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**ENDOTHELIN-1/ETB RECEPTOR SIGNALLING
DRIVES PRX1+ SKELETAL STEM CELL
SENESCENCE AND OSTEOPOROTIC BONE LOSS**

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The Hong Kong Polytechnic University

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Kong Polytechnic University and Southern
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Department of Biomedical Engineering

**Endothelin-1/ETB Receptor Signalling Drives Prx1+
Skeletal Stem Cell Senescence And Osteoporotic Bone
Loss**

ZOU Zhou

**A thesis submitted in partial fulfilment of the
requirements for the degree of Doctor of Philosophy**

August 2025

CERTIFICATE OF ORIGINALITY

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Abstract

Skeletal and vascular ageing are closely interconnected, with increasing evidence highlighting the role of vascular factors in regulating tissue-specific ageing. Endothelin-1 (ET-1), a potent vasoconstrictor, is essential for maintaining vascular tone and blood pressure. However, excessive ET-1 expression leads to endothelial dysfunction and cellular senescence, contributing to age-related diseases. Although the bone marrow is highly vascularised, the impact of elevated ET-1 secretion from the endothelial cells on the function of perivascular skeletal stem cells (SSCs) during skeletal ageing remains unclear.

Senescence of SSCs is an important but underexplored driver of osteoporosis. In this study, we identified ET-1/Endothelin B receptor (ET_BR) signalling as a key mediator of osteoporosis. In both aged and ovariectomized (OVX) mice, increased ET_BR expression was associated with bone loss and reactive oxygen species (ROS)-induced SSC senescence. Transgenic mice overexpressing ET-1 (TET-1) exhibited endothelial dysfunction and accelerated skeletal ageing, particularly in vascular-rich bone regions. Through RNA sequencing of SSCs, we found ET-1 induced senescence related to the PI3K/akt/mTOR signalling pathway. Notably, conditional knockout of ETBR in SSCs (*Prx1-Cre::ETBR^{fl/fl}*)

prevented ET-1-induced senescence, reduced ROS accumulation, and alleviated osteoporotic degeneration in OVX mice. Similarly, pharmacological inhibition of ET_BR with BQ788 similarly rejuvenated bone in both aged and OVX mice.

These findings reveal a direct ET-1/ET_BR-ROS axis that governs SSC senescence and skeletal ageing, positioning ET_BR inhibition as a promising therapeutic strategy. To translate these insights into clinical applications, we developed drug-loaded alginate-based hydrogels (Macitentan/Alg@Sr) for vascular and bone tissue regeneration. The hydrogels effectively reduced cellular senescence and inflammation. By elucidating the link between vascular dysfunction and skeletal ageing, this study advances our understanding of osteoporosis pathogenesis and identifies a precision target for combating age-related bone loss, with broad implications for the treatment of degenerative skeletal disorders.

Keywords: ET-1, ET_BR, SSC senescence, oxidative stress, osteoporosis, hydrogel

List of publications

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Chapter 1 Introduction

1.1 Background

The remarkable advancements in medical technology and public health over recent decades have significantly increased global life expectancy, with a growing proportion of the population now living well beyond their sixties^[1]. This demographic shift, while a testament to human progress, has concurrently amplified the global burden of age-related chronic diseases. Skeletal degeneration, particularly osteoporosis, represents a major challenge in this context, imposing substantial social and economic costs projected to rise steadily alongside population ageing.

Ageing is intrinsically linked to a progressive decline in physiological function across multiple organ systems. Alterations in body composition, including reductions in bone mineral density (BMD), cartilage thickness, and muscle mass (sarcopenia), alongside the accumulation of visceral fat, often commence subtly in early adulthood^[2]. This deterioration significantly impacts musculoskeletal health, culminating in a heightened risk of fragility fractures. Osteoporosis contributes to more than 1.5 million fractures annually in the United States alone^[3]. The prevalence in China underscores the scale of the

problem: national surveys reported a jump from 14.94% in 2008 to 27.96% in 2012 among the general population, with higher rates observed in rural versus urban areas and southern versus northern regions. Notably, the prevalence among individuals aged 50 or older more than doubled from 15.7% in 2006 to 34.65% by 2012^[4], highlighting ageing as a primary risk factor.

Ageing involves complex crosstalk between the vascular and skeletal systems, with vascular dysfunction emerging as a key driver of tissue-specific degeneration^[5]. Recent studies reveals that the pathological decline of distinct tissues during ageing is not isolated but involves intricate communication between organ systems. Crucially, vascular dysfunction is increasingly recognised as a key driver of tissue-specific degeneration. This is especially relevant to the skeleton, as compelling evidence points to a bidirectional relationship between vascular health and bone integrity. Shared underlying pathological mechanisms, including chronic inflammation, oxidative stress, and hormonal alterations, play a pivotal role in the development of both endothelial dysfunction (a defining characteristic of vascular ageing) and osteoporosis^[6; 7]. Recent breakthroughs utilising sophisticated 3D imaging techniques have further solidified this concept, directly visualising how vascular alterations exert spatiotemporal control over organ-specific tissue ageing^[8].

Within this framework of vascular-skeletal crosstalk, Endothelin-1 (ET-1) emerges as a molecule of significant interest. ET-1, a potent vasoconstrictor predominantly synthesised by endothelial cells, fulfils an essential physiological function in the regulation of vascular tone and blood pressure. However, its dysregulation – specifically, excessive and sustained ET-1 expression – represents a well-established and significant factor contributing to endothelial dysfunction and the development of vascular pathology. Elevated levels of ET-1 promote the development of inflammation, oxidative stress, and cellular senescence, mechanisms fundamentally implicated in the pathogenesis of numerous ageing-related cardiovascular, renal, and metabolic diseases^[9; 10].

The relevance of ET-1 to skeletal ageing stems directly from the highly vascularized nature of the bone marrow microenvironment. This perivascular niche harbours skeletal stem cells (SSCs), which play an essential role in both the ongoing maintenance of bone tissue and its regenerative capacity following injury or degeneration^[11]. While ET-1's detrimental vascular effects are well-characterised, a critical gap exists in understanding how specifically endothelial cell-derived ET-1 impacts the function of neighbouring perivascular SSCs during skeletal ageing. The consequences of increased ET-1 secretion by endothelial cells on the senescence and functional capacity of SSCs within the bone marrow microenvironment, and its subsequent contribution to age-related

bone loss conditions such as osteoporosis, remain poorly understood. This specific vascular-skeletal signalling axis represents a promising frontier for understanding the fundamental mechanisms driving osteoporosis and identifying novel therapeutic targets.

1.2 Osteoporosis: prevalence, pathophysiology, risk assessment, and evolving therapeutic landscape

Bone is far more than a static scaffold; it is a dynamic, metabolically active tissue essential for locomotion, structural support, protection of vital organs, mineral homeostasis (particularly calcium and phosphate), and haematopoiesis within the bone marrow. This functionality depends on a delicate, continuous remodelling process which maintains a balance between bone resorption mediated by osteoclasts and new bone formation carried out by osteoblasts^[12]. Osteocytes, the mature state of osteoblasts residing within the mineralised matrix, act as master regulators, sensing mechanical load and orchestrating remodelling activity through intricate signalling networks^[13]. Osteoporosis arises when this critical equilibrium is disrupted. This condition manifests as a progressive decline in bone mass coupled with diminishing microarchitectural integrity, ultimately resulting in compromised skeletal structural integrity and a heightened vulnerability to fractures occurring under minimal trauma,

commonly termed fragility (low-trauma) fractures^[14]. This debilitating condition constitutes a major global public health burden, disproportionately affecting the elderly and postmenopausal women due to hormonal shifts, but also impacting men and younger individuals with specific risk factors.

1.2.1 Pathogenesis: cellular senescence and imbalanced remodelling

The fundamental disruption underlying osteoporosis is a dysregulation of the tightly coupled bone remodelling process, orchestrated by intricate interactions across tissue, cellular, and molecular levels. At its core, bone health depends on the precise communication and coordination between three principal cell types within the dynamic bone microenvironment. Osteoblasts, derived from mesenchymal lineage precursors, are critically responsible of synthesising the organic bone matrix and facilitating its mineralisation^[15]. Counteracting the bone-forming activity of osteoblasts are the osteoclasts, large multinucleated cells derived from the haematopoietic lineage that specialise in resorbing mineralised tissue via the localised release of acids and proteolytic enzymes^[16]. Embedded within the mineralised matrix itself reside the osteocytes, comprising the vast majority of bone cells. These former osteoblasts function as sophisticated mechanosensors^[12], detecting mechanical loads and fluid flow within their lacunar-canalicular network, and responding by secreting key

signalling molecules that act as master regulators to coordinate both osteoblast and osteoclast activity, thereby maintaining skeletal equilibrium.

However, skeletal ageing triggers a profound shift marked by the progressive accumulation of senescent cells within this microenvironment^[17]. Evidence showed that bone loss due to old age often coincides with an increase of senescent bone cells in the population^[18]. This deleterious senescence phenotype extends its influence beyond mature bone cells, such as osteoblasts and osteocytes, to critically impair their essential precursors—skeletal stem cells (SSCs)—which reside within the perivascular niche of the bone marrow microenvironment. Cellular senescence constitutes a state of irreversible cell cycle arrest, instigated by diverse ageing-associated stressors including telomere attrition, persistent DNA damage, oxidative stress, mitochondrial dysfunction, and chronic inflammatory processes^[19]. Crucially, these senescent cells are not functionally quiescent; rather, they acquire a potent pro-inflammatory and tissue-degenerative phenotype termed the Senescence-Associated Secretory Phenotype (SASP)^[20]. The SASP comprises a constellation of secreted mediators, notably potent pro-inflammatory cytokines (e.g., IL-6, IL-1, TNF- α), chemokines (such as IL-8 and MCP-1), specific growth factors (including TGF- β), matrix-degrading enzymes (MMPs), and

reactive oxygen species (ROS), collectively creating a hostile microenvironment.

The secretion of this SASP exerts devastating effects on bone homeostasis. It fundamentally disrupts the delicate balance between formation and resorption by simultaneously promoting osteoclast activity and suppressing osteoblast function. SASP cytokines directly stimulate osteoclastogenesis by enhancing RANKL signalling and inhibiting the protective decoy receptor OPG, thus tipping the RANKL/OPG ratio strongly in favour of bone resorption.

Concurrently, the SASP milieu impairs osteoblast differentiation from progenitor SSCs, inhibits the synthetic activity of mature osteoblasts, and can even trigger their apoptosis. Senescent osteocytes further compound the problem by altering their expression of crucial signalling molecules—increasing inhibitors of bone formation like sclerostin and decreasing protective factors like OPG—thus perverting their role as orchestrators of remodelling into agents of imbalance. Moreover, the SASP acts beyond individual cells, propagating damage through a 'bystander effect' that induces senescence in neighbouring healthy cells and establishes a self-perpetuating, pro-inflammatory, and pro-resorptive niche within the bone marrow.

The ultimate consequence of these intertwined cellular dysfunctions is a sustained negative bone balance. Each remodelling unit experiences heightened resorption that outpaces diminished formation, leading inexorably to the progressive loss of bone mass, microarchitectural deterioration—manifested as trabecular thinning, loss of connectivity, and cortical porosity—and compromised biomechanical competence. These structural deficits define osteoporosis and create the skeletal fragility that culminates in fracture susceptibility. Importantly, the pathogenesis is often accelerated by systemic factors, particularly within the highly vascularized bone marrow microenvironment. Emerging evidence implicates endothelial cell senescence in the adjacent vasculature as a significant upstream contributor, potentially releasing potent mediators like endothelin-1 (ET-1) that further perturb perivascular SSC function and amplify osteoblast lineage senescence—a key vascular-skeletal axis directly investigated in this study. This understanding frames osteoporosis pathogenesis not just as imbalanced turnover, but as a failure of cellular function driven by senescence and microenvironmental decay, particularly in the context of vascular ageing.

1.2.2 Prevalence and impact: a growing burden

Osteoporosis constitutes a significant global public health concern, exerting a growing burden on individuals, healthcare infrastructures, and economies internationally. Its prevalence rises markedly with advancing age, making it as a primary contributor to morbidity and mortality among older adults^[21].

Supporting this trend, a comprehensive meta-analysis focusing on the Chinese population documented prevalence rates of 23.4% in women and 11.5% in men.

Furthermore, this analysis revealed an upward trend over two decades, with prevalence increasing from 16.9% prior to 2010 to 20.3% during the period 2011–2020^[22]. This demographic shift towards an older population globally ensures that osteoporosis prevalence and its consequences will continue to rise significantly. Fragility fractures constitute the primary clinical manifestation of osteoporosis, representing the disease's tangible endpoint. These fractures, occurring with minimal trauma such as a fall from standing height or even spontaneously, cause immense suffering, loss of mobility and independence, and are linked to elevated mortality rates^[23]. Within the United States, osteoporosis accounts for over 1.5 million fractures every year^[24]. These fractures affect various skeletal sites, including the spine, hip, wrist, and other locations. The personal cost involves pain, disability, reduced quality of life, and often the need for long-term care. The economic burden is staggering,

encompassing direct medical costs for hospitalisation, surgery, rehabilitation, and long-term care, as well as indirect expenditures associated with diminished workforce efficiency and the strain on caregivers, placing immense strain on healthcare resources and societal structures.

Accurately identifying individuals at high fracture risk is paramount for effective prevention and timely intervention. Fracture risk is multifactorial, influenced by an interplay of non-modifiable factors (advanced age, female sex, postmenopausal status, Caucasian or Asian ethnicity, family history of hip fracture, small body frame) and modifiable or treatable factors (low peak bone mass, vitamin D insufficiency or deficiency, inadequate calcium intake, smoking, excessive alcohol consumption, physical inactivity, low body weight, history of prior fragility fracture)^[25]. Additionally, numerous secondary causes and medications (e.g., long-term glucocorticoid therapy, endocrine disorders, eating disorders, immobilization, rheumatoid arthritis, chronic kidney disease, specific cancer treatments) significantly elevate risk^[26]. While low Bone Mineral Density (BMD) measured by Dual-Energy X-ray Absorptiometry (DXA) persists as the definitive diagnostic criterion and a robust standalone predictor of fracture probability, with epidemiological evidence consistently demonstrating an approximate doubling of fracture risk for every standard deviation reduction in Bone Mineral Density (BMD)^[27].

Nevertheless, a thorough fracture risk evaluation necessitates assessment beyond BMD measurements alone. Integrated risk assessment tools, such as FRAX®, which incorporate BMD along with key clinical risk factors, provide a more robust estimate of the projected risk for a specific person experiencing a fracture within the subsequent decade^[28]. Biochemical indicators of bone remodelling activity, such as circulating levels of serum CTX and PINP, serve as measurable parameters for assessing skeletal metabolism dynamics. These molecular markers can offer additional insights into remodelling activity and may complement risk assessment in specific clinical scenarios, but their high biological variability and unclear predictive value independent of established tools limit their routine diagnostic use; they are not substitutes for BMD measurement and clinical evaluation^[29].

The scale and impact of osteoporosis make it a considerable worldwide public health problem. It was previously believed to be a natural part of getting older, but it is now known to be both preventable and treatable. Ironically, despite significant advancements in treatment, there is a widening therapeutic gap for individuals diagnosed with osteoporosis who may be at risk for experiencing fractures. In the review by Khosla and Hofbauer in 2017^[30], they traced the evolution of the pharmacological treatment of osteoporosis. Fuller Albright demonstrated in the 1940s that oestrogen treatment could resolve the disrupted

calcium levels that occur in elderly women after ovariectomy or menopause.

The landscape of osteoporosis drug discovery underwent a significant transition around the turn of the 21st century. The field shifted its primary impetus from discoveries derived primarily from animal models and clinical observation towards a targeted focus on the development of novel therapeutic agents, such as estrogens, calcitonin, and teriparatide, alongside the innovative repurposing of established compounds like bisphosphonates. This period also saw valuable collaborative insights emerge from individuals affected by uncommon bone disorders, contributing to the development of agents including romosozumab and odanacatib.

Although significant progress has been made, concerns about the adverse effects of anti-osteoporosis medications, particularly bisphosphonates, persist. Even though various FDA-approved medications are commercially accessible, they are unable to meet all current therapeutic needs^[31]. Additionally, there was a dearth of convincing evidence to back up their longstanding drug potency, which made a lot of patients who would have benefited from drug therapy decide against taking these medications. Therefore, there is an urgent demand to progress clinical approaches to improve patient recognition and adherence to new potential drugs and continue the development of novel drugs that cause minimal side effects and are able to establish a long-term synthetic effect on

bone health. It is vital to expand our understanding of osteoporosis and bone ageing in developing novel therapeutic options. This might result in a substantial reversal of this potentially fatal illness in the aging population.

1.3 Skeletal degeneration

1.3.1 Bone physiology and pathophysiology

A thorough understanding of the intricate physiological processes governing bone formation, turnover, and their pathological disruption during ageing is fundamental to developing effective strategies against skeletal degeneration. This bone remodelling cycle ensures mechanical integrity, facilitates mineral homeostasis, and enables repair of microdamage.

The pathophysiology of bone degeneration involves a multifactorial shift towards net bone loss, characterised by an imbalance favouring osteoclastic resorption over osteoblastic formation. This imbalance may arise from diverse underlying mechanisms, such as hormonal decline (e.g., estrogen deficiency), persistent inflammatory states, compromised mitochondrial function, and the progressive build-up of senescent cells population within the bone marrow niche. Understanding these interconnected physiological processes and their pathological dysregulation, particularly the roles of cellular senescence,

oxidative stress, and vascular dysfunction, provides the essential foundation for elucidating the mechanisms driving age-related bone loss and identifying novel therapeutic targets aimed at restoring skeletal homeostasis.

1.3.1.1 Cell fate and function

Several important cell types in various positions and with different functions coordinate bone development and maintenance. The bone benefits from these cells in various ways. The bone forming osteoblasts can generate a distinctive matrix extensive of collagen known as the osteoid, which becomes mineralized in later stages hence increasing the strength of bones. A subpopulation of osteoblasts known as osteocytes develops during the ossification process when they are entrenched in the bone matrix. Another bone cell present in large quantities is the chondrocyte that forms several types of joint cartilage^[32].

Hyaline cartilage can be located in bone joints and within growth plates. During the development of the skeleton and the healing of fractures, chondrocytes are also essential for the ossification of the inner cartilage layer, in which a layer of hyaline cartilage serves as an intermediate structure before being eventually replaced by bone by association with other bone. Through the joint activities of osteoclasts and osteoblasts, this process results in the formation of the intermediate layer of hyaline cartilage, which is later replaced by bone^[33].

Two more types of skeletal cell are identified in the bone marrow: stromal cells which support the hemopoietic process as well as the bone marrow adipocytes^[34]. In contrast to the more recognizable white fat deposits, adipose tissue in the bone marrow has distinct features that set it apart. Adipocytes found in bone marrow are affected by dietary changes and ageing in both systemic and local metabolic activities such as energy metabolism, osteogenesis, and hemopoiesis^[35]. Mesenchymal stromal cells, also known as haematopoietic supporting cells, are a group of fibrous cells that provide the necessary cytokines for the differentiation of blood cells. This cell population has yet to be fully characterised, and it might include subpopulations of cells with various haematopoietic support functions^[36]. Skeletal stem cells are the common precursor cells from which all types of mature skeletal cells are derived. The periosteum, growth plate, and bone marrow all contain bone stem cells. The differentiation potential of the various skeletal stem cell types differs and seems to be related to the particular requirements of the location where they are positioned, despite the fact that there are some similarities between skeletal stem cell types, including the capacity for self-renewal and the analysis of particular markers located on cell surfaces^[37].

1.3.1.2 Bone Maintenance and Repair

An active homeostasis consisting of the resorption and formation of bone is necessary for both maintenance and repair. The continuous remodelling of bone involves coordinated cellular activities: osteoclasts resorbing aged or compromised bone tissue, osteoblasts depositing an unmineralized osteoid matrix, and the subsequent mineralisation process transforming this osteoid into new bone substance^[38]. The opposing forces of bone formation and resorption are present together during physiological remodelling, despite the fact that the net change in bone mass is small. Under specific physiological or pathological circumstances, this equilibrium can be upset, allowing the remodelling process to decouple. Under such conditions, an imbalance may arise where either bone resorption or bone formation predominates, ultimately resulting in a net alteration in bone volume. This is readily apparent on radiographic images during the diagnosis of severe osteoporosis. The pathological reduction of bone volume and quality that occurs in osteoporosis is radiologically highlighted by the bone's overall radiolucency or by the weakened bone's subsequent fracture^[39]. The pathophysiology involves rapid osteoclast resorption of bone and the concurrent senescence of osteoblasts, resulting in a state of net bone breakdown. Conversely, some athletes who repeatedly experience mechanical stress may see an increase in bone volume and density. This mechanobiological

transformation, known as Wolff's law, is hypothesised to be regulated by a transfer of current between the extracellular fluid and the osteoblast that signals the differentiation of bone progenitor cells into active osteocytes^[40].

1.3.1.3 Vasculature

Bone marrow is enriched with blood vessels. These vessels form a network and regulate the maturation and release of blood cells into the body. The blood vessels of bone are formed primarily by angiogenesis. Bone formation and angiogenesis occur concurrently through two distinct mechanisms:

intramembranous ossification or endochondral ossification^[41]. In flat bones, intramembranous ossification generates new bone tissue through direct the differentiation of mesenchymal stem cells into mature osteoblasts. These flat bones contain dense bone marrow throughout their structure, where research confirms the vasculature critically influences development and maintenance of the bone marrow microenvironment. This vascular-bone relationship proves fundamental for supporting healthy growth of haematopoietic cells and progenitor populations^[42]. Conversely, most bones - including long bones such as the femur and tibia - develop via endochondral ossification. The external surfaces of long bones feature articular cartilage composed of chondrocytes that actively secrete vascular endothelial growth factor A (VEGF-A), a potent

proangiogenic signalling molecule. The long central shaft of the bone, known as the diaphysis, is covered by the epiphysis containing the growth plate as well as the distal epiphysis^[43].

Numerous endothelial cell subtypes have been identified through in-depth studies of the bone vasculature, each with distinct molecular characteristics and unique functional duties^[44]. By examining the expression of markers and their functional traits, it is possible to distinguish these different capillary endothelial cell subgroups within the bone. Initial characterisation of endothelial cell heterogeneity within bone marrow utilised endoglin (Emcn) and CD31 expression levels to distinguish two principal subtypes. Type H endothelial cells exhibit high expression of the cell adhesion molecule CD31/Pecam1 and the salivary glycoprotein Emcn. Conversely, type L endothelial cells display low expression levels of these markers. Their anatomical distribution is distinct: type L endothelial cells predominantly form the sinusoidal vessel network within the diaphysis, while type H endothelial cells are primarily localised to the metaphysis and the endosteal region^[45].

The vascular supply to long bones reveals a specific pattern. Type L sinusoids in the diaphysis lack direct arterial input. Instead, arterial blood flow from arteries and small arterioles is received by type H vessels situated in the

epiphysis and endosteum. Consequently, blood exits the bone via the large central vein and is subsequently distributed to the type L sinusoidal network present in the epiphysis and metaphysis. This vascular architecture results in the diaphysis, unlike the metaphysis, lacking direct arterial perfusion, leading to a state of chronic hypoxia in postnatal mice. Perivascular cell associations also differ between vessel types. Type H vessels are typically surrounded by perivascular cells expressing markers such as nestin, NG2, and platelet-derived growth factor receptor (PDGFR). Mesenchymal cells expressing leptin receptor (LEPR) and PDGFR, along with CXCL12-abundant reticular (CAR) cells, are characteristically associated with type L vessels. The functional significance of type L vessel-associated mesenchymal cells, particularly in supporting haematopoietic stem-cell populations, is well documented^[46]. The precise connection between various perivascular cell populations in the bone marrow is still unknown.

Studies have found that some risk factors for osteoporosis and vascular dysfunction overlap, and they are also found associated with endothelial dysfunction^[47], indicating the potential link between vascular dysfunction and age-related bone loss. Age-related osteoporosis was found associated with reduced skeletal blood flow. Numerous physiological processes are impacted by this, such as nutrient transport, tissue metabolism, and the uptake of phosphate

and calcium ions^[48]. In an animal model of OVX-induced osteoporosis, normal ageing contributes to a depletion of type H blood vessels as well as associated osteoprogenitor cells, which could result in decreased perfusion and deterioration of bone homeostasis^[49].

1.3.2 Vascular ageing as an initiator of skeletal senescence

Ageing is a process of functional decline in living things that is time-dependent. It has been determined that there are nine cellular and molecular hallmarks of ageing^[50]. These hallmarks interconnect and simultaneously occur during ageing and often display cellular heterogeneity in aged individuals^[51]. However, it has been a huge challenge to dissect these connections to evaluate their individual roles in ageing. While chronological age may not reflect the actual biological age, some biological variables including the arterial function have been found to be linearly correlated with the chronological age^[52].

The intricate relationship between ageing and vascular ageing is particularly significant in the context of the onset of age-related cardiovascular diseases. Vascular ageing itself is characterised by modifications in both the structural integrity and functional capacity of the two primary cellular layers constituting blood vessel walls: the endothelium and smooth muscle cells. The alterations in signalling pathways hinder vascular function, in addition to other phenotypes

associated with ageing, contribute to the occurrence of structural modifications, and eventually lead to vascular stiffness and endothelial dysfunction^[53].

Building upon prior *in vitro* and *in vivo* investigations, endothelium-dependent vasodilation was found to decrease significantly in the vascular beds of old animals and humans^[54; 55]. There have been speculations that vascular ageing is closely linked with organ ageing since it has a significant role in connecting, and networking the whole-body system, yet no proof has been published until last year. In 2021, concrete evidence of 3D imaging revealed vascular control on organ-specific tissue ageing^[8], confirming the connection between vascular function and organ ageing.

1.3.3 Age-related biochemical and biomechanical changes in the bone niche

1.3.3.1 Skeletal senescence

Cellular senescence, an irreversible state where cell division no longer occurs, is brought on by a number of mechanisms. Tumour suppression, tissue repair, embryonic development, and organismal ageing are all linked to processes known as cellular senescence, feedback to a number of triggers, including external stresses and natural replicative exhaustion. The phenomenon of replicative senescence, characterised by an irreversible cessation of cell

division following a finite number of population doublings, was first documented in cultured human fibroblasts by Hayflick and Moorhead in 1961^[56]. Their seminal work established that these cells exhibit a defined proliferative lifespan *in vitro*, culminating in a permanent growth arrest state. This fundamental observation provided the initial experimental evidence for cellular ageing under controlled laboratory conditions. As a result, it was postulated that tissue ageing was brought on by cells gradually as their proliferative capacity diminished, which was necessary for replacing damaged cells that had accrued over time naturally. However, it took years to create the tools necessary to show how the buildup of senescent cells promotes ageing and dysfunction in the body.

Cellular senescence can be triggered via two distinct pathways, p53 pathway or p16^{INK4a} (p16) pathway. The former initiates the activation process of cyclin-dependent kinase inhibitor p21^{CIP1}, while the latter leads to inhibition of cyclin-dependent kinases 2 and 4, prohibiting retinoblastoma (Rb) protein phosphorylation^[57]. There has been evidence shown in the previous study that cellular senescence could lead to bone loss^[58]. Research demonstrated that either genetic or pharmacological approaches targeting senescent cell eradication or SASP synthesis suppression effectively reduced bone resorption while preserving or even enhancing bone formation^[59]. Accumulation of SASP

and senescent cells in tissues can limit their regenerative capacities in tissues and secrete pro-inflammatory mediators favouring the development and progression of several disorders in elderly^[60]. A novel approach to treating osteoporosis would be to therapeutically target senescent cells, which would concurrently halt bone resorption and promote better formation.

