively used methodology to quantitatively evaluate the integrity of cellular monolayer under *in vitro* conditions (Srinivasan et al., 2015). After exposure to different experimental conditions, the TEER of the BBB was measured with the EVOM Manual meter and STX electrodes (World Precision Instruments, Hertfordshire, UK). The two electrodes with unequal

lengths were placed vertically into the luminal and abluminal chambers without touching the sidewall. The TEER value of each insert was the average from three readings from different positions. Higher values of TEER reading suggest better-formed tight junctions between the neighbouring cells (Srinivasan et al., 2015).

The permeability of the BBB was assessed by the paracellular flux of sodium fluorescein (NaF, 376 Da) and Evan's blue labelled albumin (EBA, 67kDa). After exposed to experimental conditions, the inserts were rinsed with warm Hank's Balanced Salt Solution (HBSS, Sigma) and transferred into fresh 12-well plates containing 2 ml of HBSS. 500 µl of 50 µg/ml NaF or 165 µg/ml EBA was added into the luminal chamber of each inserts and the plates with the inserts were incubated for 1 hour at 37 ℃ in a CO_2 incubator. After incubation, 400 µl of samples was collected from the luminal and abluminal chamber, and 100 µl of each sample was pipetted into 96-well plates in triplicate. The average concentration of dye in each chamber was calculated based on the absorbance of EBA assay (610 nm) and fluorescence for NaF assay (excitation 485 nm and emission 520 nm) of the samples with the FLOUstar Omega plate reader (BMG Labtech Ltd, UK). The paracellular flux of the dye was calculated using the following formulae.

Cleared volume (µl) = Abluminal reading ×500/luminal reading.

4. Phenotypic and functional assays of BMECs

4.1. Tube formation assay

The level of tube formation capacity on growth factor reduced Matrigel (Corning, USA) was studied to evaluate the angiogenic capacity of the HBMECs. In advance of experiment, 200 µl tips and colling core were chilled in -20 ℃ freezer for at least 24

hours. Matrigel was thawed at 4℃ for overnight and pipetted into 96-well plates (50µl per well) using pre-chilled tips on a cooling core because Matrigel solidifies when the temperature goes above 12 ℃. After carefully checking and removing the bubbles, the well plates were incubated at 37℃ for 1 hour to solidify the Matrigel. Meanwhile, HBMECs were detached with trypsin and re-suspended in cell culture medium. After counting and diluting, HBMECs ($8x10^3$ cell/150µl culture medium) subjected to different experimental conditions were seeded in 96-well plates pre-coated with Matrigel and incubated at 37℃ for 4 hours. The cells were carefully seeded to avoid creating any bubbles. The formation of the tubular structures was visualized and photographed using the Digipad connected to a light microscope (Leica DFC3000 G, Leica Microsystems, Wetzlar, Germany). Using Angiogenesis Analyzer plugin ImageJ software (version 1.52k, NIH, Bethesda, MD, USA), the characteristics of the tubule networks, i.e. total number and total length of segments were analyzed.

4.2. Wound healing assay

HBMECs subjected to different experimental conditions were seeded to 6-well plates (1.5×10^5 cells/well) and grown to 95% confluence. The wound was created by scratching the cell monolayer across with a p1000 micropipette tip in one swift motion. Debris were washed away with warm PBS. The cells were incubated in complete medium at 37 ℃. Marks need to be draw on the bottom of each well using permanent marker to minimize the displacement of pictures for the same wound at different time points. Pictures of the wound were captured immediately (0 hour) and after 12, 24, 48 and 72 hours of incubation in complete media using the Digipad connected to a light microscope (Leica DFC3000 G, Leica Microsystems, Wetzlar, Germany). Wound

closure was quantified as the percentage of difference in the scratch area between 0-72 hours using the ImageJ software (version 1.52k, NIH, Bethesda, MD, USA).

4.3. LDH cytotoxicity Assay

The CyQUANT LDH Cytotoxicity Assay Kit (C20300, Invitrogen, Carlsbad, CA, USA) was used as per the manufacturer's instructions to quantify the level of cellular cytotoxicity. HBMECs (5×10^3) were seeded in triplicate in 96-well plates and incubated overnight. On the following day, the media were replaced with 100 µl of fresh media containing 10 µl sterile water (spontaneous control), 10 µl 10 × lysis buffer (maximum LDH activity control), and 10 µl of target components at different concentrations. The culture plate was then incubated for 45 min before collecting 50 µl of sample media from each well and mixing it with the reaction mixture at a ratio of 1:1 for 30 min. The reactions were then terminated by the addition of the stop solution. The absorbances were then read at 490 nm and 680 nm, and the level of cytotoxicity was calculated using the following formulae.

%Cytotoxicity= [(Compound-treated LDH activity-Spontaneous LDH activity)/(Maximum LDH activity-Spontaneous LDH activity)] × 100.

4.4. WST-1 cell viability/proliferation assay

The proliferative capacity of HBMECs was evaluated using the Cell Proliferation Reagent WST-1 (Roche, Mannheim, Austria). In principle, viable cells metabolized stable tetrazolium salt WST-1 to a soluble formazan (Yin et al., 2014). Therefore, following the incubation of the cells cultivated in 96-well plates with WST-1 reagent, the absorbance of the formazan dye exhibited a direct correlation to the number of

metabolically active cells. An equal number of cells under different experimental conditions (5,000 cells per well) were seeded into the 96-well plate and incubated for 24-48 hours. The confluency of cells needs to be under 85% to avoid under-estimated results caused by contact inhibition. The medium was subsequently replaced with 100µl fresh medium containing 10µl of WST-1 assay reagent. The plates were incubated for 2 hours at 37 ℃ prior to reading the absorbance (480 nm) by a FLUOstar Omega microplate reader (BMG Labtech Ltd, Aylesbury, UK).

4.5. Assessment of senescence-associated β-galactosidase activity

Cells were seeded in 12-well plates and cultured to around 70% confluence in completed cell culture medium. The SA-β-gal activity was evaluated using a beta-galactosidase staining kit (ab102534, Abcam, Cambridge, UK) according to the manufacturer's instructions. In brief, cells were washed with warm PBS and fixed with the fixation solution at room temperature for 10 minutes, then washed with PBS and incubated in staining solution mix at 37 ℃ for overnight. The cells were visualized and photographed using a light microscope (Leica DFC3000 G, Leica Microsystems, Wetzlar, Germany) under 20× magnification. Cells with blue dye were regarded as senescent. The number of β-gal positive cells and total cells was counted manually in four randomly chosen areas of each well before working out the percentage of SA-β-gal positive cells. The average of four areas was used in statistical analyses. Each experiment was repeated at least three times using three different biological replicates.

4.6. Immunocytochemistry

HBMECs seeded on glass coverslips were subjected to different experimental conditions before fixing (in 4% paraformaldehyde for 15 min) and permeabilizing (in 0.1% Triton X-100 for 15 min) at room temperature. After blocking with 1% bovine serum albumin in PBST (0.1% Tween20 in PBS) for 30 minutes at room temperature, the cells were incubated overnight at 4°C with primary antibodies, including ZO-1 (33-9100, Thermofisher, Waltham, MA, USA), occludin (71-1500, Thermofisher), claudin-5 (35-2500, Theromofisher), and phospho-Histone H2AX (Ser139, 9718, Cell Signaling Technology). On the following day, the cells were washed and incubated with FITC-labelled or Texas Red-labelled secondary antibodies (ab6785, an6717, ab6719, Abcam) for 1 hour at room temperature in the dark. To visualize F-actin microfilaments, the cells were blocked as above and incubated with the DyLight 594 Phalloidin (12877, Cell Signaling Tecnology) for 15 minutes and washed for 3 times. To stain nuclei, the cells were incubated with DAPI (4,6-diamidino-2-phenylindole) for 3 minutes after the secondary antibody washing. The coverslips were then mounted onto glass slides using mounting medium (Vector Laboratories, Peterborough, UK). The cells were visualized by fluorescence microscopy (Leica DFC3000 G, Leica Microsystems, Wetzlar, Germany). To quantify the accumulation of DNA damage foci indicated by γH2AX, cells were photographed under 40× magnification using the software Leica Application Suite X (3.0.015697). Nuclei with red stained foci were considered as γH2AX-positive cells. DAPI stained nuclei were counted to determine the total cell numbers. Cell counting from 3 randomly chosen areas of each slide was used to calculate the percentage of γH2AX-positive cells. Each experiment was repeated six times using three different biological replicates.

4.7. Quantitative PCR-Absolute telomere length

Genomic DNA from HBMECs was extracted with the DNeasy Blood & Tissue Kit (69504, Qiagen, USA) following the manufacturer's instructions. Briefly, the fresh cell pellet was resuspended in 200 µl PBS before adding 20 µl of proteinase K. 200 µl of buffer AL was added into the mixture, mixed thoroughly, and incubated at 56 ℃ for 10 minutes. The sample mixture was mixed with 200 ethanol and transferred into the DNeasy Mini spin column placed in a 2 ml collection tube, then centrifuged at 6000 *g* for 1 minute. The DNeasy Mini spin column was transferred into a new collection tube, and 500 µl of buffer AW was added into the column before centrifuge for 1 minute at 6000 *g*. The column was then transferred into a new collection tube, and 500 µl of buffer AW2 was added into the column before centrifuge for 3 minutes at 20,000 *g*. After adding 200 µl buffer AE, the column was transferred into a new collection tube and centrifuged at 6000 *g* for 1 minute. The DNA solution in the collection tube was collected for the following experiment. The DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific). The telomere length of the DNA samples was measured using the Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell Research Laboratories, USA). The Reference genomic DNA sample (provided by the kit) and the experiment DNA samples were mixed with the primer stock solution [telomere primer set (TEL) or single copy reference (SCR) primer set], the 2 ×TaqGreen qPCR master mix and nuclease-free water before running. The qPCR was carried out by the Stratagene MX3000P Quantitative RT-PCR System (Agilent, Santa Clara, USA) using the cycle profile in Appendix II.

The average telomere length per chromosome end was calculated as per the manufacturer's instructions using the below formula based on Cq values.

△Cq(TEL)=Cq(TEL, experiment DNA sample)-Cq(TEL, reference DNA sample)

△Cq(SCR)=Cq(SCR, experiment DNA sample)-Cq(SCR, reference DNA sample)

△△Cq=△Cq(TEL)-△Cq(SCR)

Average telomere length on each chromosome end of the sample=(935±45kb) × $2^{-\triangle\triangle Cq}$/92

4.8. Protein extraction and quantification

The cells grown in flasks were washed twice with ice-cold, sterile PBS on a cooling box before adding the cell lysis buffer (RIPA buffer) containing protease inhibitors cocktail and phosphatase inhibitor (sodium orthovanadate). The cells were scrapped down in lysis buffer and then kept at -80 for an hour. Cell lysate was collected and centrifuged at 14,000g and 4 ℃ for 15 minutes. The supernatant was collected as whole cell protein.

Protein concentration was detected with the BCA protein assay (Thermofisher, 23227) as per the manufacturer's instructions. Samples were run in duplicate in 96-well plates with albumin standards (0-2000 µg/ml). To 10 µl of samples/standards, 200 µl of bicinchoninic acid (BCA) assay reagent was added and the plate was incubated at 37 ℃ (avoid light) for 30 minutes. The absorbance of the samples was measured at 562 nm using the CLARIOstar Plus Microplate reader (BMG Labtech, Germany). The protein concentrations of the samples were calculated based on the standard curve using MARS Data Analysis Software (Version 3.32).

4.9. Western blotting

The cell lysate was centrifuged for 20 minutes at 14,000g at 4 ℃ and protein concentration was quantified using a BCA protein assay kit (section 2.6.4.). Protein samples (20-100 µg) were mixed with 4×LDS (4% lithium dodecyl sulfate) sample buffer (MPSB, Sigma, Burlington, MA, USA) in a ratio of 3:1 and heated at 75℃ for 5 minutes to open the tertiary/quaternary structures by denaturation. The protein sample mix and Amersham Rainbow Molecular Weight Markers (12-225 kDa) were run on SDS-polyacrylamide gels (see Appendix III for gel compositions) for 3 hours at 120V in Running Buffer (25 mM Tris-HCl pH 8.3, 190 mM glycine, 0.1% [w/v] SDS). After separation on 15% SDS-polyacrylamide gel (8% for ZO-1), the protein samples were transferred to a PVDF membrane in cold transfer buffer (25 mM Tris-HCl pH 8.3, 190 mM glycine, 20% [v/v] methanol). The membrane was blocked with 5% bovine serum albumin (BSA) in washing buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween-20) at room temperature for 1 hour on a rocker at 30 oscillations per minute before incubating overnight with primary antibodies (Appendix IV) in 2% BSA in washing buffer on a tube roller at 4℃. Anti-β-actin was used as an internal loading control. The membranes were then washed for 5×5 minutes with washing buffer on a rocker at 70 oscillations per minute. Washed membranes were incubated with IRDye-labeled (800CW/680CW) secondary antibodies (1:10000 diluted with 2% BSA in washing buffer) for 1 hour at room temperature in a dark box on a rocker (30 oscillations per minute). After repeating the washing step mentioned above, the membranes were scanned and the protein bands were detected using the Odyssey Fc System before analyzing their intensities via Image Studio software (Li-cor Biotechnology, Lincoln, NE, USA).

4.10. Gelatin zymography

After exposure to different experimental conditions, HBMECs were washed with warm PBS and incubated in serum-free DMEM for 20 hours before collecting and centrifuging the supernatant at 300 *g* for 10 minutes to remove the cells and debris. 20 µl of supernatant was mixed with 4 × LDS Sample Buffer in a ratio of 1:3 and run on 10% SDS-polyacrylamide gel containing 0.1% (w/v) gelatin (Appendix V) for 3 hours at 100V in the cold room. The gel was washed in 2.5% Triton X-100 in ultra-pure water for 30 minutes and then 1 hour at room temperature before incubating 16 hours at 37 ℃ in incubation buffer (50mM Tris-HCl pH7.5, 10mMCaCl2, 1µM ZnCl2, 200mM NaCl). Gels were then stained with staining solution (0.1% [w/v] Coomassie blue dye, 40% deionized water, 50% methanol, 10% acetic acid) for 1 hour and de-stained 4 times for 10 minutes each time using de-staining solution (50% deionized water, 40% methanol, 10% acetic acid). MMP activity was detected as clear bands against a dark blue background. The gels were scanned and analyzed using the Odyssey Fc System (Li-cor Biotechnology, Lincoln, NE, USA).

4.11. Proteome Profiler Array Analysis

After exposure to different experimental conditions, BMECs were washed twice with PBS and cultured with serum-free and growth factor-free media for 24 hours. Cell culture media were collected and centrifuged at 250 g for 5 minutes to remove the debris. The supernatant was then condensed 8 times by centrifugation at 4000 g for 20 minutes using a Centrifugal Filter (Merck, Taufkirchen, Germany). Proteome Profiler Human Cytokine Array Kit and Human Angiogenesis Array Kit (Ary005B and Ary007 R&D Systems, Minneapolis, MN, USA) was used as per the manufacturer's instructions to detect cytokines. Briefly, the samples were mixed with the detection

antibody cocktail, then incubated with the blocked membrane spotted with the antibodies at 4℃ overnight. After washing, the membranes were incubated with Streptavidin-HRP solution and activated by Chemi Reagent Mix. The chemiluminescent signal of the dots on the membrane was detected using Odyssey Fc system and the blotting density was measured by ImageJ software (version 1.52k, NIH, Bethesda, MD, USA).

5. Statistical analysis

The data are displayed as the mean value ± standard error of the mean (SEM) from a minimum of three independent experiments. Differences among groups were determined using an unpaired *t*-test, or one-way analysis of variance (ANOVA) followed by Tukey's post hoc-analysis. Information on specific statistical test for each experimental analysis is listed in Appendix Ⅵ. $P<0.05$ was considered as significant. GraphPad Prism 9.0 statistical software package (GraphPad Software Inc., La Jolla, CA, USA) and SPSS Statistics 27.0 (IBM, New York, USA) were used to perform these analyses.

Chapter 3: Endothelial cells display senescence-associated phenotypic changes in response to replicative and oxidative stress

1. Introduction

Ageing is a physiological process that begins during young adulthood and affects the structure and function of the vascular system over time (Celermajer et al., 1994). Endothelial dysfunction is widely regarded as the main phenotype that promotes age-dependent changes in vasculature (Widlansky et al., 2003). Accumulation of senescent ECs in vasculature is recognised as an important stimulus for the development of endothelial dysfunction during chronological ageing (Morgan et al., 2013, Cafueri et al., 2012). Triggers of cellular senescence are generally divided into two types, the attrition of telomere emerging from repetitive cell division (replicative senescence, RS) and the stress-induced premature senescence (SIPS), resulting from the accumulation of DNA damage induced by radiation, oxidative stress and genotoxic stress. Both RS and SIPS play significant roles in the development of age-related diseases (ARDs) (Aan et al., 2013). Various *in vitro* models are currently used to explore the mechanisms and possible interventions of cellular senescence, and SIPS models are more commonly applied to reduce the time duration of the experiments since RS model establishment tends to be much more labour-intensive and time-consuming (Galvis et al., 2019, Aan et al., 2013, Noren Hooten and Evans, 2017, Veronesi et al., 2023). Despite the shared traits and mechanisms of RS and SIPS, many studies have reported differences regarding phenotypes, metabolism, proteomes and intracellular proteins (Kural et al., 2016, Aan et al., 2013). However, the specific differences between RS and SIPS in endothelial cell characteristics remain poorly understood. By directly comparing the phenotypical changes regarding senescent markers and tight junction proteins in BMECs undergoing RS and SIPS, this study provides novel insights into the mechanisms of senescence-related EC dysfunction.

2. Aims

2.1. To investigate the effect of replicative senescence (RS) and stress-induced premature senescence (SIPS) on morphological and functional characteristics of brain microvascular endothelial cells (BMECs)

2.2. To identify the similar and different mechanisms contributing to RS and SIPS in endothelial cells

3. Method

The methods and materials included in this chapter are described in detail in Chapter 2 (General Methods), including the setup of replicative senescence and stress-induced premature senescence models of HBMECs, SA- β-gal staining, LDH cytotoxicity assay, western blotting and immunocytochemistry.

4. Results

4.1. Replicative senescence model of HBMECs

HBMECs were subjected to repetitive cell culture and division till they revealed no sign of proliferation for a week in complete medium with the necessary supplementary growth factors, which normally appear after passage 19. Beyond passage 16, progressively increased HBMECs were stained positive for senescence-associated-β-galactosidase, a widely established marker of senescence (Figure 6). At passage 20, above 70% of HBMECs were stained positively with SA-β-gal.

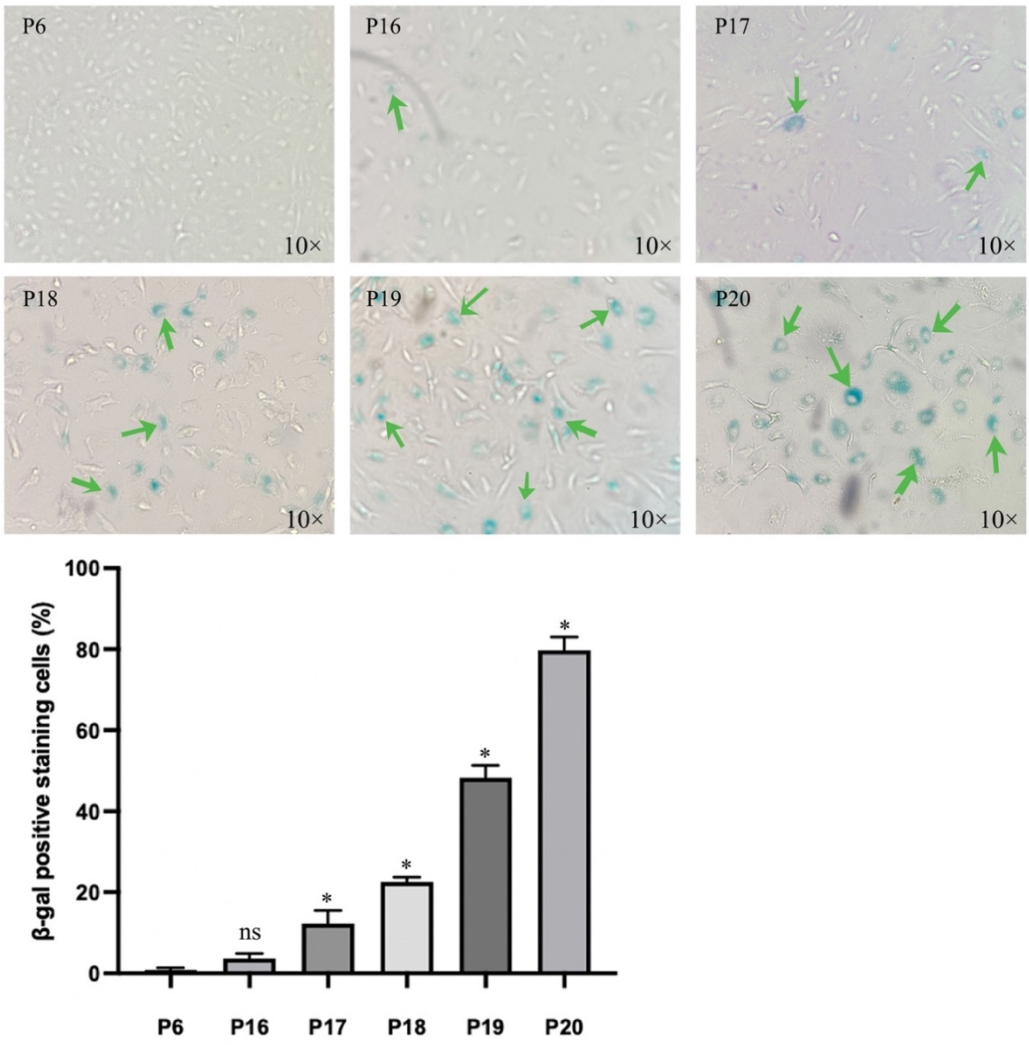


Figure 6. Repetitive division results in the accumulation of senescent HBMECs.

The green arrows indicate some of the SA-β-gal positive cells (Blue staining in the perinuclear area). Data are expressed as mean ±SEM from three independent experiments. Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to p6 BMECs, ns = not significant compared to p6 BMECs.