Ageing is associated with an elevated senescent cell population, either replicative or stress-induced senescence. Replicative senescence is a natural phenomenon caused by the shortening of telomere length that preserves the integrity of genetic materials of cells and shortens with each round of cell division until a predetermined length is reached. Beyond this critical length, the proliferation process is arrested, and cell signalling for senescence and apoptosis is triggered. Senescent cells acquire unique features associated with ageing and age-related illnesses. In contrast, stress-induced early cellular senescence is usually the result of exposure to a variety of chemical or physical agents that induce oxidative stress or oncogenic activities in cell^[57]. Nuclear DNA damage is frequently cited as the primary underlying factor in such senescence, most commonly manifested as DNA double-strand disrupts and activates the DNA damage response (DDR) pathway. This DDR signalling acts as a checkpoint to halt the continuation of the cell cycle and prevent damaged genetic material from being passed on to daughter cells^[61].

During physiological turnover and after tissue and organ damage, progenitor stem cells are essential for preserving tissue organization and homeostasis. With age, stem cells lose some of their ability to function, but not necessarily all of it. In different stem cell types from various tissues and species during normal and pathological ageing, markers of DNA damage response pathway activation have been observed, indicating that stem cells are not resistant to DNA damage accumulation and response pathway activation^[62]. Stem cell senescence and dysfunction represent fundamental drivers of age-related diseases, which can be induced by risk factors like ageing, radiation exposure, and metabolic disorders. For example, p16^{INK4a}-positive senescent MSCs advance to osteoarthritis via the release of DKK1, which activates premature bone remodelling as well as cartilage degeneration. Senescent mesenchymal stem cells (MSCs) lose their capacity to regenerate cartilage as well. Previous evidence showed that senescence is associated with compensatory overexpression of sirtuin 6 in bone marrow MSCs, which reduces stem cell senescence and confers resistance to oxidative stress. GATA6 and SOX11 are also responsible for regulating bone marrow stem cell senescence^[63].

A significant limitation within senescence research remains the absence of a singular, universal, or model-specific biomarker capable of reliably detecting senescent cells within cultures or tissue samples. Consequently, identification

currently relies on employing a combination of markers whose co-presence helps distinguish stably arrested senescent cells from transiently quiescent or terminally differentiated states. Among the most widely utilised biomarkers are senescence-associated β -galactosidase (SA- β -gal) activity and elevated expression of p16^{INK4a}[64]. SA- β -gal represents a lysosomal enzyme; crucially, at an acidic pH of 6.0, senescent cells exhibit heightened enzymatic activity, cleaving the X-gal substrate and thereby staining the cells blue. This facilitates the visualisation and labelling of senescent cells under microscopic examination. p16^{INK4a} functions as a cyclin-dependent kinase inhibitor, playing a critical role in enforcing cell cycle arrest and acting as a key tumour suppressor. Senescent cells characteristically secrete significantly higher levels of p16^{INK4a} compared to their non-senescent counterparts. Furthermore, components of the senescence-associated secretory phenotype (SASP), particularly pro-inflammatory cytokines such as interleukin-6 (Il-6) and Il-8, can be measured at both transcript and protein levels to assess senescence within tissues or cell cultures. However, the presence of SASP factors alone cannot definitively confirm senescence, as senescence induced specifically by p16^{INK4a} overexpression does not invariably lead to alterations in the transcriptional program governing SASP component production.

1.3.3.2 Cell mechanics

Ageing is a complicated process involving chronic degenerating physiological developments in living organisms. Along with ageing, significant biomechanical and biochemical modifications occur at both micro and macroscales, affecting more than just organs and tissues, cells and their organelles are also influenced. These modifications include changes in DNA transcription and epigenetics, mitochondria dysfunction and accumulation of metabolic damage, and the overall mechanical properties of cells, nuclei, and the extracellular matrix (ECM)^[65]. Furthermore, ageing affects cells' ability to detect changes in the ECM and convert them into biological signals.

Mechanical properties play critical roles in regulating biochemical functions at the intracellular and subcellular levels in response to applied external and internal forces. This regulation influences gene expression, cellular migration and adhesion, and subsequent differentiation pathways, essential for maintaining tissue homeostasis^[66]. Cell growth and death are regulated and programmed, affecting cell regeneration and inducing pathological development. Ageing is defined by progressive, chronic modifications in cellular mechanical characteristics, including cell stiffening, reduced elasticity, and weakened strength. This eventually causes abnormal cellular responses and declined functions in aged organ tissues^[51]. As abnormal cellular mechanical

changes accumulate, age-related cell malfunctioning of cells and disorders develop in the body.

Age-related musculoskeletal degenerative changes encompass diminished bone volume, reduced skeletal muscle strength, progressive joint stiffening, and fibrosis. Osteocytes are the mechanoresponsive cells of bone tissues presenting in large amounts, playing a vital role in bone remodelling and adapting to mechanical loading via regulations of osteoblast and osteoclast activity^[67].

Some changes observed in osteocytes include increased cortical diameter, reduced thickness, density, and increased porosities in the cortical bone.

Mechanical ageing of the osteocyte systems eventually results in bone volume reduction, impaired mechanosensitive signalling activity and responsiveness to mechanical loading^[68].

In the cartilage, stiffening and fibrosis result in joint pain and reduced mobility in the elderly population. Human chondrocyte elastic modulus has been found to be significantly elevated in osteoarthritis patients^[69]. Degradation of the intervertebral discs is also an indicative symptom of bone-related pains and spine stiffness in the elderly. Intervertebral discs are polyaxially articular cartilage comprising of outer collagenous ECM and fibrochondrocytic cells, as well as the nucleus pulposus core containing chondrocyte-like, notochordal

cells and a fibrous annulus fibrosus ring. Chronic degeneration with ageing leads to a significant increase of nucleus pulposus shear modulus and annulus fibrosus compressive modulus^[70]. Elevation in shear modulus suggests that the nucleus pulposus becomes stiffer and exhibits more solid-like behaviour during the ageing process.

Tendons also undergo structural and biomechanical degeneration. Tendon fibroblasts, also known as tenocytes, exhibit decreased density and cellularity together with increased elongation. In animals, the tendon mechanical strength and viscoelastic characteristics decline and become significantly stiffer with increasing age^[71]. Similarly, muscle undergo degradation and stiffening occur with age^[72]. Muscle cells and fibres are found to exhibit decreased cellular size, but with an increase in inelasticity and stiffness. These contribute to extremely high tensile strength in the cytoplasm and therefore deteriorate contraction function in ageing cells.

Ageing and cell mechanics: There are several important cellular drivers to mechanical changes and malfunction during the pathogenesis of age-related disorders. The viscosity inherent to both the cellular cytoplasm and the outer membrane, governed by factors such as the protein-to-lipid ratio and the intrinsic viscosity of cytoplasmic constituents, influences how cells respond

when subjected to an applied force. The spatial distribution and density of intracellular organelles, such as the endoplasmic reticulum (ER) and mitochondria which exhibit close physical association, demonstrate a correlation with the local organisation of the cytoskeleton. This relationship arises indirectly through the organelles' attachment to cytoskeletal elements and their participation in coupled dynamics^[73]. The concentration and activity of specific cellular proteins, including Rho guanosine triphosphatases (GTPases), cytoskeletal crosslinkers, and motor proteins, regulate these interactions and dynamics^[74]. Furthermore, water movement within the cellular environment occurs through two primary mechanisms: osmosis across membranes and transport facilitated by membranous organelles.

Cytoskeleton: The cytoskeleton comprises an intricately interwoven network of filamentous proteins essential for preserving cellular structural integrity and morphology. The cytoskeleton comprises three distinct types of protein filaments: the microfilaments (F-actin), intermediate filaments, and microtubules, which play important roles in cell division, migration, shape regulation, contractility, mechanosensation and signalling in response to external mechanical stimuli. In elderly individuals, vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and dermal fibroblasts undergo significant increases in actin filament polymerisation^[75]. This heightened

polymerisation leads to extensively reorganised cytoskeletal networks characterised by hyper-stiffness, increased density, and diminished cellular motility.

Studies have revealed that in the dermal fibroblasts from elderly donors, F-actin expression is greatly elevated, instead of G-actin (globular actin)^[76]. Therefore, the imbalanced composition of G-actin and F-actin indicates that actins condensation is amplified with ageing. Aside from presence of more F-actin, increased cytoskeletal volume, crosslinking and bundling by fascin and α -actinin, fibre density, and decreased fibre variations are also observed in the older population^[77]. Research indicates that ageing induces dysregulation of F-actin within CD4-expressing T lymphocytes, manifesting as impaired cellular activation, increased membrane rigidity, and aberrant immunological synapse formation^[78]. Aside from lymphocyte dysfunction, alveolar macrophage phagocytosis is also impaired^[79]. Furthermore, since F-actin is found just beneath the plasma membrane, the fact that actin cortex exhibits a high mechanical strength implies that F-actin plays a dominant role in the viscoelastic adaptation of cells under small stress^[76]. Mechanically rigid morphological traits in elderly adults are accompanied by changes in actomyosin contractility and actin polymerisation, both of which is governed by cytoskeleton F-actin fibre concentration and organisation, together with the

associated motor protein, myosin^[80]. Studies have concentrated on how F-actin contributes to increased membrane stiffening with age. Nevertheless, a comprehensive investigation into other critical factors remains fundamentally important; these include cell membrane mechanics and intracellular organelle localisation, both of which may participate in the mechanical alterations associated with ageing.

Likewise in *in vitro* models, when starved cells with lowered F-actin content are stimulated with external mechanical forces, such as stretching or shear stress, the cytoplasm spontaneously organises the F-actin into condensed fibres, forming the actin cap^[81]. F-actin arrangement is regulated by the ROCK (Rho-associated coiled-coil kinase) signalling cascade. Signalling activation of the pathway increases the cytoplasmic mechanical modulus by increasing physical interconnections between the nucleus, cytoskeleton, and ECM. Even though the F-actin network is highly dynamic, gradual degenerations occurs during the ageing process, affecting the homeostatic ratio of organised and less organised structures, and total F-actin content. These adjustments in F-actin organisation and content lead to elevated dysfunction in the mechanical characteristics of tissue cells. Moreover, the compromised homeostasis equilibrium and loss of cell elasticity hinder adaptation to external forces induced by natural ageing and chronic mechanical stresses, along with endogenous protein and organelle

disruption^[82], resulting in vicious cycle of mechanical and biological degenerative functions in cells.

The intermediate filament vimentin and microtubules contribute to cell mechanics. Even though no statistically significant observations were made in the vimentin and microtubules content in suspended dermal fibroblasts across age groups is observed^[76]. Intermediate filaments are the most robust cellular element responsible for protecting cellular organelles from external stress. Overexpression of intermediate filaments is associated with more than 45 types of chronic diseases caused by ageing^[83]. Microtubules primarily regulate the cell shape response to external force, especially compression. When reorganising from assembly to shortening, aged microtubules show a higher catastrophe rate than the younger ones^[84]. Ageing-related weakening of the microtubule network affects cell composition and function, including cell proliferation, movement, molecular secretion, and ligand-receptor endocytosis.

Nuclear: The nucleus, which contains genetic information, is directly engaged in the overall functionality of cells. Nucleus is traditionally regarded as a rigid organelle, but it is dynamic and being continuously exposed to various mechanical forces. Since the nucleus is attached to the surrounding cytoskeleton, mechanical signal transduction within the cell and from the

external ECM will directly act on the nucleus, affecting its morphology and function. These nuclear deformities and changes influence its reactivity to mechanical disturbances and are critical in preserving genetic materials.

The nuclear envelope has two membranes. The nuclear lamina is a thick protein architecture that lies directly under the inner nuclear membrane, and is important for the nucleus' mechanical characteristics. There are different types of lamin in the nucleus. Lamin A offers structural support to the nucleus, contributing to its rigidity and stability. Study showed that lamin A could be a marker of ageing cells^[85]. Due to lamin A overexpression, nucleus stiffness increases drastically in the dermal fibroblasts and progeria of the elderly^[86]. Similarly, in the age-related Hutchinson–Gilford progeria syndrome, identified by lamin A overexpression, the nucleus becomes stiffer and more brittle^[87]. Premature ageing^[88] and increased apoptosis^[89] are also observed. Lamin is essential for facilitating direct communication between the cytoskeleton and the cellular nucleus, mediating the transmission of external signals to the nuclear core via focal adhesions and the cytoskeletal network, ultimately reaching the nucleus^[90]. The regulation of this process involves lamin-associating proteins characterised by SUN- and KASH-domains, situated within the inner and outer nuclear membranes respectively. SUN-domain proteins establish connections with the nuclear lamina, nuclear pore complexes, and associated proteins

including emerin, while KASH proteins interact with the cytoskeletal filaments^[91]. The LINC complex, defined as the linker bridging the nucleoskeleton and cytoskeleton, comprises lamin proteins interacting with SUN and KASH domain-containing proteins. External stress transmitted through these nucleus–cytoskeleton complexes can result in intranuclear reorganisation and distortion. Recently, research has shown that manipulating the LINC complex reduces nuclear deformations caused by cellular strains^[92]. In the interior of the nucleus, chromatin also helps to resist mechanical stresses and maintains the integrity of the whole nucleus. Conformational changes in nuclear eventually affect the chromatin architecture, hindering its function, such as the release of transcription factors, the chromatin segments movement across different regions. Chromatin deformation and DNA damage are often associated with ageing and cellular senescence.

Mitochondria: Mitochondrial dysfunction exhibits a strong association with the onset and progression of age-related diseases. One hallmark of ageing and cellular senescence is the permanent cellular damage due to high concentrations of ROS, which inevitably results in functional deterioration in the entire cell^[50]. Mitochondrial morphology, dynamics and bioenergetics processes are regulated through fusion and fission^[93]. Under normal conditions, the heterogeneous population of mitochondria exhibits balanced fusion and fission activities

through homeostasis. In pathological disorders, such balance in mitochondria is disrupted. When fission is dominating, mitochondrial fragmentation occurs. This leads to an enhanced level of mitochondrial membrane permeabilisation and a reduction in membrane potential. On the contrary, when more fusion occurs, extended interconnecting mitochondrial networks form, resulting in increased mitochondrial membrane potential^[94]. Depending on the location and function, different cells have distinct mitochondrial morphologies. Stress status and cell cycle phases also influence the mitochondrial structures. Age-related mitochondrial DNA^[95] damage is often linked with the dysfunction of mitochondrial biogenesis and respiratory–electron transport chain. Such dysfunction stimulates the production of ROS, forming an atrocious positive-feedback cycle of oxidative mitochondrial malfunction. In addition to cellular damage, a high concentration of ROS in the cell also drives ECM deterioration and impairs cytoskeletal contractility.

Extracellular matrix: The ECM is a constituent outside the cell which plays an important part in tissue networking and providing a scaffold for cellular growth. It consists of proteins, polysaccharides, and water. Each tissue exhibits a unique ECM molecular makeup and structural arrangement, optimised to facilitate specific biochemical signalling and mechanical force transmission that mediates environmental cues and cellular responses. The ECM derives its essential

mechanical properties from several key constituents. Among these, fibrous proteins—including collagen, laminins, fibronectins, and elastin—contribute significantly to structural integrity. Alongside these proteins, proteoglycans such as keratan sulphate, heparan sulphate, and hyaluronic acid further enhance the ECM's biomechanical functionality. These proteoglycans are secreted primarily by fibroblasts, which serve as the principal cellular source for these crucial macromolecules within connective tissues.^[96] Fibrous proteins, their associated enzymes and proteinases regulate the organisation of matrix structure, its remodelling, and degradation rates of the ECM. Meanwhile, proteoglycans regulate homeostasis in the environment, through gel formations in the interstitial space which resist external stress. With ageing, changes occur in the ECM and its functions deteriorate usually due to an imbalance of secreted components and processes.

A large category of enzymes, consisting of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), enzymes, and crosslinking transglutaminases, govern ECM remodelling and degradation. MMPs are endopeptidases which cleave the peptide bond of non-essential amino acids. These can degrade a broad range of ECM associating proteins as well as membrane receptors., such as elastin and collagen. This regulatory function proves crucial for modulating the activation state of downstream processes

involving cell–matrix interactions, cell–cell communications, and growth factor secretion. Collagen is one of the most presented proteins in our body, constituting 30% of the overall proteins and more than 90% in the ECM^[96]. It is essential for maintaining the tissue tensile strength and mechanical stability, while also modulating cell adhesion and migration controlling their growth. Collagen fibres are primarily secreted and regulated by fibroblasts when external stretching force is detected. Collagen fibres rearrangement under pathological situations drives positive feedback signalling which affects the neighbouring cells' structure and morphology^[97]. When the tissue is exposed to repetitive stress, collagen binds to elastin, and the complex gives the tissue elastic and recoil properties. These properties can vary with different composite mixture of collagen and elastin.

As organisms age, the population of senescent cells in organ increases. Cellular senescence is often accompanied by secretion of inflammation factors, SASP. Due to inflammation, various changes occur in the ECM microenvironment, such as decreased expression of glycosaminoglycans, TIMPs, crosslinked elastin content, increased MMPs activation, plasminogen activator inhibitors, elastase activity, and mitochondrial ROS accumulation. These disrupt the appropriate organisation and stability of the collagen architecture in the ECM. In the aged population, collagen arrangement is loose and collagen fibres

become improperly crosslinked. This phenomenon is accompanied by increased tissue stiffness and fibrosis, caused by poor collagen solubility and degradation^[98], as well as activated Wnt signalling. As a result of losing structural support and mechanical properties, organ functions are impaired in various organs due to easily deformed blood vessels and weakened tissue^[82].

1.3.3.3 Bioengineering tools for cellular mechanical studies

To explore cellular mechanical properties across different scales, diverse bioengineering tools have emerged. These technologies fall broadly into two categories: those involving direct physical interaction with cells to assess their mechanical responses, and those examining subcellular mechanical characteristics through the introduction of beads or particles into cellular environments.

The atomic force microscope (AFM) is a commonly used instrument for measuring cell mechanics which uses a probe-based technique to scan the cell surface, capable of producing 3D images with high resolution^[99]. With a cantilever, AFM generates an indentation force on the cell surface, then displacement of the tip is measured which can be used to calculate the mechanical interactions in cells with a suitable modelling equation. The instrument's high sensitivity enables the measurement of cellular subcellular

properties, such as those of the nucleus and cytoplasm. An advantage of AFM is that the indentation force can be easily manipulated by the cantilever tip and its spring constant, making it able to generate force ranging from pico-Newtons to nano-Newtons. Mechanical parameters including Young's modulus and viscoelastic properties can be obtained.

Microfluidic devices are an emerging technology characterised with high efficiency and throughput. A microfluidic device containing large vesicles mimicking cells for micropipette aspiration has been fabricated to study cell membrane mechanics^[100]. The flow of fluids inside microfluidic devices can be easily manipulated to assess cell deformations and elastic modulus.

1.4 Skeletal regeneration

1.4.1 Challenges in aged/pathological bone regeneration

The intrinsic biological alterations accompanying ageing or chronic pathological states present formidable barriers to effective bone regeneration, creating a microenvironment fundamentally hostile to the natural healing processes. Central to this impairment is the profound dysfunction within the skeletal stem cell (SSC) compartment^[101]. SSCs, serving as the essential source of osteoblast progenitors, undergo a significant reduction in population size,

proliferative ability, and differentiation capability. This impairment is predominantly caused by the build-up of senescent cells within this compartment. Senescent SSCs enter a phase of permanent cessation of cell division yet retain metabolic activity. They release a powerful mixture of inflammatory cytokines, chemokines, proteases, and reactive oxygen species (ROS), components collectively termed the senescence-associated secretory phenotype (SASP). This SASP actively disrupts the bone marrow microenvironment, inducing a state of persistent, low-grade inflammation. This inflammatory milieu directly suppresses osteoblast activity and enhances osteoclast formation, consequently tipping the bone remodelling equilibrium towards increased bone resorption^[102]. Concurrently, excessive ROS generation within this milieu inflicts oxidative damage on proteins, lipids, and DNA, further impairing SSC function and viability, compromising the viability of newly formed bone tissue, and perpetuating a cycle of cellular damage and dysfunction.

Compounding these cellular deficits is the significant impairment of the vascular network supplying the bone. Vascular insufficiency, characterized by endothelial dysfunction, reduced capillary density, and impaired perfusion, leads to local hypoxia and diminished delivery of essential oxygen, nutrients, circulating progenitor cells, and systemic factors critical for cell survival,

proliferation, and differentiation. This compromised vascular supply not only starves the regenerative process but also disrupts crucial signalling pathways between endothelial cells and SSCs/pericytes within the perivascular niche, which are vital for maintaining stemness and coordinating repair^[103]. The synergistic effect of diminished and dysfunctional SSCs burdened by senescence, operating within an inflamed and oxidatively stressed niche further crippled by inadequate vascular support, creates a profoundly inhibitory environment that severely limits the efficacy of conventional regenerative approaches, necessitating novel strategies that directly target these root causes of regeneration failure.

1.4.2 Therapeutic targeting of the bone marrow niche and senescence

Rejuvenating the ageing or osteoporotic bone marrow niche represents a paradigm shift in regenerative strategies, moving beyond merely augmenting the activity of mature effector cells to directly addressing the fundamental cellular and microenvironmental dysfunctions that impede healing. Central to this approach is the targeted mitigation of the senescence burden, particularly within the SSC population, which serves as the essential reservoir for osteoblast progenitors^[104]. Therapeutic approaches that precisely target the removal of senescent cells (senolytics) or counteract their harmful senescence-associated

secretory phenotype (senomorphics) are increasingly recognised as vital strategies for overcoming this obstacle to tissue regeneration.

Furthermore, effective niche rejuvenation necessitates counteracting inhibitory signalling pathways that originate from within the niche itself. A primary source of such inhibitory cues is the dysregulated vasculature characteristic of aged or diseased bone. Endothelial cell dysfunction, frequently presenting as senescence within the vascular compartment, results in the dysregulated release of vasoactive mediators and signalling molecules—including endothelin-1 (ET-1)—that directly disrupt SSC function, induce oxidative stress, and facilitate the paracrine propagation of senescence^[105]. Therefore, strategies aimed at normalising vascular function or specifically blocking the action of these inhibitory vascular-derived mediators (e.g., ET-1 receptor antagonists) are integral to reversing the niche pathology. By simultaneously tackling SSC senescence and quenching the inhibitory signals emanating from sources like the dysfunctional vasculature, this combined therapeutic approach seeks to actively remodel the bone marrow microenvironment, transforming it from a hostile, pro-degenerative space back into one conducive to SSC renewal, osteoblast differentiation, and ultimately, successful bone regeneration. This represents a crucial frontier in developing effective treatments for skeletal

conditions where conventional approaches falter due to the profoundly compromised endogenous regenerative capacity.

1.4.3 Biomaterial platforms for bone tissue engineering

The formidable challenge of achieving functional bone regeneration within the compromised microenvironments characteristic of ageing or pathological states necessitates innovative strategies that extend beyond conventional biological approaches. In this context, synthetic and natural biomaterial scaffolds have emerged as highly promising platforms, offering sophisticated solutions to overcome the intrinsic barriers to healing. Hydrogels, formed from cross-linked hydrophilic polymer networks, are of particular importance owing to their distinctive physicochemical characteristics and exceptional adaptability^[106]. These materials can be precisely engineered to replicate critical features of the natural extracellular matrix, thereby furnishing vital structural support and establishing a three-dimensional framework supporting cell adhesion, migration, and proliferation. Furthermore, hydrogels function as highly versatile delivery platforms, enabling the precisely controlled and localised administration of a wide spectrum of therapeutic agents directly to the site of injury or defect^[107]. This includes the encapsulation and sustained presentation of viable cells, such as progenitor cells or genetically modified cells; bioactive

molecules, encompassing growth factors, cytokines, and signalling peptides; and pharmacological agents, such as small molecule drugs or targeted inhibitors. Furthermore, hydrogels can be functionalised with specific ions, such as strontium or calcium, known to exert osteogenic or osteoinductive effects.

Beyond mere delivery, these biomaterial platforms possess the inherent capacity to actively modulate the local tissue microenvironment. Their composition, mechanical properties, degradation kinetics, and surface functionality can be precisely tailored to influence critical processes such as inflammation, angiogenesis, and stem cell fate decisions. For instance, hydrogels can be designed to sequester pro-inflammatory cytokines, present anti-inflammatory signals, release reactive oxygen species (ROS)-scavenging compounds, or provide pro-angiogenic cues. This ability to locally reshape a hostile niche—counteracting chronic inflammation, mitigating oxidative stress, and promoting vascularisation—is paramount for enhancing regeneration in settings where endogenous healing mechanisms are severely impaired, such as in aged bone or osteoporotic fractures. Consequently, biomaterial scaffolds, and hydrogels in particular, represent a cornerstone of advanced bone tissue engineering, providing a multifunctional platform to deliver complex therapeutic regimens and actively engineer a microenvironment favourable for the restoration of

skeletal integrity^[108]. Their application is central to developing next-generation therapies aimed at overcoming the specific regeneration deficits outlined previously.

1.5 Endothelin signalling: from vascular activity to skeletal ageing

1.5.1 The Endothelin family

The endothelin family comprises three structurally similar 21-amino-acid peptides: endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3).

These potent vasoactive peptides are produced by the proteolytic cleavage of their respective pre-proendothelin precursors and exert their biological effects primarily by binding to two G-protein-coupled receptors, ETA and ETB^[109].

While all three isoforms share a common molecular architecture, their expression patterns and physiological roles are distinct. ET-1, the most widely studied and abundant isoform, is constitutively secreted by vascular endothelial cells and is a key regulator of vascular tone, cell proliferation, and fibrosis^[110].

ET-2, expressed mainly in the kidneys and intestine, is implicated in ovarian function and fluid homeostasis^[111]. ET-3 plays a critical role in the development of enteric neurons and melanocytes, with mutations in its pathway linked to Hirschsprung's disease^[112].

Within the context of skeletal degeneration and ageing, our research is particularly focused on ET-1 for several compelling reasons. Unlike ET-2 and ET-3, ET-1 is not only a potent vasoconstrictor but also a robust and versatile cytokine that is highly expressed within the bone microenvironment. Furthermore, the established link between vascular calcification and osteoporosis in the elderly positions ET-1, a central player in both vascular biology and bone metabolism, as a prime mechanistic candidate linking systemic cardiovascular ageing to skeletal frailty^[113]. Therefore, while the endothelin family has diverse functions, it is the dysregulation of the ET-1 pathway, sitting at the crossroads of vascular health and skeletal homeostasis, that presents a particularly promising and coherent target for understanding and potentially mitigating the molecular drivers of skeletal ageing.

1.5.2 ET-1: biosynthesis, regulation, and physiological roles

Endothelin-1 (ET-1) functions as a potent vasoactive peptide, chiefly synthesised by vascular endothelial cells that line blood vessels across the cardiac and circulatory system^[110]. Although multiple cell types contribute to its production—including pulmonary, renal, and colonic epithelial cells, macrophages, monocytes, enteric glia, choroid plexus, and specific neuronal and reactive glial cells in the central nervous system—the principal

investigative emphasis remains on its endothelial derivation. This focus stems from ET-1's indispensable function in vascular physiology, where its endothelial origin underpins its core regulatory mechanisms. The peptide's synthesis within these specialised cells remains a primary subject of research due to its profound influence on vascular tone and homeostasis. Consequently, while ET-1 exhibits a broad cellular expression profile, its endothelial biosynthesis remains the dominant paradigm in current scientific inquiry, reflecting its critical role in maintaining vascular integrity and function. This widespread cellular expression underscores ET-1's fundamental involvement in regulating perfusion across virtually all organs.

Within endothelial cells, ET-1 synthesis and release occur continuously, primarily governed at the transcriptional level. Several key stimuli significantly modulate ET-1 expression, notably haemodynamic shear stress, tissue hypoxia, thrombin exposure, and vasoactive compounds including angiotensin II (ANGII). Among these, shear stress—the tangential force generated by blood flow interacting with the endothelial surface—holds critical importance for sustaining vascular homeostasis. This mechanical stimulus achieves homeostasis by finely modulating the equilibrium between ET-1 synthesis and the production of vasodilatory nitric oxide (NO)^[114]. Conversely, hypoxia, a common feature in pathologies like tumours, significantly upregulates ET-1

expression by activating hypoxic response elements within its promoter, thereby accelerating disease escalation^[115]. Transforming growth factor-beta (TGF- β) constitutes a significant modulator of endothelin-1 biosynthesis, wherein activation of the ALK5/Smad3 signalling cascade within endothelial cells upregulates preproET-1 transcription through engagement of AP-1 and Smad binding elements within the gene promoter, thereby fostering endothelial maturation and function.

The biosynthesis of ET-1 within endothelial cells occurs through a sophisticated dual-mechanism secretory process governing its release. The constitutive pathway entails the continuous secretion of ET-1 from small vesicles, allowing for basal interaction with endothelin receptors and contributing to the regulation of vasomotor tone. The regulated pathway responds acutely to external stimuli by releasing stored ET-1 from specialised endothelial organelles known as Weibel-Palade bodies. Upon release, ET-1 acts in a highly localised manner. ET-1 released from the abluminal (basal) aspect of endothelial cells predominantly engages endothelin type A receptors (ETA; ET_AR) expressed on neighbouring vascular smooth muscle cells, inducing potent vasoconstriction. While endothelin type B (ETB; ET_BR) receptors are also present on smooth muscle in some vascular beds and can mediate constriction (either solely or in concert with ET_AR, depending on the vessel

type), ET_BR on endothelial cells themselves can bind ET-1 in an autocrine fashion. Activation of the endothelial ET_BR frequently mitigates vasoconstrictive effects by inducing the release of vasodilatory substances such as nitric oxide and prostacyclin. The intracellular trafficking pathways for the receptor-ligand complex exhibit marked divergence between receptor subtypes: following ligand binding, the ET-1/ETA complex characteristically undergoes endocytosis into endosomal compartments, succeeded by receptor recycling back to the plasma membrane. This recycling mechanism permits ETA receptor antagonists to effectively counteract ET-1-mediated physiological responses. Conversely, the ET-1/ETB complex is internalised and subsequently directed towards degradation within lysosomes, a process that renders ETB receptor antagonists incapable of displacing the bound ligand. Detectable concentrations of both the mature, biologically active big ET-1 and its inactive precursor molecule circulate within the plasma compartment. Big ET-1 is further processed into the active mature peptide by endothelin-converting enzymes (ECE) present on vascular smooth muscle cells, adding another layer of regulation to this potent signalling system. This intricate network of biosynthesis, stimulus-dependent regulation, targeted secretion, specific receptor binding, and complex ligand-receptor trafficking underscores the sophisticated mechanisms governing ET-1 signalling, which is central to

maintaining vascular haemostasis and whose dysregulation underpins numerous cardiovascular pathologies. Understanding these detailed regulatory mechanisms in both health and disease states provides crucial insights for developing targeted therapeutic interventions.

1.5.3 Endothelin receptors: expression and signalling diversity

The expression and functional roles of endothelin receptors, ET_AR and ET_BR, exhibit significant diversity across tissues and species, underpinning the complex physiological and pathological actions of endothelin signalling. In adult mouse tissues, a distinct expression pattern is observed whereby ET_AR localisation occurs primarily within vascular smooth muscle cells, whereas ET_BR expression is largely confined to endothelial cells^[116; 117]. Analysis of messenger RNA (mRNA) levels demonstrates that ET_AR expression is notably prominent within cardiac and pulmonary tissues relative to other organs, while comparatively reduced levels are detectable within the central nervous system (CNS). This differential expression pattern underscores the fundamental significance of the endothelin signalling pathway, specifically ETA-mediated signalling, for cardiovascular function. This importance is further evidenced by the observation that ETA mRNA constitutes one of the most prevalent G-protein-coupled receptor (GPCR) transcripts found within cardiac tissue.

Conversely, ET_BR demonstrates their highest expression density within the brain, specifically concentrated in neuronal regions as confirmed by radioligand binding studies.

The relative expression ratio of ET_A to ET_B receptors exhibits considerable variation across different human tissues^[118]. This receptor expression diversity extends significantly to the skeletal system and is highly relevant to bone pathology. While ET_AR is expressed on osteoblasts and can inhibit bone formation, the expression of ET_BR is observed to be significantly prominent across SSCs residing in the bone marrow niche, as well as on osteoblasts and endothelial cells constituting the bone's vascular network^[119]. The signalling pathways engaged by these receptors dictate their impact on bone homeostasis. Activation of the ET_AR typically involves coupling to G α _q/11 proteins. This coupling stimulates phospholipase C beta (PLC β), resulting in the generation of inositol triphosphate (IP3) and diacylglycerol (DAG). These second messengers subsequently trigger intracellular calcium mobilisation and protein kinase C (PKC) activation. Within the context of bone biology, this signalling cascade has been associated with the promotion of osteoblast apoptosis and the inhibition of osteoblast differentiation. In contrast, ET_BR signalling exhibits greater complexity. ET_BR activation can potentially couple to G α _{i/o} proteins, leading to the inhibition of adenylate cyclase and a consequent reduction in

cyclic adenosine monophosphate (cAMP) levels. Alternatively, ET_BR can also couple to G_{αq}/11 proteins. The specific G-protein coupling and downstream signalling pathway engaged by ET_BR are highly dependent on the cellular context in which the receptor is expressed.

This endothelin-driven cascade represents a critical mechanism directly linking dysregulated endothelin signalling on SSCs, to the imbalance in bone remodelling, microarchitectural deterioration, and net bone loss characteristic of age-related and pathological osteoporosis. Therefore, understanding the specific expression and signalling diversity of ET receptors is fundamental to elucidating the molecular basis of bone degeneration and developing targeted interventions through endothelin antagonists.

1.5.4 Endothelin Receptor Antagonists: BQ123 and BQ788

To dissect the distinct roles of the two primary endothelin receptor subtypes—ETA and ETB—highly selective peptide antagonists are indispensable pharmacological tools. Among these, BQ123 and BQ788 are widely used in research due to their well-characterised and profound receptor specificity.

BQ123 is a cyclic pentapeptide functioning as a potent, competitive antagonist with exceptional selectivity for the ETA receptor. Its specificity for ETA over ETB is remarkably high, with studies demonstrating a selectivity ratio ranging

from approximately 1,000-fold to over 20,000-fold depending on the tissue and species examined^[120; 121]. This makes BQ123 an unparalleled tool for cleanly isolating ETA-mediated pathways, which are of paramount interest in skeletal ageing due to the receptor's expression on osteoblasts and osteoclasts and its established role in promoting bone resorption and modulating formation^[122].

BQ788 is a modified tripeptide recognised as a selective competitive antagonist for the ETB receptor. It is frequently cited as having a 100- to 300-fold greater affinity for ETB over ETA in various pharmacological assays, particularly in rodent models and certain cell lines^[121; 123]. Despite this variability, BQ788 remains the antagonist of choice for probing ETB function in most experimental systems, as it consistently and effectively blocks ETB-mediated responses, such as the clearance of ET-1 from the circulation and specific intracellular signalling cascades^[124].

With respect to potential clinical or systemic adverse effects arising from the use of these selective antagonists, it is crucial to consider the physiological consequences of blocking distinct endothelin pathways. For BQ123, as ETA activation is a fundamental mediator of vasoconstriction and vascular smooth muscle proliferation, its antagonism predictably leads to vasodilation^[125]. In a therapeutic context, this can manifest as a significant and potentially

problematic decrease in systemic vascular resistance and arterial blood pressure, particularly in normotensive individuals or those with compromised vascular autoregulation^[126]. Furthermore, by selectively inhibiting the dominant vasoconstrictor ETA pathway, BQ123 may unmask or potentiate ETB-mediated effects, which in some vascular beds can include the release of nitric oxide and prostacyclin, leading to further vasodilation^[127]. This haemodynamic shift requires careful monitoring, as it could induce reflex tachycardia or, in susceptible patients, provoke organ hypoperfusion^[128].

For BQ788, the adverse effect profile is intrinsically different and dominated by the consequences of ETB receptor blockade. Administration of BQ788 predictably results in a rapid and sustained increase in plasma ET-1 concentrations^[123]. This elevated ET-1 is then free to act exclusively on unopposed ETA receptors, leading to a paradoxical exacerbation of ETA-mediated effects. Clinically, this could translate into acute vasoconstriction, increased systemic and pulmonary vascular resistance, and a resultant rise in arterial blood pressure^[126]. This effect is particularly hazardous in conditions characterised by pre-existing endothelial dysfunction or hypertension.

Additionally, as ETB activation contributes to natriuresis and diuresis in the kidney, its inhibition may promote sodium and water retention, potentially worsening fluid balance in patients with cardiac or renal impairment^[129]. Thus,

while BQ788 is a valuable research tool, its clinical translation is complicated by this self-defeating pharmacological action, which could worsen the very pathologies an endothelin antagonist might aim to treat.

1.5.5 ET-1 in vascular dysfunction and the ageing bone niche

ET-1 is an emerging target of interest in age-related studies. Endothelial cells can secrete a variety of biologically active substances into the niche, including ET-1, responsible for regulating inflammation, vascular tone and cell growth. ET-1 is recognised as a potent vasoconstrictor. When diabetes, high blood pressure, and ageing progress, it is upregulated^[130]. This peptide mediates its effects through two distinct G-protein coupled receptor subtypes, designated ET_AR and ET_BR^[131]. Both ET_AR and ET_BR receptors are activated by phospholipase C, contributing to the increased myosin kinase phosphorylation and cytosolic calcium, resulting in the prolonged effect of smooth muscle contraction and dilation. The net impact of ET-1 production is determined by the balanced activity of ET_A and ET_B receptors. Under normal physiological circumstances, ET-1 is secreted at low basal levels, functioning primarily through paracrine signalling mechanisms. However, under pathological conditions, ET-1 is secreted in large amounts. Elevated secretion and heightened biological activity of ET-1 can precipitate endothelial dysfunction.

This pathological state is strongly associated with the development of conditions including hypertension and cardiovascular disease, pathologies whose prevalence escalates with ageing. An increased plasma ET-1 concentration is also observed in elderly humans^[132]. Previous research has demonstrated that endothelin-1 (ET-1) significantly stimulated both reactive oxygen species (ROS) generation and hypertrophic cell enlargement^[133].

It has been extensively discussed how ET-1 controls osteoblast activity and helps maintain bone homeostasis. It was discovered that mice lacking ET-1 had craniofacial deformities, low bone mass, and mandibular hypoplasia. This suggests that ET-1 potentially plays a role in the intramembranous ossification of flat bones. Trabecular bone mass and long bone volume were markedly decreased in mice following specific inactivation of ET_AR in osteocalcin-positive osteoblasts^[134]. This implies that ET-1 is a key regulatory compound in the postnatal development of long bones and may be involved in the endochondral ossification procedure. Additionally, male and female mice responded differently to ET_AR knockdown. This gender disparity does suggest that ET-1 signalling and sex hormones, particularly androgens, interact to mediate bone metabolism. To obtain concrete proof from castration and ovariectomy models, more research is necessary.

Uncertainty exists regarding how ET-1 overexpression affects age-related bone remodelling. There has been much discussion about ET-1's function in regulating osteoblastic activity and preserving bone homeostasis. The focus of research has been on determining how ET-1 affects chondrocytes, osteoblasts, and osteoclasts. It was shown that exogenous ET-1, even at low doses, promoted osteoblast precursor differentiation and proliferation^[135]. ET-1 has been implicated in facilitating intramembranous ossification within flat bones. When ET_AR was specifically deactivated in osteocalcin expressing osteoblasts, the volume and quality of trabecular bone in the mice were substantially reduced^[134]. The evidence regarding ET-1 function in osteoclasts is less clear than that for osteoblasts. In one study, overexpressing ET-1 inhibited cell motility and reduced bone resorption caused by osteoclasts^[136], whereas another study reported increased bone resorption by osteoclasts via ET_AR^[137]. Nevertheless, ET-1 in bone is important for regulating bone remodelling through osteoblasts and osteoclasts. Chondrocytes in the joints, along with synovial inflammation and fibrosis, are all directly impacted by ET-1. Except in cases of disease or normal ageing, articular chondrocytes hardly ever express ET-1. Age and a series of cytokines and growth factors, such as platelet-derived growth factor-BB (PDGF-BB), TGF- β , TNF- α , and IL-1, also affect the amount of ET receptor expression on articular chondrocytes^[138]. ET_AR is the primary ET-1

binding site. There are roughly twice as many ET-1 binding sites on older chondrocytes as there are on younger chondrocytes^[139].

However, the information on bone precursor cells is limited. MSCs are important osteoprogenitors in the bone formation process, residing in highly vascularised bone marrow within a close niche with epithelial cells. The accumulation of endothelial senescent cells promotes SASP generation and overexpression of ET-1^[140], which poses significant local and systemic implications for the tissue microenvironment. The endogenous and exogenous effects of signalling ET-1 are usually coupled in action. The impact of secreted ET-1 in the bone niche and the resultant altered microenvironment on SSCs growth warrants further exploration.

1.6 Research gap and significance: targeting the vascular-skeletal axis

Despite the established role of vascular ageing in skeletal decline and the known impact of ET-1 on vasculature, the specific role of EC-derived ET-1/ET_BR signalling in directly driving perivascular SSC senescence and bone loss, and its viability as a focus for therapeutic intervention in osteoporosis, remained a critical unanswered question.

There are two major challenges in the current age-related osteoporosis studies: the absence of early, non-radiographic diagnostic criteria and the lack of long-term synthetic treatment with minimal side effects. The lack of reliable early diagnostic indicators for osteoporosis means individuals at elevated fracture risk often escape timely identification, precluding opportunities for early preventative interventions. Most clinical determining symptoms are only visible through radiographic analysis when severe bone loss has already occurred. Therefore, many may miss the best opportunity to intervene and treat the disorder promptly. In terms of the fundamental research at the cellular level, the role of progenitor cells in the bone niche is not well understood. While the capacity of SSCs for self-renewal and differentiation into mature bone cell lineages is established, the specific nature of their interactions with other cell populations within the bone microenvironment remains poorly understood. Beyond their intrinsic regenerative properties, the precise crosstalk and signalling dynamics between SSCs and neighbouring cells in this specialised niche are not yet fully elucidated. There is also a large knowledge gap in the relationship between ET-1 and bone stem cells. Research on ET-1 remains predominantly focused on its role in vascular pathophysiological aspects, while investigations into the relationship between ET-1 and the skeletal system remain comparatively scarce.

Therefore, in this study, we propose to target these research gaps through the following actions. The initial phase of this research will examine whether endothelial cells exhibit dysregulated expression of ET-1 and whether this contributes to structural bone degeneration in ageing individuals. Subsequently, we aim to elucidate the contribution of senescent bone cells, specifically senescent stem cells, to age-associated bone loss and delineate the molecular mechanisms underpinning this relationship. Second, we will explore the underlying mechanism and the possibility of establishing a senescence model via ET-1, and determine the similarities or differences compared to the conventional senescence models, such as the replicative senescence model and H₂O₂-induced stress model, in terms of both biological and mechanical characteristics. Third, it is vital to identify a novel target in the biochemical processes that could slow or prevent bone loss due to old age by reducing the aggregation of senescent bone cells in the elderly to restore bone health.

1.7 Hypothesis, objectives, and therapeutic translation

This study hypothesized that endothelial dysfunction leading to increased ET-1 secretion activates ET_BR on perivascular skeletal stem cells (SSCs), driving senescence through ROS accumulation and thereby promoting osteoporosis, and that blocking this axis would represent a novel therapeutic strategy.

1) Investigate ET-1/ET_BR involvement in age/OVX-related bone loss; 2) Define the ROS-mediated senescence mechanism in SSCs; 3) Assess the therapeutic effect of ET_BR deletion/inhibition in vivo; 4) Develop and test targeted ET_BR antagonist delivery systems for regeneration.

Chapter 2 ET-1/ET_BR Signalling Induces SSC

Senescence And Bone Loss

2.1 Introduction

Bone constitutes a rigid yet dynamic system facilitating essential mechanical and homeostatic functions, encompassing locomotion, load bearing, organ protection, and serving as a reservoir for calcium and marrow^[141]. Skeletal integrity relies fundamentally on the equilibrium between bone resorption and formation processes^[142], crucial for preserving both bone strength and volume. Disruptions within the bone microenvironment can ultimately precipitate bone disorders. Osteoporosis, a systemic skeletal condition defined by reduced bone mass and microarchitectural deterioration^[143], places a substantial burden on ageing populations^[14], especially postmenopausal women. This disease pathogenesis stems from an imbalance in bone remodelling^[144], involving the dynamic interplay between osteoclast-driven bone resorption and osteoblast-mediated bone formation^[145].

Current therapies targeting these effector cells mainly focus on slowing the progression of the disease with few targets aimed at bone restoration, underscoring the need to explore upstream mechanisms driving cellular

dysfunction. Emerging evidence implicates cellular senescence, a state of irreversible growth arrest marked by pro-inflammatory secretome release, as a critical yet underexplored contributor to skeletal ageing^[140]. Senescent cells accumulate in ageing bone, disrupting tissue homeostasis and propagating a toxic microenvironment through SASP^[18]. However, the triggers of skeletal stem cells (SSCs) senescence and their interplay with vascular ageing remain poorly investigated.

ET-1 is a potent vasoconstrictor^[146], elevated in ageing and endothelial dysfunction, which emerges as a compelling candidate bridging vascular and skeletal disorders. Best known for its roles in hypertension and cardiovascular disease^[130], ET-1 signals through ET_AR and ET_BR to regulate diverse processes, including oxidative stress and cell survival^[133]. SSCs reside in a perivascular niche, directly exposed to endothelial-derived factors. Age-related endothelial senescence drives ET-1 overproduction, creating a localised milieu conducive for paracrine disruption of SSCs function^[147]. While low ET-1 doses promote osteoblast precursor activity, chronic ET-1 exposure—as seen in ageing—may paradoxically induce SSCs senescence via reactive oxygen species (ROS) accumulation, a hallmark of stem cell exhaustion^[132]. In bone, ET-1 modulates osteoblast-osteoclast crosstalk^[148], yet its influence on SSCs—the osteoprogenitors responsible for lifelong bone formation—remains unknown.

Here, we unveiled ET-1/ET_BR signalling as a linchpin mechanism coupling vascular ageing to osteoporosis. We demonstrated that ET-1 overexpression in aged and ovariectomized (OVX) mice drives ET_BR upregulation in SSCs, triggering ROS-mediated senescence and impairing osteogenic capacity. Genetic knockout of ET_BR in Prx1⁺ SSCs or pharmacological inhibition with the antagonist BQ788 rescues senescence, restores bone formation, and reverses skeletal deterioration. By identifying ET_BR as a master regulator of Prx1⁺ SSCs fate, this work refined osteoporosis pathogenesis through the lens of stem cell-vascular crosstalk and positioned ET-1 blockade as a potential therapeutic strategy to rejuvenate aged bone.

This chapter comprises of two major models: the *in vivo* bone degeneration model in mice and the *in vitro* cell culturing model. The animal model will help to identify the biological and mechanical phenomena and characteristics of bone in aged mice, beneficial for finding correlations between characteristics, e.g., longitudinal relationship of plasma ET-1 concentration, presence of ET receptors and reactive oxygen species with age. The animal model is also responsible for validating the treatment effect of our target. The cell culturing model allows specific demonstration of ET-1 overexpression on MSCs fate, advantageous in investigating the role of individual ET receptors and possible signalling pathways. The cell culturing model also serves as a pilot study for

the treatment effect of ET receptor inhibitors before administration them on the animal model.

2.2 Methodology

2.2.1 Human single-cell RNA sequencing analysis

Public scRNA-seq datasets were utilised to investigate endothelin signalling in the human skeletal microenvironment. The analysis integrated data from four Gene Expression Omnibus (GEO) accessions, selected to encompass a spectrum of bone states. The dataset GSE169396 included unsorted cells from the femoral head of two healthy adult donors (aged 31 and 45), providing a baseline of normal adult bone cell heterogeneity. To represent pathological states, we incorporated GSE147287, which contains CD271+ bone marrow mononuclear cells from an osteoporosis patient, and GSE147390, consisting of ALPL-high/CD45-low cells from an osteopenia patient. The inclusion of both osteopenia and osteoporotic samples was a deliberate strategy to identify pathways active across the continuum of bone loss, rather than solely in its most severe form. Furthermore, we included data from GSE143753, derived from human embryonic limb development. Whilst primarily developmental, this dataset was valuable as it provided a well-defined reference of primordial skeletal progenitor states; its integration allowed us to verify that core skeletal

stem cell identities, as defined by key markers such as PRRX1, PDGFRA and THY1, were consistently identified following harmonisation, serving as an internal control for the integration process.

Digital gene expression (DGE) matrices were obtained from Gene Expression Omnibus (GEO). Analysis of all scRNA-seq data was performed using the Seurat R package, version 4.5.0. To integrate the datasets and identify shared biological states across them, the functions *FindIntegrationAnchors* and *IntegrateData* were employed. This integration process utilised the top 2,000 highly variable genes to anchor the datasets and establish a common correlation structure. Following successful integration, unsupervised clustering was applied to the integrated data matrix to categorise individual cells into distinct clusters according to their transcriptional profiles. For visualisation purposes, principal component analysis (PCA) was conducted on the integrated data. The top 30 principal components derived from the PCA were subsequently used as input for non-linear dimensionality reduction. This reduction was achieved using the t-distributed stochastic neighbour embedding (t-SNE) algorithm, projecting the high-dimensional data into a two-dimensional space for visual interpretation. Statistical analysis to identify differentially expressed genes between the defined cell clusters was carried out using the Wilcoxon rank-sum test. This test

was implemented within Seurat's *FindMarkers* function. Expression of marker genes was visualised using the *FeaturePlot* and *VinPlot* functions.

2.2.2 Natural ageing mouse model

All experimental animals were housed under controlled conditions featuring a constant ambient temperature of 25°C and a regulated 12-hour light/dark cycle. They were provided with ad libitum access to standard laboratory feed and drinking water. Female Balb/c mice, procured from CAF (The Hong Kong Polytechnic University), were maintained until reaching the designated ages of 6 months and 18 months for the study. All procedures involving animals received formal approval from the Animal Subjects Ethics Sub-Committee (ASESC 19-20/13-BME-R-GRF) and were conducted in strict compliance with the guidelines stipulated under the Animal (Control of Experiments) Ordinance Cap. 340, operating under licence number (20-257) in DH/HT&A/8/2/4 Pt.4.

2.2.3 ET-1 overexpression transgenic mouse model

The transgenic mouse model utilised in this investigation, specifically the TET-1 line characterised by endothelial cell-specific overexpression of Endothelin-1 (ET-1), was generously provided by Professor Sookja Kim Chung of the University of Hong Kong. This valuable model was generated through microinjection techniques, incorporating murine ET-1 cDNA along with an

SV40 polyadenylation sequence, under the transcriptional control of the Tie-1 promoter to ensure endothelial specificity. Prior to their use in the experimental procedures detailed within this thesis, rigorous genotyping was performed on all animals to confirm the presence of the transgene and ensure the genetic homogeneity of the cohort employed, as referenced^[149]. The experimental cohort comprised four TET-1 transgenic mice and four age-matched wildtype control mice, all of which were female and aged 9 months at the commencement of the study.

2.2.4 Ovariectomy-induced bone loss

Female C57BL/6J mice were purchased from GemPharmatech Co., Ltd and raised in Southern University of Science and Technology. All animal procedures conducted in this study received formal approval from the Animal Ethics Committee of the Southern University of Science and Technology (Approval No. SUSTech-JY202303086). These procedures were conducted in strict compliance with the national standard governing the ethical review of laboratory animal welfare (GB/T 35892-2018) and were performed under licence (Licence No. Larc-2022-155). Female C57BL/6J mice with 14-week-old were anesthetized and bilaterally ovariectomized (OVX group) or sham

operated (control group). Blood and leg bone samples were harvested at 22-week-old for further analysis.

2.2.5 Micro-computed tomography (μ CT) analysis of tibia subchondral bone

Tibial bones harvested from mice underwent micro-computed tomography (μ CT) scanning utilising the Viva CT40 system (Scanco Medical AG, Switzerland). Image acquisition parameters were configured with a nominal resolution of $18\mu\text{m}$ and an isotropic voxel dimension of $10.5\mu\text{m}$. Scanning was performed employing an X-ray source voltage of 70 kV in conjunction with a 1.0 filter. Following data acquisition, volumetric image datasets underwent standardised reconstruction procedures using the SkyScan recon software. Subsequent quantitative analysis of the reconstructed datasets for each experimental cohort was conducted using the SkyScan CT-analyzer software package. The region of interest in trabecular bone selected for analysis was 150 slices (1.575mm) of the metaphyseal secondary spongiosa, originated 50 slices (0.525mm) below the growth plate. Within this ROI, the parameter bone volume fraction (BV/TV) was calculated and recorded for each specimen.

2.2.6 Quantification of plasma serum ET-1 concentration

The concentration of endothelin-1 (ET-1) within mouse plasma samples was determined utilising a commercially available Endothelin-1 ELISA kit (Abcam, ab133030), strictly adhering to the manufacturer's supplied protocol. Whole blood was collected from the experimental animals on the designated terminal experimental day, coinciding with sacrifice. These collected blood samples underwent centrifugation at 4°C for a duration of 15 minutes, applying a relative centrifugal force of 1,600g. Following centrifugation, the supernatant plasma fraction was carefully harvested. This isolated plasma was subsequently employed as the sample matrix for the precise quantification of ET-1 concentration via the ELISA procedure. The resultant ET-1 concentration data, derived from the plasma samples, are presented as the mean $\pm$ standard deviation (mean SD).

2.2.7 Histology analysis of mice tibia bone

Tissue samples underwent fixation and embedding according to established laboratory protocols. Serial sections of 5 μ m thickness were prepared using a microtome. To evaluate the histomorphology of knee joint sections, samples mounted on glass slides were stained with haematoxylin and eosin (H&E) following standard staining protocols.