4.2. Stress-induced premature senescence model of HBMECs

H_2O_2 has been the most commonly used component to induce premature senescence (Petrova et al., 2016). To determine the optimal condition that yields high levels of premature senescence without compromising overall viability, HBMECs were cultured in a complete media containing different concentrations of H_2O_2 (100, 400

and 1000 μM) for 24, 48, or 72 hours. A further 1, 6, or 12 days of incubation under normal experimental conditions followed these treatments. Exposure to incremental concentrations (100, 400 and 1000 μM) of H_2O_2 for increasing periods of time (24, 48 and 72 hours) to induce oxidative stress resulted in a higher prevalence of SA-β-galactosidase positive rate in cell populations subjected to 400 μM of H_2O_2 for 48 hours before a further culture in normal conditions for 12 days, which contributed around 30% senescent rate. Neither higher concentrations of H_2O_2 above 400 μM nor extending exposure time above 48 hours was able to increase the percentage of SA-β-gal positive cells (Figure 7). The LDH cytotoxicity assay detected an increase of 1.13% cytotoxicity with 400 μM H_2O_2 (Figure 7E). The cell viability rates, assessed by the trypan blue exclusion test on day 12 after exposure to H_2O_2, remained similar between the treatment and control groups, being 96.19% vs 95.52%, respectively.

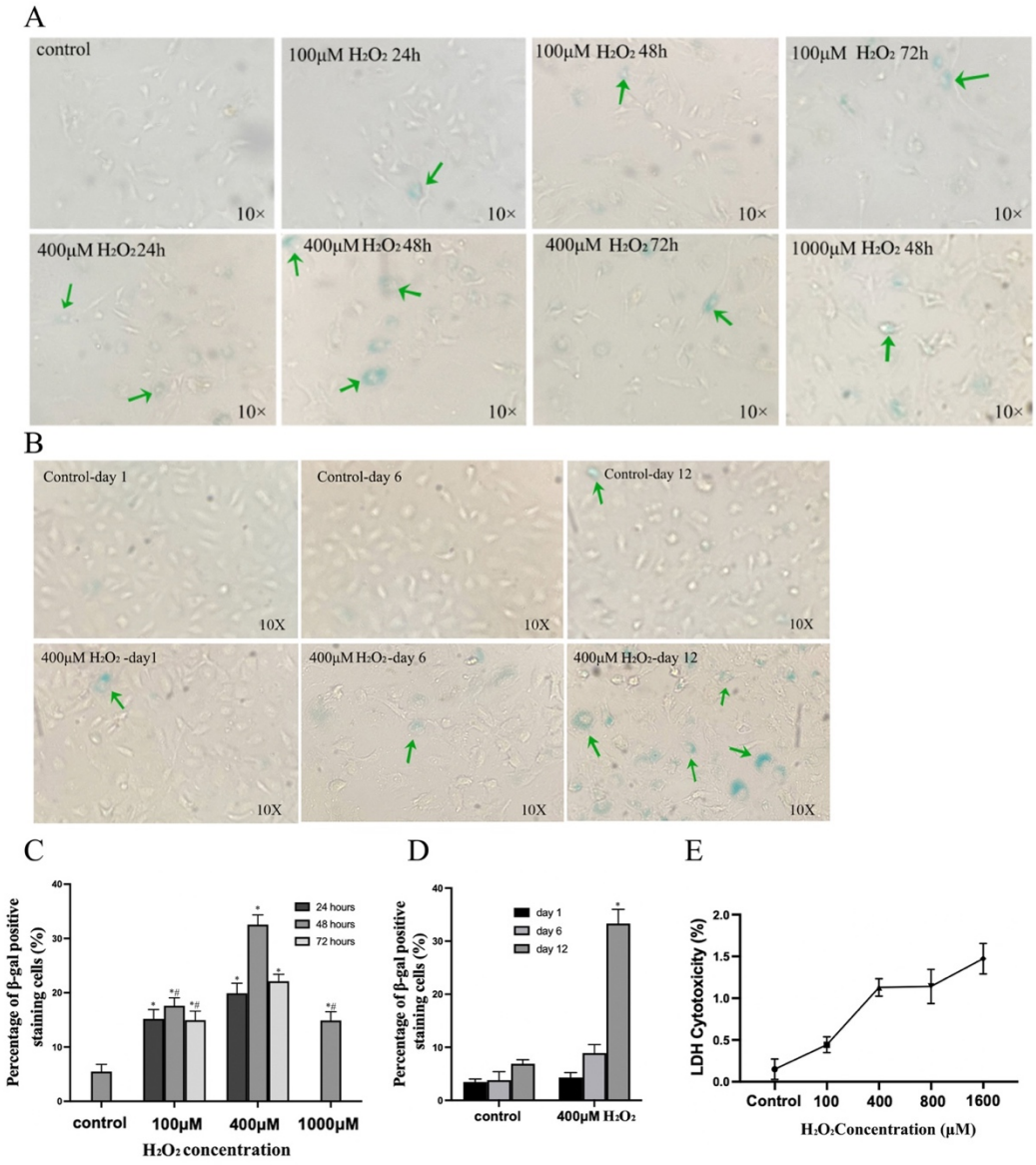


Figure 7. Time- and concentration-dependent effects of H_2O_2 on HBMECs.

(A) Senescent cells with positive SA-β-gal staining emerged in cells treated with increasing concentrations of H_2O_2 for 24-72 h. (B) Twelve days appeared to be an essential time period for the development of senescence following exposure to 400 μM of H_2O_2 for 48 hours. (C, D) Percentage of cells reaching senescence, as ascertained by SA-β-gal positive staining, in response to different concentrations of H_2O_2 and post-H_2O_2 incubation period. (E) Level of LDH cytotoxicity in response to different concentrations of H_2O_2. The green arrows indicate some of the SA-β-gal positive cells in different experimental groups. Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to control group.

4.3. RS and SIPS induce morphological changes in HBMECs

HBMECs subjected to repetitive cell culture or H_2O_2 displayed larger morphology compared to the young cells. However, in RS HBMECs, the majority of the cells were evenly enlarged and elongated, and the cobblestone appearance completely disappeared, while a considerable amount of cells in the SIPS group remained smaller sizes and showed inerratic arrangement and the rest of the cells were enlarged and flattened instead of elongated (Figure 8A). The actin cytoskeleton staining confirmed the enlarged morphology, and showed the formation of thick stress fibers in both RS and SIPS HBMECs (Figure 8B).

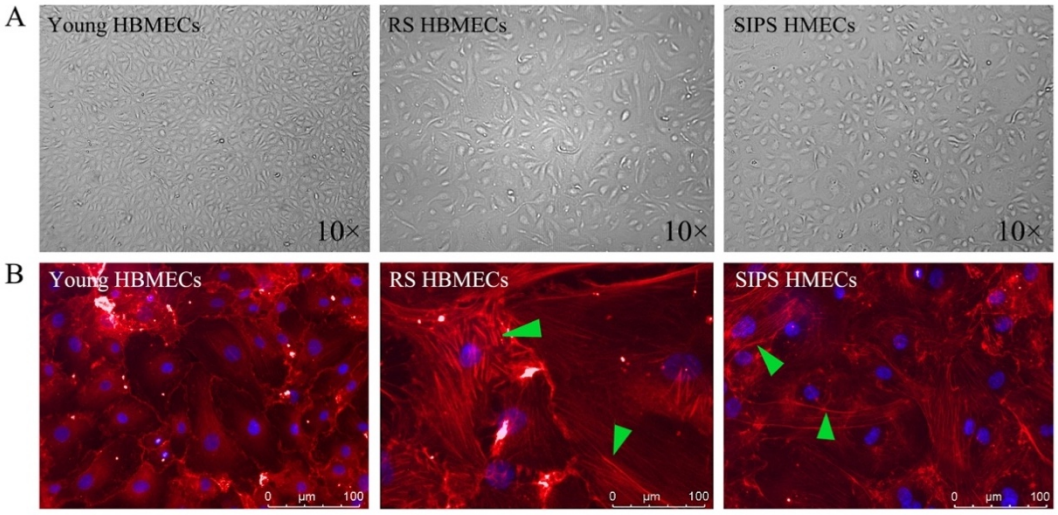


Figure 8. Morphological changes and actin stress fiber formation in senescent HBMECs. Exposure of HBMECs to replication stress or oxidative stress induces alterations in cellular morphology (A) and re-organization in actin cytoskeleton (B) compared to young cells. Replicative senescent (RS) HBMECs exhibit homogeneously enlarged and elongated morphology, while only a minority of stress-induced premature senescent (SIPS) HBMECs have enlarged and flattened morphology. Thickened actin stress fibers were widely detected in almost all cells in both types of senescence.

4.4. RS and SIPS HBMECs exhibit similar senescence-associated heterochromatic foci but different percentage

Accumulation of DNA damage was observed in the nuclei of both RS and SIPS HBMECs, as evidenced by greater γH2AX staining compared to the young cells (Figure 9). However, the percentage of cells with positive γH2AX foci was relatively lower in the SIPS group compared to the RS group (Figure 9), which is consistent with the results of β-gal staining. Enlarged and irregulated nuclei were observed in both RS and SIPS HBMECs compared to the young cells.

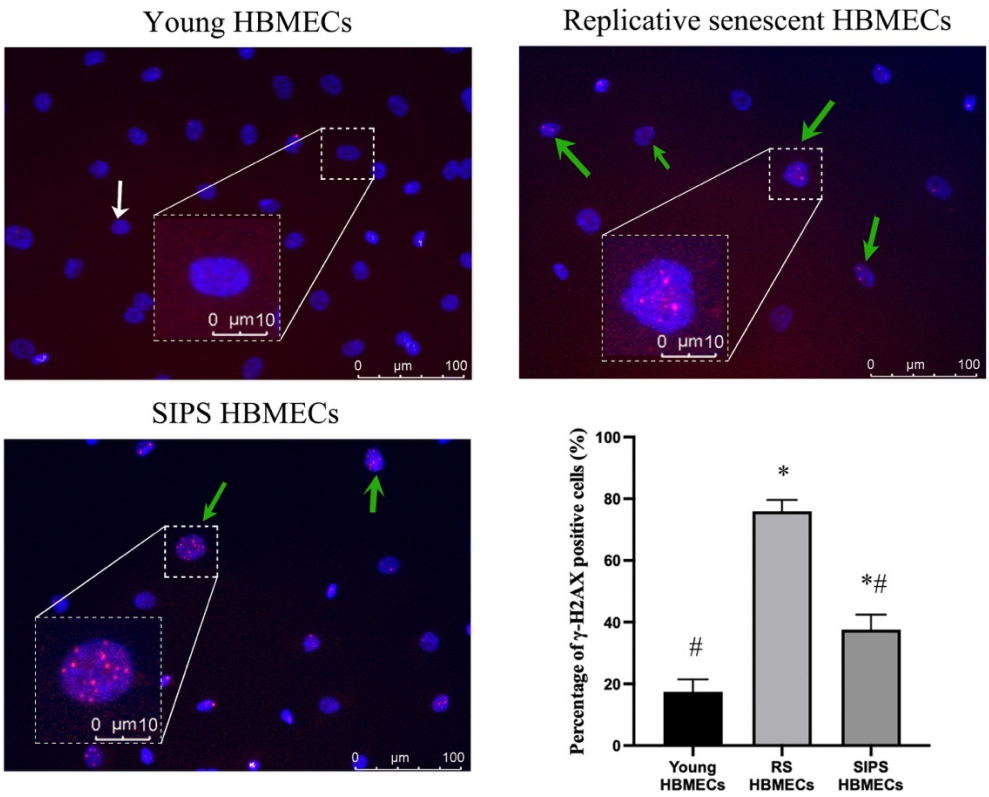


Figure 9. DNA damage accumulation in replicative senescence and SIPS BMECs.
Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to control group, #p< 0.05 compared to RS group.

4.5. Both RS and SIPS are strongly correlated with the activation of the p16 pathway

Repetitive cell division and exposure to oxidative stress evoked significant increases in the expression of cyclin-dependent kinase inhibitor p16 (Figure 10), which leads to the arrest of cell cycle.

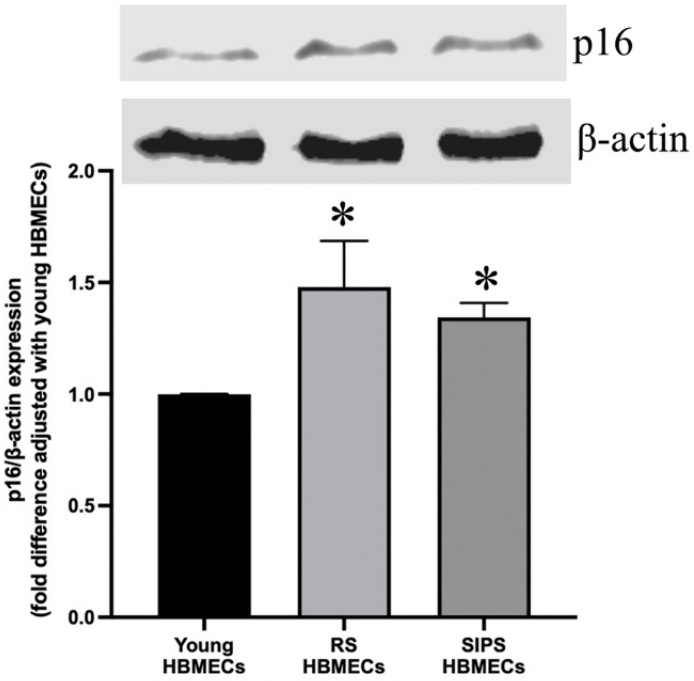


Figure 10. Effects replication stress or exposure to oxidative stress on p16 expression. Significantly elevated expressions of cyclin-dependent kinase inhibitor p16 were observed by Western blotting analysis in replicative senescent and stress-induced premature senescent HBMECs compared to the young HBMECs without any treatment. Data are presented as mean ± SEM from three independent experiments. *$p < 0.05$ compared to the young HBMECs group.

4.6. Modification of the expression and localization of junctional proteins in HBMECs by RS and SIPS

The key function of endothelial cells is forming the mechanical barrier between blood and tissue parenchyma, and this function is highly dependent on the formation of tight

junction complexes (Rodrigues and Granger, 2015). The total expression and the localisation of the three crucial tight junction proteins were evaluated in this study. When HBMECs reached replicative senescence, the total expressions of ZO-1, occludin and claudin-5 all showed a significant drop, especially in the case of claudin-5. However, SIPS did not affect the expression of ZO-1 and occludin, it even increased the expression of claudin-5 (Figure 11).

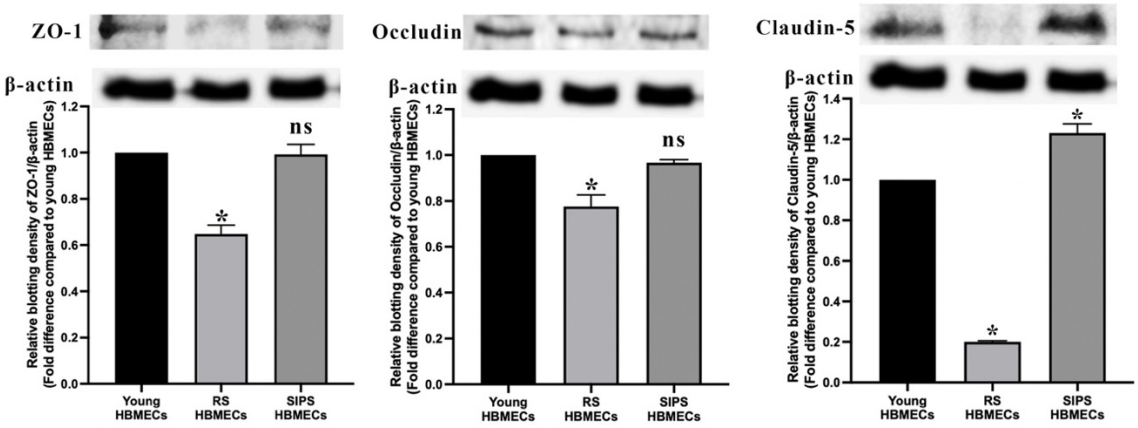


Figure 11. Effects replication stress or oxidative stress on the expressions of tight junction proteins.

Levels of tight junction protein expressions significantly drop in replicative senescent HBMECs, but not in stress-induced premature senescent cells. Data are presented as mean ± SEM from three independent experiments. *$p < 0.05$ compared to young HBMECs group, ns = not significant compared to young HBMECs.

Disruption in the plasma membrane staining of ZO-1 was observed in both RS and SIPS HBMECs when compared to the consistent positive staining at the cell-cell contact. Additionally, increased ZO-1 localisation in the perinuclear area was noticed in SIPS cells. Occludin and claudin-5 appear to be located in the cytoplasm instead of the plasma membrane in HBMECs from different groups (Figure 12).

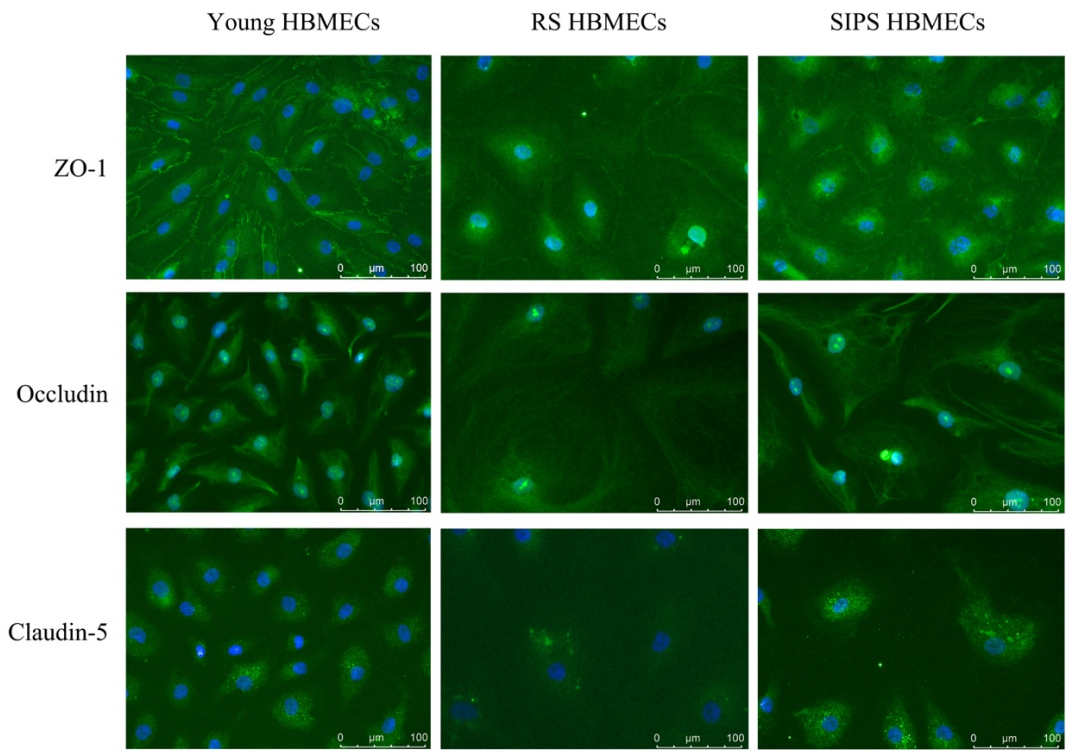


Figure 12. Senescence affects the subcellular distribution of tight junction proteins. Both replicative senescence and stress-induced premature senescence disturb the plasma membrane localisation of ZO-1.

5. Discussion

Results from this study shown that continuous cell division and sublethal level of oxidative stress both resulted in accumulation of senescent BMECs in the cell population, evidenced by increased SA-β-galactosidase activity, γH2AX positive staining, and cyclin-dependent kinase inhibitor p16 expression. BMEC senescence initiated by different triggers both displayed enlarged morphology, increased thickened actin stress fiber, and disrupted plasma membrane localization of zonula occludens-1 (ZO-1). However, replication stress induced senescent BMECs shown significant decreased total expressions of the tight junction proteins, including ZO-1, occludin and claudin-5, while ZO-1 and occludin levels remained unaffected and claudin-5 level shown further elevation in oxidative stress induced senescent BMECs.

Accumulation of senescent cells has been widely determined to be a direct contributor to organismal ageing and ARDs (Calcinotto et al., 2019, Shakeri et al., 2018). Novel treatments targeting senescent cells have been widely investigated in preclinical settings as anti-ageing strategies. Aged mice benefited from the selective elimination of senescent cells with senolytics, evidenced by improved physical functions and extended lifespan (Xu et al., 2018). Vascular ageing constitutes one of the most significant pathological hallmarks of many age-related vascular diseases (Ding et al., 2022), and senescent endothelial cells have great potential to serve as both prognostic biomarkers and therapeutic targets. Senescent ECs, characterised by β-gal positive staining and the upregulation of cyclin-dependent kinase inhibitors, have been widely detected in the lesion of various ARDs, including atherosclerosis, hypertension, and Alzheimer's disease (Holdt et al., 2011, Chu et al., 2024, Garwood et al., 2014).

It was observed that primary cells undergoing a certain number of divisions entered a status of irreversible cell cycle arrest, known as RS (Hayflick and Moorhead, 1961), or cell cycle arrest triggered by acquired challenges such as oxidative stress, known as SIPS (Toussaint et al., 2002). A growing body of research indicates that despite significant similarities, there are differences in terms of mechanisms, phenotypes and functional changes between RS and SIPS (Kural et al., 2016, Bielak-Zmijewska et al., 2014, Wang et al., 2021). The gene expression analyses of human diploid fibroblasts have identified several genes that are specifically involved in the process of premature senescence rather than replicative senescence (Pascal et al., 2005). In the human body, both RS and SIPS can exist simultaneously, but the dominant senescent type may differ in response to age and health conditions. Several risk factors, such as environmental pollution, certain medications, obesity, cigarette smoke or alcohol, can

result in the overproduction of oxidative stress in the vascular system, which acts as the most common stimulus of SIPS (Pizzino et al., 2017, Yang et al., 2024).

Results from our study indicate that RS and SIPS HBMECs share certain traits, including DNA damage accumulation, increased SA-β-gal activity, cyclin-dependent kinase inhibitor p16 expression, and disrupted membrane ZO-1 expression. However, they differ regarding their senescent status and the expression of junction proteins. HBMECs developed replicative senescence gradually along with the continued cell division, eventually reaching a status that over 70% of cells went senescence. In contrast, acute oxidative stress induced only approximately 30% of cellular senescence, and increasing either the hydrogen peroxide concentration or exposure time failed to further elevate the senescent rate. It has been demonstrated that lower doses of H_2O_2 can induce cellular senescence, while higher doses of H_2O_2 are more likely to cause cellular apoptosis instead (Childs et al., 2014). In this study, the acute exposure of BMECs to H_2O_2 resulted in DNA damage lesions. However, possibly due to the different status of the cells, such as different antioxidant capacities, phases in the cell cycle, or genomic stability, some cells repaired the damages during temporary cell cycle arrest and re-enter the cell cycle, while other cells became senescent to avoid the propagation of damaged DNA. Enlarged and irregular nuclei were observed in both RS and SIPS groups, suggesting that nuclear morphological changes may serve as viable markers for assessing cellular senescence. Larger sizes and folds in the nuclear envelope in senescent cells were indicated to be associated with the alteration in chromatin composition, distension of centromeres and nuclear envelope protein dysfunction (Heckenbach et al., 2022, Martins et al., 2020, Criscione et al., 2016).