For immunofluorescence detection of specific proteins, tissue sections underwent antigen retrieval followed by quenching of endogenous peroxidase activity. After blocking non-specific binding sites, sections were incubated with primary antibodies overnight at 4°C. The primary antibodies employed included: Endothelin 1 (1:1000; Abcam, ab2786), Endothelin type A Receptor (1:1000; Abcam, ab117521), Endothelin type B Receptor (1:1000; Abcam, ab117529), 4-Hydroxynonenal (4HNE) antibody (1:200; Abcam, ab46545; Invitrogen, MA5-27570), p16INK4a antibody (1:200; Abcam, ab211542), and PRRX1 antibody (1:500; Invitrogen, PA5-106700). Positive signals were visualised using fluorescent secondary antibodies: Donkey anti-Rabbit IgG H&L (DyLight® 488), Goat anti-Mouse IgG (H+L) (Alexa Fluor® 488), and Goat anti-Mouse IgG (H+L) Superclonal™ (Alexa Fluor® 594). Co-staining for ETBR and PRRX1 utilised the LinKine™ AbFluor™ 488 Labeling Kit (Abbkine, KTL0520) and LinKine™ AbFluor™ 594 Labeling Kit (Abbkine, KTL0540). Negative control sections underwent identical processing steps with omission of the primary antibodies. Quantitative analysis, assessing either the percentage of positive cells or fluorescence intensity, was performed where applicable. Fluorescence images were captured using a Leica TCS SPE Confocal Microscope and a Nikon ECLIPSE Ti2-E microscope at 20x and 40x magnifications.

Immunohistochemistry employed the DAB Substrate Kit (Vectorlabs, SK-4100) for visualisation. Sections were incubated overnight at 4°C with the following primary antibodies: p16 antibody (1:400, Abcam, ab189034), p21 antibody (1:400, Abcam, ab188224), p53 antibody (1:400, Abcam, ab188224), and sp7 antibody (1:1000, Abcam, ab209484). Tartrate-resistant acid phosphatase (TRAP) activity was detected using a working solution prepared with Sodium Acetate Trihydrate (Sigma, S9513), Sodium Tartrate Dihydrate (Sigma, S4797), Fast Red violet LB salt (Sigma, F3381), Naphthol AS-MX phosphate (Sigma, N4875), N,N-Dimethylformamide (Sigma, D4551), and Manganese(II) Chloride tetrahydrate (Sigma, M3634). Sections were counterstained with haematoxylin (Baso, BA4021A). Brightfield images of immunohistochemistry and TRAP-stained sections were acquired using an Olympus BX53 Microscope at 10x magnification.

Images were quantified with ImageJ (version 1.54g). For fluorescence images, parameters including mean grey value, area, median, min and max intensity values were measured for region of interest (ROI). A blank area (with no signal) near ROI was measured and subtracted from ROI's mean grey value for corrected intensity. Signal threshold and background area were kept consistent. For DAB-stained images, parameters area, area fraction and mean grey value

were measured. Threshold was carefully adjusted to highlight brown signals while minimising background.

2.2.8 MSCs senescence model

Commercially-available murine mesenchymal stem cells (mMSCs) cell line was obtained (OriCell Strain Balb/c bone mesenchymal stem cell; Cyagen, MUCMX-01101). Throughout the length of study, MSCs were frequently characterized via flow cytometry using specific markers as recommended by the producing manufacturer (CD29⁺, CD44⁺, Sca-1⁺ >70%; CD117⁻ <5%).

MSCs were cultivated in 75 cm² flasks utilising Dulbecco's Modified Eagle Medium (DMEM 1x, Gibco; C11995500BT) supplemented with 10% fetal bovine serum (Gibco; 10270106) and 1% penicillin-streptomycin (Gibco; 15140122). Cultures were maintained under standard incubation conditions of 37°C and 5% CO₂. Throughout the duration of the study, the identity and characteristics of the MSCs were regularly verified using flow cytometry, employing specific markers as stipulated by the manufacturer's recommendations.

Senescence Induction: MSCs are treated separately with either H₂O₂ or ET-1. The negative control in this study will consist of untreated MSCs. H₂O₂ is an established oxidative stress-induced senescence model which has been applied

in studies on senescent cells for a long time, it serves as a positive control in this study. At around 80% confluency, either H₂O₂ (Merck, #1.07298.0250) or ET-1 (Sigma, B157-200UG), is introduced into the culture medium for senescence induction. Without replacing culture medium, MSCs are treated for 24 hr at a concentration of 100µM H₂O₂ or 100nM ET-1. Finally, cells are harvested using 0.05% Trypsin-ethylenediaminetetraacetic acid (Gibco, 25300-062).

Pre-treatment of ET-1 antagonists BQ123 and BQ788: After seeding cells and incubating to a confluency of around 80%, treatments will be introduced prior to the addition of ET-1. BQ123 (Sigma, B150-200UG) and BQ788 (Sigma, B157-2000UG) are pre-treated for 30 min, followed by senescence induction using ET-1 for 24 hr.

Cell viability is determined by trypan blue staining where equal volume of trypan blue (Gibco, 15250-061) and cells are mixed. Percentage of cells-stained trypan blue positive and negative will be determined using the hemocytometer by manual counting. Cells with viability > 90% are considered in good quality and used for experiments.

Cell size is estimated with the Countess II FL Automatic Cell Counter (Invitrogen). 10 μ l of cells (1×10^4 cells/mL) is loaded onto the disposable cell counting chamber slide (Invitrogen #C10228) for each test.

RNA sequencing was performed by Personal Biotechnology Co., Ltd. (China).

Samples were prepared by lysing untreated, ET-1 induced and BQ788 pre-treated ET-1 induced murine MSCs, three biological repeats were collected in each group.

2.2.9 Evaluation of cellular senescence

Cellular lysates were prepared for quantitative polymerase chain reaction (qPCR) analysis. Total RNA extraction was performed utilising the Total RNA Kit I (Omega Bio-Tek) strictly according to the manufacturer's guidelines. The purity and concentration of the isolated RNA were assessed spectrophotometrically using a Nanodrop instrument. Only RNA samples meeting stringent quality criteria, specifically exhibiting absorbance ratios (A260/280 and A260/230) approximating 2.0, were deemed suitable and selected for further processing. Subsequently, complementary DNA (cDNA) synthesis was carried out via reverse transcription of the qualified RNA samples, employing the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Finally, qPCR amplification was performed on the generated cDNA

templates. This amplification utilised specific primer sets designed for the target genes of interest: p16INK4a, ET-1, ETAR, and ETBR (Sigma-Aldrich, sequences detailed in Table 1), alongside primers targeting the reference gene GAPDH. The quantification of relative mRNA expression levels for the target genes was determined by comparing the cycle threshold (Ct) values obtained during the exponential phase of amplification. These Ct values were normalised to the corresponding Ct values of the GAPDH reference gene within each sample. The fold change in mRNA expression for the target genes was then calculated relative to the expression level of GAPDH, using the comparative Ct method ($\Delta\Delta C_t$ method).

Table 1 List of primers

Primer	Foward	Reverse
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-GGGGTCGTTGATGGCAACA-3'
p16	5'-GAACTCTTTCGGTCGTACCC-3'	5'-CGAATCTGCACCGTAGTTGA-3'
ET-1	5'-TTTCCCGTGATCTTCTCTCTGC-3'	5'-CTGAGTTCGGCTCCCAAGAC-3'
ET ^A R	5'-ATGAGTATCTTTTGCCTTGCGG-3'	5'-GTCTTCCATGTGGCTGCTTAG-3'
ET ^B R	5'-AAGCCACGCTGTCACTTCTC-3'	5'-GAGGAACGCATCAGACTGGA-3'

Cellular proliferation rates were quantified using the colorimetric MTT assay, performed with Cell Proliferation Kit I (Roche, 11465007001) according to the manufacturer's protocol. Cells were harvested and subsequently resuspended in complete culture medium at a standardised density of 2×10^4 cells per millilitre. A precise volume of 100 microlitres (μ l) of this cell suspension was

dispensed into each well of a 96-well microplate. Following an initial 24-hour incubation period to facilitate cell attachment, experimental treatments, namely H₂O₂ or ET-1, were administered to the designated wells. Upon completion of the treatment phase, 10 µl of the MTT reagent solution was introduced into every well. The plates were then incubated for a further 3-hour period to allow for formazan crystal formation. After this incubation, the supernatant medium containing the MTT reagent was carefully aspirated, taking meticulous care not to disturb the insoluble purple formazan crystals deposited at the base of the wells. To solubilise these crystals, 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of the resulting solution within each well was subsequently measured using a BioTek microplate reader, with the optical density recorded at a wavelength of 590 nanometres (nm). The cellular proliferation rate for each experimental condition was ultimately calculated relative to the baseline absorbance value obtained from control wells containing cells seeded for only the initial 24-hour period prior to any treatment.

Cellular senescence was visualised using the Senescence-Associated β-Galactosidase (SA-β-gal) Staining Kit (Cell Signaling Technology, #9860), performed at pH 6.0. Cells were plated at a density of 1×10^4 cells per well in 12-well culture plates. Following the staining procedure, samples were sealed with Parafilm® to create a closed environment and incubated at 37°C without

supplemental CO₂ for a duration of 16-18 hours (overnight). Visualisation of stained samples was subsequently performed using bright-field microscopy. Senescent cells were identified by the development of a distinct blue chromogenic precipitate within their cytoplasm. Micrographs were acquired using a Nikon Eclipse 80i Microscope (Nikon, Japan) to document the staining results.

2.2.10 Evaluation of mitochondrial oxidative stress

MSCs were plated within 12-well plates containing circular glass coverslips. Following a 24-hour incubation period to allow cell adherence, the existing culture medium was aspirated. Each well, ensuring full coverage of the coverslips, then received 1 mL of a working solution of MitoSOX Red reagent (ThermoFisher, M36008). This solution was prepared by diluting the reagent to a final concentration of 5 μ M using Hanks' Balanced Salt Solution (HBSS) supplemented with calcium and magnesium. Cells were subsequently incubated with this working solution for precisely 10 minutes at 37°C, while crucially maintaining conditions of darkness throughout this period to prevent photobleaching. Following incubation, the MitoSOX working solution was carefully removed. The cells were then subjected to three sequential washes using fresh HBSS (with calcium and magnesium) to eliminate residual, unbound

dye. The coverslips, now containing the stained MSCs, were carefully mounted onto glass microscope slides using ProLong Gold Antifade Mountant (ThermoFisher, P36935) containing DAPI nuclear counterstain. After allowing the mounting medium to cure and dry appropriately, the prepared slides were examined using fluorescence microscopy. Image acquisition was performed using a Leica TCS SPE Confocal Microscope. The resulting fluorescence images were subsequently analysed quantitatively using the ImageJ software package to assess mitochondrial superoxide levels.

2.2.11 Flow cytometry

Cells were separated using the cell sorter (BD FACSAria III) according to their expression of uPAR (Abcam, ab103791). The integrated real-time flow cytometry software analysis was used to configure the sample sorting process. At the outlet, uPAR positive and negative cells were collected in separate tubes. Cellular signals were detected and analysed using flow cytometry analysis. The cell complexity versus size (SSC vs FSC) scatter plot was used to visualize the signalling graph and determine the range of signal for the cells of interest. Due to mMSCs being a commercialised cell line with innate green fluorescence, the FITC signalling graph was also examined in order to cross-validate for the

specific events that correspond to the cells in the samples. APC signalling graph was used to identify the uPAR-positive cells marked by red fluorescence.

2.2.12 Mechanical performance

Cellular stiffness assessment employed a Bruker Catalyst atomic force microscope (AFM) integrated with a Nikon inverted microscope. Silicon nitride cantilevers from the MLCT series, possessing a nominal spring constant of 0.02 N/m, were utilised for these measurements. Prior to data acquisition, calibration of the probe's sensitivity and spring constant was performed directly within the culture medium. To identify suitable measurement locations on individual cells, the nucleus was first located using the inverted microscope's bright-field imaging capability. The indentation point was selected as the midpoint between the nucleus centre and the cell boundary. Given that cells typically exhibit viscoelastic properties, they can be approximated as linearly elastic materials under conditions of low strain (<10–15%) and slow loading rates (1 $\mu\text{m/s}$), as referenced^[150]. For each cell analysed, five distinct force-indentation curves were recorded from points surrounding the chosen indentation location. The Young's modulus values were subsequently derived using Bruker's Nanoscope Analysis 1.9 software, applying Sneddon's modification of the Hertzian model for data fitting. To minimise potential confounding factors, all stiffness

measurements were conducted on cells maintained at room temperature and completed within a one-hour timeframe following their removal from incubation. Throughout the AFM measurement process, no observable alterations in cell morphology occurred. A total of fifty individual measurements were compiled for subsequent analysis.

2.2.13 Statistical analysis

All experimental data are presented as mean values accompanied by the standard deviation (SD). Comparative analyses between groups for signal intensities and histomorphometry parameters employed either unpaired two-tailed t-tests or one-way/two-way analyses of variance (ANOVA), selected based on the experimental design and data structure. Where ANOVA identified statistically significant overall differences between groups, appropriate post hoc tests were performed to delineate specific group differences. The relationship between circulating plasma ET-1 concentration and bone volume fraction was assessed using Pearson's correlation coefficient. Statistical significance was defined as a p-value less than 0.05 ($p < 0.05$). The symbol 'n' denotes the number of biologically independent replicates or samples within each experimental group. Statistical computations and graphical representations were generated

using GraphPad Prism software (Version 10.3.1, GraphPad Software, CA, USA).

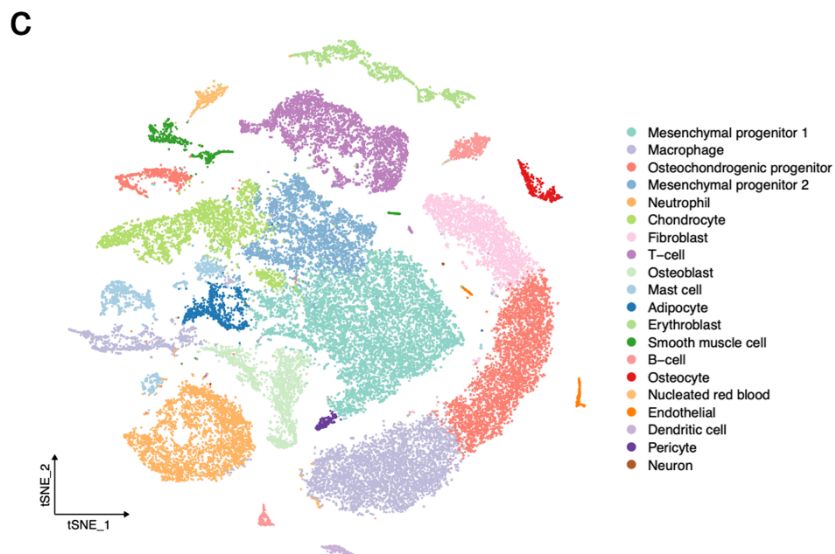
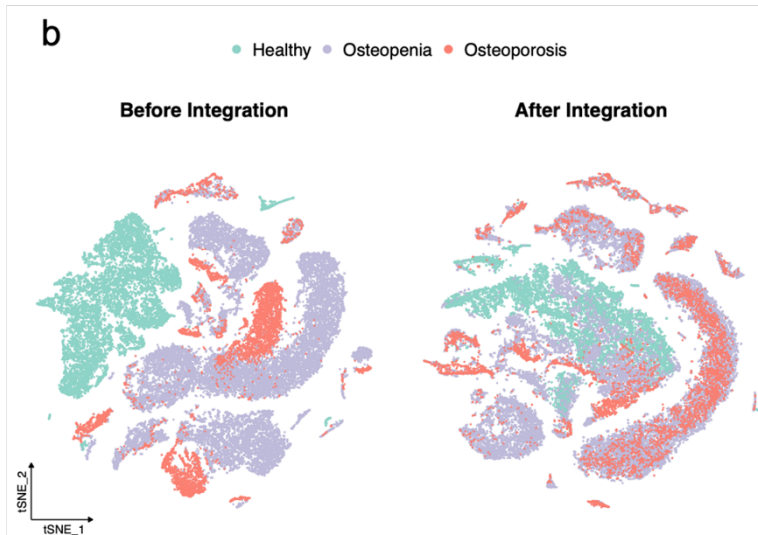
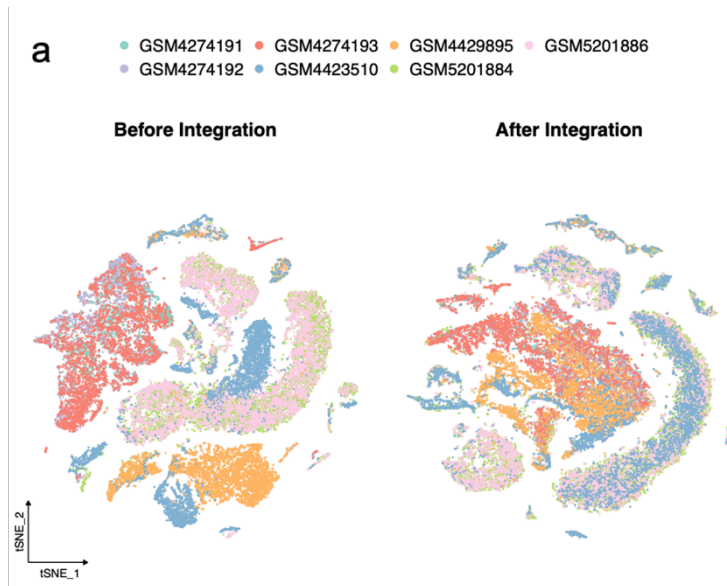
2.3 Results

2.3.1 Increased ET_BR expression in human osteoporotic skeletal progenitor cells

To delineate the role of endothelin signalling in skeletal ageing, we leveraged the human single-cell RNA sequencing datasets from GEO. After harmonizing datasets using the top 2,000 highly variable genes, we performed unsupervised clustering to resolve bone cell heterogeneity in healthy individuals and patients with osteopenia/osteoporosis (Fig. 1a and b). Using this integrated data, unsupervised clustering was performed to categorise cells into distinct cell clusters based on transcriptomic similarity. This revealed distinct populations of osteolineage cells, immune subsets, and SSCs (Fig. 1c).

We specifically examined the expression of skeletal progenitor cell marker genes PDGFRA, THY1, PRRX1 and Endothelin-1 related genes. It was observed that ET_BR gene (EDNRB) expression in bone highly overlapped with the mesenchymal stem cell expression (Fig. 1d). This suggested that the majority of EDNRB was expressed by the mesenchymal progenitor cells in the bone tissue. We further analysed the expression level of ET_BR across the different cell clusters in bone. EDNRB expression, but not EDNRA, significantly increased in pathological bone patients in the mesenchymal

progenitor cells only ($p < 0.001$), with no significant changes observed in osteoblasts, osteoclasts, or immune subsets (Fig. 1e and f). This pathology-specific upregulation positions EDNRB as a biomarker of SSCs dysfunction in bone loss. In the previous study, ET-1 overexpression has shown a strong correlation with vascular dysfunction by inducing vascular cell senescence. Similarly in bone, ET-1 overexpression in pathological bone might lead to increased levels of bone cell senescence and reduction of the stem cell pool. The upregulated ET-1 expression might be highly related to bone loss in pathological conditions, possibly through an increased ET_B R expression in the mesenchymal stem cells.



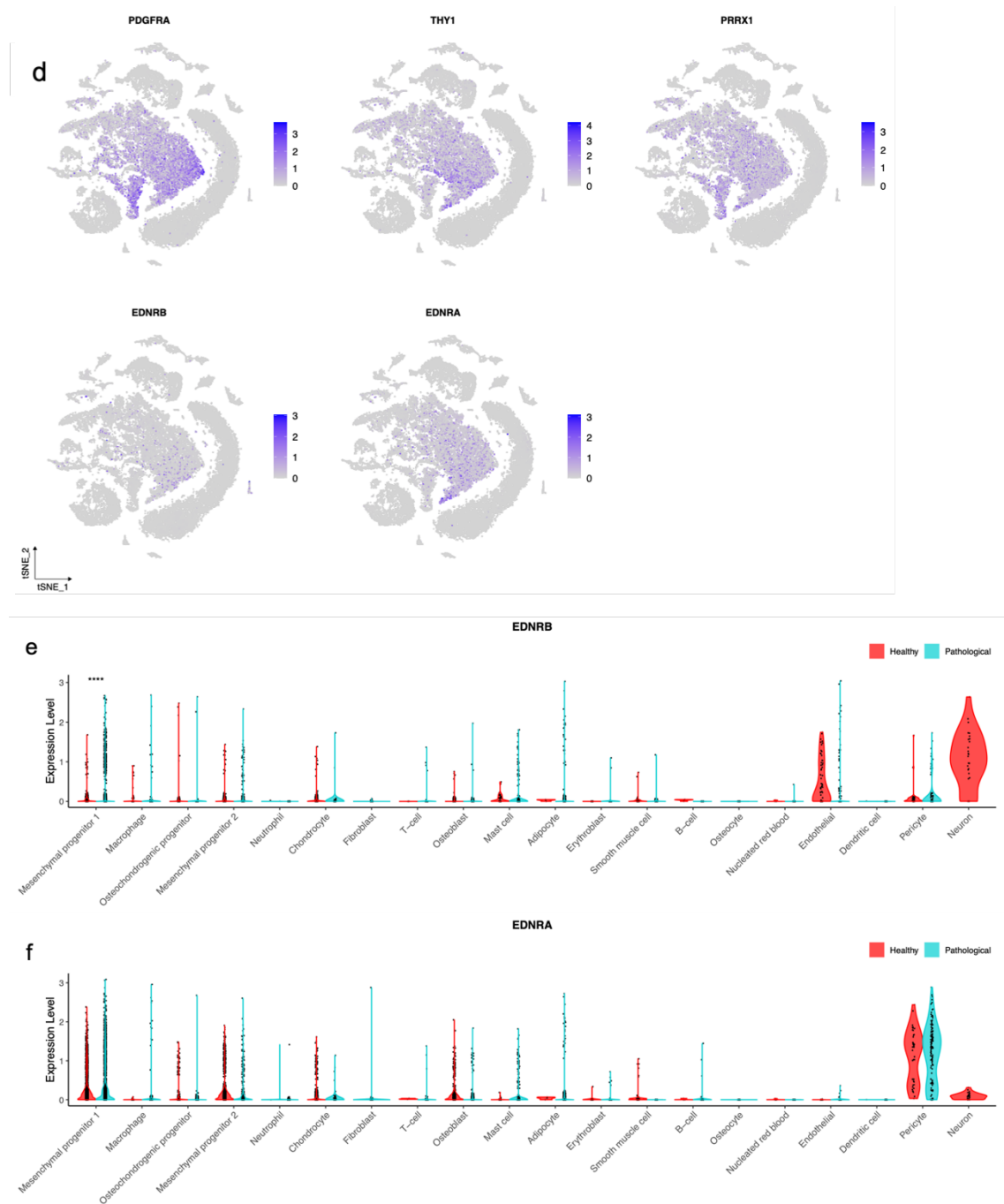


Figure 1 Single-cell sequencing analysis of skeletal stem cell markers and endothelin receptor expressions in human (a) TSNE plots showing sample distribution before and after data integration. Colour by accession number. (b) TSNE plots showing sample distribution before and after data integration. Colour by disease. (c) TSNE plot showing cell clusters. (d)

TSNE plots showing the expression of PDGFRA, THY1, PRRX1 and EDNRB genes. (e)

Violin plot showing the expression of EDNRB between healthy and pathological cells of each cell type. (f) Violin plot showing the expression of EDNRA between healthy and pathological cells of each cell type.

2.3.2 ET-1/ET_BR signalling drives SSCs senescence and osteoporotic bone loss via ROS accumulation in aged mice model

To define the role of ET-1 in skeletal ageing, we first established its systemic and local dynamics in young (6-month-old) and aged (20-month-old) mice.

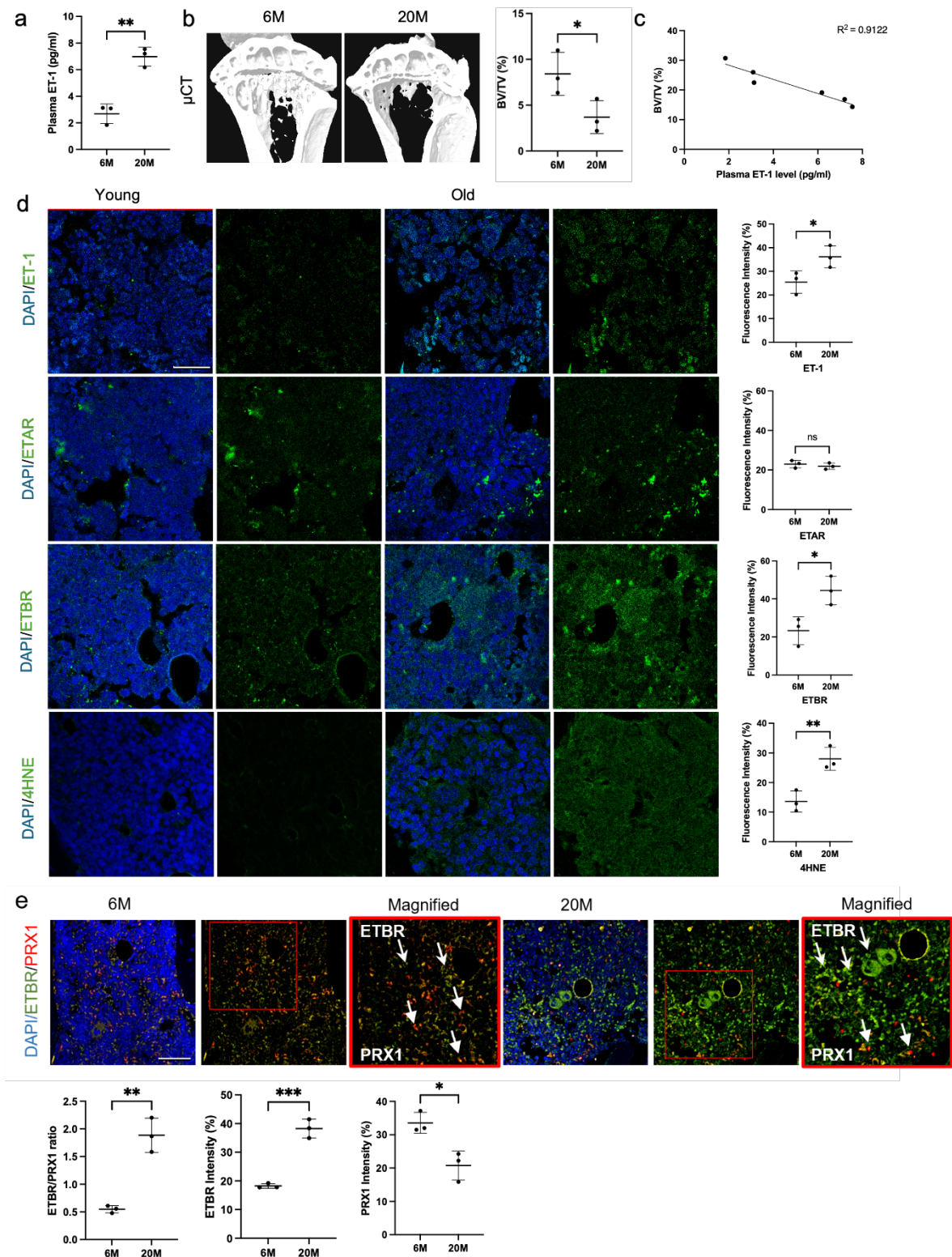
Systemic and local ET-1 expression was assessed using ELISA and immunostaining respectively comparing the young and aged mice. Plasma ET-1 level increased by 2.6-fold in aged mice ($p < 0.01$, Fig. 2a), paralleling significant trabecular bone loss was observed as indicated by BV/TV ($p < 0.001$, Fig. 2b). ET-1 concentration inversely correlated with BV/TV ($R^2 = 0.91$, $p < 0.0001$, Fig. 2c), suggesting a strong association between ET-1 and age-related skeletal senescence.

Aged bone exhibits ET_BR-selective upregulation and oxidative stress.

Immunostaining revealed local ET-1 overexpression ($p < 0.05$) and an increase in ET_BR expression ($p < 0.05$) within aged bone marrow, while ET_AR expression remained relatively constant (Fig. 2d). This receptor-specific change coincided

with the observations made in human single-cell RNA sequencing data. There was a two-fold rise in the lipid peroxidation marker 4HNE ($p < 0.01$, Fig. 2d), confirming ROS accumulation as a hallmark of aged bone.

Co-stain of ET_BR and PRX1 was performed to analyse the SSCs-specific effect of ET-1 overexpression. The ET_BR/PRX1 ratio significantly increased in aged mice ($p < 0.01$, Fig. 2e). Lowered PRX1 expression indicated a reduction in SSCs pool ($p < 0.001$, Fig. 2e). Increased expression of senescent markers p16^{INK4a} ($p < 0.01$), p21^{Cip1} ($p < 0.05$) and p53 ($p < 0.05$) was observed in aged mice (Fig. 2f). Expression of sp7 decreased ($p < 0.0001$, Fig. 2f), showing a reduction of osteoblasts due to the decline in progenitor differentiation in aged bone.



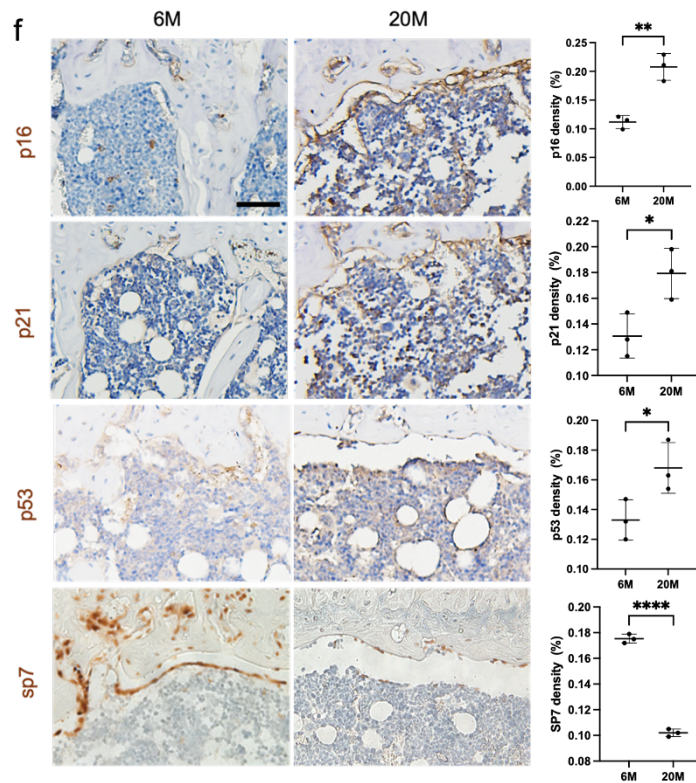


Figure 2 Expression of ET-1, its receptors, ROS and senescent cells in age-related mice

bone loss. (a) Plasma ET-1 concentration of 6M (n = 3) and 20M (n = 3) mice. (b)

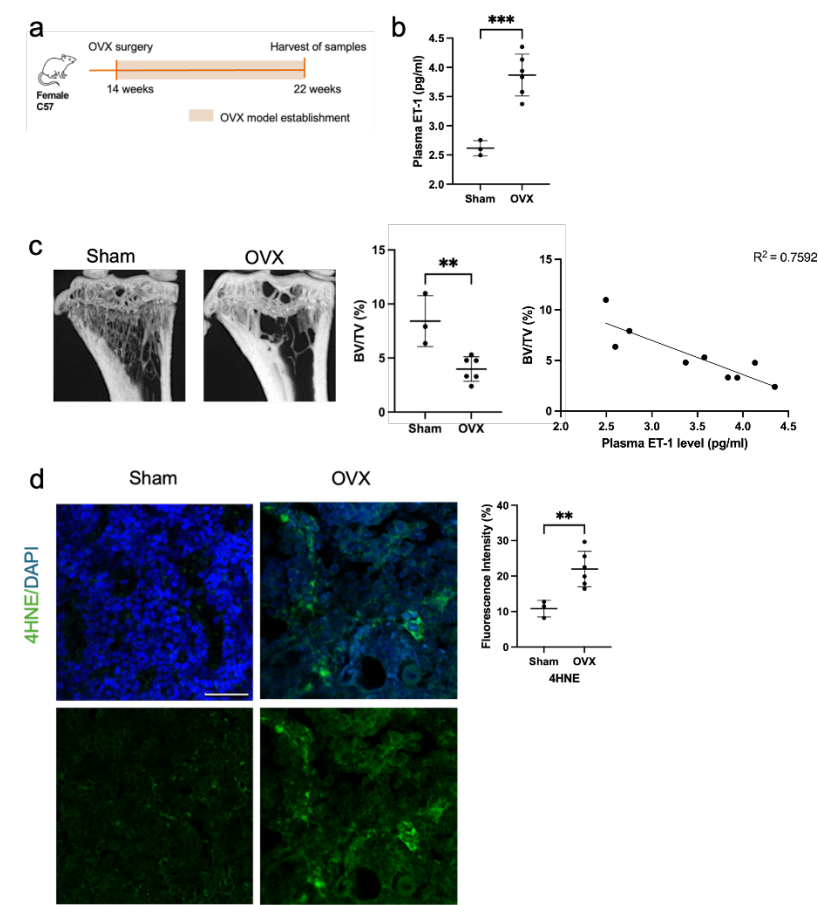
Representative images and quantification of μ CT from 6M and 20M mice. (c) Relationship between BV/TV and plasma ET-1 level. (d) Representative images of immunofluorescence and quantification of ET-1 ETAR, ETBR and 4HNE in primary spongiosa bone of ageing model with DAPI (blue) for nuclear identification. Scale bar, 25 μ m. (e) Representative images of immunofluorescence staining and quantification of ETBR and PRX1 co-stain with DAPI (blue) for nuclear identification. Scale bar, 200 μ m. (f) Representative images and quantification of p16, p21, p53 and sp7 in ageing model. Scale bar, 100 μ m.

2.3.3 Increased SSCs senescence and ROS accumulation through ET-1/ET_BR signalling in OVX-induced mice bone loss

To investigate the endocrine alterations associated with ET-1 overexpression in pathological bone loss, we utilized the ovariectomy (OVX) model in female mice, a well-established method for inducing postmenopausal osteoporosis (Fig. 3a). This surgical intervention successfully recapitulated key features of the disease state. The plasma ET-1 level increased in OVX mice ($p < 0.001$, Fig. 3b), and significant trabecular bone loss was observed as indicated by BV/TV ($p < 0.0001$, Fig. 3c). Immunostaining of 4HNE showed ROS accumulation ($p < 0.01$, Fig. 3d), and ET_BR/PRX1 ratio significantly increased in OVX mice ($p < 0.0001$, Fig. 3e). Increased expression of senescent markers p16^{INK4a} ($p < 0.001$), p21^{Cip1} ($p < 0.05$) and p53 ($p < 0.0001$) was observed in OVX mice (Fig. 3f). Expression of sp7 decreased in OVX mice ($p < 0.0001$, Fig. 3f).

The pattern of changes observed in this OVX-induced pathological model – encompassing elevated ET-1, increased ROS, heightened senescence markers, elevated ET_BR signalling in SSCs, reduced osteoblast activity, and significant bone loss – closely mirrored the alterations previously documented in our model of natural skeletal ageing. This striking similarity strongly suggests a common pathological pathway. Both ageing-related bone loss and the

pathological bone loss induced by OVX appear to be directly driven by ET-1 overexpression acting predominantly through the ET_BR signalling pathway within the skeletal stem cell niche.



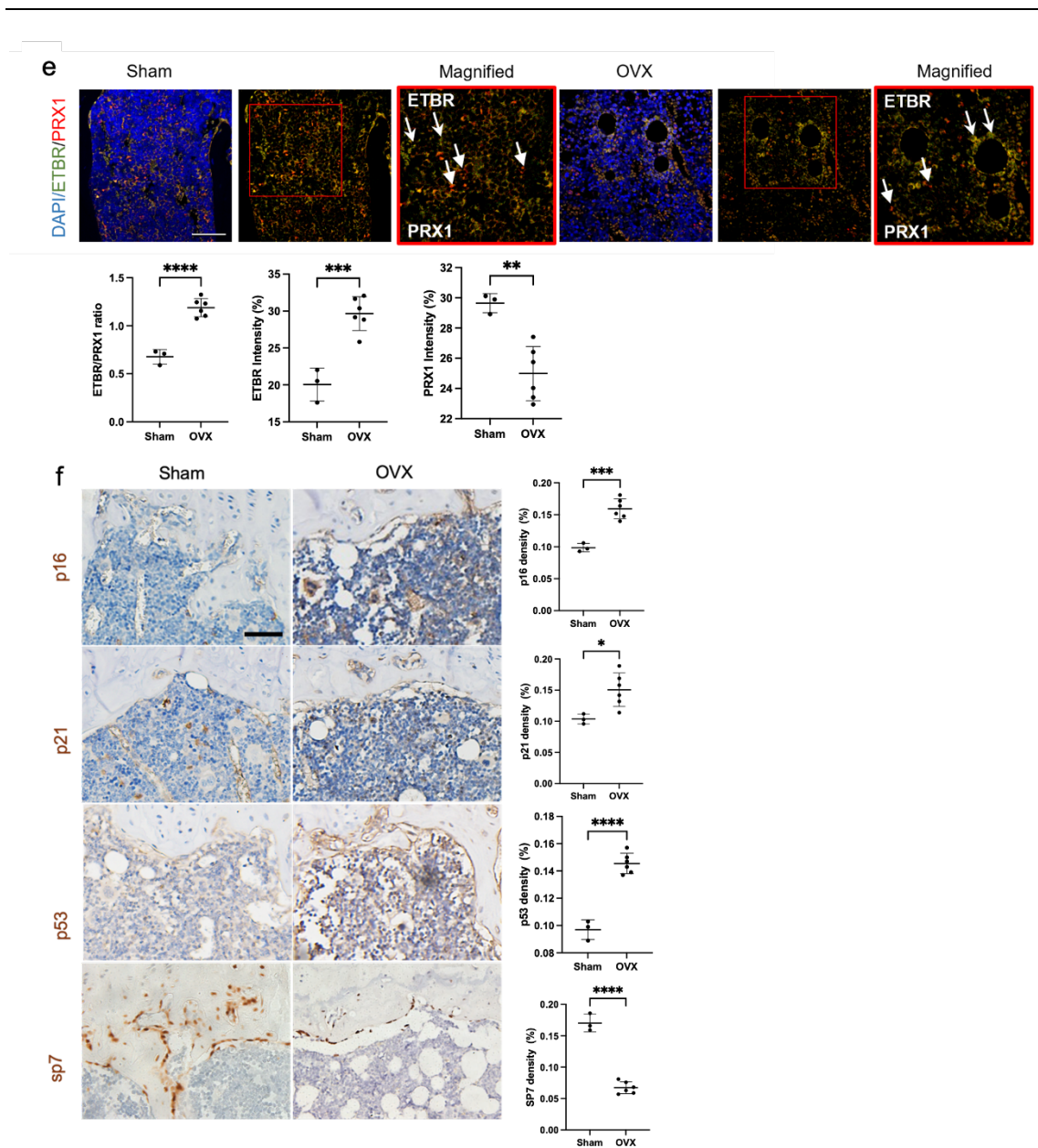


Figure 3 Expression of ET-1, its receptors, ROS and senescent cells in OVX-induced mice

bone loss. (a) Schematic illustration of OVX-induced bone loss mice model establishment. (b)

Plasma ET-1 concentration of Sham (n = 3) and OVX (n = 3) mice. (c) Representative images

and quantification of μ CT from OVX model.(d) Representative images of

immunofluorescence and quantification of 4HNE in primary spongiosa bone of OVX model

with DAPI (blue) for nuclear identification. Scale bar, 50 μ m. (e) Representative images of

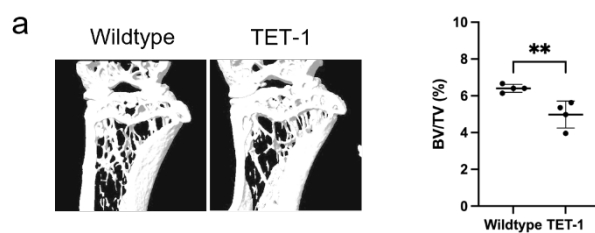
immunofluorescence staining and quantification of ETBR and PRX1 co-stain with DAPI (blue) for nuclear identification. Scale bar, 200 μ m. (f) Representative images of immunohistology staining and quantification of p16, p21, p53 and sp7 in primary spongiosa bone. Scale bar, 100 μ m.

2.3.4 Overexpression of endothelial ET-1 resulted in SSCs senescence accumulation

It was observed that systemic and local ET-1 concentrations in bone are strongly related to bone loss in aged mice. To validate whether bone loss is indeed caused by the accumulation of ET-1 secreted by the endothelial cells, a mouse model with transgenic endothelial cells overexpressing ET-1 (TET-1) was utilized in this experiment to determine the impact of ET-1 on bone degeneration. TET-1 was proven previously to display bone pathogenic phenotypes from the age of 9 months^[151].

To establish causality between endothelial ET-1 overexpression and skeletal ageing, we employed a transgenic mouse model (TET-1) with endothelial-specific ET-1 upregulation^[151]. TET-1 mice exhibited profound trabecular bone loss at 9 months, with BV/TV declining by 2.7% ($p < 0.01$, Fig. 4a). Mirroring natural ageing, TET-1 bone marrow showed a 3-fold increase in ET-1 ($p < 0.0001$) and 2-fold upregulation of ET_BR ($p < 0.001$), while ET_AR remained

relatively constant (Fig. 4b). Pathological hallmarks of ageing, oxidative stress as indicated by increased 4HNE expression ($p<0.05$) in TET-1 mice (Fig. 4b). $ET_B R/PRX1$ ratio significantly increased in TET-1 mice ($p<0.01$, Fig. 4c). Increased expression of senescent markers $p16^{INK4a}$ ($p<0.01$), $p21^{Cip1}$ ($p<0.05$) and $p53$ ($p<0.01$) was observed in TET-1 mice (Fig. 4d). Expression of $sp7$ decreased in TET-1 mice ($p<0.01$, Fig. 4d). These results confirm endothelial ET-1 as a driver of skeletal decline. Collectively, these results – encompassing structural bone loss, elevated ET-1 and $ET_B R$, increased oxidative stress, enhanced $ET_B R$ signalling in SSCs, induction of senescence, and suppression of osteoblast activity – provide definitive evidence that endothelial cell-derived ET-1 overexpression is a primary driver of skeletal decline, recapitulating the hallmarks of age-related bone degeneration.



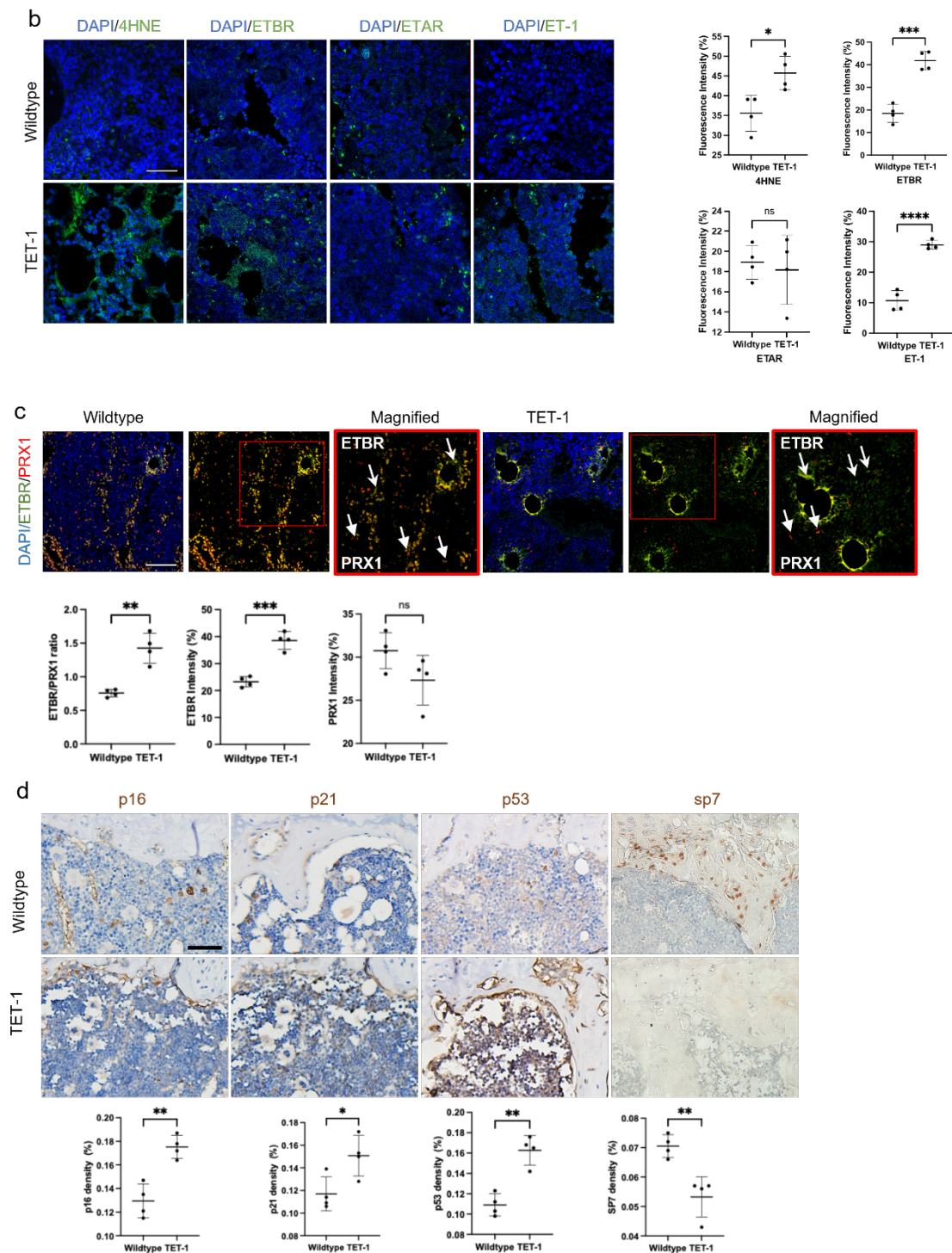


Figure 4 Transgenic mouse overexpressing ET-1 in endothelial cells displayed bone loss and MSCs senescence. (a) Representative images of μ CT and quantification from wildtype (WT) (n = 4) and TET-1 (n = 4) mice. (b) Representative images of immunofluorescence

staining and quantification of ET-1 ETAR, ETBR and 4HNE in primary spongiosa bone with DAPI (blue) for nuclear identification. Scale bar, 25 μ m. (c) Representative images of immunofluorescence staining and quantification of ETBR and PRX1 co-stain with DAPI (blue) for nuclear identification. Scale bar, 200 μ m. (d) Representative images of immunohistology staining and quantification of p16, p21, p53 and sp7. Scale bar, 100 μ m.

2.3.5 ET-1 induces ROS accumulation and MSCs senescence *in vitro* via ET_BR

A cell culture model was used to investigate the direct effect of ET-1 overexpression on MSCs. To confirm which ET receptors played a more dominant role in ET-1 induced senescence in MSCs, cells were exposed to pre-incubation of receptor antagonists BQ123 and BQ788 30min for ET_AR and ET_BR respectively prior to the treatment of ET-1.

ET-1 and antagonists' concentrations and duration of treatment were determined by comparing proliferation rates (Fig. 5a-c) and degree of senescence induced (Fig. 5d-f). Final concentration of 100nM ET-1 treated for 24 h showed the best senescence induction effect and 2 μ M of antagonists showed the best senescence alleviation effect, these concentrations were used for later experiments.

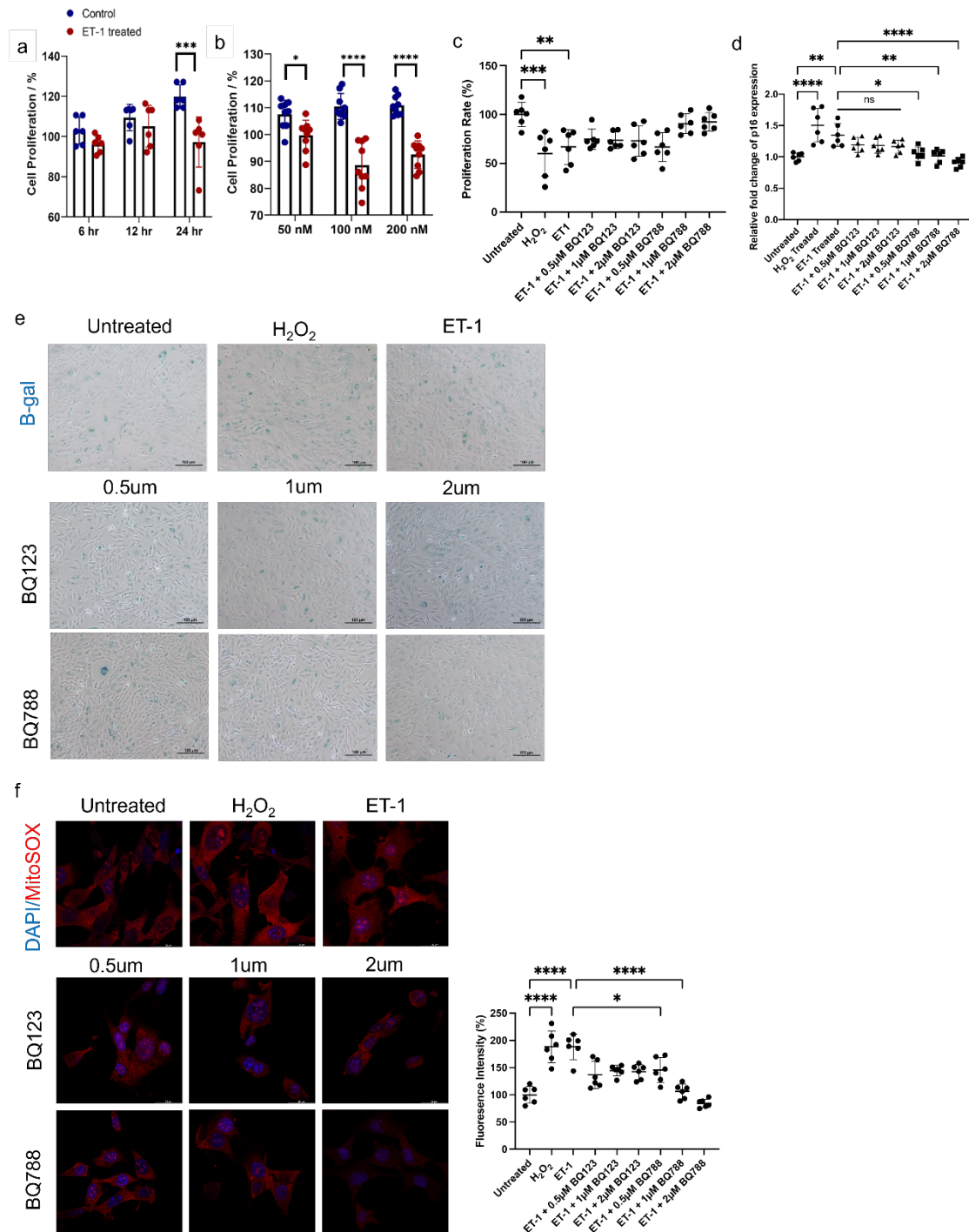


Figure 5 Determining viable concentration of ET-1 and antagonists in MSCs. (a)

Proliferation rates of cells were treated with 100 nM ET-1 for various time. (b)

Proliferation rates of cells were treated with various concentrations of ET-1 for 24 h. (c-f)

Cells were treated with 100 μ M H₂O₂, 100 nM ET-1 or blocker together with ET-1. Blockers were added 30 min before ET-1 stimulation for 24 h. (c) Proliferation rate of MSCs. (d) mRNA expression of p16^{INK4a} via qPCR. (e) Representative images of β -gal staining (blue). Scale bar, 100 μ m. (f) Representative images showing mitochondrial superoxide production (red) by MitoSOX staining and quantitative. MSCs were grown on coverslips. Scale bar, 25 μ m. Cells were counter-stained with DAPI (blue).

ET-1 treatment in MSCs reduced proliferation by 33% ($p < 0.01$, Fig. 6a) and triggered senescence via p16^{INK4a} upregulation (1.3-fold, $p < 0.001$, Fig. 6b), which is also confirmed by β -galactosidase staining (Fig. 6c), and ET-1 exposure elevated mitochondrial superoxide by 88% ($p < 0.001$, Fig. 6d). ET_BR blockade with BQ788 rescued proliferation (92% of control, $p < 0.05$), suppressed p16^{INK4a} ($p < 0.01$), reduced ROS accumulation ($p < 0.001$) and β -galactosidase activity, whereas ET_AR inhibition with BQ123 had no obvious effects in alleviating ET-1 induced senescence (Fig. 6a - d). This identifies ET_BR as the dominant mediator of ET-1-induced SSCs senescence.