One of the key roles of BMEC is to form the mechanical barrier between the blood and the interstitium of the central nervous system to control the transit of substances. Tight junctions are major determinants of BBB integrity. They physically seal the paracellular space between adjacent endothelial cells (Liu et al., 2012). In this study, the total expression and distribution of tight junction proteins, ZO-1, occludin and claudin-5, were assessed to investigate the impact of cellular senescence from different triggers on BMEC phenotypes. Replicative senescent BMECs displayed disintegration of ZO-1 expression on the plasma membrane and decreased levels of all junction proteins. In contrast to RS HBMECs, total expressions of junction proteins, ZO-1, occludin, and claudin-5 in HBMECs did not decrease in response to oxidative stress, but the continuous membrane staining signal of ZO-1 showed severe disruption in SIPS cells. Previous studies focusing on endothelial cell senescence and tight junction (TJ) protein expression mostly indicated compromised levels of the TJ proteins and increased permeability of the endothelial monolayer despite the different triggers of senescence (Krouwer et al., 2012, Salvador et al., 2021). However, oxidative stress has been shown to compromise BBB integrity by inducing alterations in the localisation of junction proteins without significantly affecting their total expressions in BMECs (Lee et al., 2004, Enciu et al., 2013). Since phenotypic alterations have been observed in BMEC senescence, which also varies with the triggers of the senescent process, further investigations regarding the mechanisms and functional changes will be conducted to explore potential interventions for BMEC senescence in the following research.

6. Conclusion

Replicative stress emerging from repetitive passaging and enhanced oxidative stress can both accelerate BMEC senescence in a somewhat similar manner. However, RS and SIPS BMECs display different characteristics in tight junction protein expressions.

Chapter 4: Delay of endothelial replicative senescence protects blood-brain barrier from age-related disruption

1. Introduction

BBB dysfunction during programmed ageing or pathological ageing can be a major contributor to age-associated central nervous system (CNS) diseases (Knopp et al., 2023). Endothelium covers the entire inner surface of all blood vessels and carries multiple crucial functions, including vascular tone regulation, vascular permeability modulation, angiogenesis and inflammation adjustment (Widlansky et al., 2003). Brain microvascular endothelial cells (BMECs) form a monolayer that interacts with astrocytes, and pericytes to selectively restrict the passage of compounds between the blood and the central nervous system, named the BBB (Segarra et al., 2021). Although various types of cells contribute to the formation of BBB, BMECs, capable of forming tight junctions to prevent paracellular leakage, are regarded as the main cellular component of the BBB (Bayraktutan, 2019). Tight junctions are multiprotein junctional complexes that consist of several transmembrane proteins and tight junction-associated scaffolding proteins, notably occludin, claudin-5, and zonula occluden-1 (ZO-1) (Lochhead et al., 2020). Therefore, any biological scenario capable of affecting the viability or function of BMECs during ageing process may trigger endothelial dysfunction as an early event and consequently compromise BBB integrity (Kim and Cheon, 2024). Hence, the identification of mechanisms involved in the initiation or progression of senescence-related endothelial dysfunction is of crucial importance to develop novel strategies that can delay or prevent BBB dysfunction and improve lifelong health and wellbeing.

Endothelial cell senescence is implicated in both the structural and functional deterioration of the endothelium (Vasile et al., 2001). Consistent detection of senescent ECs, defined by permanent cell cycle arrest, shortened telomere length and positive staining for SA-β-gal, in atherosclerotic plaques attest to the intrinsic role

played by EC senescence in the formation and progression of vascular pathologies (Song et al., 2020). Increasing evidence suggests that the acquisition of an irreversible senescence-associated secretory phenotype (SASP), accompanied by secretion of several cytokines, chemokines, and matrix metalloproteases (MMPs) may help spread senescence to the neighbouring cells and renders the microenvironment amenable to ageing and age-related diseases (ARDs) development (da Silva et al., 2019, Salminen et al., 2012). The activation of p38MAPK/NF-κB pathway appears to be the main stimulus (Salminen et al., 2012, Freund et al., 2011). Indeed, selective inhibition of p38MAPK activity has been shown to collapse the senescence-associated cytokine network in human fibroblasts and to delay replicative senescence induced by telomere dysfunction (Freund et al., 2011, Iwasa et al., 2003). Similarly, specific targeting of NF-κB, a transcription factor that regulates the release of inflammatory cytokines and ROS, has been shown to improve endothelial function in healthy older adults to the levels observed in young controls (Pierce et al., 2009). Suppressed cellular proliferation, decreased SA-β-gal activity and the expression of senescent pathway proteins, such as p16, p21 and p53, in mouse aortic ECs subjected to inflammatory cytokine interleukin-17A by an NF-κB inhibitor further confirmed the role of NF-κB in EC senescence (Zhang et al., 2021).

In addition to targeting specific mechanisms involved in cellular senescence, the elimination of senescent cells by senolytics may also attenuate or even eradicate the impact of senescent cells on both the neighbouring cells and the remote tissue. The combination of dasatinib and quercetin (D+Q) has been the most commonly used senolytics in recent experimental and clinical studies (Hickson et al., 2019, Novais et al., 2021, Amor et al., 2020). While displaying no significant effects on non-senescent cells, D+Q selectively eliminated senescent cells in skin and adipose tissues (Amor et

al., 2020). Studies specifically focusing on ECs have shown that D+Q alleviates LPS-induced senescence in human umbilical vein ECs through inactivation of a pathway involving the MAPK/NF-κB axis (Fan et al., 2022b). However, the mechanism of D+Q in replicative EC senescence-related BBB dysfunction remains unexplored.

In light of the above, using a cell culture model of chronological ageing, the current study has explored the impact of senescence on the morphological and functional characteristics of BMEC in the absence or presence of D+Q or senomorphics targeting p38MAPK/NF-κB. Using a well-established in vitro model of human BBB (Kadir et al., 2021), the specific mechanisms through which D+Q and p38MAPK inhibition exert their cerebral barrier-protective effects were investigated. Taking together the data generated provided important novel insights into the prophylactic and therapeutic role of senotherapeutics in controlling EC senescence-mediated BBB dysfunction and associated vascular complications.

2. Aims

2.1. To assess how BMECs senescence affects BBB integrity and to explore the molecular mechanisms involved in potential changes in BBB integrity and function, including alterations in MMP activation, and tight junction protein expression and localisation

2.2. To determine whether the delay of BMECs senescence by senotherapeutics can attenuate age-related BBB disruption

3. Methods

The methods and materials included in this chapter were described in detail in Chapter 2 (General Methods), including the setup of replicative senescence model,

trypan blue cell count, in vitro model of BBB establishment and assessment, SA- β-gal staining, tube formation, WST-1 proliferation assay, wound healing assay, western blotting, immunocytochemistry, qPCR-absolute telomere length measurement, gelatin zymography and proteome profiler.

4. Results

4.1. Late passage endothelial cells exhibit typical characteristics of cellular senescence

Given the non-specificity of markers used to detect senescence in *in vitro* settings, in the present study, a series of markers targeting cellular morphology, cell cycle progression and metabolic activity were simultaneously studied. Initial studies looking at the changes in endothelial phenotype showed that HBMECs subjected to repetitive cell culture acquired larger, flattened and heterogeneous morphology and developed thick actin stress fibers traversing the cells at passage ≥19. Passage 16 HBMECs started to display enlarged morphology and generate stress fibers without staining positive by SA-β-gal (Figure 13). Following studies exploring the effects of p38MAPK inhibitor, BIRB796, NF-κB inhibitor, QNZ or a cocktail of senolytics, D+Q on phenotypic changes showed that treatments with those agents from passage 16 to passage 20, except for QNZ, lead to deceleration of morphological changes and stress fibers formation (Figure 13).

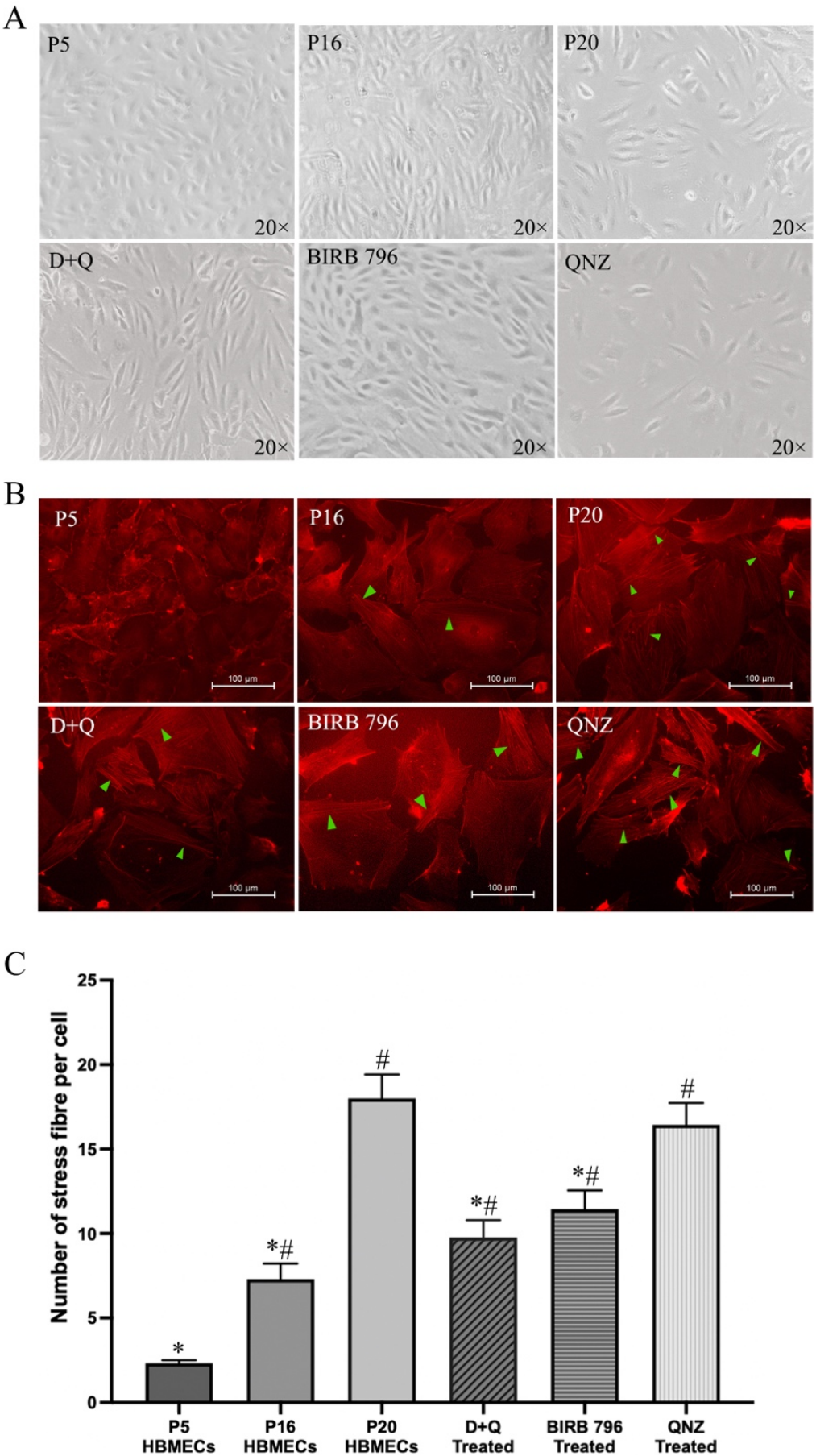
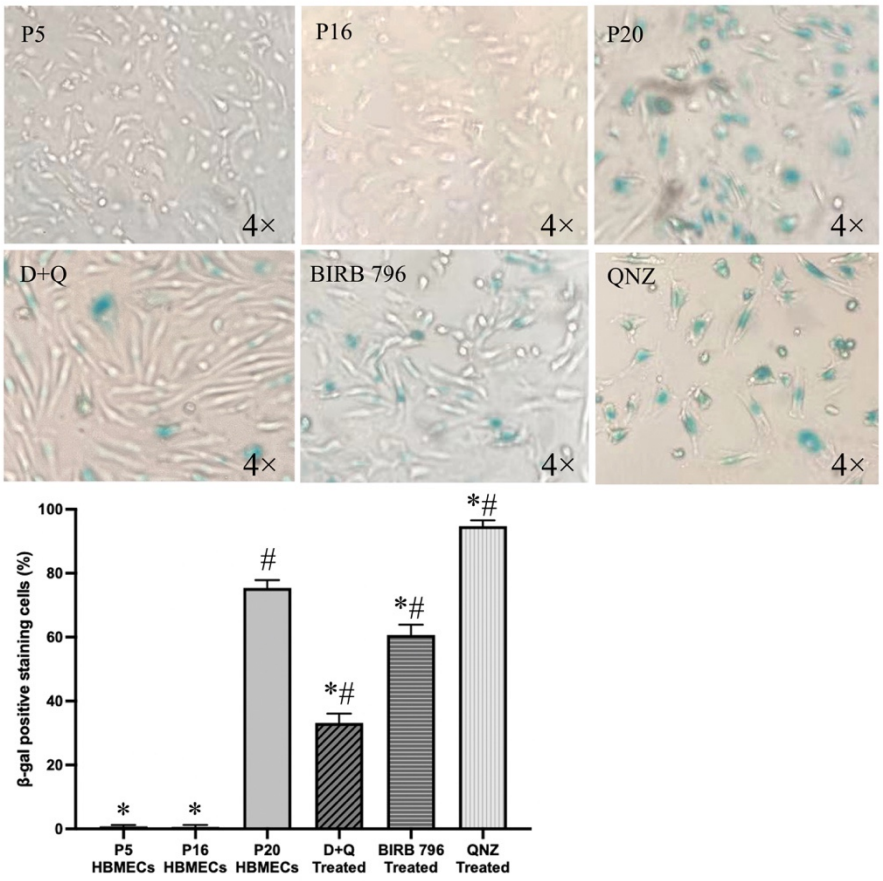

A
P5
P16
P20
D+Q
BIRB 796
QNZ
20×
B
P5
P16
P20
D+Q
BIRB 796
QNZ
100 µm
C
Number of stress fibre per cell
25
20
15
10
5
0
*
*#
#
*#
*#
#
P5 HBMECs
P16 HBMECs
P20 HBMECs
D+Q Treated
BIRB 796 Treated
QNZ Treated

Figure 13. Morphological changes and actin stress fibers formation along with the increase of passage number in BMECs.

Exposure of human BMECs to an in vitro model of chronological ageing, mimicked by replicative senescence, induces profound alterations in cellular morphology (A) and cytoskeletal organisation compared to young cells (B). Compared to young cells (p5), relatively old (p16) and senescent cells (p20) possess larger and flattened morphology and thick actin fibers. Treatment of p16 cells with inhibitors for p38MAPK (BIRB796), or a cocktail of senolytics (dasatinib and quercetin; D+Q) until p20 attenuated the effect of replicative senescence on both morphology (A) and cytoskeletal organisation as ascertained by reductions in the number of stress fibers indicated by white arrows (B, C). Numbers of total stress fiber were counted manually from minimum 20 cells in four randomly chosen areas of each slide and divided by cell numbers. Scale bar: 100 μm. The images of cellular morphology and stress fibers were captured using ×20 and ×40 magnification. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

Subsequent studies aiming to provide further evidence for HBMECs senescence demonstrated significantly higher SA-β-gal activity in late-passage (p20) versus low passage (p5) cells (Figure 14A). To evaluate the status of senescence from multiple aspects, apart from SA-β-gal staining, γH2AX expression, a sensitive marker of double-stranded DNA breaks and p16 expression, a cyclin-dependent kinase inhibitor, was investigated in this study. As with the SA-β-gal activity, passage 20 HBMECs exhibit higher accumulation of γH2AX positive foci and increased p16 expression, compared to younger counterparts (Figure 14B). Consistent with morphological studies, treatments with BIRB796 and D+Q, drastically reduced the number of SA-β-gal positive cells and generation of stress fibers, prevented the accumulation of γH2AX positive foci and suppressed p16 expression (Figure 13, 14, 15). Interestingly, inhibition of the NF-κB pathway with QNZ significantly diminished the accumulation of DNA damage, evidenced by reduced γH2AX positive foci, but failed to reduce the number of SA-β-gal positive cells or stress fibers generation (Figure 14).

A


P5
P16
P20
D+Q
BIRB 796
QNZ
4×
β-gal positive staining cells (%)
100
80
60
40
20
0
*
*
#
*#
*#
*#
P5 HBMECs
P16 HBMECs
P20 HBMECs
D+Q Treated
BIRB 796 Treated
QNZ Treated

B

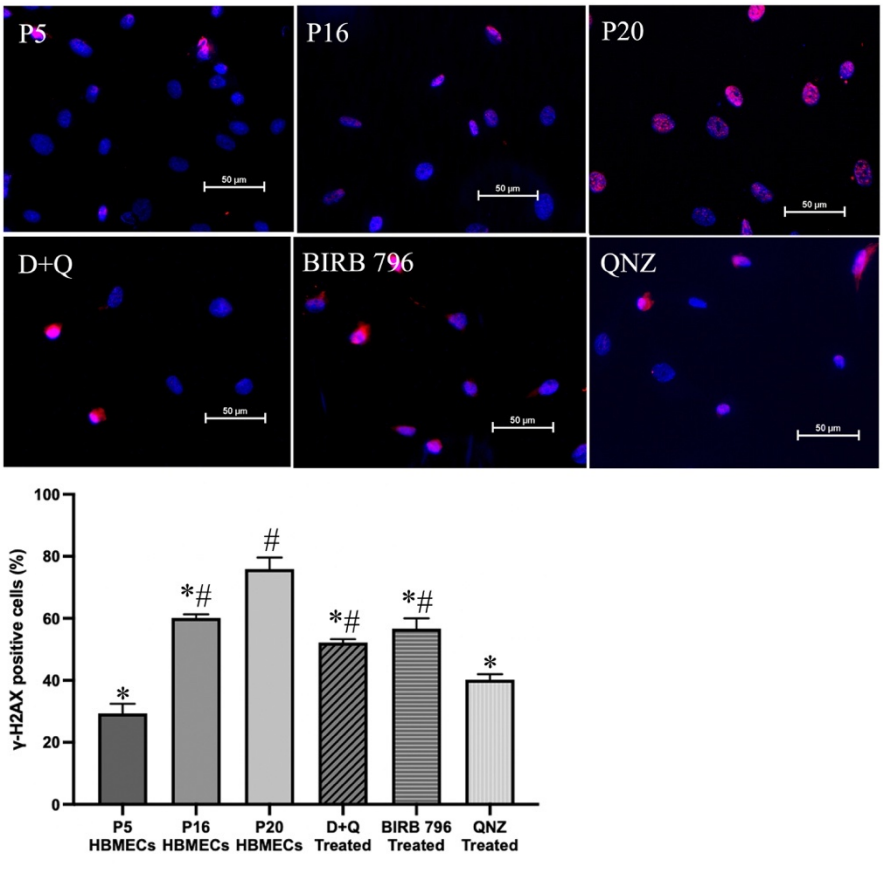

P5
P16
P20
D+Q
BIRB 796
QNZ
50 µm
γ-H2AX positive cells (%)
100
80
60
40
20
0
*
*#
#
*#
*#
*
P5 HBMECs
P16 HBMECs
P20 HBMECs
D+Q Treated
BIRB 796 Treated
QNZ Treated

Figure 14. The number of SA-β-gal positive cells and γH2AX positive cells increases by replicative senescence.

Exposure of human BMECs to replicative cell culture markedly increased the number of SA-β-gal positive cells in late passage BMECs (p20) compared to young (p5) and mid-passage (p16) cells. DNA damage foci accumulated in the senescing process, evidenced by the progressively increased number of γH2AX positive staining cells along with the increase of passage number. Treatments of p16 cells with senomorphics targeting p38MAPK (BIRB796) or a cocktail of senolytics (D+Q) until p20 diminished the number of cells stained positively with SA-β-gal or γH2AX. Treatment with QNZ, an NF-κB inhibitor, significantly elevated the number of SA-β-gal positive cells, but decreased the number of γH2AX positive cells. The images of SA-β-gal and γH2AX staining were captured using ×20 or ×40 magnification. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

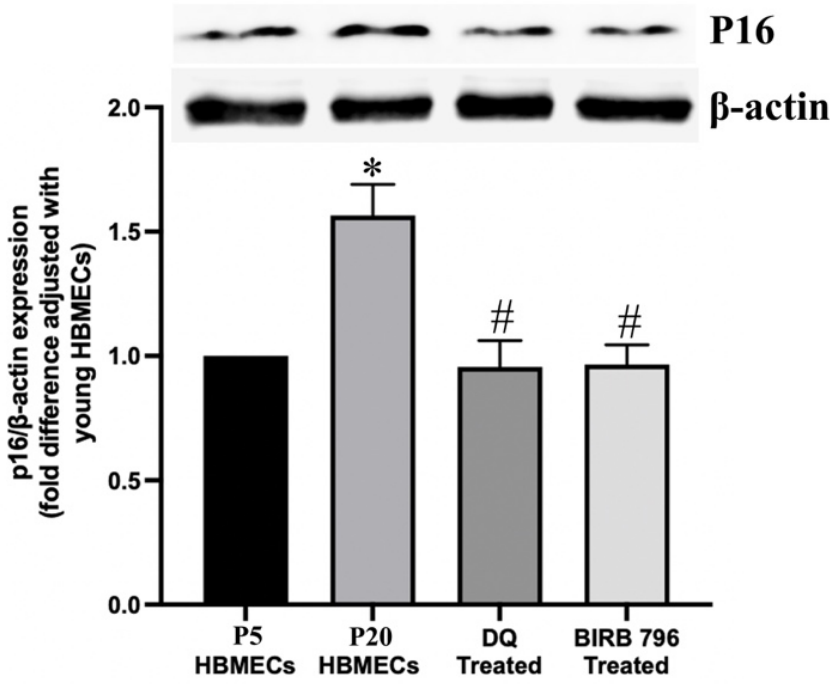


Figure 15. Treatments with senotherapeutics suppressed the senescence-evoked expression of p16.

Significantly increased expression of cyclin-dependent kinase inhibitor p16 was observed by western blotting in p20 HBMECs compared to p5 cells. Treatments of passage 16 cells with senolytics (D+Q) and p38MAPK inhibitor (BIRB796) until passage 20 diminished the replicative stress-induced p16 expression. Data are expressed as mean ±SEM from three

independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

Total cell count using trypan blue showed that treatment with QNZ significantly diminished the number of viable HBMECs compared to controls, i.e. p16 cells (Figure 16). Because of this potential apoptotic effect, QNZ was not pursued in the subsequent experiments.