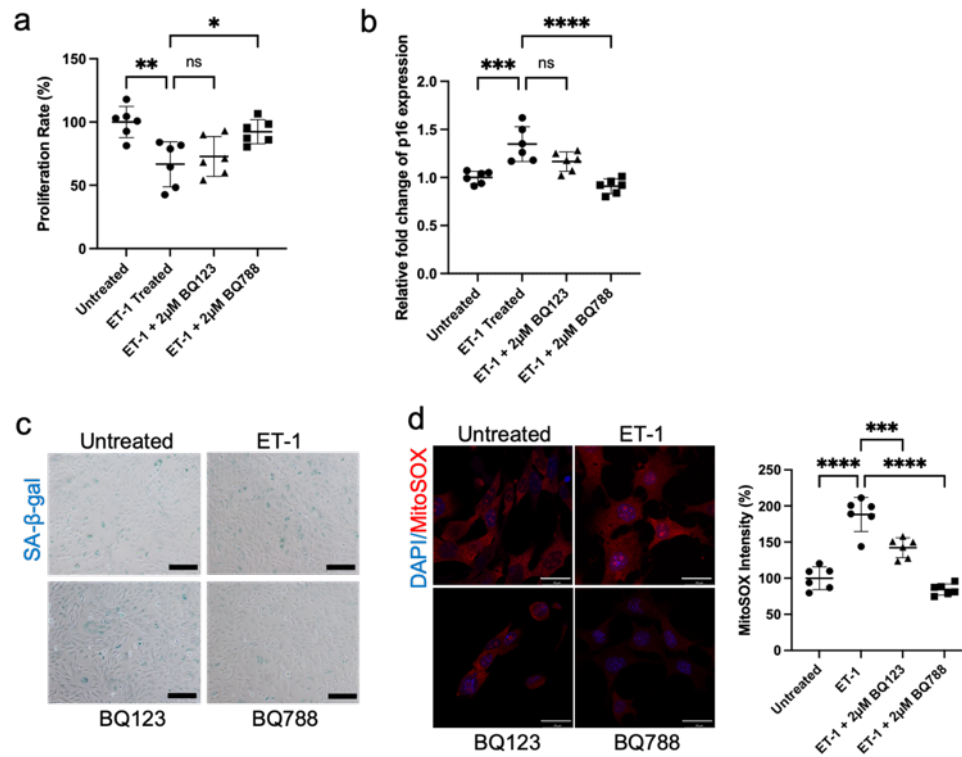
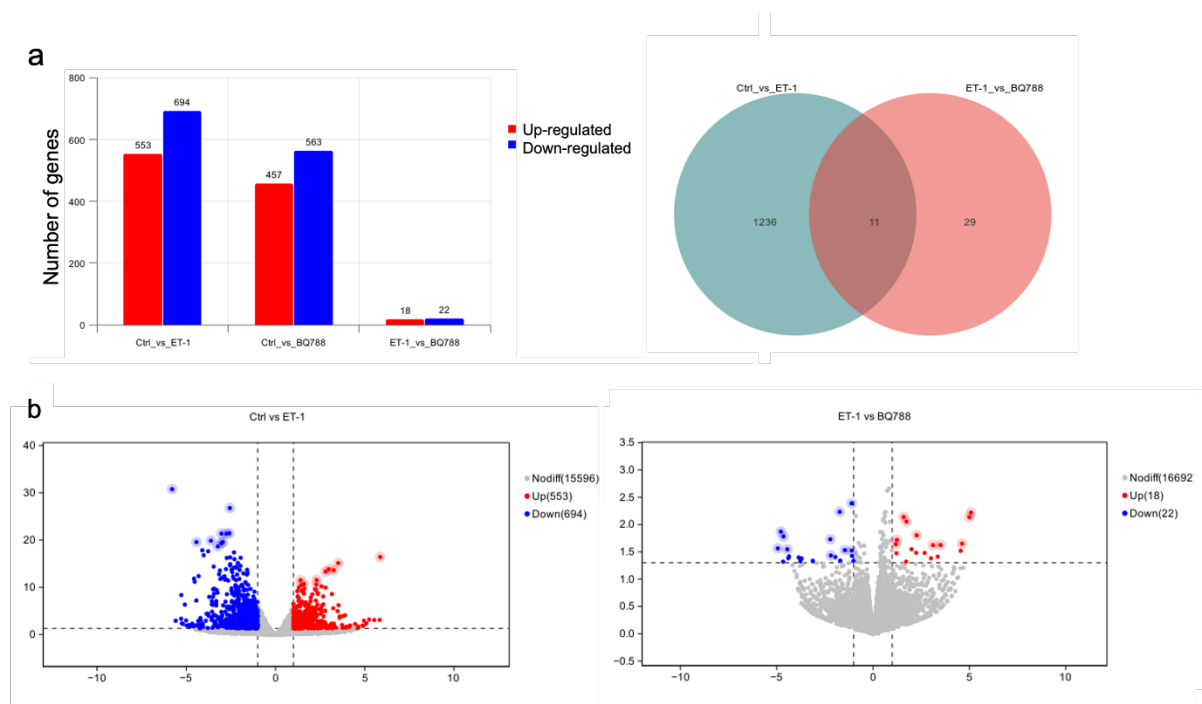


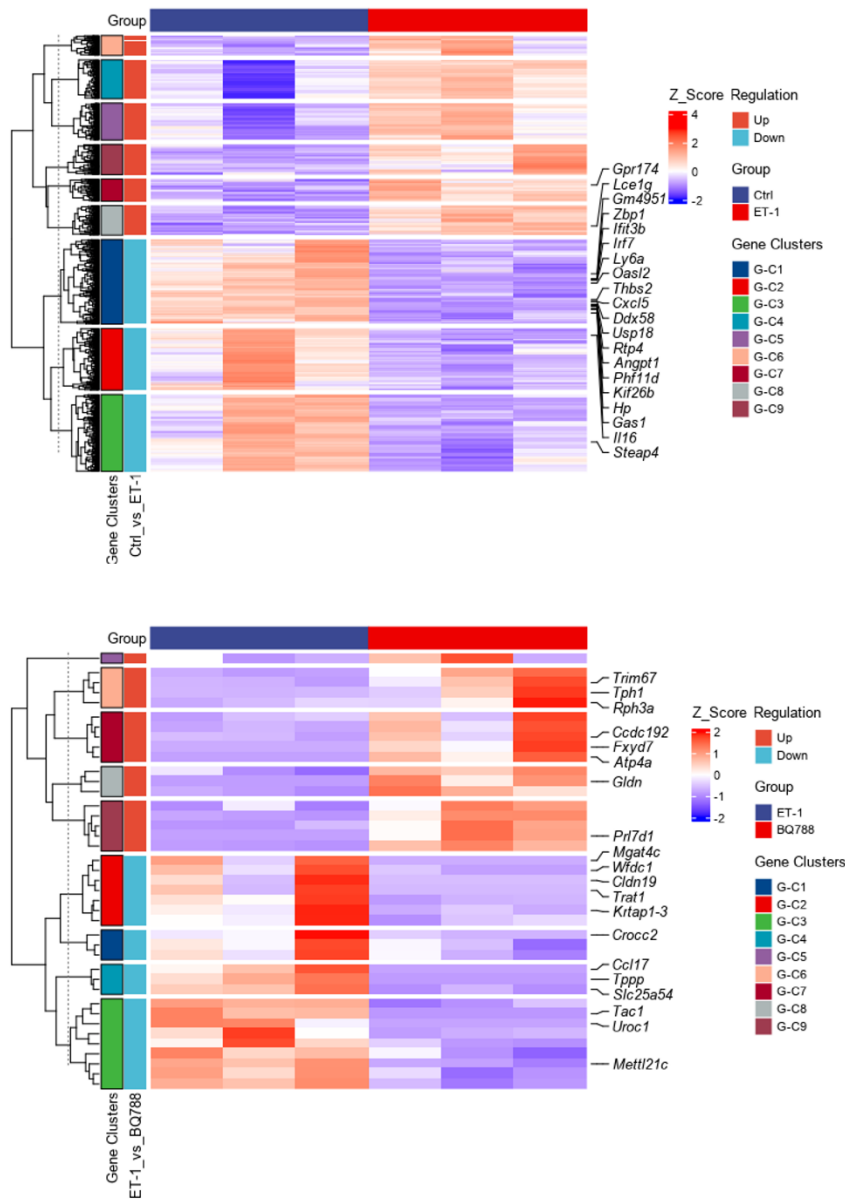
Figure 6 ET-1 induced stimulation of mitochondrial oxidative stress and senescence in MSCs, selective ET_BR blocker reduced such effects. (a - h) Cells were treated with 100 μM H₂O₂, 100 nM ET-1 or blocker together with ET-1. Blockers were added 30 min before ET-1 stimulation for 24 h. (a) Proliferation rate of MSCs. (b) mRNA expression of p16^{INK4a} via qPCR. (c) Representative images of β-gal staining (blue). Scale bar, 100 μm. (d) Representative images showing mitochondrial superoxide production (red) by MitoSOX staining and quantitative. MSCs were grown on coverslips. Scale bar, 25 μm. Cells were counter-stained with DAPI (blue).

2.3.6 RNA-sequencing showed ET_BR-mediated MSCs senescence via the PI3K-Akt-mTOR pathway

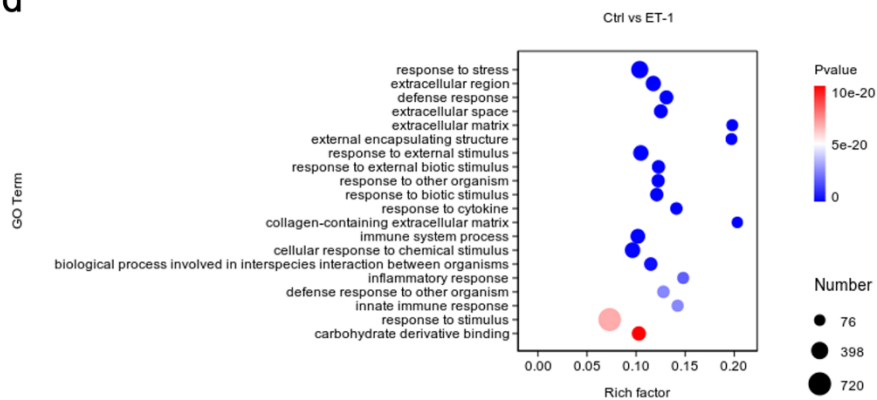
We performed bulk RNA-sequencing on ET-1 induced senescent MSCs and BQ788 treated MSCs. Fig. 7a-b summarise differentially expressed genes (DEGs) across the three groups, top 20 DEGs were labelled in the heatmap (Fig. 7c). Gene ontology (GO) indicated that ET-1 overexpression triggered responses to stress, inflammation and defence, affecting the cell cycle, DNA replication, PI3K-Akt and p53 pathways (Fig. 7d-e). The involved pathways were consistent with our prior observations in murine and cell models. Their activation of the pathways accelerates cellular senescence by promoting DNA damage and SASP.



c



d



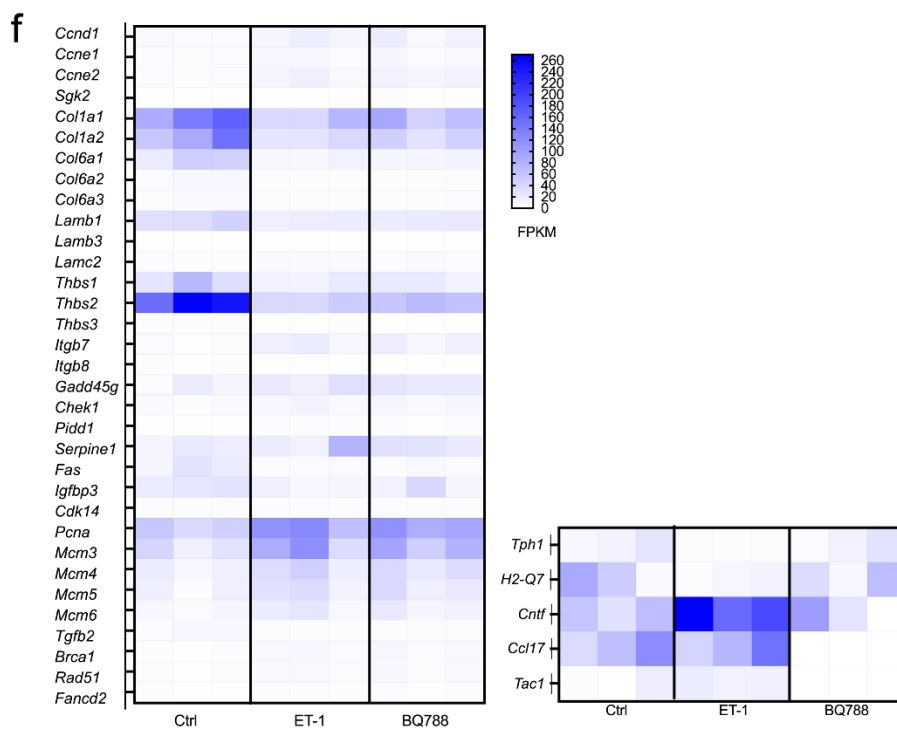
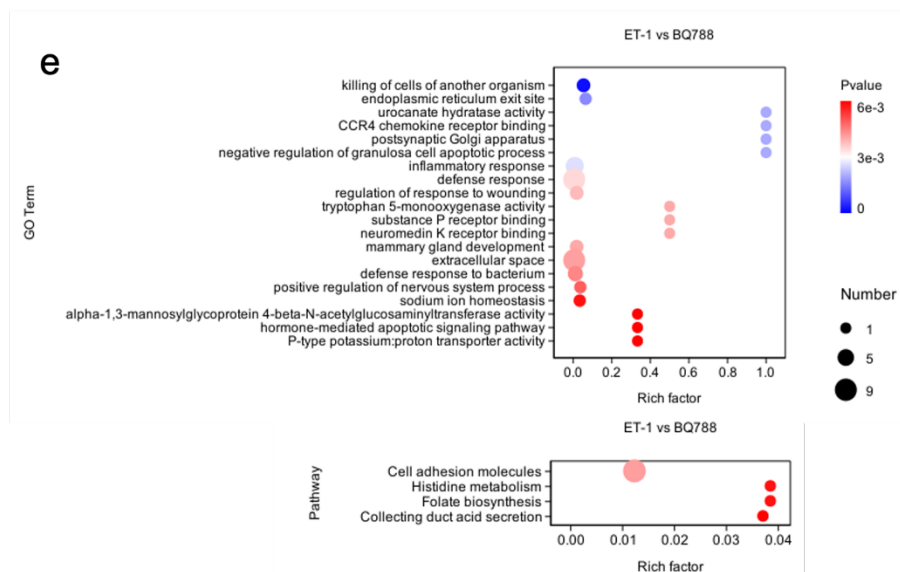
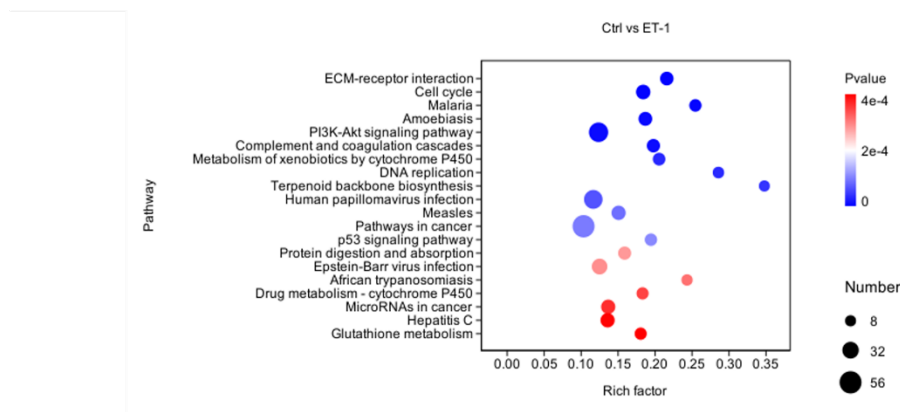


Figure 7 RNA sequencing analysis of ET-1 induced MSCs before and after BQ788

inhibition. (a) Overview of differentially expressed genes (DEGs) from RNA-seq between Ctrl (n=3), ET-1 induced senescence (n=3) and BQ788-treated (n=3) MSCs. (b) Volcanic maps of DEGs (c) DEGs cluster heatmap. (d) GO classification of top 20 biological processes and KEGG pathway enrichment bubble chart after ET-1 induced senescence. (h) GO classification of top 20 biological processes and KEGG pathway enrichment bubble chart after treating senescent cells with BQ788. (i) Heatmap of DEGs.

Transcriptomic profiling of endothelin-1 (ET-1) challenged murine mesenchymal stem cells (MSCs) revealed that ET-1/ETB receptor signalling orchestrates cellular senescence through the synergistic dysregulation of the PI3K-Akt and p53 pathways, concurrently degrading the extracellular matrix (ECM) critical for bone integrity (Fig. 7f). ET-1 exposure induced significant upregulation within the PI3K-Akt signalling pathway, including key proliferative drivers *Ccnd1*, *Ccne1*, and *Ccne2*, alongside the survival kinase *Sgk2*. This pro-proliferative signal occurred simultaneously with the profound suppression of numerous ECM structural components and regulators essential for tissue architecture and stem cell niche maintenance. Significantly downregulated genes included collagen isoforms (*Colla1*, *Colla2*, *Col6a1*, *Col6a2*, *Col6a3*), laminin subunits (*Lamb1*, *Lamb3*, *Lamc2*), thrombospondins (*Thbs1*, *Thbs2*, *Thbs3*), and integrins (*Itgb7*, *Itgb8*). This degradation creates a

pathological microenvironment permissive for senescence by disrupting integrin-mediated signalling, which normally suppresses unrestrained PI3K-Akt activation and provides essential survival cues. Concomitant activation of the p53 signalling pathway was evidenced by the upregulation of DNA damage response elements (*Gadd45g*, *Chek1*, *Pidd1*) and the master senescence executor *Serpine1* (PAI-1), while pro-apoptotic factors like *Fas* and growth inhibitors such as *Igfbp3* were suppressed, enabling cellular persistence in a senescent state rather than apoptosis. The suppression of thrombospondins is particularly consequential, as these proteins sequester latent TGF- β and inhibit angiogenesis; their loss likely fuels the senescence-associated secretory phenotype (SASP). ET-1 also forced aberrant cell cycling, evidenced by the upregulation of G1/S transition promoters (*Cdk1*, *Pcna*) and minichromosomal maintenance complexes (*Mcm3*, *Mcm4*, *Mcm5*, *Mcm6*), coinciding with the downregulation of the growth-inhibitory cytokine *Tgfb2*, collectively promoting replicative stress. Furthermore, the observed upregulation of DNA repair genes within the AGE-RAGE signalling pathway (*Brca1*, *Rad51*, *Fancd2*) signifies a state of genomic instability and compensatory, yet ultimately ineffective, repair mechanisms in response to ET-1-induced damage, likely stemming from the combined effects of replicative stress and ECM degradation disrupting redox balance.

Pharmacological blockade of the endothelin receptor B with BQ788 effectively counteracted this senescence program, demonstrating reversal through multimodal mechanisms centred on restoring cellular defence and resolving inflammation (Fig. 7f). BQ788 treatment significantly upregulated genes suppressed by ET-1, *Tph1* (tryptophan hydroxylase), suggesting serotonin-mediated cytoprotection; and the histocompatibility complex *H2-Q7*, potentially improving immune surveillance of damaged cells. Conversely, BQ788 downregulated ET-1-induced pro-inflammatory and pro-senescence factors, notably *Cntf* (ciliary neurotrophic factor), whose suppression directly attenuates JAK-STAT signalling implicated in SASP development, the chemokine *Ccl17* (promoting immune cell infiltration), and the neuropeptide precursor *Tac1* (substance P). This coordinated transcriptional shift indicates BQ788 promotes resolution of inflammation and reduces the SASP burden. The restoration of defence mechanisms (*Hp*, *Lyz2*) and the induction of potential cytoprotective pathways (*Tph1*) directly counteract the oxidative and inflammatory stresses central to ET-1-induced senescence.

2.3.7 Comparing ET-1 induced MSC senescence with replicative and H₂O₂-induced senescence

2.3.7.1 uPAR as a senescence biomarker of MSCs

The urokinase-type plasminogen activator receptor (uPAR) is a cell-surface protein which is available in larger amounts during senescence. It was first reported as a senescence biomarker in the lung adenocarcinoma cells^[152]. To investigate whether it can be used as a marker in MSCs, the population of high uPAR expressing (uPAR⁺) MSCs is quantified and isolated using the flow cytometry method. Then, the p16^{INK4a} expression was compared across the samples for both replicative and stress-induced senescence groups. Results proved that uPAR is a senescence marker of MSCs.

In the replicative senescence group, the percentage of uPAR⁺ cells are relatively constant for both early and late passage MSCs at around 40% (Fig. 8a). After isolating the cells based on uPAR expression, an elevated expression of p16^{INK4a} was observed in the early passage (~p15) uPAR⁺ MSCs and the late passage (~p35) uPAR⁺ MSCs; a decreased expression of p16^{INK4a} was observed in the early passage uPAR⁻ MSCs and the late passage uPAR⁻ MSCs (Fig. 8b). In the stress-induced senescence group, the percentage of uPAR⁺ cells increased for both H₂O₂ and ET-1 treated MSCs (Fig. 8c). After isolating the

cells based on uPAR expression, an elevated expression of p16^{INK4a} was observed in the heterogenous ET-1 treated and its population of uPAR⁺ MSCs; similar trends were observed in the heterogenous H₂O₂ treated and its population of uPAR⁺ MSCs (Fig. 8d). Cellular sizes of uPAR⁺ MSCs are significantly larger than the uPAR⁻ MSCs (Fig.8e).

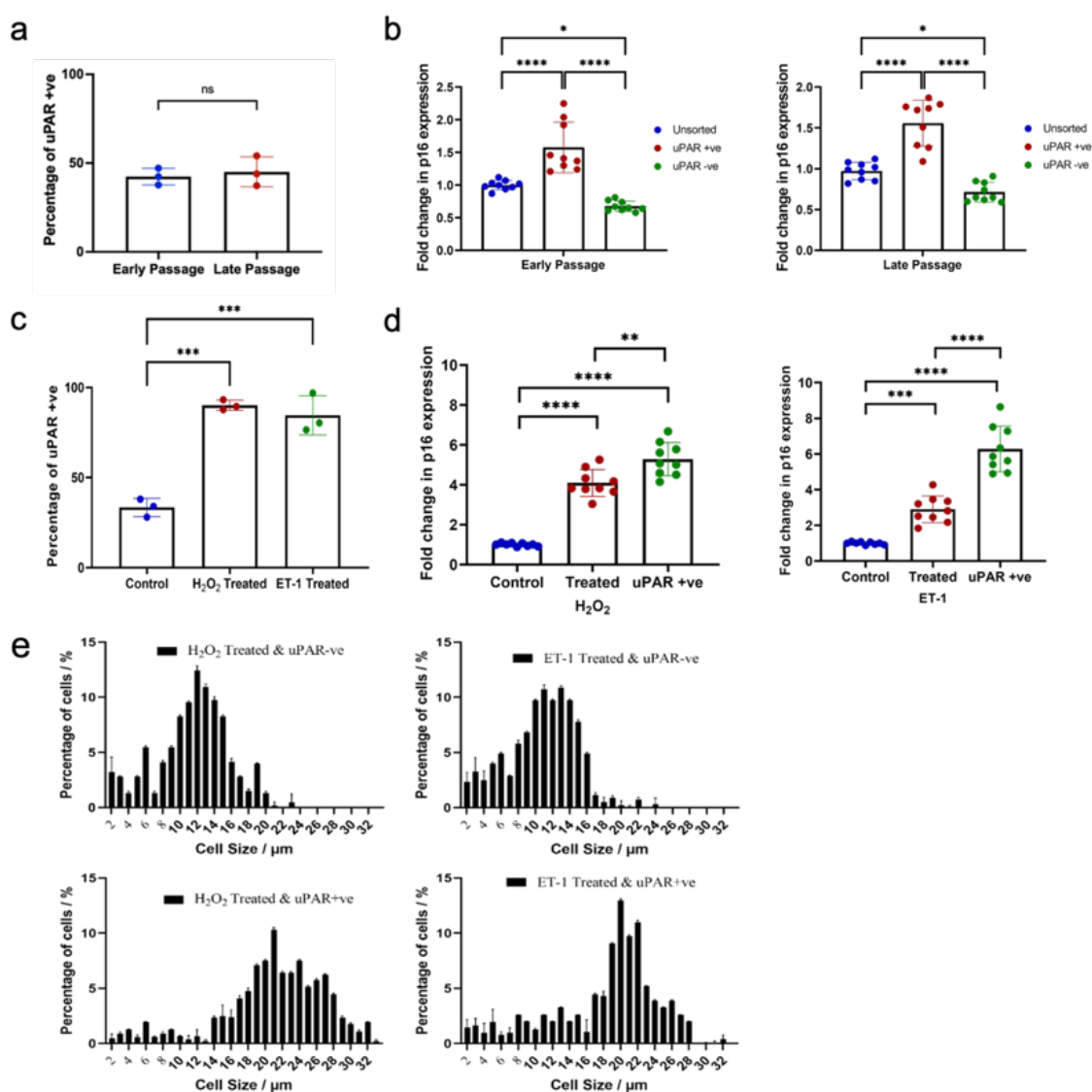


Figure 8 uPAR is a senescence biomarker of MSCs. (a) percentage of uPAR⁺ MSCs in early and late passages as determined via flow cytometry. (b) mRNA expression of p16INK4a via qPCR in early and late passages of MSCs. (c) percentage of uPAR⁺ MSCs in H₂O₂ and ET-1 induced senescent MSCs as determined via flow cytometry. (d) mRNA expression of p16INK4a via qPCR. (e) Estimation of cell sizes. Cells were treated with either 100nM ET-1 or 100μM H₂O₂; uPAR⁺ cells were isolated using the cell sorter.

The urokinase plasminogen activator surface receptor (uPAR) has been identified as a marker of senescent cells (SnCs)^[152]. As uPAR has not been tested on MSCs before, we also validated its potential to select senescent MSCs *in vivo*. In aged mice model, the expression of uPAR⁺ MSCs increased (Fig. 9a). In TET-1 mice model, the expression of uPAR⁺ MSCs also increased (Fig. 9b).

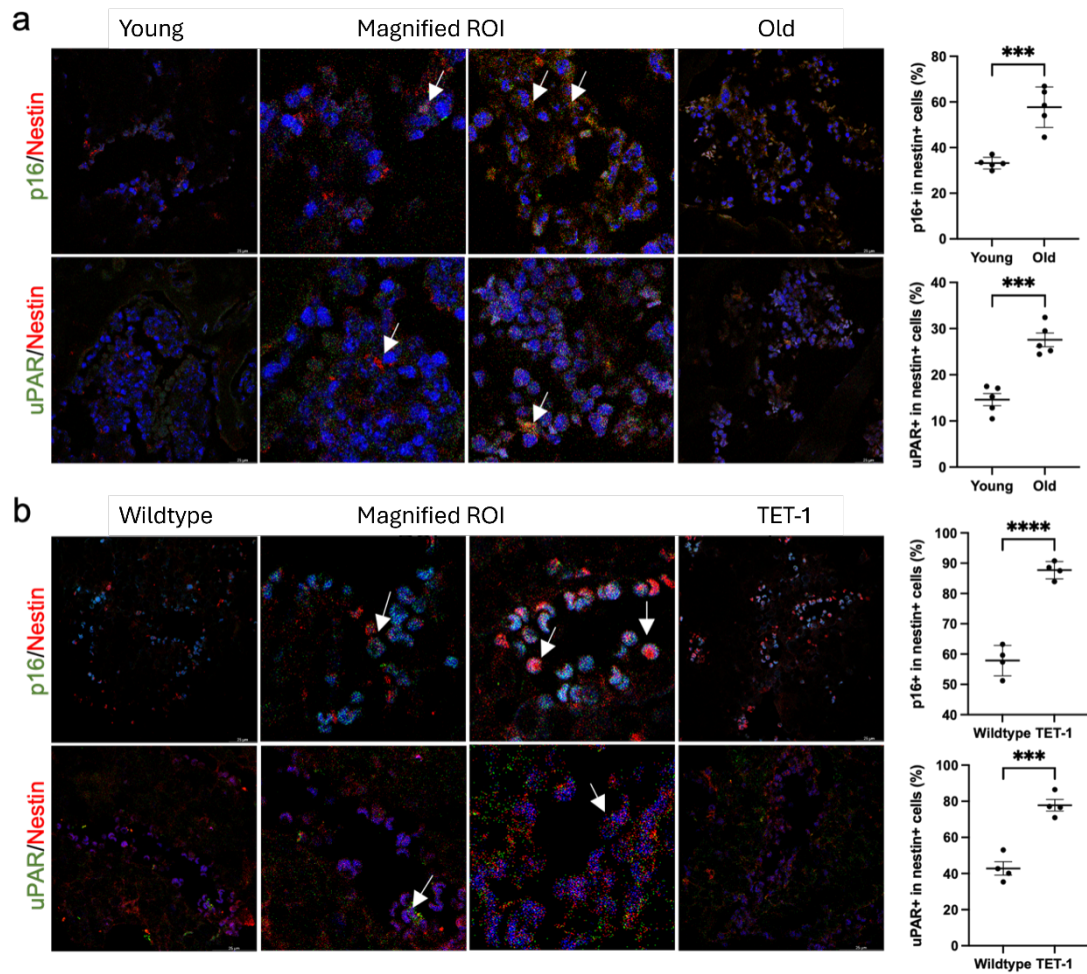


Figure 9 Increased MSCs senescence in aged mice and TET-1 mice bone. Representative images of immunofluorescence and quantification analysis of p16INK4a (green) and uPAR (green) overlapping with nestin (red) in primary spongiosa bone of (a) young (3 - 6M, n = 5) and aged (≥ 9 M, n = 5) mice; (b) Wildtype (n = 4) and TET-1 (n = 4) mice. Scale bar, 25 μ m. Each data point represents one female balb/c mouse.