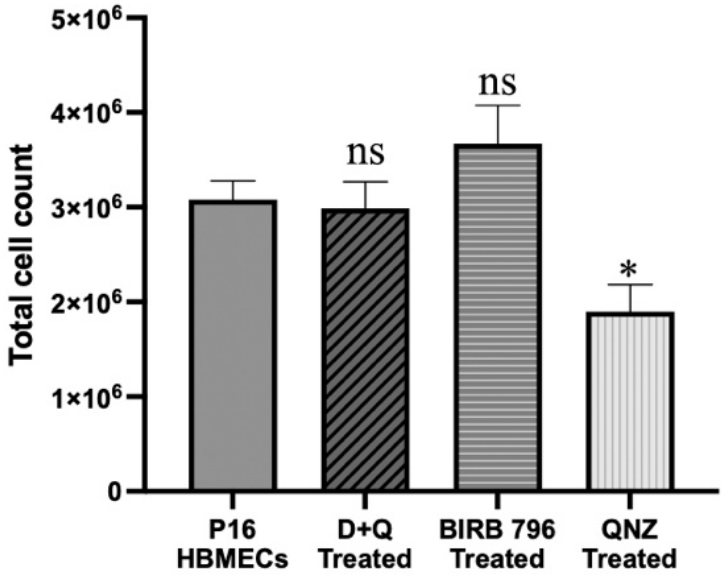


Figure 16. Impact of senotherapeutics on viable cell numbers.
Treatment with a senolytics cocktail (D+Q) and p38MAPK inhibitor (BIRB796) did not affect the total number of p16 cells in the T75 flask. However, treatment with QNZ significantly reduced the number of viable cells compared to the p16 cells in the control group. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p16 control BMECs, ns= not significant compared to p16 control group.

Scrutiny of cell proliferation as an index of cell cycle progression revealed a significant decline in HBMECs proliferative capacity with increasing passages. Despite halting these decreases, treatments with BIRB796 and D+Q failed to return the proliferation rates to the levels seen in low-passage cells (Figure 17). From this point on, HBMECs with high proliferative capacity and less than 5% SA-β-gal

positive staining were defined as young cells. HBMECs that showed no sign of proliferation for a week in culture with complete medium and had over 70% SA-β-gal- and γH2AX-positive staining were defined as senescent.

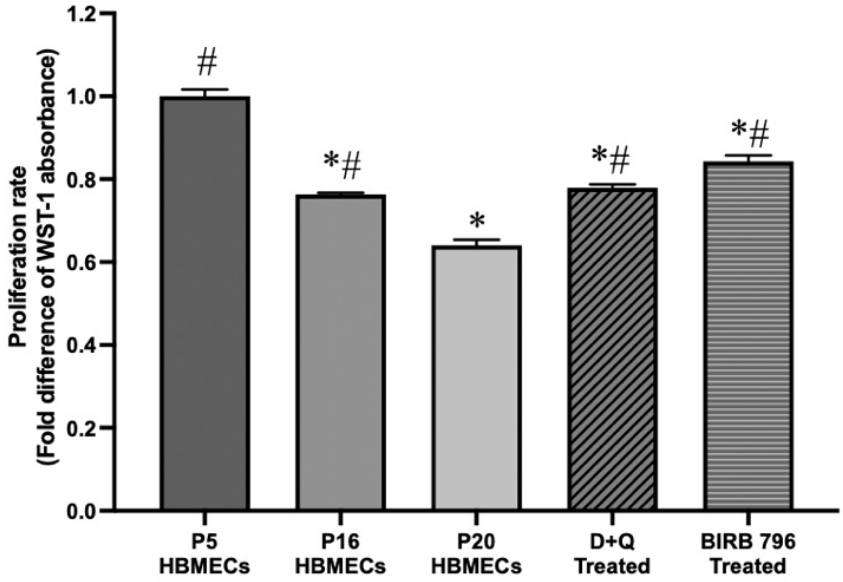


Figure 17. Senotherapeutics reduce the impact of senescence on BMEC proliferative capacity.

The proliferation rate of BMECs, assessed by the WST-1 assay, decreased with increasing passage numbers. Treatments of p16 cells with senomorphics targeting p38MAPK (BIRB796) or senolytics (D+Q) to p20 significantly decreased the suppressive effect of senescence on proliferation. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

Considering that telomeres play an essential role in preserving the integrity of chromosomes during the cell division cycle and undergo programmed shortening in culture conditions (Lopez-Otin et al., 2013), the telomere length was investigated by quantitative PCR and shown to be substantially shorter in senescent versus young cells. The inability of BIRB796 or D+Q to influence telomere length suggested that telomere was not the target of these agents (Figure 18).

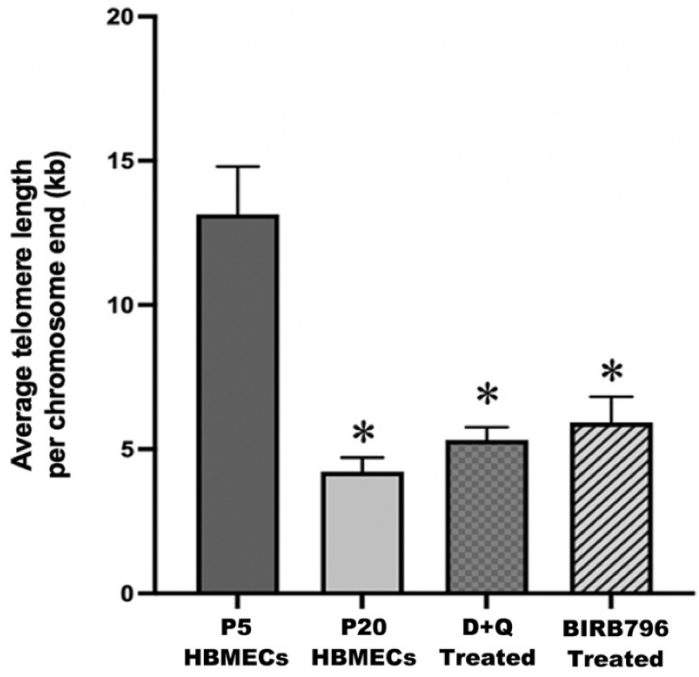


Figure 18. Replicative senescence is associated with telomere attrition.
Compared to young HBMECs (p5), the average telomere length is markedly reduced in senescent cells (p20). Treatments of p16 cells with p38MAPK inhibitor (BIRB796) and a cocktail of senolytics (D+Q) until p20 failed to negate the effect of senescence on telomere length. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs.

4.2. Senescent endothelial cells lose their angiogenic potential

Angiogenesis is a vital process by which new blood vessels form and thus contribute to the growth, development and regeneration of the damaged tissue (Simons, 2005). As evidenced by significant decreases in number and length of new tubules appeared on Matrigel, senescence negatively affected the capacity of ECs for angiogenesis. Here, suppression of p38MAPK by BIRB796, rather than elimination of senescent cells via D+Q, appeared to be more effective in diminishing the impact of senescence on tubule formation *in vitro* setting (Figure 19).

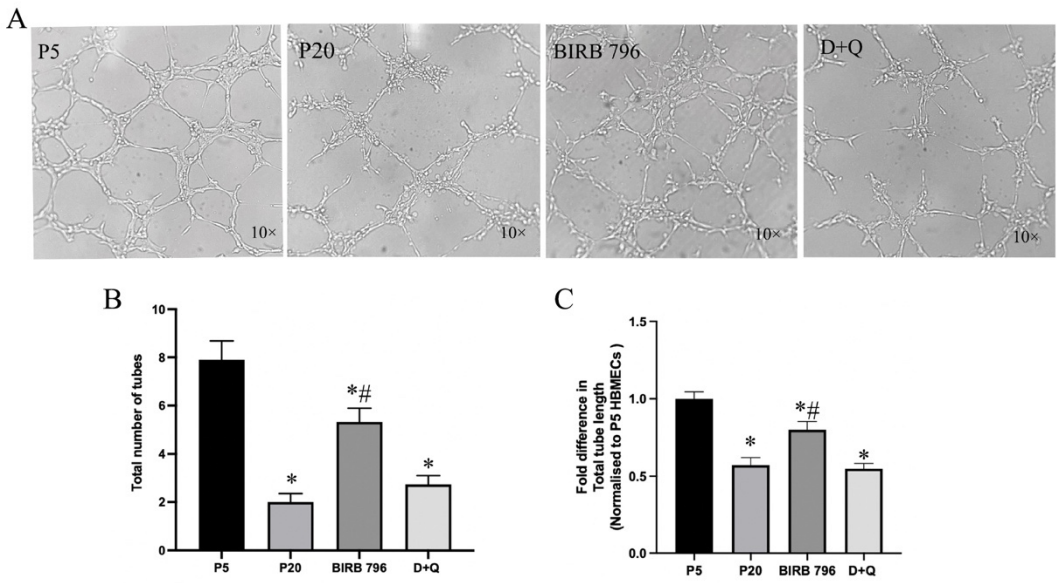


Figure 19. Senescence adversely affects the angiogenic capacity of BMECs.
Senescent (p20) BMECs displayed significantly fewer and shorter tubules on Matrigel compared to young (p5) cells. Treatment with p38MAPK inhibitor (BIRB796), increased the number and total segment length of tubules. In contrast, treatment with a cocktail of senolytics (D+Q) didn't significantly affect the tube number and total segment length (A-C). The tubules were visualised using light microscope under 10 × magnification. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

4.3. Senescent endothelial cells have impaired wound healing capacity

The wound healing assay is a widely recognised *in vitro* technique for the evaluation of cell migration using live cell imaging. Additionally, it also evaluates the status of various cellular functions (Jonkman et al., 2014). The wound healing capacity depends on the production of essential cytokines to recruit the cells, the proliferation and migration of the cells, and the remodelling of the extracellular matrix (Mayya et al., 2021). Declined wound healing ability was observed with senescent ECs, demonstrated by significantly slower wound closure compared to the young ECs. Neither inhibition of p38MAPK by BIRB796 nor senolytics cocktail D+Q was able to restore the wound healing capacity of the late passage ECs (Figure 20).

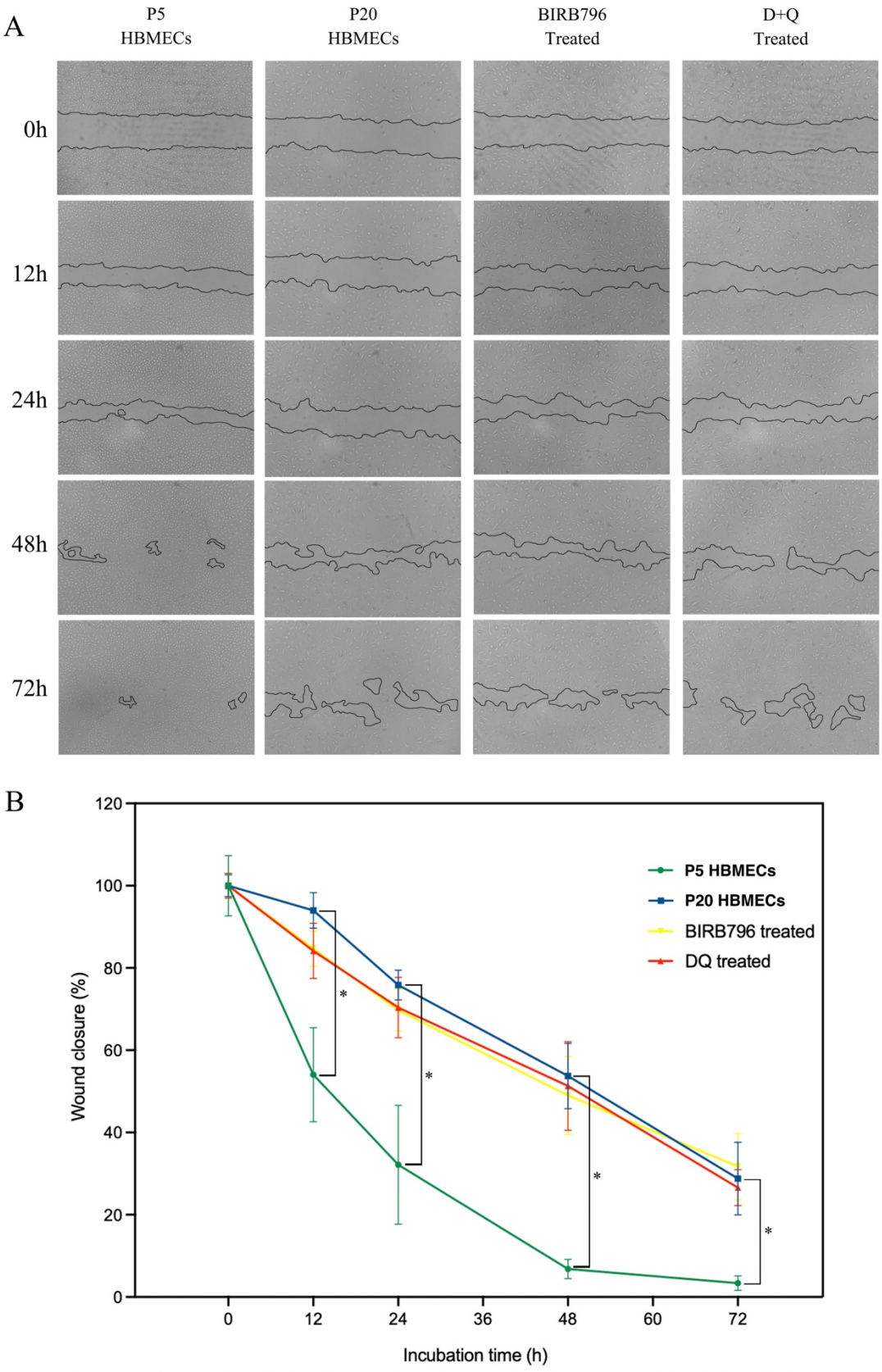


Figure 20. Senescent HBMECs displayed reduced migratory capacity.

Compared to young HBMECs (p5), senescent HBMECs (p20) showed significantly impaired migratory capacity (A), as evidenced by the extended time required to repair the wound

inflicted on HBMEC monolayer (B). Treatments with both p38MAPK inhibitor (BIRB796) and a cocktail of senolytics (D+Q) failed to negate the impaired migration ability caused by senescence. Data are expressed as mean ±SEM from three independent experiments. *$p < 0.05$ compared to p5 BMECs.

4.4. Cellular senescence significantly affects the expressions of angiogenic factors

To further explore the mechanisms of senescence-induced impairment of angiogenesis and wound repair ability of HBMECs, a membrane-based array to detect the relative levels of selected angiogenic factors was performed with the cell culture media of young and senescent HBMECs. In line with the declined angiogenic and migratory capacity, senescent HBMECs display dramatically decreased release of several critical growth factors, including angiopoietin-2, placental growth factor (PLGF), fibroblast growth factor (FGF), and heparin-binding EGF-like growth factor (HB-EGF); declined expression of pro-angiogenic and pro-migratory proteins and peptides, including angiogenin, endothelin-1 and endoglin. In addition, significantly increased levels of the anti-angiogenic regulator, thrombospondin-1 (Figure 21).

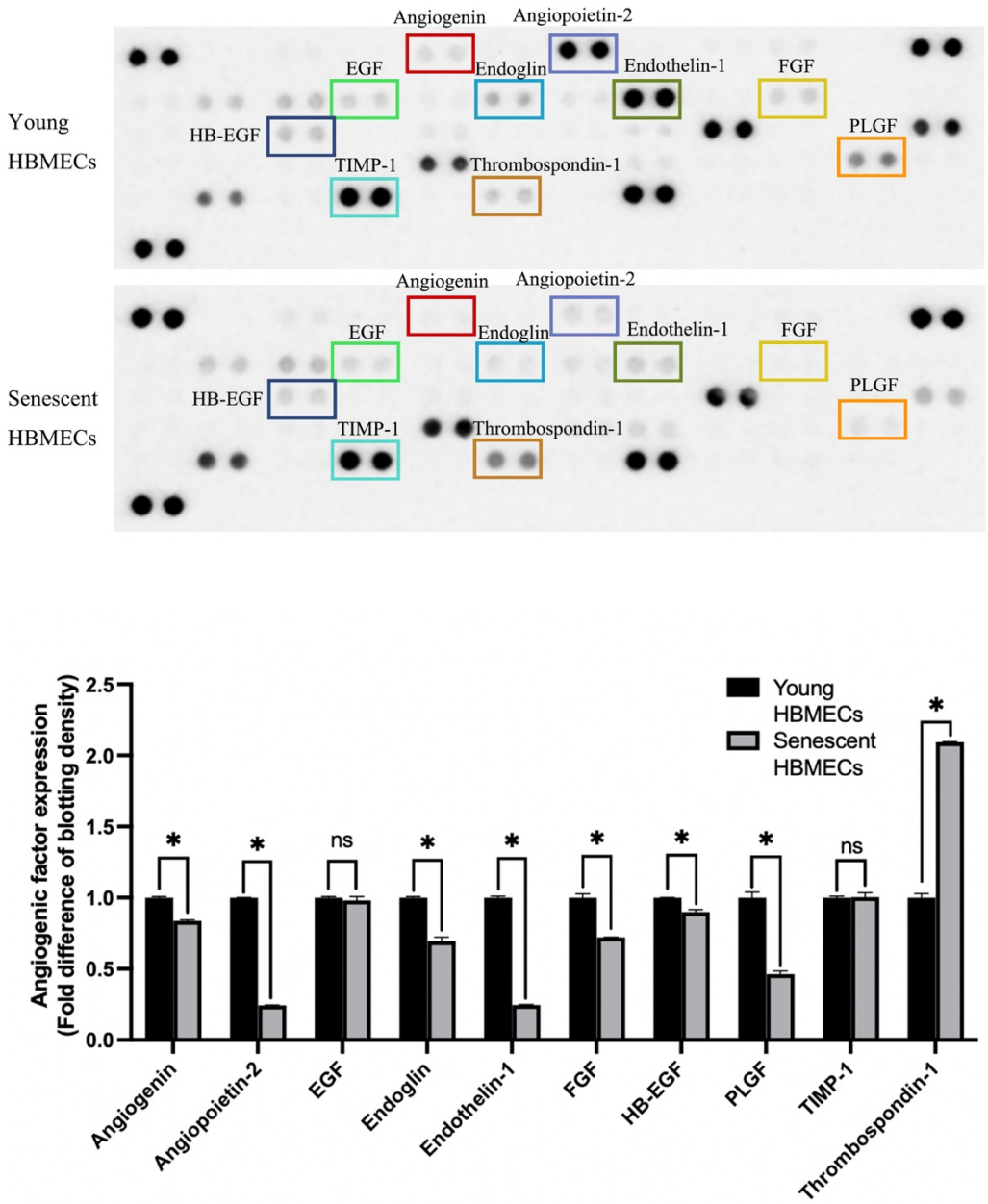


Figure 21. Senescence-induced upregulation and downregulation of angiogenic factors in HBMECs.

Senescent HBMECs express significantly lower quantities of pro-angiogenic factors, including angiogenin, angiopoietin-2, endoglin, endothelin-1, FGF, HB-EGF, PIGF, and higher levels of anti-angiogenic factor, thrombospondin-1. The expressions of EGF and TIMP-1 remain unaffected in senescent HBMECs. The expression of angiogenic factors was quantified using ImageJ based on the densitometric analysis of the dot blot duplicates. Data are presented as mean ± SEM (n=2). *p< 0.05 compared to young HBMECs group, ns= not significant compared to young HBMECs group.

4.5. Senescent endothelial cells fail to form functional BBB

Through lateral migration and consequent replacement of dead or dying ECs, resident ECs play an important role in preventing structural and functional vascular damage (Bayraktutan, 2019). The data generated up to this point suggested that senescent HBMECs were unlikely to be as protective or functional as their younger counterparts. The overt inadequacy of senescent HBMECs to form a functional model of human BBB, evidenced by decreased transendothelial electrical resistance (TEER) and increased paracellular flux of sodium fluorescein (NaF) across the barrier, supported this hypothesis. Interestingly, treatments with BIRB796 and D+Q completely neutralised the disruptive effects of senescence on the cerebral barrier. Contrary to the increased permeability of NaF, the flux of Evans blue-labelled albumin (EBA), a high molecular weight permeability marker, remained unchanged across all experimental conditions, thereby implying the formation of relatively small inter-endothelial cell openings between senescent HBMECs (Figure 22).

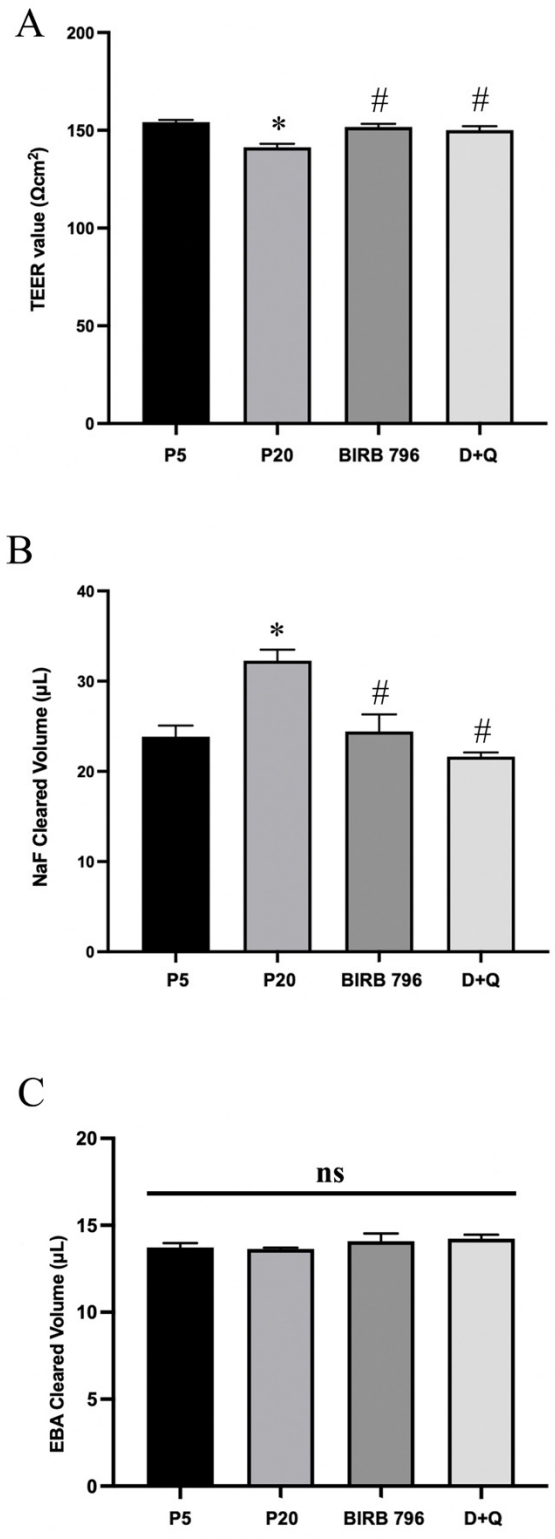


Figure 22. The presence of senescent BMECs adversely affects blood-brain barrier integrity and function.