2.3.7.2 ET-1 induced cellular senescence shares similarities with H₂O₂ and replicative senescence

In both *in vitro* and *in vivo* models, it was observed that ET-1 stimulates the production of ROS in bone cells, and this form of cellular senescence is similar to the oxidative stress induced by H₂O₂. We further investigated the biophysical and biochemical characteristics of SnCs induced by ET-1 and H₂O₂, and also cross-checked with replicative SnCs.

Two biophysical characteristics are of interest, cell stiffness and size. Cell stiffness is measured by AFM and quantified via the Young's modulus. In replicative senescence, cell stiffness is relatively the same for both early and late passages of MSCs. On the other hand, cell stiffness increased for ET-1 induced senescence and H₂O₂ induced senescence (Fig. 10a). An increase of cell size is observed for replicative senescence and stress-induced senescence via ET-1 and H₂O₂ (Fig. 10b). In terms of ET-1 and receptors mRNA expression, replicative senescence and H₂O₂ induced senescence showed an increased expression of ET-1 only, while ET-1 induced senescence showed increased expression of ET-1 along with its receptors A and B (Fig. 10c). The summary radar chart based on the characteristics investigated in this study

shows that ET-1 induced senescence falls between H_2O_2 induced and replicative senescence, with more similarities with H_2O_2 induced senescence (Fig. 10d).

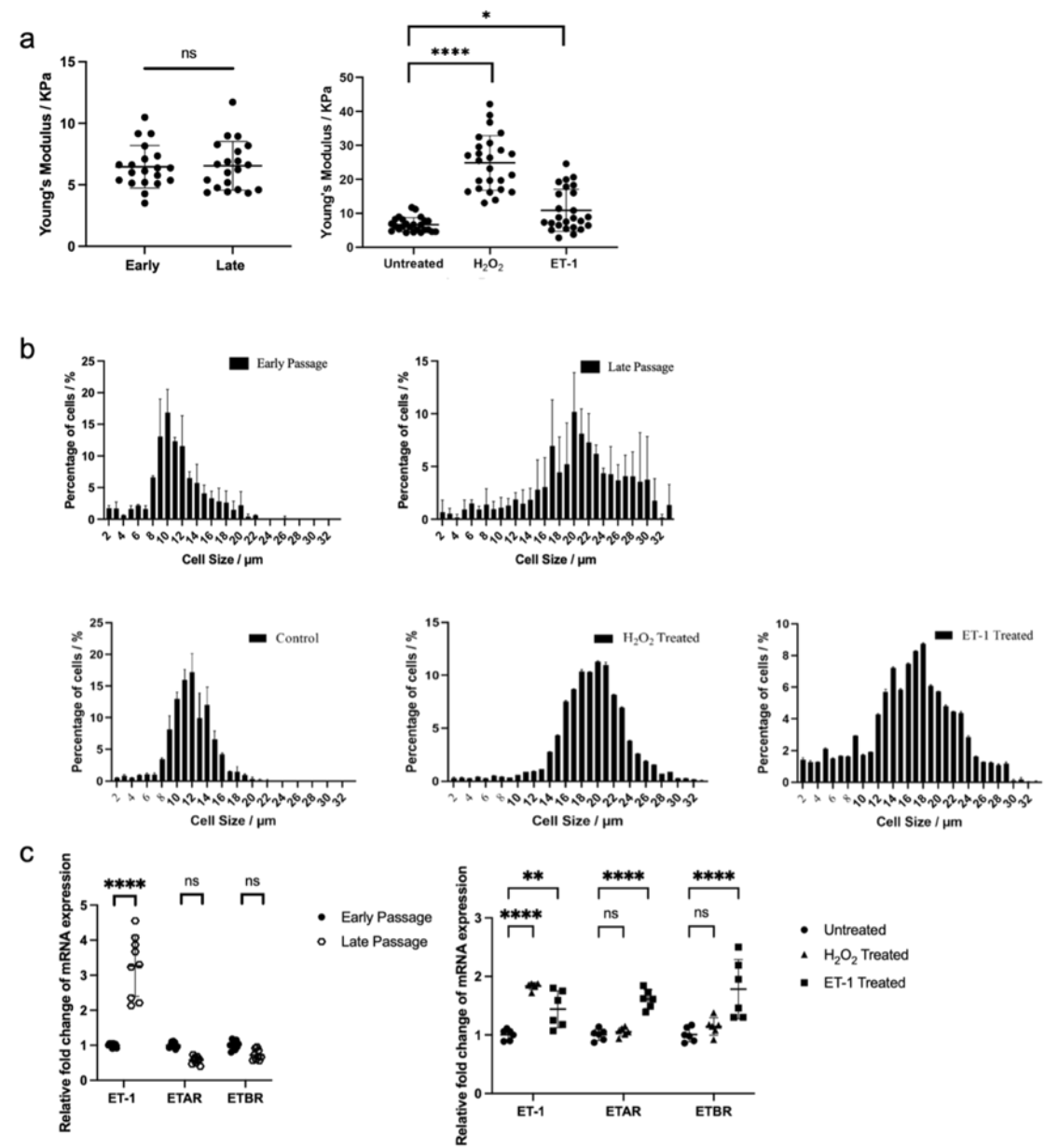




Figure 10 Biophysical and biochemical characteristics of ET-1 and H₂O₂ induced cellular senescence in MSCs. (a) Cell stiffness (b) Cell size distribution and (c) ET-1 and receptors expression in replicative and stress induced senescent MSCs. Each data point represents one female balb/c mouse. (d) Summary radar comparing ET-1 induced senescence with replicative and H₂O₂-induced senescence.

2.3.7.3 ET-1 affects MSCs lineage and fate

MSCs are precursor cells with the potent ability of differentiating into several mature lineages. In response to different sets of transcriptional factors, micro-environmental triggers, and signals, MSCs can undergo osteogenesis and differentiate into chondrocytes (cartilage), osteocytes (bone) or adipogenesis into adipocytes (fat). The mRNA expression of osteogenesis and adipogenesis markers suggests that ET-1 treated MSCs are more prone to the adipogenesis lineage rather than undergoing osteogenesis (Fig. 11a). The size of adipocytes in bone primary spongiosa of aged mice (Fig. 11b) and TET-1 mice also increased (Fig. 11c).

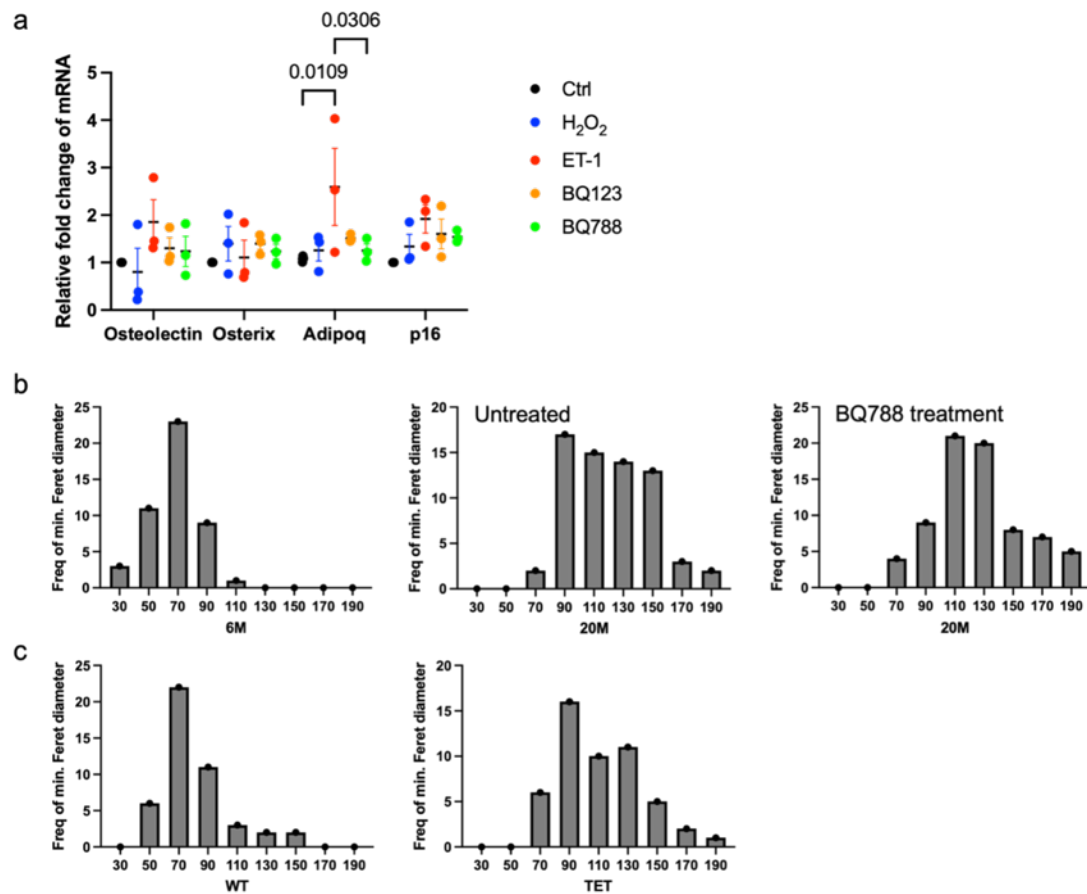


Figure 11 ET-1 induces adipogenesis and suppresses osteogenesis. (a) mRNA expression of osteogenesis and adipogenesis markers via PCR. (b-c) Size distribution of adipocytes in bone primary spongiosa of aged mice and TET-1 mice models. Each data point represents one female balb/c mouse.

2.4 Discussion

As the most potent endogenous vasoconstrictor, ET-1 has been widely studied in vascular biology for its roles in vasoconstriction, inflammation, and fibrosis through ET_AR^[153; 154]. In contrast, endothelial ET_BR stimulates nitric oxide

production and clears ET-1 from blood circulation^[155]. Within bone, ET-1 signalling has been implicated in regulating osteoblast proliferation, bone formation, and osteoclast activity predominantly via ET_AR under physiological conditions^[156; 157], though its precise role in skeletal ageing remains poorly characterized. Our study advances this understanding by identifying ET-1 as a critical mediator of skeletal ageing. We mechanistically link age-associated vascular dysfunction within the bone marrow niche to SSCs senescence and subsequent osteoporotic bone loss. Specifically, we demonstrated that overexpression of ET-1 in aged bone marrow upregulates ET_BR on SSCs. The ET-1/ET_BR signalling triggers intracellular ROS accumulation, culminating in senescence via p53-p21^{CIP1} and p16^{INK4a} pathways.

We showed that increased ET-1 expression with ageing correlates with bone loss in old mice due to an abundance of small vessels within the bone marrow. ET-1, which is derived from the endothelium, affects the blood vessel-rich bone in addition to causing endothelial dysfunction. We found that ET_BR, not ET_AR, is the primary cause of senescence in SSCs exposed to ET-1. After ET_BR is specifically blocked using BQ788, ROS secretion, the number of senescent cells, and bone volume are all effectively decreased in old mice. This study provides insight into vascular-related causes of skeletal change in ageing. It is important for understanding the pathogenesis and management of age-related

skeletal diseases. The findings can help in developing mechanism-based drugs specifically targeting the elderly. The aligned observations with other researchers in the capability of uPAR in selecting senescent MSCs, larger cellular size and stiffer MSCs also provided more evidence in the field.

In addition to the commonly used senescence biomarkers, we validated the potential of uPAR in marking senescent MSCs. uPAR controls the signalling of the plasminogen activation system expressing plasmin at the cell surface. Plasmin is essential for cleavage of extracellular matrix components, including the degradation of fibrinolysis and activation of matrix metalloproteases^[158]. uPAR is also involved in signalling pathways responsible for cell motility, invasion, proliferation, and survival. Its expression is upregulated during inflammatory responses and senescence in cells^[159]. It was observed that after senescence-inducing treatment in the mouse model, the amount of uPAR positive cells elevated. The uPAR selected cell population displayed a higher expression of p16^{INK4a} and β -galactosidase positive signals. This population also contained cells with larger size as compared to the remaining none selected population.

H₂O₂ is one of the well-established oxidative agents that leads to oxidative stress-induced senescence^[160]. From our data, the stimulus of H₂O₂ generated a

strong and fast oxidative stress that induced MSCs senescence. H₂O₂ was volatile and could result in large population cell loss due to death. We found that ET-1 induced senescence in MSCs is similar to the H₂O₂ induced senescence. Other than biomarker changes in the induced senescence model, we also observed some physical changes in MSCs. Senescent MSCs exhibits larger cell sizes and become stiffer. Increase in cellular size has been long known as a feature of senescence, a possible result of changes in DNA content and nucleocytoplasmic ratio when cell cycle is arrested. The correlation between increased stiffness and cellular senescence has been demonstrated in several types of cells, for example, the epithelial cells ^[161]. When we evaluated the stiffness of individual MSCs in bone with atomic force microscopy (AFM), we observed the same correlation. The Young's modulus showed that stiffness in MSCs increased from 6.641KPa in control cells to 24.84KPa in H₂O₂ induced senescent MSCs and 10.88KPa in ET-1 induced senescent MSCs. Both physical features can be potential selection factors for senescent cells. In contrast, ET-1 induced senescence was less sensitive and produced milder effects for the same dosage and treatment duration. Significantly less cell loss was also observed during senescence induction. Therefore, in the future, ET-1 may serve as a better model for senescence induction as we could have better control of the degree of senescence induction, also preventing large-scale cell loss.

The finding that ET_BR, rather than ET_AR, plays the dominant role in mediating SSCs senescence is highly significant. While ET_AR classically drives vasoconstriction, our data aligns with previous evidence implicating ET_BR in oxidative stress amplification across diverse tissues^[162; 163; 164].

Mechanistically, ET_BR activation in SSCs induced mitochondrial ROS overproduction, depleting cellular antioxidant defences. RNA-sequencing analysis further revealed activation of the PI3K-AKT-mTOR signalling pathway downstream of ET_BR engagement. While the precise mechanistic link between PI3K-AKT-mTOR and ROS accumulation requires further investigation, we hypothesise this pathway may potentiate mitochondrial dysfunction or suppress antioxidant responses, thereby accelerating the senescence program. Importantly, our data position ET-1/ ET_BR signalling as an upstream trigger for the generation of such senescent SSCs populations, offering a novel, druggable node for intervention.

The molecular interdependencies revealed by cellular RNA sequencing explain the potency of ET-1/ET_B signalling in driving murine MSC senescence. These findings have significant implications for osteoporosis, where increased MSC senescence population contributes to bone loss. The specific suppression of bone-critical ECM genes (*Colla1*, *Colla2*, *Lamb1*, *Lamb3*) directly impairs osteoid formation and matrix integrity, providing a direct mechanistic link to

ET-1-driven osteoporotic pathology. The downregulation of thrombospondins likely exacerbates this by permitting abnormal vascularization in bone.

BQ788's ability to reverse these transcriptional signatures, particularly by restoring defence mechanisms and suppressing inflammation, positions ETB antagonists as a promising novel therapeutic strategy. Key genes, including *Hp* and *Lyz2* (indicators of restored defence), *Thbs1* and *Colla1* (markers of ECM integrity), and *Cntf* and *Ccl17* (SASP indicators), serve as mechanistically grounded biomarkers for assessing therapeutic efficacy in future studies targeting senescent SSCs in age-related bone loss. Validation in human osteoporotic SSCs and the development of targeted delivery systems for ETB antagonists represent critical next steps for clinical translation.

Chapter 3 Reducing Bone Loss And Enhancing Skeletal Regeneration Through Intraperitoneal Injection Of ET_BR Inhibitor And Specific-PRX1⁺ SSC ET_BR Knockout In Ovx Mice

3.1 Introduction

The progressive decline in skeletal integrity associated with ageing and various pathological states, most notably osteoporosis and impaired fracture healing, represents a colossal burden on global health systems and individual quality of life. Characterised by an insidious shift in the fine-tuned equilibrium between bone formation by osteoblasts and bone resorption by osteoclasts towards net bone loss, this phenomenon manifests as debilitating fractures, chronic pain, reduced mobility, and significant morbidity and mortality. While current therapeutic modalities, primarily anti-resorptive agents like bisphosphonates and denosumab, and the anabolic agent teriparatide (intermittent PTH), offer some clinical benefit, they are often hampered by significant limitations. These include modest efficacy gains in certain high-risk populations, potential adverse effects (e.g., osteonecrosis of the jaw, atypical femoral fractures with prolonged bisphosphonate use), the finite duration of anabolic therapy, and, crucially, their fundamental inability to fully restore the intrinsic regenerative capacity of

the skeletal tissue itself. As shown in the previous chapter, in the ageing skeleton and under conditions of chronic stress, SSCs exhibit a marked decline in their number, intrinsic function, and overall regenerative potential. This SSC impairment is a complex phenotype driven by a confluence of cell-intrinsic alterations and detrimental microenvironmental cues. Within this complex interplay of SSC dysfunction, ROS accumulation, and senescence, a compelling molecular target has emerged: the Endothelin B Receptor.

Evidence suggests that activation of ET_BR signalling in SSCs contributes directly to the pathological processes undermining skeletal homeostasis. Mechanistically, ET-1 binding to ET_BR on SSCs can trigger intracellular signalling cascades involving sustained activation of NADPH oxidases (NOX enzymes), particularly NOX4, leading to the excessive generation of intracellular superoxide and other ROS. This ET_BR-driven ROS surge contributes significantly to the oxidative burden within SSCs, accelerating DNA damage response pathways and directly activating key senescence-inducing programs like the p53-p21 and p16^{INK4a} - Rb pathways. The resulting senescent SSCs not only lose their proliferative and regenerative capacity but also actively contribute to a pro-osteoclastic, anti-osteogenic, and pro-inflammatory bone marrow microenvironment via their SASP, further fuelling bone loss and impairing healing. Thus, ET_BR emerges not just as a mediator of

vascular effects, but as a critical intracellular amplifier of oxidative stress and a direct instigator of SSC senescence within the bone compartment. Inhibiting this specific pathway offers a targeted strategy to ameliorate SSC dysfunction at its core molecular drivers – reducing ROS production and mitigating the accumulation and detrimental impact of senescent SSCs.

BQ788 (N-cis-2,6-Dimethylpiperidinocarbonyl-L- γ -methyllleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) is a highly potent, selective, and competitive peptidergic antagonist of the ET_BR. Its pharmacological profile is characterised by exceptional specificity for ET_BR over ET_AR (typically IC₅₀ for ET_BR in the low nanomolar range with >1000-fold selectivity), making it an ideal tool for dissecting the specific roles of ET_BR signalling *in vivo* and *in vitro*. While BQ788 has been extensively used in cardiovascular and renal research, its therapeutic potential within the skeletal system, particularly concerning SSC function and bone regeneration, remains largely unexplored territory with profound implications. The central hypothesis underpinning the work described in this chapter is that systemic pharmacological blockade of ET_BR using intraperitoneal administration of BQ788 will effectively antagonise ET-1/ET_BR signalling in SSCs and the bone marrow microenvironment. This inhibition is anticipated to confer significant therapeutic benefits: (1) Reduction of intracellular ROS accumulation within SSCs; (2) Attenuation of senescence

induction in SSCs and/or enhanced clearance of existing senescent SSCs; (3) Overall improvement in bone mass and microarchitecture by rebalancing bone remodelling towards net formation. By addressing the fundamental SSC dysfunction at the level of ROS and senescence through ET_BR blockade, BQ788 therapy offers a novel mechanistic approach to enhance bone quality and regenerative potential.

However, while systemic pharmacological inhibition offers broad accessibility to the bone marrow compartment and stromal niche, it inherently acts on all ET_BR-expressing cells. While this is potentially beneficial for modulating the overall inflammatory and oxidative microenvironment, discerning the precise contribution of SSC-intrinsic ET_BR signalling to the observed therapeutic effects requires a complementary genetic approach. To achieve this, we employ an inducible, SSC-specific knockout strategy. Utilising mice harbouring LoxP sites flanking the critical exons of the *Ednrb* gene (encoding ET_BR) and crossing them with transgenic mice expressing tamoxifen-inducible Cre-recombinase under the control of the *Prx1* promoter (Prx1-Cre^{ERT2}), allows for highly specific ablation of ET_BR function within the PRX1⁺ SSC population and its osteogenic progeny *in vivo* upon tamoxifen administration. The *Prx1* promoter drives expression predominantly in limb bud mesenchyme during development and is reactivated postnatally in a subset of adult bone marrow

SSCs crucial for bone formation and regeneration. This targeted knockout model enables us to isolate the functional consequences of disrupting ET_BR signalling specifically within the skeletal stem/progenitor lineage itself. Comparing and contrasting the effects of the genetic SSC-specific knockout with those induced by systemic pharmacological knockdown via Adeno-Associated Viral (AAV) vector will provide unparalleled mechanistic insights. It will allow us to dissect the relative contributions of SSC-intrinsic ET_BR signalling versus microenvironmental or systemic effects mediated by ET_BR on other cell types (e.g., endothelial cells, immune cells, neurons) to the overall therapeutic outcomes. This chapter details a comprehensive investigation into the therapeutic efficacy and mechanistic underpinnings of targeting ET_BR signalling for mitigating age-related and pathological bone loss and enhancing skeletal regeneration.

3.2 Methodology

3.2.1 Animal model

The animals were nursed in an environment with a stable temperature of 25°C, a 12/12 cycle of light and dark, and unlimited access to food and water. The approval of animal procedures was given by (1) the Southern University of Science and Technology (SUSTech-JY202303086); (2) the Animal Subjects

Ethics Sub-Committee (22-23/551-BME-R-CRF) and performed according to the guidelines under the Animal (Control of Experiments) Ordinance Cap. 340, licenced (20-257) in DH/HT&A/8/2/4 Pt.4. All animals used in this chapter were female C57BL/6J mice.

3.2.2 Transgenic PRX1⁺ ET_BR knockout mice model

ET_BR^{fl/fl} mice were purchased from Cyagen Biosciences Inc (Guangdong, China). *Prx1-Cre* mice were provided by Professor Xiao Guozhi (Southern University of Science and Technology). *Prx1-Cre::ET_BR^{fl/fl}* mice were bred for further experiments after genotyping (Supplementary Fig. 8, ET_BR: 5'-ATT TTC TTA TCC ACC GAG GCC AA-3', 5'-CTC CGA TCT GAC ACT TTT CAT CTT-3'; Prx1-cre: 5'-GAT CTC CGG TAT TGA AAC TCC AGC-3', 5'-GCT AAA CAT GCT TCA TCG TCGG-3') using Quick Genotyping Assay Kit for Mouse Tail (Beyotime, D7283M). Female *Prx1-Cre::ET_BR^{fl/fl}* mice and *ET_BR^{fl/fl}* mice were selected for OVX surgery at the age of 14 weeks old. Samples were harvested at 22-week-old for further analysis.

3.2.3 AAV-induced global ET_BR knockdown mice model

Female C57BL/6J mice were purchased from GemPharmatech Co., Ltd and raised in Southern University of Science and Technology. Female C57BL/6J mice were given AAV through single intraperitoneal injection at the dose of

5×10^{11} at the age of 14 weeks old. After 4 weeks of induction, mice were sacrificed and samples were harvested for further analysis.

3.2.4 BQ788 treatment in OVX mice model

To induce osteoporosis in the young mice, ovariectomy (OVX) surgery was performed. Female C57BL/6J mice were randomly grouped into SHAM, OVX and treatment groups (pre-OVX and post-OVX treatment). In week 14, the SHAM group mice received operations on skin and muscle incisions only, while mice in the OVX and post-OVX treatment group received OVX surgery. 4 weeks after surgery, the mice in SHAM and OVX received placebo treatment (Saline), and the post-OVX treatment group received BQ788 treatment (1mg/kg per day) for another 4 weeks. The pre-OVX treatment group received OVX surgery in week 18 and BQ788 treatment (1mg/kg per day) starting 3 days before the surgery. All treatments were administered through intraperitoneal injection. All mice were sacrificed on week 22 and micro-CT analysis was conducted on both leg limbs.

3.2.5 ET_BR antagonist treatment in aged mice

Female Balb/c mice were raised to the age of 19 months old. The first μ CT scan was performed to collect the baseline data. Through randomisation, mice were divided equally into two different groups, 3 for placebo and 3 for BQ788

treatment. BQ788 (MCE HY-15894A) was introduced through intraperitoneal injection at the dose of 1mg/kg daily for 1 month. After treatment, a second μ CT scan was performed.

3.3 Results

3.3.1 Alleviated bone loss in OVX-induced PRX1⁺ SSCs-specific ET_BR knockout transgenic mice

In the *in vitro* ET-1 senescence model, we demonstrated the potential of BQ788 in rescuing cellular senescence in MSCs. To understand whether ET_BR was responsible for mediating ET-1 induced bone loss, we adopted an PRX1⁺ SSCs-specific ET_BR knockout transgenic mice model (*Prx1-Cre::ET_BR^{fl/fl}*) with OVX surgery performed (Fig. 12a). Conditional ET_BR knockout reduced plasma ET-1 by 23% ($p<0.05$, Fig. 12b). Comparing the μ CT images, preserved trabecular bone volume ($p<0.01$, Fig. 12c) was observed in conditional ET_BR knockout mice. Bone ROS accumulation decreased by 55% ($p<0.0001$, Fig. 12d). Immunohistology showed reduced expression of senescent markers p16^{INK4a} ($p<0.0001$), p21^{Cip1} ($p<0.001$) and p53 ($p<0.001$) after knockout (Fig. 12e and g). Expression of osteoblast increased (sp7, $p<0.0001$), and osteoclasts reduced (TRAP staining, Fig. 12f and g). These verify ET_BR's role in osteoporosis treatment.