Co-culture of senescent (p20) BMECs with astrocytes and pericytes in an in vitro model of human BBB decreased barrier integrity (A) and increased paracellular flux of low molecular weight (NaF) but not high molecular weight (EBA) permeability marker across the barrier (B,

C). Treatments with senomorphics BIRB796, a p38MAPK inhibitor and a cocktail of senolytics (dasatinib and quercetin, D+Q) neutralised the deleterious effects of senescence on both BBB integrity and function (A-C). Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

4.6. Replicative senescence distinctly affects tight junction protein expression and localisation and activates MMP-2

To determine whether possible changes in tight junctional complex formation and the extracellular matrix (ECM) might explain the abovementioned angiogenesis and BBB-related findings, subcellular localisation and abundance of ZO-1, occludin and claudin-5, and the MMPs were investigated. Intermittent interruptions in plasma membrane staining and significantly declined abundance of ZO-1 offered some explanation for the impaired integrity and function of the BBB established with senescent HBMECs. The abundances of occludin and claudin-5 were significantly lower in senescent HBMECs compared to their young counterparts. However, unlike ZO-1, occludin and claudin-5 were found to be located mostly to cytoplasm and nuclei in both young and senescent cells (Figure 23). Although treatments with BIRB796 and D+Q could partially prevent the loss of ZO-1 from the plasma membrane in the cells undergoing senescence, the abundance of ZO-1 remained significantly lower compared to the young HBMECs. Neither BIRB796 nor D+Q altered the subcellular localisation of occludin or claudin-5 observed in senescent cells despite elevating the abundance of claudin-5 (Figure 23).

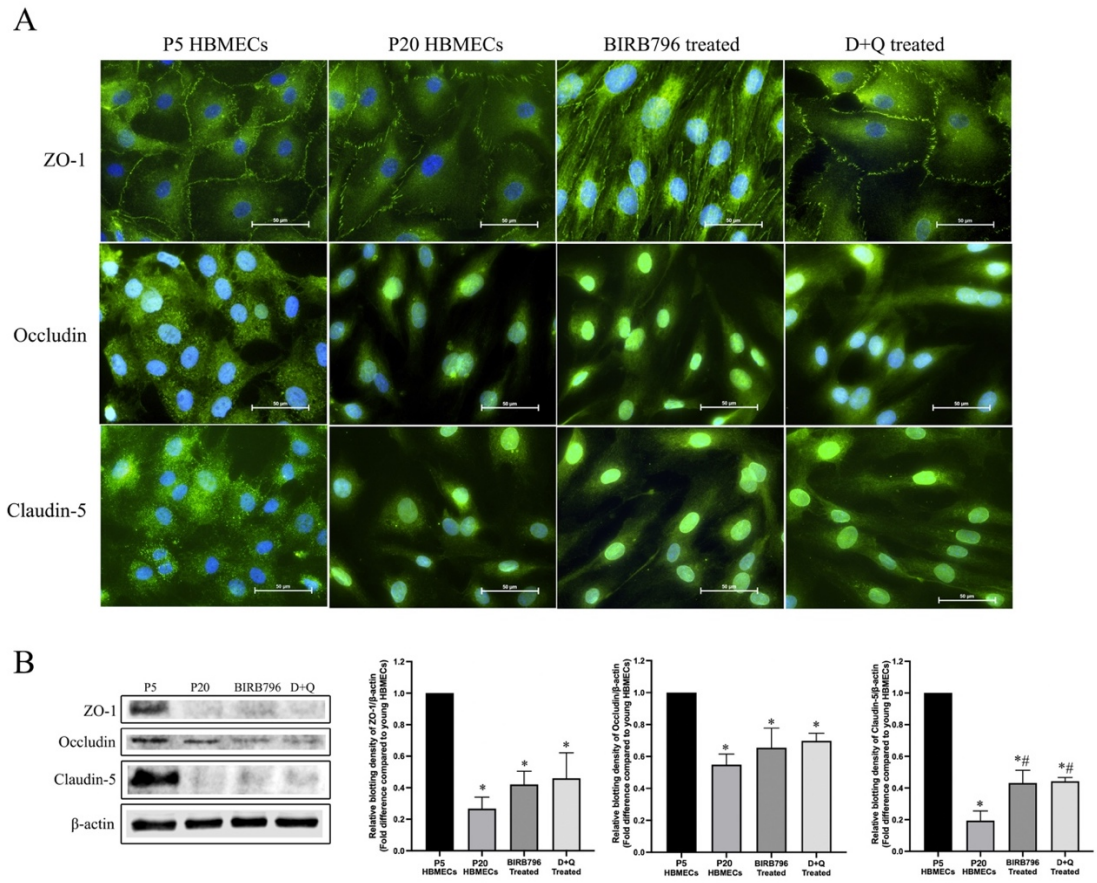


Figure 23. Senescence is associated with disruptions in tight junction protein localisation and expression.

Compared to young (p5) cells, the plasma membrane localisation of ZO-1 is interrupted in senescent BMECs, which was somewhat prevented by treatments with senomorphic BIRB796, a p38MAPK inhibitor or a cocktail of senolytics (D+Q). Unlike ZO-1, both occludin and claudin-5 appear to localise to cytosol and nuclei, and treatments with either agent did not greatly influence their subcellular localisation (A). Significantly decreased expressions of TJ proteins, ZO-1, occludin and claudin-5 were observed in senescent BMECs compared to their young counterparts. Treatments with BIRB796 and D+Q partially returned the level of claudin-5 to those seen in p5 cells. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

The gelatin zymography images only detected pro- and activated MMP-2 in this study. Replication stress significantly raised the expression of active MMP-2. Treatments with BIRB796 or D+Q significantly suppressed the activation of MMP-2 while the levels of pro-MMP-2 remained unaffected (Figure 24).

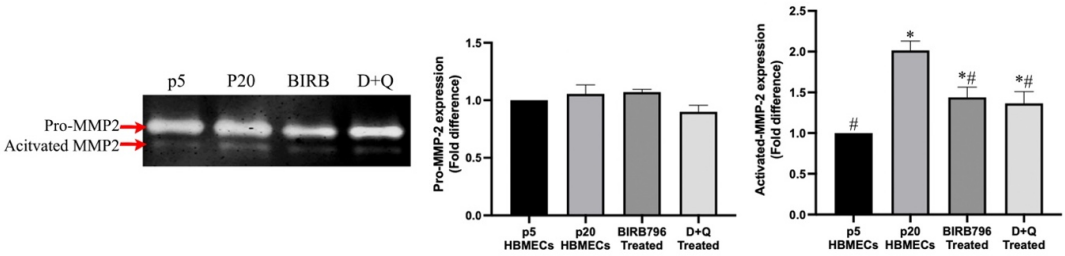


Figure 24. Senescence promotes the activation of MMP-2.

Despite the unaffected pro-MMP-2 level in all experimental groups, a significantly elevated level of activated MMP-2 was detected with senescent BMECs (p20) compared to the young cells (p5), treatments with p38MAPK inhibitor (BIRB796) and a cocktail of senolytics (D+Q) suppressed the replicative stress-evoked MMP-2 activation. Data are expressed as mean ±SEM from three independent experiments. *p< 0.05 compared to p5 BMECs. #p< 0.05 compared to p20 BMECs.

5. Discussion

Extending the findings presented from the previous chapter, this study further demonstrated that replicative senescent BMECs displayed shortened telomere length and significantly impaired angiogenesis and migration capacity. In addition, the impairment observed in the integrity of BBB established with senescent BMECs, ascertained successively by decreases in TEER values and increases in paracellular flux, revealed the close correlation between endothelial senescence and BBB dysfunction. Treatments of relatively late passage BMECs with a cocktail of senolytics (dasatinib and quercetin), or senomorphics targeting the p38MAPK pathway until passage 20 rendered these cells more resistant to senescence and preserved BBB characteristics by restoring the membrane localization and expression of tight junction proteins and inhibit the activation of MMP2.

BBB regulates the passage of an array of selective components between brain parenchyma and systemic circulation (Bayraktutan, 2019, Segarra et al., 2021). Due to its pivotal roles in nutrient supply and waste disposal, it is essential to monitor the

function of BBB at all times to conserve neurovascular homeostasis. Increased permeability of the BBB is implicated in chronological ageing and age-related neurological disorders (Farrall and Wardlaw, 2009, Jia et al., 2019). Continual detection of senescent cells at specific sites of age-related pathologies, like atherosclerosis or Alzheimer's disease, confirms the idea that cellular senescence contributes to organismal ageing and age-related pathologies (Minamino et al., 2002, Yu et al., 2024). Although formed through complex interactions between cells composed of the neurovascular unit and the basement membrane, dysfunction of BMECs alone may be sufficient to compromise BBB integrity during the ageing process (Jia et al., 2019). Bearing these in mind and using a cell culture model of chronological ageing, the current study specifically assessed the impact of BMEC senescence on BBB integrity and function.

Given the non-specificity of commonly used markers for cellular senescence, this study, as indicated before, employed a panel of interrelated markers aiming to detect changes in cell phenotype, cell cycle progression and metabolic activity. Since accurate identification of specific mechanisms involved in BMEC senescence and dysfunction is critically important for strict modulation of ageing-related cerebrovascular alterations and clinical conditions, such as vascular dementia, this comprehensive approach was of paramount importance (Graves and Baker, 2020). Acquisition of a flattened and enlarged morphology in cells that ceased to proliferate, in culture media supplemented with necessary growth factors, confirmed that senescence occurred in BMECs after about 19-20 population doublings. Marked reductions in protein degradation and RNA turnover, leading to increases in intracellular RNA and protein content may, to a degree, account for the cellular enlargements observed in this study. Persistent DNA damage response arising from

the exhaustion of DNA replication cycles and telomere shortening due to repetitive cell culture may result in the cell cycle arrest by activating cyclin-dependent kinase inhibitors, p21 and p16, and thus blocking the cell cycle at G1-S interphase (Kim et al., 2017, Rayess et al., 2012). Further scrutiny of cellular morphology by analyses of cytoskeletal organisation through actin microfilament staining verified that senescent cells took up a larger phenotype and developed thick actin stress fibers traversing the cells. The cytoskeleton primarily helps cells maintain their shape and internal organisation, which enables them to perform essential functions like proliferation and motility. Under normal circumstances, the cortical actin bands participate in the formation of tight junctional complexes and regulate paracellular flux. Detection of remarkably higher SA-β-gal staining, γ-H2AX deposition coupled with permanent DNA damage, and thickened actin stress fibers in cells manifested morphological changes substantiates the notion that senescence is a complex multifactorial process (Lopez-Otin et al., 2013).

Despite the availability of nutrients, growth factors and space to grow into, the permanent cell cycle arrest indicated that senescent cells also develop functional abnormalities. These are often associated with survival and cell division. Given that proliferation and migration of BMECs are important prerequisites for the replacement of the dead or dying ECs, age-dependent progressive loss of BMECs and their functionality are linked to perturbation of vascular integrity and homeostasis. Accumulation of metabolically active senescent BMECs in cerebrovasculature can further worsen vascular impairment by adversely affecting the function of the neighbouring cells and circulation EPCs through secretory agents, including ROS, cytokines and chemokines that they synthesise and release (Hohn et al., 2017).

Attenuation of angiogenesis and migration by senescent BMECs may also exacerbate cerebrovascular damage. Observation of fewer and shorter tubule formation on Matrigel with senescent BMECs proved that senescence adversely affects angiogenic potential. The extended duration of wound closure on BMECs monolayer indicated that senescent cells have diminished migratory and proliferative capacity. The impaired angiogenesis and migratory capacity of endothelial cells may impair wound repair and new vessel development under physiological and pathological conditions (Suh et al., 2023). The mechanism of age-related impairment of angiogenic capacity in endothelial cells may be associated with the inhibition of certain pro-angiogenic factors (Chang et al., 2007, Xia et al., 2012). Consistent with those studies, decreased expression of pro-angiogenic and pro-migratory factors and increased levels of anti-angiogenesis factors were detected in the culture media of senescent BMECs compared to their young counterparts. Out of 55 angiogenesis-related proteins targeted by multianalyte assays, 10 proteins detected were selected for density quantification. Among several different molecules in the vascular endothelial growth factor (VEGF) family, a family of secreted polypeptides that are essential for vessel development, PLGF was specifically detected in BMECs and found to dramatically decrease when entered senescence, which may contribute to the impaired angiogenesis by directly suppressing endothelial cell proliferation and migration (Holmes and Zachary, 2005, De Falco, 2012). Interestingly, amongst the two members of the epidermal growth factor (EGF) family, while significant downregulation was observed for HB-EGF in senescent BMECs, EGF levels remained unchanged between young and senescent BMECs. HB-EGF is reported to promote migration and angiogenesis of endothelial cells via the activation of phosphoinositide 3-kinases (PI3Ks) and extracellular signal-regulated kinases (ERK) signalling pathways, and through stimulation of EGF

receptor (EGFR) expression (Mehta and Besner, 2007, Rao et al., 2020). The angiogenesis factors analysis provides potential supplementary targets to ameliorate age-related angiogenic dysfunction. Failure of senescent BMECs to establish a functional model of BBB when co-culture with astrocytes and pericytes, provided further evidence for the dysfunctionality of senescent cells. In support of a recent study documenting that mixing replicative senescent EPCs with young BMECs elicits BBB damage in *in vitro* settings (Reskiawan et al., 2022), this study also showed a substantial breakdown in integrity and function of BBB established with senescent BMECs and attributed this to the alteration of junction proteins and extracellular matrix of senescent cells. Considering that ZO-1 mediates the connections between the actin cytoskeleton and other tight junction proteins, the disruptions may conceivably have a severe impact on overall BBB function (Fanning et al., 1998). Decreased expression of the unexpected cytoplasmic and nuclear localisation of claudin-5 and occludin in senescent BMECs may also contribute to BBB dysfunction. Cytoplasmic and nuclear localisation of occludin has previously been noted in other cerebral cells, such as astrocytes and neuroepithelial cells. In these cells, occludin appeared to play crucial roles relating to RNA metabolism and nuclear function (Morgan et al., 2018). In another study, non-plasma membrane localisation of tight junction proteins has been correlated with decreased telomerase activity in senescent cells (Huang et al., 2010). While substantially reduced telomere length in senescent BMECs may shed some light on the non-plasma membrane localisation of occludin and claudin-5 observed in this study, these findings cannot satisfactorily explain the complete disappearance of these proteins in BMEC plasma membrane. Apart from affecting the distribution of tight junction proteins, senescent BMECs also released

higher levels of activated MMP-2, which participates in the degradation of key extracellular matrix components and consequently impairs the BBB integrity.

To assess whether manipulation of senescence may extend the life span and proliferative capacity of ECs, we treated relatively old but non-senescent BMECs (p16) with a cocktail of senolytics, D+Q or the inhibitors of p38MAPK or NF-κB. In the present study, attenuation of senescence-related changes in cellular morphology, proliferative capacity, tubulogenesis, SA-β-gal activity, γH2AX staining and complete restoration of BBB function with BIRB796 proposes p38MAPK signalling pathway as a potential therapeutic target for the management of chronological senescence. Inhibition of p38MAPK may also halt the spread of senescence to the neighbouring cells through suppression of SASP via a mechanism involving NF-κB transcriptional activity (da Silva et al., 2019). Hence, treatments with an NF-κB inhibitor, i.e. QNZ, rather unsurprisingly diminished the impact of senescence on γH2AX positive staining. However, significant drops in number of viable cells in QNZ-treated experimental groups casts serious doubt on the applicability of NF-κB inhibitors as efficacious therapeutics.

Selectively removal of senescent cells by senolytics is thought to subside the burden of these cells on their microenvironment (neighbouring cells) and remote tissue and improve cerebrovascular function as a consequence. The combination of D+Q is regarded as an effective cocktail of senolytics with significant anti-ageing effects (Kirkland and Tchkonia, 2020). While dasatinib induces apoptosis and attenuates the viability of senescent adipocytes (Amor et al., 2020), quercetin displays potent anti-inflammatory, antioxidant and immunoprotective effects (Farag et al., 2021). Once combined, D+Q exerts further beneficial effects, reduces the secretion of pro-

inflammatory cytokines, prevents the accumulation of macrophages, and potentiates the proliferative capacity of progenitor cells to reduce the burden of senescent cells (Hickson et al., 2019, Fan et al., 2022a, Johmura et al., 2021). In accordance with these observations, treatments with D + Q, in the current study, markedly delayed BMEC senescence and improved their proliferative and barrier-forming capacity.

6. Conclusion

In conclusion, this experimental study supports the concept that ECs go into senescence during ageing process, which ultimately damages the integrity of the BBB. The study reports that inhibition of p38MAPK and removal of senescent cells by senolytics successfully delay the senescence of BMEC and fully restore BBB integrity and function. Building upon these findings, the next chapter will further investigate the activation pattern of the p38MAPK/NF-κB pathway and focus on stress-induced premature senescence to provide an in-depth and comprehensive understanding of this pathway in the ageing process.

Chapter 5: Senotherapeutics targeting the p38MAPK/NF-κB pathway protect endothelial cells from oxidative stress-mediated premature senescence

1. Introduction

The accumulation of senescent cells in vivo constitutes one of the main causes of organismal ageing and age-related diseases (ARDs). The widespread distribution of senescent cells in a variety of organs and lesions, such as atherosclerotic plaques, in aged versus young and healthy subjects provides evidence for this notion and pinpoints senescent cells as a potential therapeutic target (Baker et al., 2011). Senescent cells lose their ability to self-renew and fail to conduct their normal function (Lewis-McDougall et al., 2019). In addition, they secrete a cocktail of cytokines, chemokines and growth factors, known as senescence-associated secretory phenotypes (SASP), which spread senescence to the neighbouring cells and promote ageing and ARD development (Salminen et al., 2012). Hence, the early detection and elimination of senescent cells and SASP may be of huge therapeutic importance in the delay or prevention of ARDs (Birch and Gil, 2020).

The blood-brain barrier (BBB) is a highly specific semi-permeable barrier between the blood and the brain parenchyma. It regulates the selective passage of solutes, nutrients, and chemicals from the blood to the brain and vice versa. Brain microvascular endothelial cells (BMECs) make up the most important cellular constituents of the BBB. They cover the entire inner surface of cerebral microvasculature and tightly control the paracellular flux through tight junctions (Pong et al., 2020).

Although chronological ageing adversely affects the physiological characteristics of the cerebral endothelium and the BBB, these structural and functional changes are augmented in a variety of neurodegenerative conditions, notably vascular dementia, Alzheimer's disease, and stroke (Rajeev et al., 2022, Bomboi et al., 2010, Numazaki

et al., 2023). Here, the increased availability of senescent BMECs in the microvasculature may promote a pro-inflammatory microenvironment and play a key role in age-related BBB dysfunction (Knopp et al., 2023). A diverse range of stimuli, such as replication stress, mitochondrial dysfunction, genotoxic stress, oxidative stress, and oncogenic activation, can trigger cellular senescence through the activation of one of the two mechanisms, namely telomere shortening due to replicative exhaustion and DNA damage followed by the DNA damage response (DDR) due exposure to different acute sub-lethal stresses, notably oxidative stress (Blagosklonny, 2023, Coleman et al., 2010). Oxidative stress stems from an imbalance between the synthesis and metabolism of reactive oxygen species (ROS). ROSs accelerate telomere shortening, oxidase DNA bases, break down DNA strands, and as a result, potentiate DDR activity (Barnes et al., 2019). These, in turn, activate various mechanisms that lead to cellular senescence via the activation of cell-cycle checkpoints and mitochondrial dysfunction, which then produce more ROSs and help maintain this vicious circle in an active state (Guachalla and Rudolph, 2010).

The current evidence indicates that p38MAPK/NF-κB is a key signalling pathway in the context of senescence. As illustrated in (figure 25), p38MAPK/NF-κB is activated in response to enhanced oxidative stress, DDR, and pro-senescence stimuli (Wood et al., 2009, Yasuda et al., 2022). Once activated, p38MAPK elevated the release of several SASP components, e.g., IL-6, IL-8 and IL-1β, and, as a consequence, accelerates cellular senescence (Alimbetov et al., 2016). Similarly, the transcription factor NF- κB regulates the expression of genes that mediate the release of several pro-inflammatory factors, such as IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (GM-CSF) (Salminen et al., 2012). Given the prominent role of the p38MAPK/NF-κB pathway in a variety of major cellular events including

proliferation, senescence, and apoptosis (Coulthard et al., 2009), the present study aimed to investigate the potential protective effect of targeting this particular pathway in oxidative stress-induced senescence and ensure BBB function.

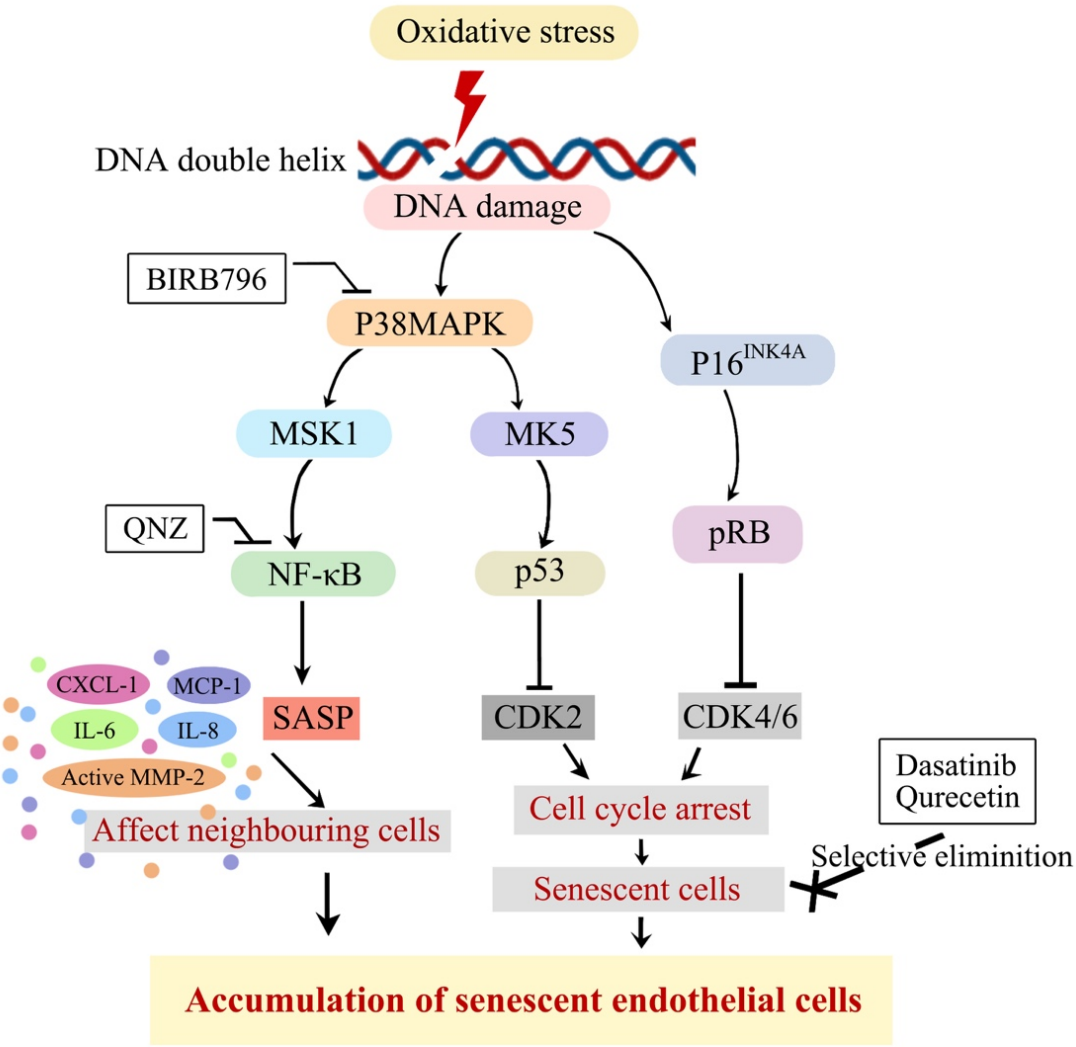


Figure 25. A schematic representation of mechanisms involved in oxidative stress-mediated induction of cellular senescence.

Excessive bioavailability of oxidative stress promotes DNA damage and activates p38MAPK/p53 and p16/RB pathways which, in turn, inhibit the CDK2 and CDK4/6 enzymatic activities and induce cell-cycle arrest. Activation of the p38MAPK/NF-κB pathway also promotes senescence through the release of SASP. Therapeutic strategies targeting the p38MAPK/NF-κB pathway or senescent cells themselves effectively attenuate the accumulation of senescent cells. Figure adopted from (Ya and Bayraktutan, 2024).

2. Aims

2.1. To explore how oxidative stress modulates BMEC senescence and BBB integrity

2.2. To determine the role of MMP activation, junction protein expression and localisation, and secretory cytokines in oxidative stress-induced endothelial dysfunction and BBB disruption

2.3. To determine whether the selective clearance of senescent endothelial cells can protect the BBB from oxidative stress-induced dysfunction

2.4. To specifically evaluate the involvement of the p38MAPK/NF-κB signalling pathway in oxidative stress-induced BBB disruption

3. Methods

The methods and materials included in this chapter were described in detail in Chapter 2 (General Methods), including the setup of the premature senescence model, in vitro model of BBB establishment and assessment, SA- β-gal staining, tube formation, WST-1 proliferation assay, western blotting, immunocytochemistry, qPCR-absolute telomere length measurement, gelatin zymography and proteome profiler.

4. Results

4.1. H_2O_2-induced morphological changes and nuclear damage in senescent cells

Exposure to H_2O_2 led to enlarged cell morphology and increases in SA-β-gal activity and γH2AX staining, in HBMECs 12 days after the cessation of exposure. Treatments with an inhibitor of p38MAPK (BIRB796) or NF-κB (QNZ) or the combination of

dasatinib and quercetin (D+Q) attenuated the impact of H_2O_2 on all these elements (Figure 26).

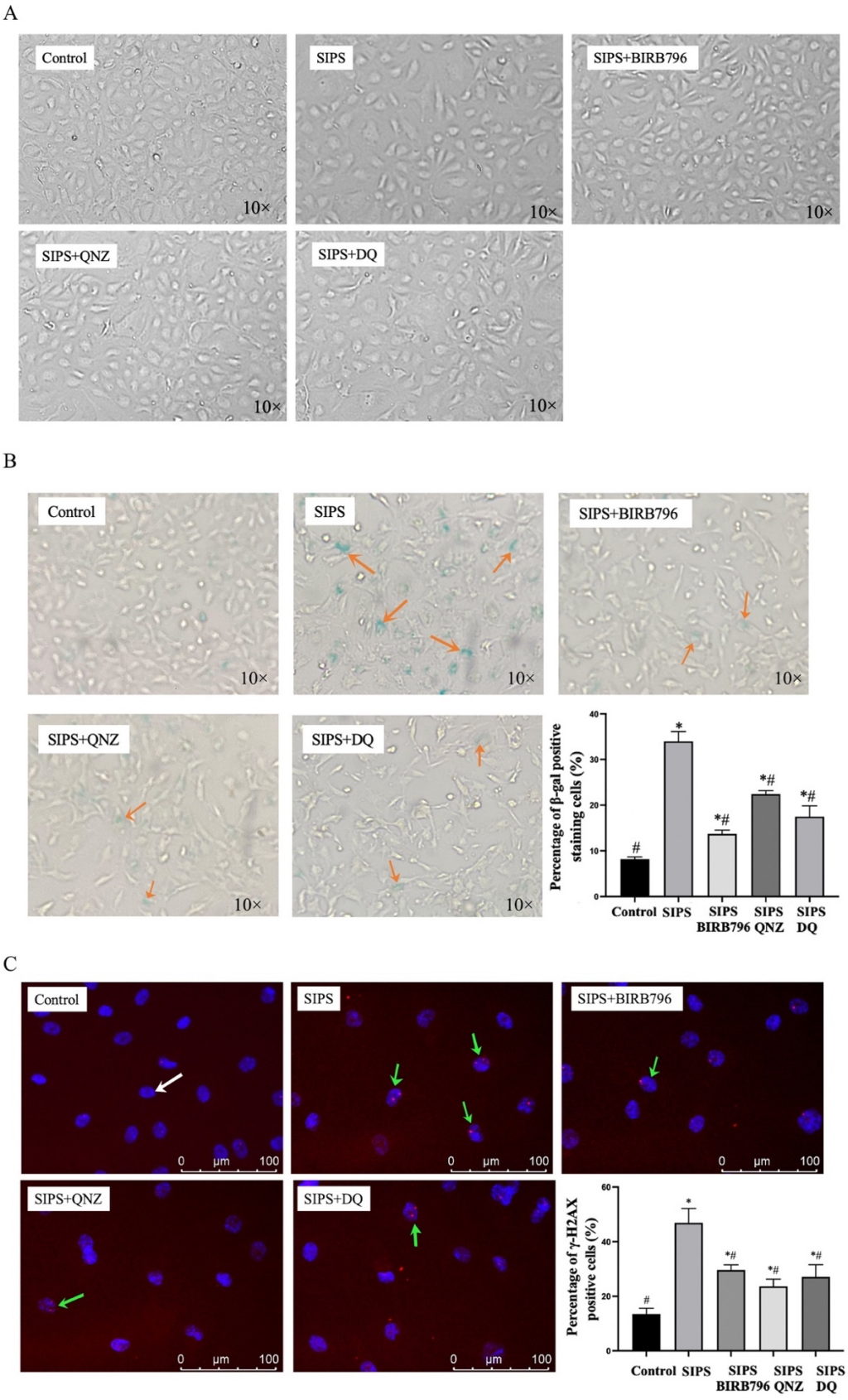


Figure 26. Effect of H_2O_2 treatment on BMECs morphology, SA-β-gal activity and γH2AX foci formation.

Exposure to H_2O_2 led to enlarged cellular morphology (A) and elevated the number of cells stained positive for SA-β-gal (B, orange arrow) and γH2AX (C, green arrow), where normal nuclear stained with DAPI is illustrated by the white arrow. Data are presented as mean ± SEM from three independent experiments. *$p < 0.05$ compared to the control group. #$p < 0.05$ compared to the SIPS group.

4.2. H_2O_2 evoked an increase in p16 expression

Exposure of HBMECs to H_2O_2 evoked significant increases in the expression of cyclin-dependent kinase inhibitor p16, which was selectively suppressed by treatments with an inhibitor of NF-κB (QNZ) and D+Q but not treatment with a p38MAPK inhibitor, BIRB-796 (Figure 27A). However, no significant difference was observed in telomere length between the control cells and those subjected to H_2O_2 in the absence or presence of BIRB-796, QNZ, or D+Q, implying that oxidative stress-mediated SIPS may be developed by DDR through a telomere-independent mechanism (Figure 27B).

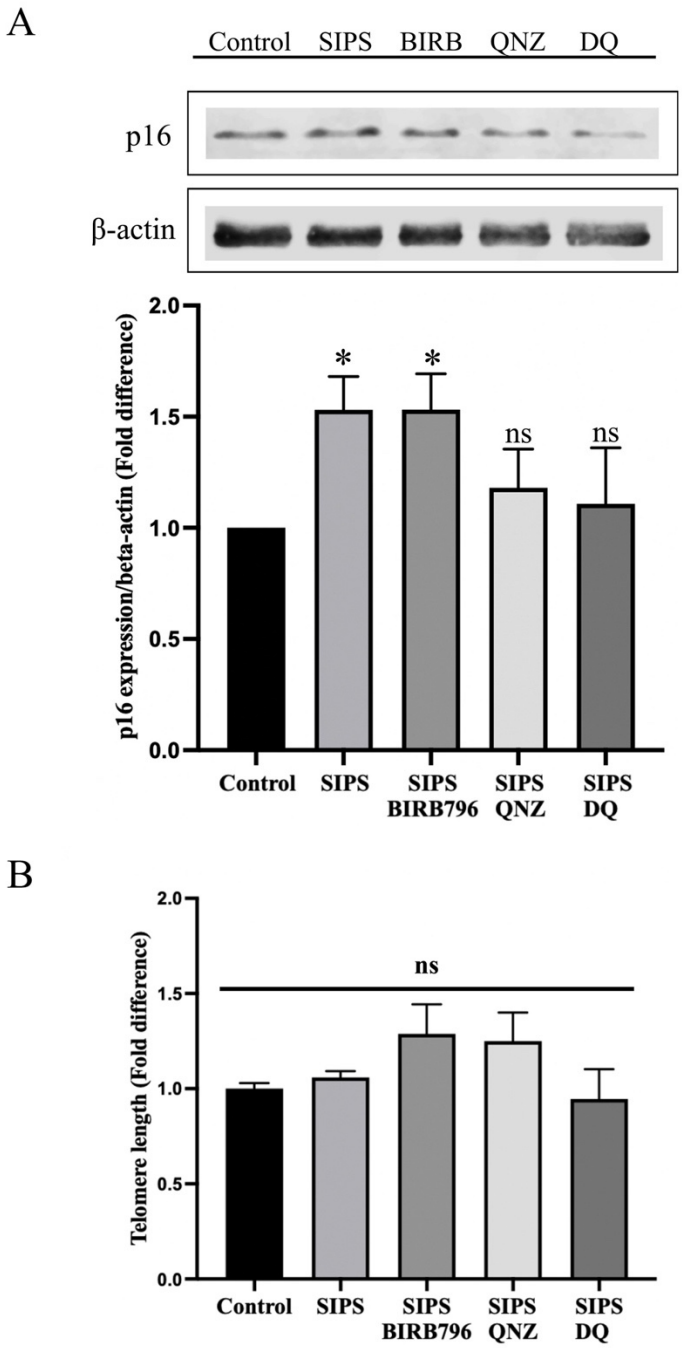


Figure 27. Effect of H_2O_2 exposure on p16 expression and telomere length.

A significant increase in the expression of cyclin-dependent kinase inhibitor p16 was detected by Western analyses in prematurely senescent cells that were selectively suppressed by treatments with an inhibitor of NF-κB (QNZ) and D+Q (A). No significant difference was observed in telomere length in any of the experimental groups in the absence or presence of inhibitors (B). Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to the control group, ns= not significant compared to the control and/or SIPS group.

4.3. Impact of H_2O_2 on p38MAPK and NF-κB phosphorylation

To determine whether exposure to oxidative stress may phosphorylate p38MAPK and/or NF-κB in HBMECs in a time-dependent manner, the cells were treated with 400 μM of H_2O_2 for up to 24 hours. These led to significant increases in p38MAPK and NF-κB phosphorylation within 5 minutes and 30 minutes, respectively (Figure 28A).

While BIRB796 supressed the activation of both p38MAPK and NF-κB in cells subjected to H_2O_2 for 30 minutes, treatment with QNZ only supressed the activation of NF-κB without affecting that of p38MAPK, indicating that NF-κB acts as a downstream effector to p38MAPK (Figure 28B).

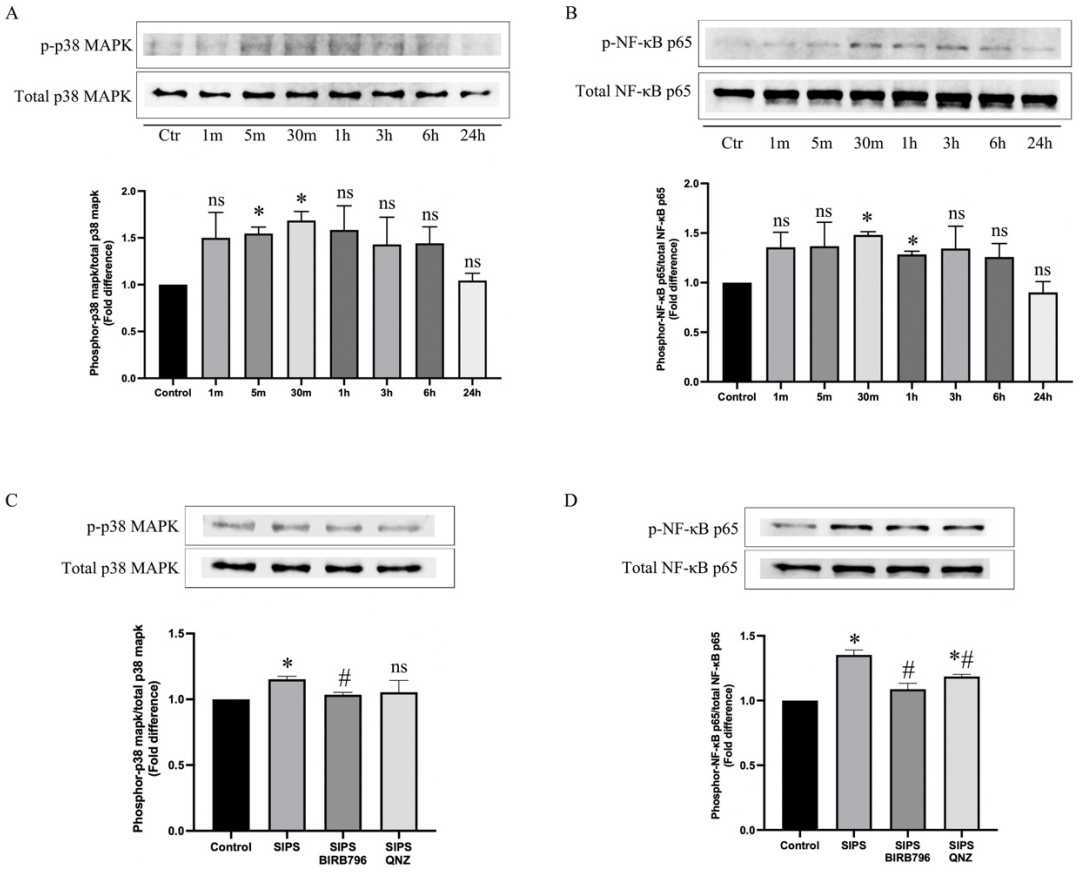


Figure 28. Time-dependent activation of p38MAPK/NF-κB pathway by H_2O_2.

Representative images showing the impact of H_2O_2 on p38MAPK and NF-κB phosphorylation. H_2O_2 evoked p38MAPK (A) and NF-κB (B) phosphorylation in a time-dependent manner. The increases observed in both p38MAPK and NF-κB activity within 30 minutes of exposure to H_2O_2 were significantly suppressed by BIRB796, a p38MAPK inhibitor where the impact of QNZ was specific to NF-κB activity (C, D). Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to the control group, ns= not significant compared to the control and/or SIPS group.

4.4. Stress-induced premature senescent HBMECs lead to BBB disruption

Similar to replicative senescent ECs (Ya et al., 2023), prematurely senesced HBMECs also failed to form fully functional barriers as ascertained by decreases in TEER and increases in the paracellular permeability of NaF, a low molecular weight paracellular flux marker. The selective elimination of senescent ECs by D+Q and inhibition of p38MAPK and NF-κB significantly attenuated the barrier-disruptive effect of SIPS (Figure 29).

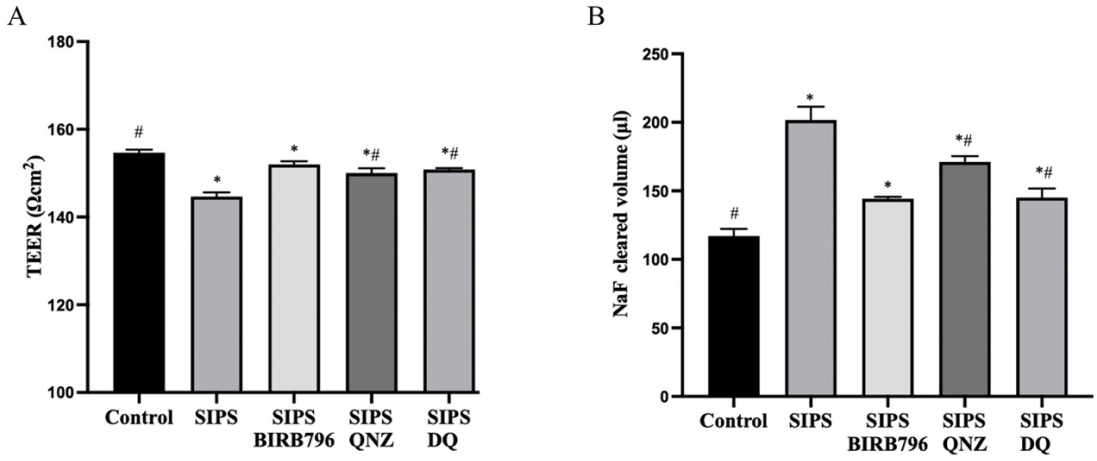


Figure 29. HBMECs senescence adversely affect the characteristics of the blood-brain barrier.

An in vitro model of human BBB established with human astrocytes, pericytes and SIPS HBMECs led to significantly decreased transendothelial electrical resistance (TEER) (A) and increased paracellular flux (B) of a low molecular weight permeability marker, sodium fluorescein (NaF). Treatments with BIRB796 (p38MAPK inhibitor), QNZ (an NF-κB inhibitor), and a combination of dasatinib and quercetin (DQ) markedly reduced the impact of

SIPS on both parameters. Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to control group. #p< 0.05 compared to the SIPS group.

4.5. Premature senescent HBMECs lack angiogenic capacity

A tubulogenic assay measuring the ability of endothelial cells to form tubules on Matrigel showed that SIPS adversely affected the capacity of HBMECs to form capillary-like structures. Although the inhibition of both p38MAPK and NF-κB, as well as the elimination of senescent cells by D+Q, negated the SIPS-mediated decreases in the number and length of the tubules observed, the magnitude of improvements was much better with BIRB796, a p38MAPK inhibitor (Figure 30).

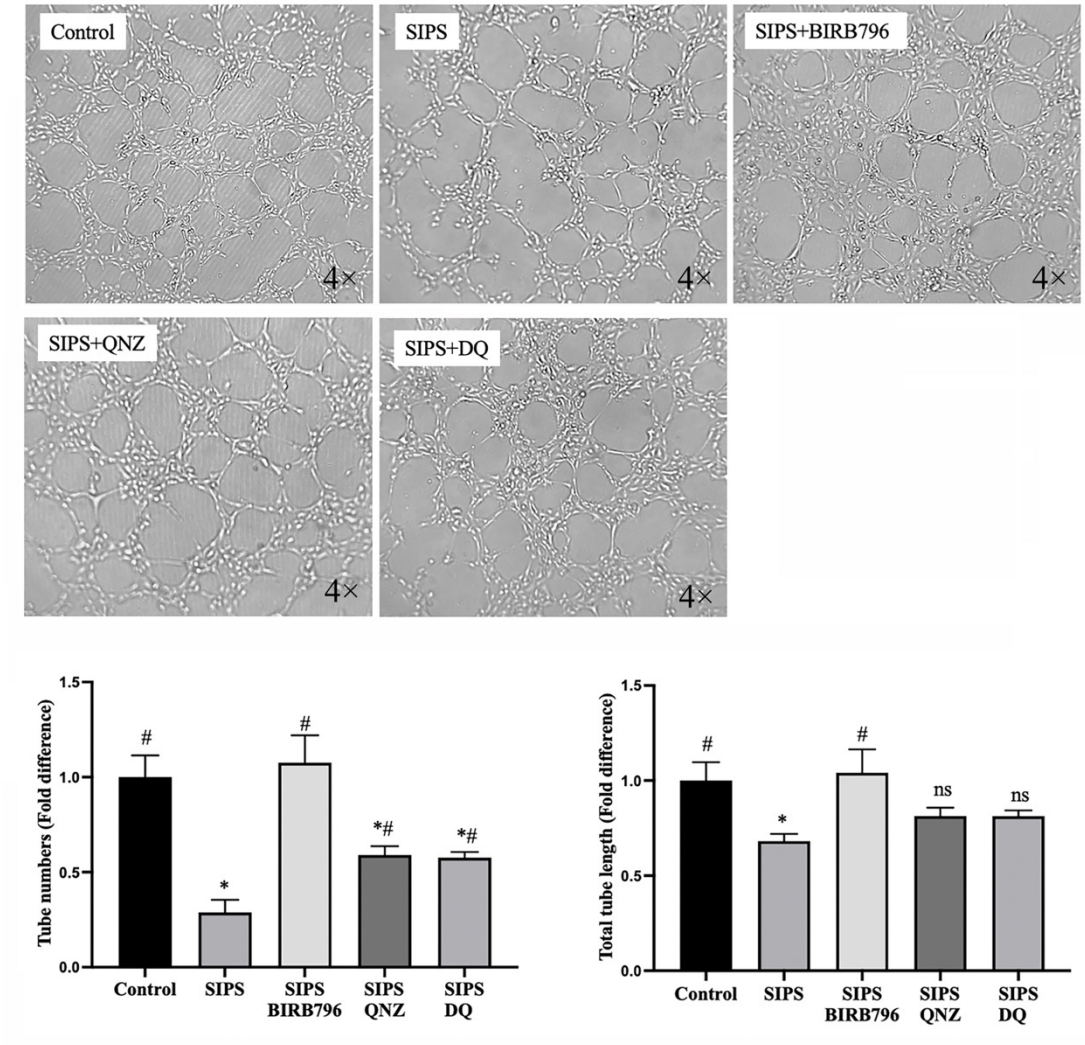


Figure 30. Senescence diminishes the angiogenic capacity of HBMECs.
Cells subjected to H_2O_2 formed significantly fewer and shorter tubules on Matrigel compared to those cultured under normal conditions. Treatment with BIRB796 (p38MAPK inhibitor) completely restored the angiogenic capacity, while treatments with QNZ (NF-κB inhibitor), and a cocktail of dasatinib and quercetin (DQ) were not as effective despite attenuating the impact of H_2O_2. Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to control group. #p< 0.05 compared to the SIPS group, ns = not significant compared to the control and/or SIPS group.

4.6. SIPS distinctly affects tight junction protein expression and localisation and activates matrix metalloproteinase-2

Investigation of the subcellular mechanisms involved in SIPS-induced BBB disruption showed that, while SIPS did not alter ZO-1 protein expression, it elevated those of occludin and claudin-5. Interestingly, albeit insignificant compared to the SIPS group, co-treatments with BIRB796, and QNZ caused further increases in HBMEC claudin-5 levels. In SIPS HBMECs, ZO-1 showed disruptive plasma-membrane staining and appeared to localise to the cytoplasm and perinuclear area. Treatments with BIRB796, QNZ and D+Q restored plasma-membrane localisation of ZO-1 in HBMECs. In SIPS HBMECs, occludin appeared in the cytosol in a somewhat tubular fashion, which was not greatly affected by any of the treatment regimens. In contrast, claudin-5 congregated largely in the perinuclear region of cells exposed to SIPS in the absence or presence of the abovementioned senotherapeutics (Figure 31A-B).

To investigate whether the breakdown of the extracellular matrix may contribute to SIPS-induced BBB disruption, the levels of pro- and active MMPs were examined in the control versus the SIPS HBMECs by gelatin zymography. In this study, only pro-MMP-2 and active MMP-2 could be detected. While, pro-MMP-2 levels remained the same in all the experimental groups, SIPS significantly raised the level of active MMP-

2. Treatments with p38MAPK/NF-κB pathway inhibitors and D+Q markedly reduced these increases without normalising the levels of pro-MMP-2 (Figure 31C).

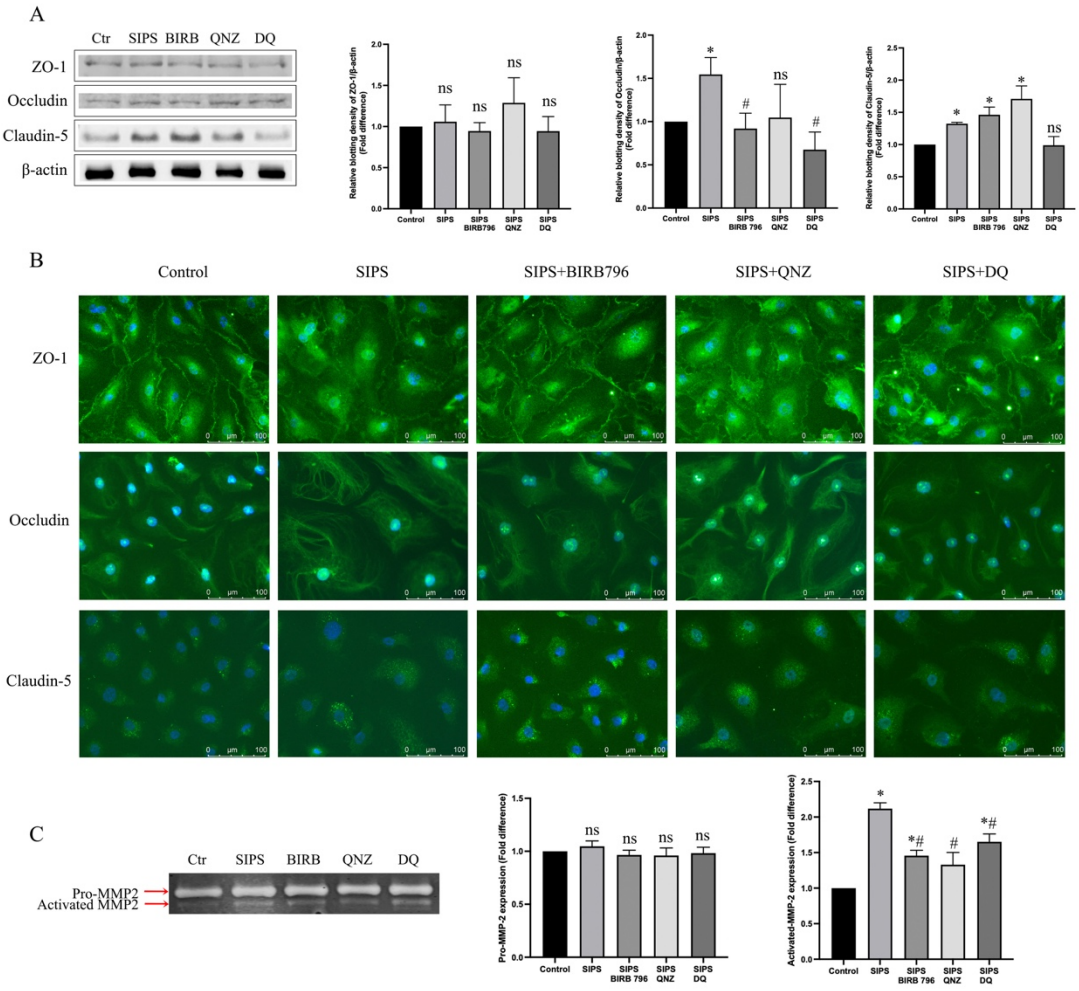


Figure 31. Stress-induced premature senescence affected the subcellular distribution of tight junction proteins.

Compared to controls, H_2O_2 increased the total expression of occludin and claudin-5 without affecting that of ZO-1 (A). Treatment with H_2O_2 perturbed plasma-membrane localisation of ZO-1. Co-treatment of cells with H_2O_2 and an inhibitor for p38MAPK (BIRB796), NF-κB (QNZ), or a combination of dasatinib and quercetin (DQ) restored the plasma-membrane expression of ZO-1 (B). While levels of pro-MMP-2 remained unaffected in all experimental groups, a significantly increased level of activated MMP-2 was observed in the SIPS group. Treatments with BIRB796, QNZ and DQ suppressed the oxidative stress-evoked MMP-2 activation (C). Data are presented as mean ± SEM from three independent experiments. *p< 0.05 compared to control group. #p< 0.05 compared to the SIPS group, ns = not significant compared to the control and/or SIPS group.

4.7. Oxidative stress promotes the expression of senescence-associated secretory phenotype

HBMECs exposed to H_2O_2 acquired SASP, as evidenced by increases in the expression of pro-inflammatory elements, monocyte chemoattractant protein-1 (MCP-1), CXC motif chemokine ligand 1 (CXCL-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), IL-8 and macrophage migration inhibitory factor (MIF), and a decrease in plasminogen activator inhibitor-1 (PAI-1) level compared to control cells (Figure 32).

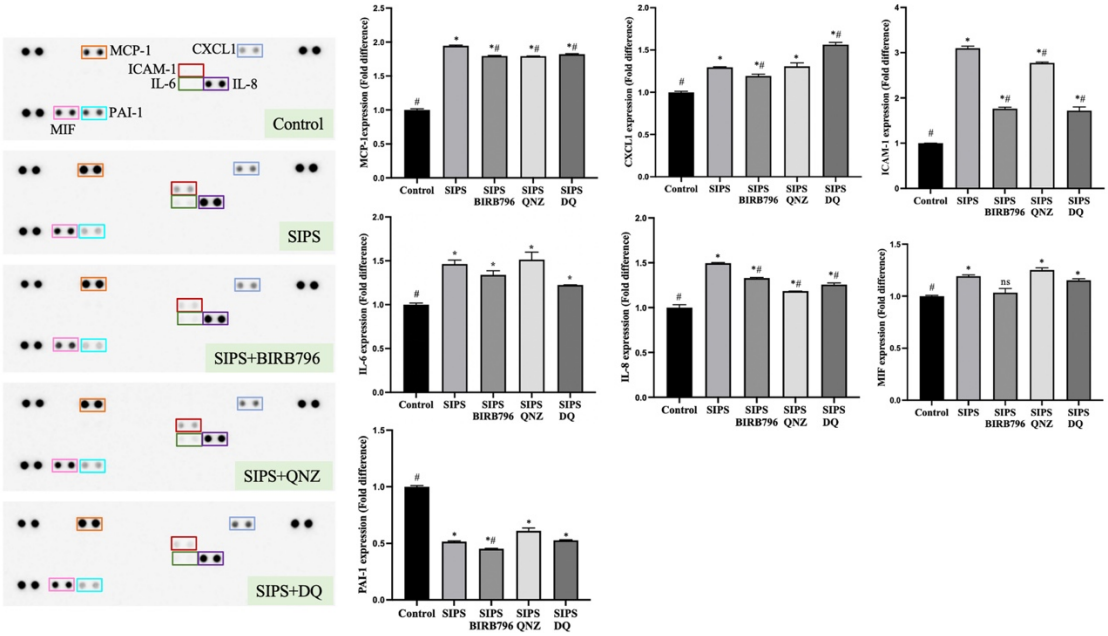


Figure 32. Senescence-induced adoption of pro-inflammatory phenotypes in human brain microvascular endothelial cells.

Cells exposed to H_2O_2 secreted significantly higher levels of MCP-1, CXCL-1, ICAM-1, IL-6, IL-8 and MIF and lower levels of PAI-1. Inhibition of p38MAPK (by BIRB796) and NF-κB (QNZ) and treatment with a cocktail of dasatinib and quercetin (DQ) partially suppressed the overexpression of the MCP-1, ICAM-1 and IL-8. Treatment with BIRB796 also suppressed the expression of CXCL-1. The expression of cytokine was quantified using ImageJ based on the densitometric analysis of the blot duplicates. Data are presented as mean

± SEM (n=2). *p< 0.05 compared to control group. #p< 0.05 compared to the SIPS group, ns = not significant compared to the control and/or SIPS group.

The selective clearance of senescent cells by D+Q and the inhibition of p38MAPK and NF-κB diminished SIPS-promoted secretion of MCP-1, ICAM-1, and IL-8. The impact of SIPS on CXCL-1 expression was specifically suppressed by BIRB796. Treatment with D+Q caused further elevations in CXCL1 levels (Figure 32). None of the therapeutic options had markedly influenced the effect of SIPS on IL-6 or MIF expressions. In contrast, BIRB796 caused a further decrease in PAI-1 levels compared to control cells and those subjected to H_2O_2 alone.

5. Discussion

Consistent with the findings in previous chapters, oxidative stress-induced premature senescent BMECs displayed higher β-galactosidase activity, γH2AX staining, p16 expression, impaired tubulogenic and BBB forming capacity. Furthermore, premature senescence disrupted plasma membrane localisation of the tight junction protein ZO-1, elevated MMP2 activity and pro-inflammatory cytokine release. Inhibition of p38MAPK by BIRB796 and NF-κB by QNZ and the elimination of senescent cells by a combination of dasatinib and quercetin attenuated the effects of H_2O_2 on senescent markers, suppressed release of the pro-inflammatory cytokines, restored tight junctional unity and improved BBB function.

The accumulation of senescent cells in vivo may contribute to the pathogenesis of various ARDs. Specifically, accumulation of BMECs in cerebral vasculature is likely to evoke structural and functional damage to BBB and may contribute to the pathogenesis of several neurovascular conditions associated with the ageing process, including stroke (Sweeney et al., 2018, Pan and Nicolazzo, 2018). BBB breakdown,

evidenced by increased permeability to radiocontrast agents, is also a common occurrence during the process of physiological ageing (Montagne et al., 2015). BMECs play a key role in the establishment and maintenance of BBB in that they not only restrict paracellular flux through the formation of tight junction complexes that seal the gaps between adjacent cells but also limit transcellular transport. Moreover, BMECs help adjust the cerebral flow, control the transmigration of immunocytes in the central nervous system, and secrete a wide range of active compounds, e.g., nitric oxide, endothelin-1, cytokines, chemokines, and enzymes that regulate immune responses, vascular tone, coagulation, angiogenesis, and neurogenesis (Andjelkovic et al., 2023, Wu et al., 2017).

To corroborate whether the presence of senescent HBMECs prompts significant impairment in BBB integrity and function, a triple culture model of human BBB composed of astrocytes, pericytes, and young or senescent HBMECs was employed in this study. To mimic oxidative stress-induced senescence, cells were subjected to H_2O_2 (400 μM) for 48 h, and those cells displaying enlarged morphology and nuclear damage (ascertained by γH2AX staining), as well as higher SA-β-gal activity, were considered as senescent. Since the prevalence of cells possessing these characteristics was considerably higher in cells kept in normal culture conditions for 12 days after exposure to H_2O_2, these particular cells were used to set up the BBB, which revealed marked decreases in TEER values and increases in the paracellular flux compared to the BBB model set up using young HBMECs. These indicated that, through their physical presence and/or the compounds they release, senescent ECs evoke significant impairments in both the integrity and function of the BBB, which in turn propose these cells as potential therapeutic targets to maintain neurovascular homeostasis.

Previous studies focusing on the correlation between EC senescence and BBB characteristics attributed barrier hyperpermeability and dysfunction in both human umbilical vein EC (HUVEC) monolayers and a multicellular model of human BBB, containing replicative senescent BMECs, to changes in the distribution and availability of the tight junction proteins, including ZO-1, occludin and claudin-5 (Krouwer et al., 2012, Ya et al., 2023). The co-culture of senescent HUVECs with non-senescent cells led to dramatic decreases in occludin and claudin-5 expression between senescent cells and along the entire periphery of non-senescent cells lining senescent HUVECs (Ya et al., 2023, Abdullah et al., 2015). Similarly, the co-culture of young HBMECs with replicative senescent outgrowth ECs, a functional subtype of endothelial progenitor cells capable of differentiating into mature endothelial cells, substantially diminished ZO-1 expression between adjacent cells and suppressed the tubulogenic capacity (Abdulkadir et al., 2020, Reskiawan et al., 2022). In our study, disrupted plasma-membrane staining and increased cytoplasm staining of ZO-1 was observed in prematurely senescent HBMECs, and treatments with BIRB796, QNZ, and D+Q prevented the re-distribution of ZO-1. The level of expression and subcellular localisation of occludin and claudin-5 did not differ in cells treated with H_2O_2 in the absence or presence of the abovementioned inhibitors. Despite the observation of an initial increase of 1.13% cytotoxicity with 400 μM H_2O_2 in HBMECs (Figure 7), cellular viability in the long run appeared to be unaffected. Indeed, 12 days after exposure to H_2O_2, the cellular viability rates, assessed by a trypan blue exclusion test, remained similar between the H_2O_2 and control groups, (96.19% vs 95.52%, p=0.5675). This implies that the differences observed in BBB integrity and function after exposure to H_2O_2 do not stem from cytotoxicity and ensuing reductions in cell numbers.

In addition to differences in tight junctional complex formation, the current study shows that an increase in MMP-2 activity may also be instrumental in senescence-mediated BBB breakdown. MMPs are a group of endopeptidases that are expressed as inactive zymogens and are subsequently activated by other extracellular proteases, hypoxia, or oxidative stress. Once activated, MMP-2 plays a key role in the regulation of blood vessel formation and remodelling, as well as tissue repair and regeneration (Rempe et al., 2016). However, MMP-2 can also compromise vascular integrity and trigger BBB leakage. We observed that senescent endothelial cells released higher levels of activated MMP-2 into the culture medium, which were neutralised by BIRB796, QNZ and D+Q treatments. It is possible that the acquisition of SASP and the accompanying elevated release of a series of inflammatory mediators by senescent HBMECs, notably MCP-1, CXCL1, ICAM-1, IL-6, IL-8, and MIF, may also accentuate the barrier-disruptive effects of MMP-2 through the involvement of different mechanisms (Abdullah et al., 2015, Rakkar et al., 2014). The overproduction of MCP-1, CXCL1, ICAM-1, IL-6, and IL-8 in senescent endothelial cells is also documented in previous studies, which suggests persistent DNA damage as the causal effect in the formation of SASP (Hampel et al., 2006, Giuliani et al., 2023, Korybalska et al., 2013). Once established, SASP reinforces the senescent state, spreads it to the neighbouring cells and participates in chronic inflammation, tissue remodelling, and immune modulation (Malaquin et al., 2019). The observation of a higher level of γH2AX positive staining in HBMECs cultures for 12 days after exposure to H_2O_2 compared to the control cells, confirmed the link between persistent DNA damage and the acquisition of SASP.

Migration, growth, and differentiation of ECs are required for the formation of new blood vessels, a.k.a. angiogenesis, which is a crucial element in neurovascular

hemodynamic recovery after ischaemic brain injury. To explore whether the angiogenic capacity of HBMECs declines with senescence, a tube-formation assay that measures the ability of ECs, seeded on Matrigel at subconfluent densities to form capillary-like structures, was conducted. Decreases in the total number and total length of the tubules observed in the senescent HBMECs group prove that SIPS adversely affects angiogenesis-related factors. These include ECs' adhesive, proliferative, migratory, and angiogenic cytokine and proteolytic enzyme secretory capacity, which are required for overall tubulogenic activity (Vailhe et al., 2001).

Recent evidence suggests that environmental stresses, such as ROSs, can induce premature cellular senescence without critical telomere shortening (Debacq-Chainiaux et al., 2010). The present study confirms this finding and reports an increased expression of p16, a cyclin-dependent kinase inhibitor, in HBMECs exposed to H_2O_2. Through its action on CDK4/6 kinase, p16 prevents the phosphorylation of retinoblastoma (Rb) family proteins and suppresses the release of E2F, a translation factor known to be crucial for cell-cycle progression. These, in turn, trigger G1 cell-cycle arrest and consequently promote senescence (Frippiat et al., 2003). This study also reports that oxidative stress induces cell-cycle arrest and the transcription of senescence-associated genes through the activation of the p38MAPK/NF-κB signalling pathway components in a time-dependent fashion, implying both p38MAPK/NF-κB as potential targets to delay or attenuate SIPS. Given that senescence cannot be reversed by any of the current clinical interventions, this may be of significant therapeutic value. In the present study, the attenuation of senescence-related changes in SA-β-gal activity and γH2AX staining and the partial restoration of tubulogenesis and BBB characteristics with BIRB796 emphasise the importance of the p38MAPK signalling pathway in the management of premature senescence

induced by environmental stresses. Suppression of p38MAPK may also prevent the spread of senescence to the neighbouring cells through a paracrine mechanism (da Silva et al., 2019). Hence, treatments with QNZ expectedly reduced the impact of SIPS on SA-β-gal activity, γH2AX staining, and partially improved tubulogenesis and BBB integrity and function. By removing the burden of senescent cells on their microenvironment and remote tissue, senolytics are expected to improve neurovascular function. Dasatinib is a small-molecule tyrosine kinase inhibitor that evokes apoptosis and abates the viability of senescent cells (Amor et al., 2020). Quercetin, on the other hand, is a flavoprotein with potent anti-oxidant and immune-protective effects (Farag et al., 2021). When used together, D+Q makes up a senolytics cocktail with great anti-ageing potential (Kirkland and Tchkonia, 2020). In support of the notion, treatment with D+Q diminished the SA-β-gal activity and γH2AX staining in prematurely senescent HBMECs and enhanced their tubulogenic capacity and ability to form a functional BBB. D and Q decrease the secretion of pro-inflammatory cytokines and major SASP factors in a more effective manner (Johmura et al., 2021). In accordance with these observations, treatment with D+Q markedly reduced the secretion of MCP-1, ICAM-1 and IL-8 in the current study. Albeit insignificant, a similar trend was also observed in the IL-6 levels. The inhibition of the p38MAPK/NF-κB pathway components displayed similar effects on these SASP elements, supporting the hypothesis that this pathway on its own may be sufficient for the development of SASP (Freund et al., 2011). However, greater decreases in ICAM-1 and IL-8 levels with BIRB796 and the inefficacy of QNZ in subsiding CXCL1 expression suggest that additional downstream effectors, other than NF-κB, may also be involved in the p38MAPK-mediated appearance of SASP. It is of note that the pro-inflammatory cytokines IL-6, IL-8, MCP-1, and CXCL1 stimulate the release of other

inflammatory factors, trigger the recruitment and activation of immune cells, and accelerate cellular senescence in the vasculature that collectively promotes atherosclerotic disease development and plaque vulnerability (Georgakis et al., 2021, Korbecki et al., 2022, Zhang and Dhalla, 2024). Interestingly, despite reducing SIPS-induced increases in p16 expression, neither QNZ nor D+Q brought its levels closer to that seen in control cells. BIRB796 had no effect on p16 expression. Taken together, these findings bestow a prominent role on SASP in oxidative stress-evoked BMEC premature senescence.

6. Conclusion

This study suggests that enhanced oxidative stress status during the ageing process may contribute to the premature senescence of endothelial cells and, consequently, compromise the integrity and function of BBB. The inhibition of p38MAPK/NF-κB pathway components and the selective elimination of senescent cells from the vasculature may help protect the BBB from environmental damage promoted by oxidative stress and the ensuing inflammatory responses.

Chapter 6: General Discussion

1. General discussion

BBB is a selective semipermeable barrier that regulates the passage of essential nutrients and metabolic waste, protects the brain from neurotoxins and pathogens, and maintains the homeostasis of the CNS (Knox et al., 2022, Segarra et al., 2021). It is well-documented that several neurological diseases are age-dependent, with BBB dysfunction serving as a key factor in the associated pathological processes (Viswanathan and Greenberg, 2011, Maier et al., 2014). For instance, early disruption of BBB in aged rats has been shown to contribute to larger infarct areas and worse functional outcomes after the middle cerebral artery occlusion (MCAO) surgery (DiNapoli et al., 2008). In addition, a correlation between the severity of BBB impairment and the progression of cognitive decline has also been observed in the elderly population (Bowman et al., 2018), which further emphasises the significance of BBB function in healthy ageing. In this context, investigations into the mechanisms underlying age-related alterations in BBB and identifying potential therapeutic targets are crucial for preventing and managing neurological disorders. Despite the increasing number of studies investigating the mechanisms of BBB dysfunction in age-related neurological diseases, specific strategies to prevent BBB ageing remain unclear.

Age-related BBB dysfunction may develop from several pathologies, including oxidative stress, chronic inflammation, cellular senescence, basement membrane degradation, and altered metabolism (Knox et al., 2022, Alkhalifa et al., 2023). The accumulation of senescent BMECs in the CNS is considered a fundamental alteration in age-related BBB dysfunction (Gulej et al., 2023). In this regard, the studies presented in this thesis were designed to explore the role of BMEC senescence in BBB function and identify potential therapeutic targets to protect BBB from age-related damage.

Telomere shortening derived from continuous cell division is widely acknowledged as a key trigger of cellular senescence and organismal ageing (Vaiserman and Krasnienkov, 2020). To mimic this physiological process, BMECs were passaged repetitively until no observation of cell proliferation in fresh complete media, and the cellular senescence was confirmed by a panel of interrelated markers, including 70% SA-β-gal positive staining, accumulation of DNA damage foci, enlarged morphology, decreased proliferation capacity, attritted telomere and elevated expression of the cyclin-dependent kinase inhibitor p16. BMECs exhibit several crucial functions in the maintenance of BBB integrity, including the formation of tight junction complexes, interaction with other cells, repair of injury, and regulation of the extracellular matrix (Abdullahi et al., 2018, Procter et al., 2021). The *in vitro* model of human BBB established with astrocytes, pericytes and senescent BMECs displayed a significant decrease in TEER and an increase in paracellular flux compared to the ones composed with young BMECs, supporting the notion that accumulation of senescent BMECs alone can cause severe BBB damage. The mechanistic analysis suggested that the reduced expression and altered distribution of ZO-1 and the activation of MMP-2 in senescent BMECs may underlie the age-related BBB dysfunction.

Another pivotal role of BMEC is the formation, renewal and repair of cerebral blood vessels under both physiological and pathological conditions. This function is highly dependent on the proliferation, migration and angiogenesis of cells (Zeng et al., 2021). Furthermore, balanced synthesis and release of angiogenic factors are vital for the angiogenesis and migration of endothelial cells (Ateeq et al., 2024). For example, growth factors such as VEGF, PLGF, FGF or EGF play essential roles in endothelial cell proliferation, differentiation and migration (Ferrara, 1999). Additionally, modulators of the extracellular matrix (ECM), including tissue inhibitor

metalloproteinase-1 (TIMP-1), MMPs or angiopoietins, participate in the remodelling of ECM, promoting vessel sprouting and interacting with other angiogenic factors (Quintero-Fabian et al., 2019). This thesis evaluated the angiogenic potential of BMECs in different experimental conditions, and senescent BMECs exhibited significantly declined tubulogenic capacity and slower wound healing rates compared to their young counterparts. What's more, dramatically declined production of crucial angiogenic proteins, notably angiogenin, angiopoietin-2, endoglin, endothelin-1, FGF, HB-EGF and PLGF, and significantly increased expression of anti-angiogenesis factor, thrombospondin-1 in the culture media of senescent BMECs compared to the young cells, may account for the compromised angiogenic capacity. The decreased proliferative ability, evidenced by lower WST-1 absorbance, may also contribute to the delayed wound repair in senescent BMECs. This thesis provided evidence for senescence-induced endothelial dysfunction, including impaired barrier formation, angiogenesis and wound healing, and investigated the potential mechanisms underlying these effects.

The free radical theory, a well-regarded theory of ageing, suggests that ageing and ARDs result from the accumulation of oxidative damage on cellular macromolecules and the deficiency in antioxidant capacity (Polidori and Mecocci, 2022, Harman, 1992). ROS, the by-products of oxidative phosphorylation during mitochondrial respiration, have detrimental and beneficial effects on various physiological processes (Gemma et al., 2007). Excessive ROS production results in DNA damage accumulation, DDR activation, and eventual cell cycle arrest. In addition to endogenous sources, exogenous factors, such as environmental pollutants, heavy metals or cigarette smoking, can also generate ROS through their degradation, metabolism, or inducing mitochondrial dysfunction (Pizzino et al., 2017). A previous

study from our group reported elevated levels of ROSs in senescent endothelial cells compared to young cells (Reskiawan et al., 2022). The excessive production of ROS in senescent cells, and the promoting effect of ROS on cellular senescence, established a positive feedback loop that accelerates BBB ageing. In ageing/senescence-related *in vitro* studies, H_2O_2 is a commonly applied inducer of cellular senescence because of its time and cost efficiency compared to the replicative senescence model (Wang et al., 2013). Many studies have reported phenotypical and proteomic differences between replicative senescence and SIPS (Dierick et al., 2002). In this context, this thesis performed a comparative analysis of these two models of cellular senescence to evaluate whether SIPS and replicative senescence exhibit identical phenotypes.

SIPS was induced by exposing young BMECs to 400μM H_2O_2 for 48 hours and under normal experimental conditions for a future 12 days. This revealed a set of similar changes to those induced by replicative senescence, including enlarged morphology, increased SA-β-gal activity, DNA damage accumulation, actin stress fibre formation and elevated expression of cyclin-dependent kinase inhibitor p16. However, in line with the findings of other studies, no significant telomere attrition was observed in SIPS cells, confirming a telomere-independent senescent pathway in BMECs subjected to oxidative stress (Sikora et al., 2011, Herbert et al., 2008). Interestingly, despite the existence of cellular senescence confirmed by a panel of markers, the expressions of tight junction proteins, ZO-1, occludin and claudin-5, remain unaffected by oxidative stress. Moreover, we observed that exposure to H_2O_2 induced senescence in approximately 30% of BMECs, deemed by both SA-β-gal and γH2AX staining. This rate was considerably lower than that observed in replicative senescence models, and further increases in the exposure time or H_2O_2 concentration did not elevate the senescent rate. However, all H_2O_2-exposed BMECs displayed enlarged

morphology and formation of stress fiber, regardless of the percentage of SA-β-gal or γH2AX positive staining. Given these different characteristics observed between replicative senescence and SIPS, the impact of SIPS on BBB function needs further investigation.

Oxidative stress has been shown to evoke an instant increase in endothelial monolayer permeability as an acute insult (Lee et al., 2006, Niwa et al., 2001), but the long-term effects of oxidative damage on barrier integrity remain unclear. We determined that oxidative stress can permanently alter endothelial cell function by promoting their senescence. In addition, BBB formed by oxidative stress-treated BMECs displayed a significantly decreased TEER and increased permeability to NaF, indicating that 30% of SIPS BMECs were sufficient to induce BB disruption. Furthermore, analysis of tight junction proteins, extracellular matrix and secretory phenotypes shows SIPS-evoked BBB disruption is associated with the disrupted membrane distribution of ZO-1, activation of MMP-2 and excessive production of pro-inflammatory cytokines.

Given the critical role of senescent BMECs in BBB dysfunction, therapeutic strategies targeting these cells have great potential for protecting the CNS from age-related neurological disorders. Traditional translational research, aimed at discovering novel therapeutics, sequentially assesses the mechanisms of the target disease through preclinical, experimental models, and at least three phases of clinical trials, which inevitably requires massive amounts of time, effort and investments (Wehling, 2008). Likewise, the mechanisms of cellular senescence have been investigated for several decades, while hundreds of senolytics and senomorphics continue to face challenges in the transition from preclinical research to clinical application (Chaib et al., 2022).

Under this circumstance, reverse translational study may be employed to guide the design of laboratory studies (Shakhnovich, 2018). Drug repurposing, a type of reverse translational study, has been proven to be a time- and cost-effective strategy to address clinical challenges (Pinzi et al., 2024).

Dasatinib, a tyrosine kinase inhibitor that was approved by the Food and Drug Administration (FDA) for the treatment of leukaemia in 2006 (Administration, 2006), was determined to be capable of selectively eliminating senescent cells in 2015, and has since gained increasing attention for its role as senolytics in pre-clinical studies and clinical trials (Zhu et al., 2015, Farr et al., 2024, Islam et al., 2023). Dasatinib exerts further beneficial effects when combined with quercetin, which has been extensively studied as a senolytics cocktail with significant anti-ageing potential (Nambiar et al., 2023).

Senescent cells induce secondary senescence and dysfunction of the neighbouring cells via direct intercellular communication and the spread of SAPS (Campisi, 2005), which explains the beneficial effects of selective elimination of senescent cells by senolytics (Kudlova et al., 2022). This thesis employed the cocktail of senolytics, dasatinib combined with quercetin (D+Q) in both replicative senescence and SIPS BMECs to assess whether targeting senescent cells can effectively protect BBB against age-related dysfunction. The attainment of significantly improved BBB integrity and function, accompanied by decreased expression of senescent markers, ameliorated angiogenic capacity and suppressed activation of MMP-2 in BMECs, highlights the therapeutic potential of D+Q. In addition, treatment with D+Q partially inhibits the oxidative stress-evoked expression of several pro-inflammatory cytokines

and SASP factors, which can be major promotors of BMEC dysfunction and BBB disruption (Abdullah et al., 2015, Yang et al., 2022).

The p38MAPK/NF-κB pathway is the key regulatory pathway for the initiation and modulation of SASP (Freund et al., 2011). P38MAPK has been indicated as a stress-response protein kinase that can be activated by various stress, including oxidative stress, inflammation or genetic stress (Canovas and Nebreda, 2021). NF-κB acts as one of the downstream effectors of p38MAPK to amplify SASP expression (Salminen et al., 2012). In addition, p38MAPK directly participates in DNA damage response and cellular senescence by phosphorylating cyclin-dependent kinase inhibitors such as p53 or p16 (He et al., 2020, Canovas and Nebreda, 2021). As regards the significance of DDR and SASP in cellular senescence and the pivotal role of the p38MAPK/NF-κB pathway in these mechanisms, this thesis explored the activity of this pathway during the senescence process and whether its inhibition could affect the senescence progression and function in BMECs. In replicative senescent BMECs, inhibition of p38MAPK significantly attenuated the repetitive division-induced accumulation of senescent markers compared to the untreated cells. P38MAPK inhibition also markedly ameliorated the proliferative and angiogenic capacity of BMECs and enhanced the integrity of BBB. However, NF-κB inhibitor (QNZ) notably decreased the total cell count of BMECs during treatment from passage 16 to passage 20, and significantly increased the percentage of SA-β-gal positive cells compared to the untreated cells.

In the final part of this thesis, we observed that the p38MAPK/NF-κB signalling pathway is activated by H_2O_2 after 5 minutes of exposure, suggesting the essential role of this pathway in the initiation of SIPS. Inhibition of both p38MAPK and NF-κB

effectively attenuated the oxidative stress-induced senescent features, including SA-β-gal positive staining, γH2AX foci and SASP expression, while restoring the tubulogenic capacity and BBB characteristics of BMECs compared to control cells. The protective effect of p38MAPK/NF-κB inhibition in BBB integrity may be attributed to the suppression of MMP-2 activation, restored membrane localisation of ZO-1 and the reduced expression of pro-inflammatory cytokines. In contrast to replicative senescence, the inhibition of p38MAPK suppressed the development of SIPS without decreasing p16 expression, suggesting that oxidative stress may upregulate p16 expression through a p38MAPK-independent pathway (Wang et al., 2018, Huang et al., 2022). Taken together, the findings of this thesis demonstrate that inhibition of p38MAPK is capable of delaying BMEC senescence and protecting BBB function against replicative and oxidative stress. However, NF-κB inhibition exhibited mixed effects, depending on the trigger of senescence.

In conclusion, this thesis has determined that the progression of senescence in BMECs can compromise the integrity and function of BBB via disruption of tight junction proteins, activation of MMP-2 and excessive production of SASP. During the ageing process, the consistent cell division from tissue renewal and the enhanced oxidative stress status both have the potential to accelerate cellular senescence in a similar manner. This thesis illustrates that the selective elimination of senescent cells and the inhibition of the p38MAPK signalling pathway are both capable of preventing BMEC senescence and restoring BBB function. Inhibition of the NF-κB pathway displayed controversial effects between replicative senescent and SIPS BMECs.

2. Limitations

This thesis has some limitations. Despite using a variety of antibodies of occludin and claudin-5, as well as coating the coverslips with Matrigel or fibronectin, we were unable to visualise these particular proteins on the BMEC plasma membrane. Although the staining patterns observed were in full agreement with the manufacturer's immunocytochemistry results for the respective proteins, the lack of plasma membrane staining was unexpected and deserves further attention in future studies.

While this study demonstrated that RS and SIPS BMEC exhibited similar phenotypic alterations and functional impairments, the direct correlation between cellular senescence and these changes remains unsubstantiated due to the intricate mechanisms involved in the experimental conditions, such as oxidative stress-induced damage and long-term culture effects from the non-physiological environments.

Inhibition of NF-κB induced cell death and increased the percentage of SA-β-gal positive cells in RS BMECs while preventing cellular senescence evoked by oxidative stress in SIPS experiments. However, due to the potential cytotoxic effect of the NF-κB inhibitor (QNZ) on passage 16 BMECs, the concentration of this agent was reduced from 1 μM to 100 nM in SIPS experiments. Treatment of the BMECs with 100 nM QNZ from passage 16 to passage 20 would have provided more definitive evidence of the adverse or protective effects of QNZ on replicative senescence.

The p53/p21 and p16/pRB pathways play essential roles in initiating and reinforcing cell cycle arrest during senescence (Kang et al., 2015). After several attempts using different antibodies and protocols, this study failed to detect p53 protein expression levels via Western blot under RS or SIPS conditions. Given the essential function of

this pathway in DNA damage response and cellular senescence, additional assessments of p53 mRNA expression via qPCR, RT-PCR, or the protein levels of the downstream effector p21 could have offered a more comprehensive understanding of the senescence process in BMEC.

This study explored how BMEC senescence and senotherapeutics influence the integrity and function of an *in vitro* model of the human BBB. Despite well-documented interactions amongst the different cellular layers, this model does not reflect the complexity of the *in vivo* barrier. Additionally, in our studies, only BMECs were subjected to RS or SIPS and treated with senotherapeutics. Although this experimental design helped investigate the specific correlations between senescent BMECs and age-related BBB dysfunction, it did not factor in the impact of senescence, oxidative stress, or senotherapeutics on the other cells in the BBB model, namely astrocytes and pericytes.

3. Future research

This thesis demonstrates that targeting senescent BMECs has significant therapeutic potential in age-related BBB damage. To further illustrate that the phenotypic alterations and BBB dysfunction are directly linked to cellular senescence, $p16^{INK4}$ transfected BMECs may be utilised to establish the BBB, and to perform the morphological and functional assay, as p16 can specifically induce cell cycle arrest without triggering other potential cellular damage, such as apoptosis (Li et al., 2005, Katayama et al., 2001).

This thesis provides evidence for the beneficial effects of the senolytics cocktail, D+Q, on an *in vitro* BBB model, as well as on the angiogenic and wound repair ability of BMEC against age-related challenges. Nevertheless, *in vivo* studies and subsequently,

clinical trials are required to validate the anti-ageing effects of D+Q in the cerebral vascular system. The BBB penetration and the senolytic role of these two agents in Alzheimer's disease have been indicated in a phase 1 feasibility trial (Gonzales et al., 2023), which supports the potential therapeutic value of D+Q in age-related neurological disorders. Age-related BBB dysfunction has been observed in the elderly population with cerebral vascular diseases such as stroke or vascular dementia (Andjelkovic et al., 2023, Montagne et al., 2015, Abdullahi et al., 2018). Exploration of whether D+Q treatment can prevent or ameliorate cognitive loss and cerebral artery stenosis in individuals with vascular risk factors is urgently needed. Alongside the safety and feasibility study, the optimal administration dose and frequency for individuals with different degrees of senescent burdens and health conditions must also be determined.

The crucial role of the p38MAPK signalling pathway in BMEC senescence and the consequent BBB dysfunction was further elaborated in this thesis. However, the p38MAPK signal needs to be specifically activated by genetic manipulation, such as transfection with MAPK14/p38α, MKK3/MKK6 plasmids (Paillas et al., 2012, Wang et al., 2016), or chemical activators such as anisomycin (Chen et al., 2022), and inhibited by siRNA or CRISPR interference to further illustrate these effects. Furthermore, the suppression of CXCL1 levels with the p38MAPK inhibitor and the inefficacy of the NF-κB inhibitor indicated additional downstream effectors other than NF-κB of p38MAPK-mediated SASP expression. Further experiments, such as phospho-proteomics profiling, could uncover more specific signalling networks involved in p38MAPK-mediated senescence in BMECs (Gerritsen and White, 2021). Furthermore, p38MAPK inhibition revealed superior effects in promoting angiogenesis in both RS and SIPS BMECs, compared to the untreated group as well

as other treatment groups. The angiogenesis profiler, which demonstrated the upregulated and downregulated angiogenic factors in the senescence process, should be run with p38MAPK inhibitor treatment to identify the factors affected that played a role in the restoration of the angiogenic capacity of BMECs.

To minimise impacts on healthy cells, senotherapeutic strategies must be optimized to selectively eliminate senescent cells. Several molecules and proteins have been identified as highly or specifically expressed on the surface of senescent cells. Therapeutic approaches, such as antibody-conjugated or ligand-coated nanoparticles, can be designed based on these markers to enhance the specificity of anti-ageing treatments and reduce potential adverse effects (Rossi and Abdelmohsen, 2021).

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Appendices

Appendix I

Equipment and chemicals

Materials	Company
Apparatus	
Amersham Hybond P 0.2 PVDF Membrane	GE Healthcare
Cell scrapers	Falcon
Centrifuge IEC central CL3	Thermo Scientific
Class II microbiological safety cabinet	Esco Lifesciences
Cryogenic vials	Starlab
Digital dry bath	LifeScience
Digital tube roller	Fisher Scientific
EVOMX meter	World Precision Instruments
Falcon tubes-50ml, 15ml	BD Biosciences
Flat-bottom microplates-96 well white	Nunc
FLUOstar omega plate reader	BMG Labtech
Glass coverslip	Cover glass
Glass slides	Thermo Scientific
Microcentrifuge Minispin	Eppendorf
Mr frosty freezing Container	Nalgene
NanoDrop 2000 UV	Thermo Scientific
Odyssey Fc Imaging System	LI-COR Biosciences
Orbital plate shacker	Stuart Scientific
Temperature control centrifuge 5417R	Eppendorf
Tissue culture CO2 incubator	Sanyo

Tissue culture flasks -T75, T25	Corning Life Sciences
Tissue culture plates -6, 12, 24, 48, and 96 well	Corning Life Sciences
Transwell 12-well 0.4μm pore polyester inserts	Corning Life Sciences
Water bath	Fisher Scientific
Tissue culture materials	
Astrocyte medium	ScienCell
Dimethyl sulfoxide	Sigma-Aldrich
Endothelial cell medium	ScienCell
Foetal bovine serum (FBS)	Sigma-Aldrich
Human brain astrocytes	Neuromics
Human brain microvascular endothelial cells	Neuromics
Human brain microvascular pericytes	Neuromics
Material	Company
Pericyte medium	ScienCell
Tripsin 10×	Sigma-Aldrich
Trypan blue	Invitrogen
Activators/senotherapeutics	
Hydrogen peroxide solution 30% (w/v)	Sigma-Aldrich
QNZ	Selleckchem
Dasatinib	Selleckchem
Doramapimod (BIRB796)	Selleckchem
Quercetin	Sigma-Aldrich
Chemicals	
16% Formaldehyde (w/v)	Thermo Scientific
Bovine serum albumin	Fisher Scientific

Broad range prestained protein markers	Proteintech
DAPI(4,6-diamidino-2-phenylindole)	Sigma-Aldrich
Ethanol	Fisher Scientific
Evan's blue	Sigma-Aldrich
Gelatin	Sigma-Aldrich
Glycine	Fisher bioreagent
Hydrochloric acid	Fisher Scientific
Methanol	Loba Chemie
Phosphate buffered saline tablet	Sigma-Aldrich
Protease inhibitor cocktail	Sigma-Aldrich
RIPA lysis buffer, 10×	Sigma-Aldrich
Sodium Chloride	Fisher Scientific
Sodium fluorescein	Sigma-Aldrich
Tris	Fisher Scientific
Triton X-100	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Antibodies	
Claudin-5	ThermoFisher
FITC-conjugated Mouse secondary antibody	Abcam
FITC-conjugated Rabbit secondary antibody	Abcam
gamma-H2AX (rabbit)	Abcam
Goat Anti-Rabbit IgG H&L (Texas Red)	Abcam
NF-B p65 (rabbit)	Cell Signaling technology
Occludin	ThermoFisher
p16	Abcam

p38MAPK (rabbit)	Cell Signaling technology
Phospho-Histone H2A.X (Ser139) (rabbit)	Cell Signaling technology
Phospho-NF-B p65 (mouse)	Cell Signaling technology
Phospho-p38MAPK (mouse)	Cell Signaling technology
Rhodamine phalloidin	Cell Signaling technology
Zonula occludens-1	ThermoFisher
β-actin (mouse)	Abcam
Assay kit	
Dneasy Blood&Tissue kit	Qiagen
Nucleon BACC1 Genomic DNA Extraction kit	GE Healthcare
Pierce BCA Protein Assay Kits	ThermoFisher
Proteome Profiler Human Cytokine Array Kit	Bio-techne
Proteome Profiler Human Angiogenesis Array Kit	Bio-techne
β-galactosidase staining kit	Abcam

Appendix II

Thermal cycling profile of the Telomere Length Quantification qPCR Assay

Step	Temperature	Time	Number of cycles
Initial denaturation	95 ℃	10 minutes	1
Denaturation	95℃	20 seconds	32
Annealing	52℃	20 seconds	
Extension	72℃	45 seconds	
Data acquisition	Plate reading		

Appendix III

Western blotting gel compositions (per gel)

	Stacking gel (ml)			Resolving gel (ml)
	8%	10%	15%	
Ultra-pure H2O	1.7	1.3	0.5	1.36
30% Acrylamide/Bis-acrylamide (29:1)	1.3	1.7	2.5	0.34
1 M Tris pH 8.8	1.9	1.9	1.9	-
1 M Tris pH 6.8	-	-	-	0.26
10% SDS	0.05	0.05	0.05	0.02
10% APS	0.05	0.05	0.05	0.02
TEMED	0.003	0.002	0.002	0.002

Appendix IV

Protein molecular weights and antibody concentrations.

Target protein	MW/kDa	Concentration (dilution ratio)
P16	16	1:2000
ZO-1	225	1:1000
Occludin	52	1:1000
Claudin-5	23	1:1000
β-actin	43	1:4000
P38MAPK	40	1:2000
Phospho-p38MAPK	43	1:1000
NF-κB	65	1:1000
Phspho-NF-κB	65	1:1000

Appendix V

Gelatin zymography SDS PAGE gel compositions (per gel).

	Stacking gel (ml)	Resolving gel (ml)
Ultra-pure H2O	1.3	1.36
30% Acrylamide/Bis-acrylamide (29:1)	1.7	0.34
1 M Tris pH 8.8	1.9	-
1 M Tris pH 6.8	-	0.26
5% (w/v) gelatin	0.1	-
10% SDS	0.05	0.02
10% APS	0.05	0.02
TEMED	0.002	0.002

Appendix VI

Details of statistical analysis

Figure	Panel	Statistical test
4	(A-E)	Unpaired *t*-test
6	-	Unpaired *t*-test
7	(C-D)	Unpaired *t*-test
9	-	One-way ANOVA followed by Tukey's post-hoc
10	-	Unpaired *t*-test
11	-	Unpaired *t*-test
13	C	Unpaired *t*-test
14	A-B	Unpaired *t*-test
15	-	Unpaired *t*-test
16	-	Unpaired *t*-test
17	-	Unpaired *t*-test
18	-	One-way ANOVA followed by Tukey's post-hoc
19	B-C	One-way ANOVA followed by Tukey's post-hoc
20	B	Unpaired *t*-test
21	-	Unpaired *t*-test
22	A-C	One-way ANOVA followed by Tukey's post-hoc
23	B	Unpaired *t*-test
24	-	Unpaired *t*-test
26	B-C	Unpaired *t*-test
27	A	Unpaired *t*-test
	B	One-way ANOVA followed by Tukey's post-hoc

28	A-D	Unpaired *t*-test
29	A-B	One-way ANOVA followed by Tukey's post-hoc
30	-	Unpaired *t*-test
31	A and C	Unpaired *t*-test
32	-	Unpaired *t*-test