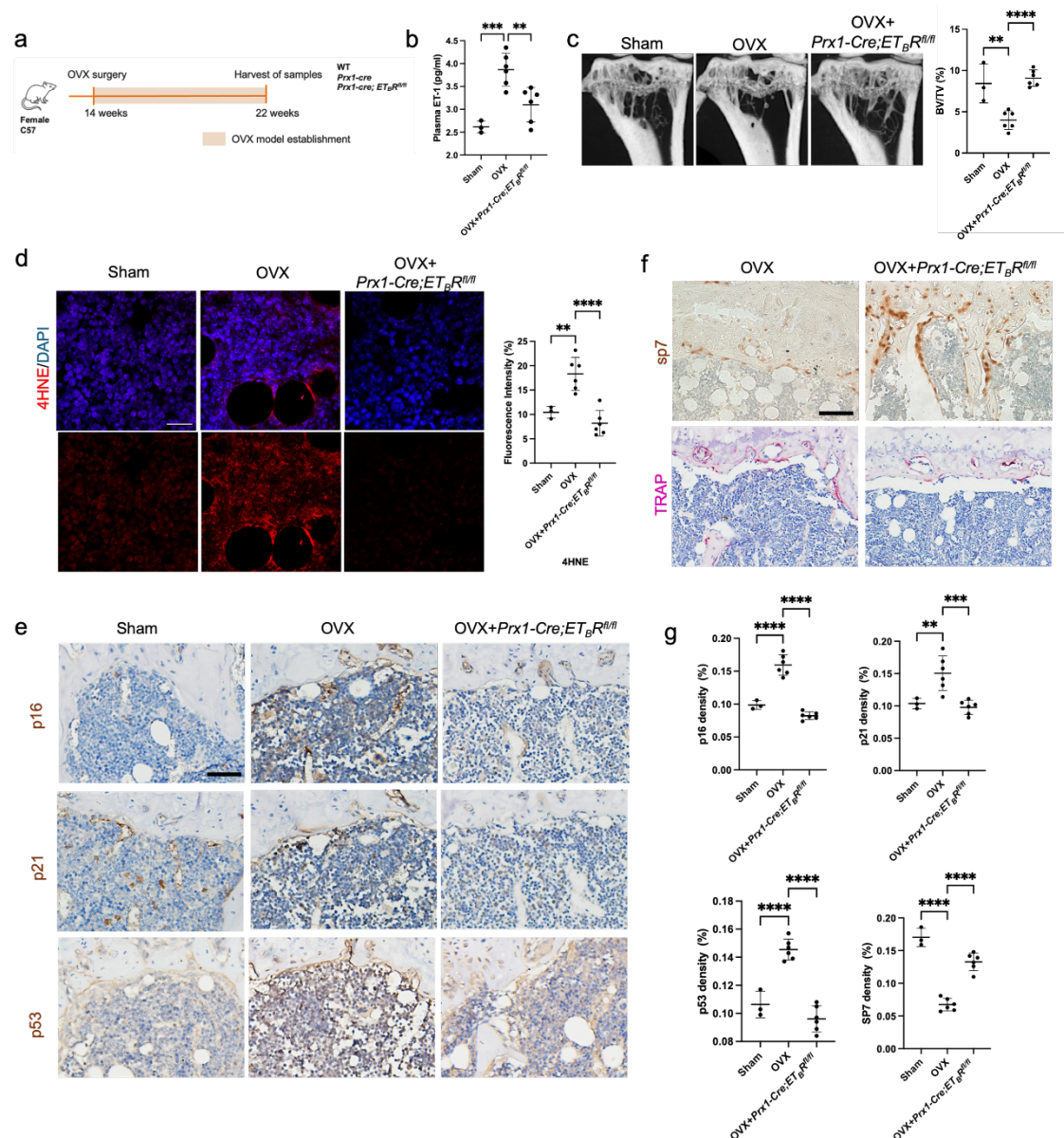


Figure 12 Enhanced bone regeneration in OVX-induced MSC-specific ET_BR knockout transgenic mice. (a) Illustration of OVX establishment in *Prx1-Cre;ET_BR^{fl/fl}* mice. (b) Plasma ET-1 concentration of untreated sham (n = 3), OVX-induced (n = 6) and OVX-induced *Prx1-Cre;ET_BR^{fl/fl}* (n = 6) mice. (c) Representative images and quantification of μCT analysis of tibial subchondral bone (d) Representative images of immunofluorescence staining and quantification of 4HNE in primary spongiosa bone of mice with DAPI (blue) for nuclear

identification. Scale bar, 50 μ m. (f) Representative images of immunohistology staining of sp7 and TRAP. Scale bar, 100 μ m. (e) Representative images of immunohistology staining of p16, p21 and p53. Scale bar, 100 μ m. (g) Quantification of p16, p21, p53 and sp7 staining.

However, in contrast to the success of the SSC-specific genetic knockout, an alternative strategy employing systemic knockdown of ET_BR using adeno-associated virus (AAV) delivery proved ineffective. Systemic AAV-mediated knockdown failed to recapitulate the protective effects observed in the cKO model (Fig. 13). This critical result highlights the importance of cell-type specificity in targeting the ET-1/ET_BR pathway for osteoporosis treatment. It suggests that systemic ET_BR inhibition may disrupt essential physiological functions of the receptor in other cell types or tissues, potentially counteracting any potential benefit within the bone, or that the specific cellular context and signalling within SSCs necessitates precise targeting.

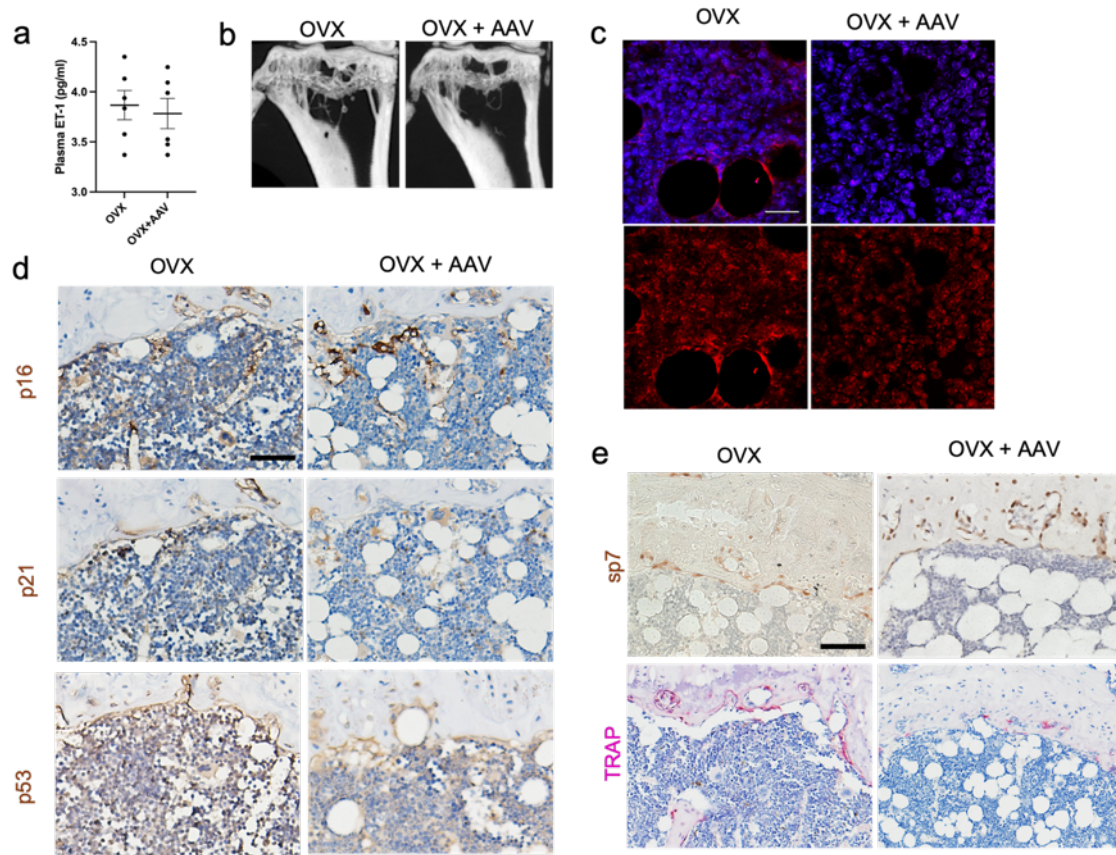
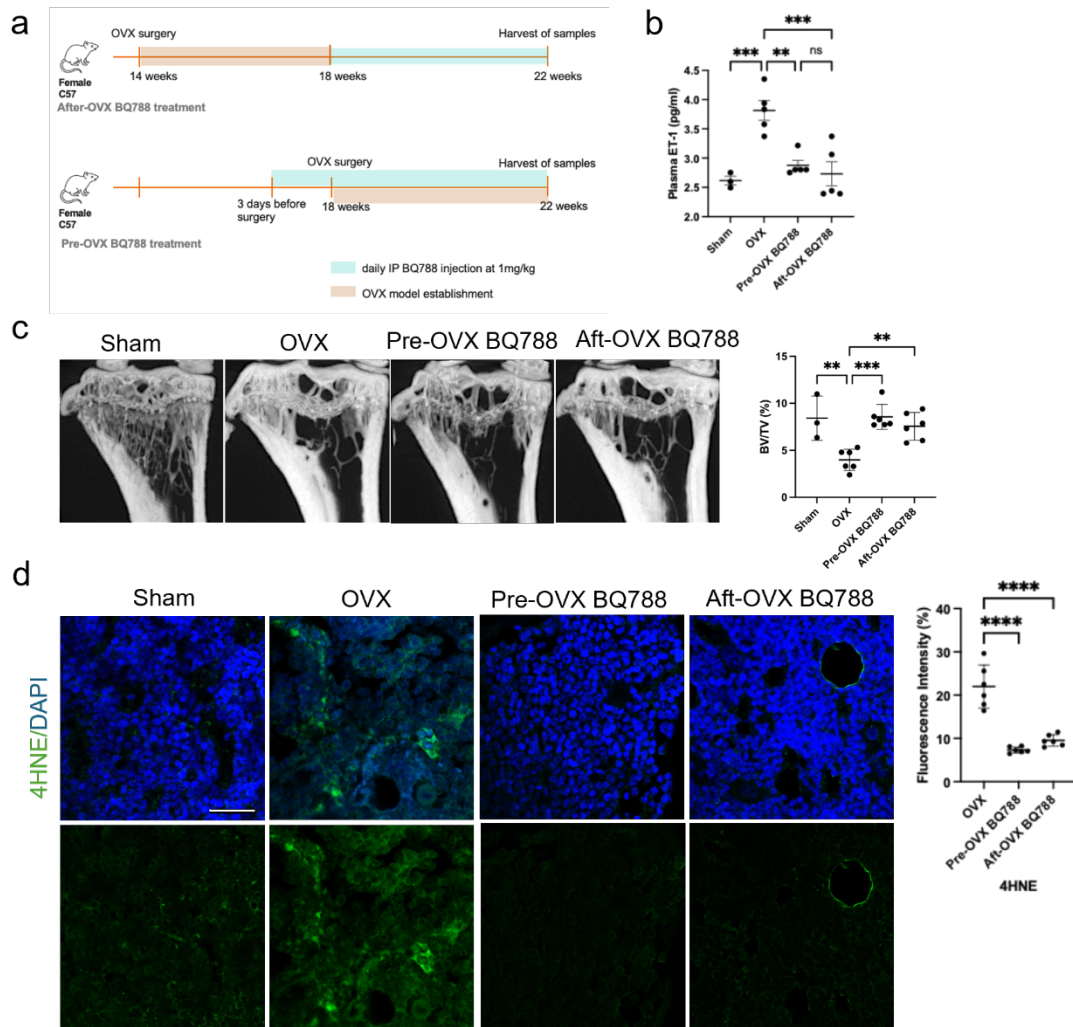


Figure 13 AAV-induced systemic ETBR knockdown failed to alleviate SSCs senescence and bone loss. (a) Plasma ET-1 concentration of OVX-induced (n = 6) and OVX-induced AAV treated (n = 6) mice. (b) Representative images of μ CT analysis of tibial subchondral bone (c) Representative images of immunofluorescence 4HNE staining in primary spongiosa bone of mice with DAPI (blue) for nuclear identification. Scale bar, 50 μ m. (d) Representative images of immunohistology staining of sp7 and TRAP. Scale bar, 100 μ m. (e) Representative images of immunohistology staining of p16, p21 and p53. Scale bar, 100 μ m.

3.3.2 Both preventive and curative treatment of ET_BR antagonist BQ788 in OVX-induced mice model alleviate bone loss

To understand whether preventive or curative treatment of BQ788 exhibits a better effect, OVX surgery was performed on 3M female mice. The preventive treatment group was given BQ788 starting 3 days before the surgery, while the curative treatment group was treated 1 month after the surgery (Fig. 14a).

Plasma ET-1 in OVX mice was reduced by 26% in preventive treatment ($p < 0.01$) (Fig. 6b) and 30% in curative treatment ($p < 0.001$, Fig. 14b). The trabecular bone volume was preserved after preventive treatment ($p < 0.01$), whereas bone volume in curative treatment was less preserved in comparison with OVX mice ($p = 0.0625$, Fig. 14c). Bone ROS accumulation decreased by 63% ($p < 0.0001$) and 55% ($p < 0.0001$) respectively in preventive and curative treatments (Fig. 14d). Immunohistology showed reduced expression of senescent markers p16^{INK4a} ($p < 0.0001$, $p < 0.01$), p21^{Cip1} ($p < 0.001$, $p < 0.01$) and p53 ($p < 0.0001$, $p < 0.0001$) after both treatments (Fig. 14e-f). Expression of osteoblast was increased ($p < 0.0001$, $p < 0.0001$), and osteoclasts were reduced after treatments (Fig. 14e-f). Considering the aforementioned results, preventive treatment demonstrated a better effect in alleviating osteoporosis.



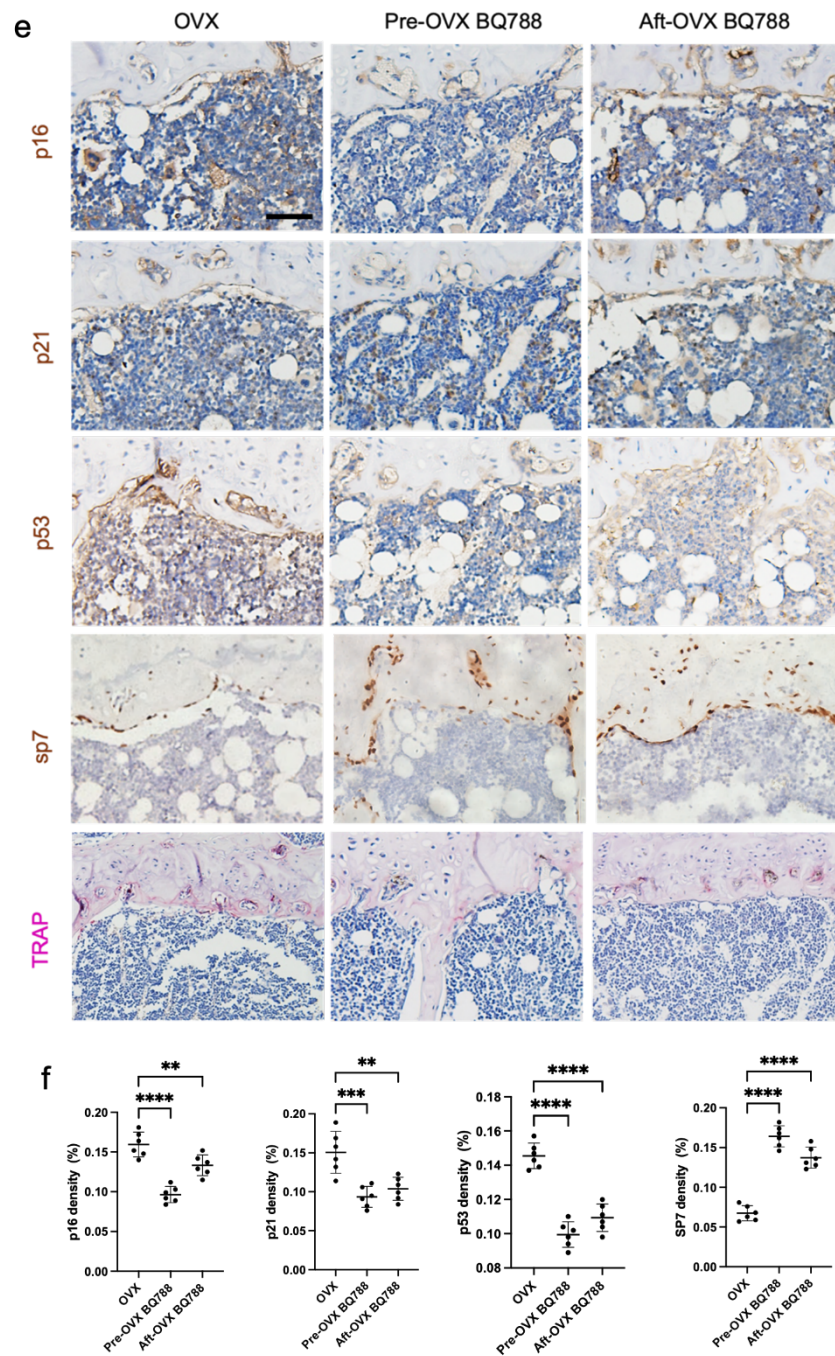


Figure 14 Preventive and treatment effects on OVX-induced bone loss. (a) Schematic illustration of OVX establishment and treatment timeline to study preventive and curative treatment effects of BQ788. (b) Plasma ET-1 concentration of untreated sham ($n = 3$), OVX-induced ($n = 6$), pre-surgery BQ788 treatment ($n = 6$) and after-surgery BQ788 treatment ($n =$

6) mice. (c) Representative images and quantification of μ CT analysis of tibial subchondral bone before and after treatment. (d) Representative images and quantification of 4HNE immunofluorescence staining in primary spongiosa bone of OVX mice. Scale bar, 50 μ m. (e) Representative images of immunohistology staining of p16, p21, p53 and sp7 in primary spongiosa bone of OVX mice. Scale bar, 100 μ m. (f) Quantification of p16, p21, p53 and sp7 immunohistology staining.

3.3.3 ET_BR antagonist improves subchondral bone changes in aged mice

To determine whether BQ788 was beneficial for restoring bone volume during the ageing process, we treated 19M old mice with intraperitoneal injection of ET_BR antagonist BQ788 at the dose of 1mg/kg daily for a total period of 1 month (Fig. 15a). BQ788 reduced plasma ET-1 by 34% ($p < 0.01$, Fig. 15b).

Comparing the μ CT images before and after treatment, trabecular bone volume was preserved to a certain extent ($p = 0.091$, Fig. 15c). Bone ROS accumulation decreased by 28% ($p < 0.01$) (Fig. 15d). Immunohistology showed reduced expression of senescent markers p16^{INK4a} ($p < 0.05$), p21^{Cip1} ($p < 0.01$) and p53 ($p < 0.01$) after treatment (Fig. 15e). Expression of osteoblast increased ($p < 0.05$), and osteoclasts decreased (Fig. 15e). These results position ET_BR inhibition as a viable strategy to mitigate skeletal ageing.

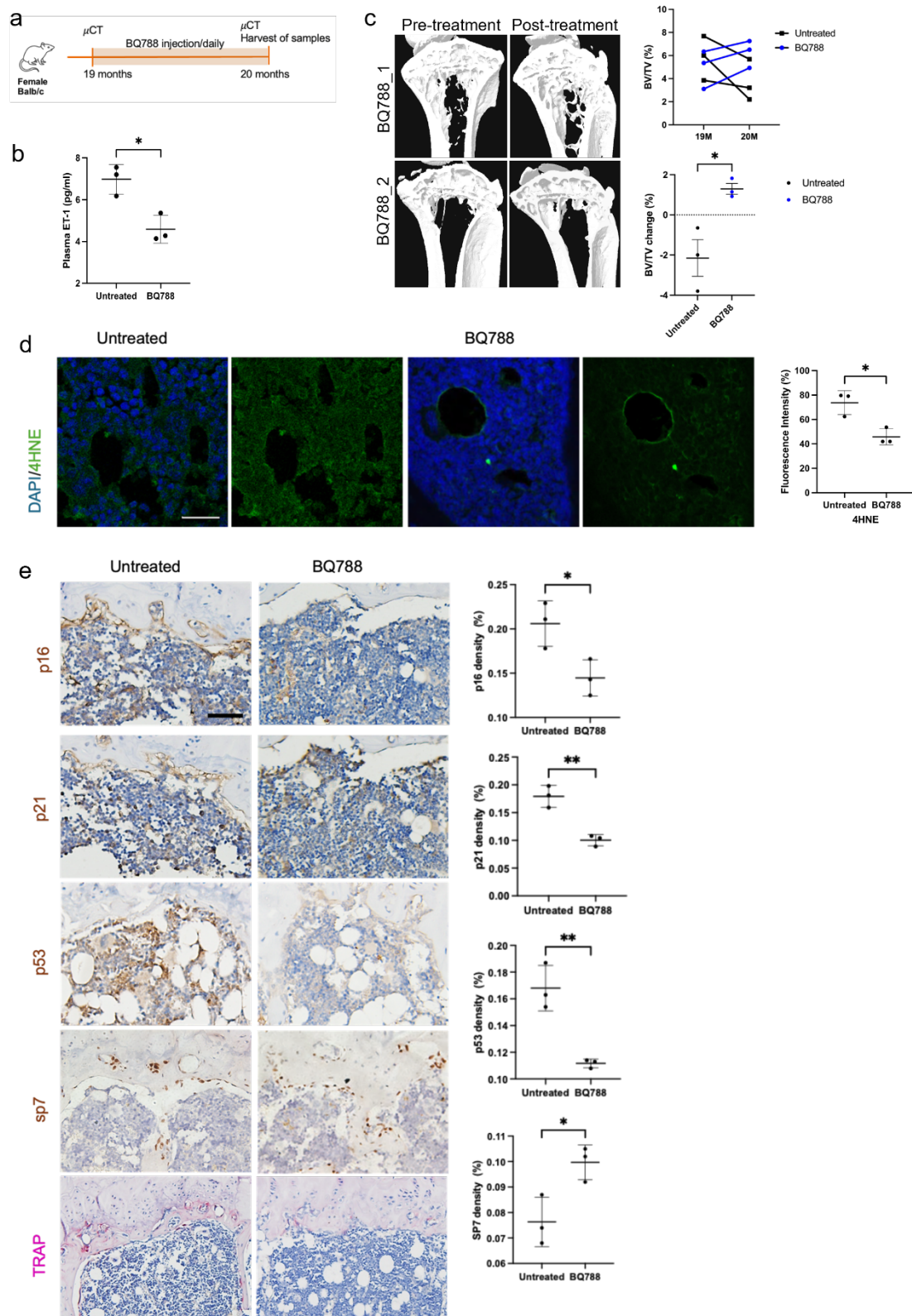


Figure 15 Bone loss due to ageing could be rescued by specific endothelin receptor B

inhibition. (a) Schematic illustration of treatment in aged mice. (b) Plasma ET-1 concentration of untreated (n = 3) and BQ788-treated (n = 3) aged mice. (c) Representative images and quantification of μ CT analysis of tibial subchondral bone and respective BV/TV (%) change before and after treatment. (d) Representative images and quantification of 4HNE immunofluorescence staining in primary spongiosa bone of aged mice. Scale bar, 25 μ m. (e) Representative images and quantification of p16, p21, p53 and sp7 immunohistology staining in primary spongiosa bone of aged mice. Scale bar, 100 μ m.

3.4 Discussion

The therapeutic potential of targeting this axis is underscored by our findings that local genetic ET_BR knockout or pharmacological ET_BR inhibition using BQ788 potentially attenuated SSCs senescence, restored the osteogenic capacity of the SSC pool, and significantly reversed trabecular bone loss in aged mice. Crucially, BQ788 treatment not only reduced the burden of senescent SSCs but also restored bone volume, suggesting that inhibiting senescent cell formation may effectively rejuvenate the osteogenic niche – an approach potentially superior to current antiresorptive or anabolic therapies by targeting the root cause of age-related osteoblast dysfunction. Our observations gain clinical

relevance from reports of elevated plasma ET-1 levels in elderly osteoporotic patients^[165].

However, several considerations regarding therapeutic translation warrant attention. While BQ788 serves as a valuable proof-of-concept research tool, its clinical utility is likely limited by pharmacodynamic and pharmacokinetic constraints^[166]. Specifically, although the precise plasma half-life of BQ788 remains uncharacterized, its documented instability in aqueous solution implies rapid systemic clearance. Furthermore, the lack of clinical studies^[167], and its peptidomimetic structure raises concerns regarding potential off-target effects and suboptimal drug-like properties for human therapeutics. Consequently, the development of longer-acting, non-peptidic, and tissue-selective ET_BR antagonists represents a crucial direction for future investigation. Furthermore, while our murine model recapitulates key features of osteoporosis, skeletal ageing is multifactorial. ET-1/ET_BR inhibition may prove most effective as part of a combinatorial strategy, potentially integrated with senolytics (to clear existing senescent cells), antioxidants (to mitigate residual oxidative stress), or agents targeting parallel drivers such as chronic inflammation or intrinsic mitochondrial dysfunction, which were not fully addressed here.

By establishing ET-1 as a paracrine driver orchestrating SSCs senescence through ET_BR, this work fundamentally redefines osteoporosis pathogenesis through the lens of dysfunctional vascular-stem cell crosstalk within the ageing bone marrow niche. Our results position ET_BR antagonists as a novel class of "geroprotective" therapies capable of disrupting the vicious cycle linking vascular ageing to skeletal decline. Future investigations should focus on elucidating the systemic effects of ET-1 in bone homeostasis beyond the niche, refining selective ET_BR antagonists with improved pharmacokinetic properties, and rigorously validating this therapeutic strategy in clinically relevant models, including large animals and human SSCs systems. Ultimately, translating these findings could revolutionize the management of age-related bone loss, shifting the paradigm from palliative care to proactive stem cell rejuvenation.

This observed discrepancy between the established pharmacological principle and our experimental data presents a compelling point for discussion. While prevailing literature robustly demonstrates that acute ET_B receptor blockade with BQ788 leads to a rapid and sustained increase in circulating ET-1 due to the inhibition of its primary clearance pathway^[123], our chronic one-month administration in aged and OVX mice yielded the opposite result—a significant reduction in plasma ET-1 concentrations. This divergence suggests that the long-term modulation of the endothelin system in a pathophysiological context

engages compensatory mechanisms that override the acute pharmacokinetic effect. A primary explanation may lie in the presence of a potent negative feedback loop. Chronic ETB inhibition and the subsequent initial rise in bioactive ET-1 could lead to a downregulation of endothelin-converting enzyme (ECE) activity or a suppression of prepro-ET-1 transcription within the vascular endothelium and other tissues over time, ultimately reducing the net synthesis and release of the peptide. Furthermore, the pathological milieu of aged and oestrogen-deficient bone, characterised by chronic inflammation and oxidative stress, may uniquely influence this regulation. It is plausible that BQ788, by altering the balance of ET-1 signalling, indirectly ameliorates the inflammatory drivers that are themselves potent stimulators of ET-1 production in these models. Consequently, the reduction in systemic inflammation we observed may be the primary event, leading to a decreased stimulus for ET-1 synthesis that surpasses the impact of impaired clearance. This finding underscores the critical distinction between acute receptor pharmacology and chronic in vivo adaptation, suggesting that in the context of skeletal ageing and oestrogen deficiency, prolonged ETB receptor antagonism may modulate the system at the level of ligand production rather than merely receptor engagement or ligand clearance.

Chapter 4 A Dual-Targeting Hydrogel Therapeutic for Modulating Endothelin Signalling in Age-Related Tissue Degeneration

4.1 Introduction

4.1.1 ET-1/ETB axis as a driver of age-related bone-vascular crosstalk

The foundational work of this thesis has rigorously delineated a pathogenic cascade wherein dysregulated ET-1 signalling, with a particular focus on the ETB receptor pathway, functions as a critical mechanistic link between vascular dysfunction and skeletal frailty in ageing. Our data substantiate that the aged microenvironment is characterised by a loss of coordinated endothelin signalling, contributing to a state of chronic, low-grade inflammation and impaired cellular homeostasis. The observed paradoxical reduction in circulating ET-1 following chronic BQ788 administration in our murine models is a pivotal finding. It suggests that prolonged ETB receptor antagonism does not merely block a clearance pathway but actively engages complex systemic and local feedback mechanisms, potentially downregulating prepro-ET-1 synthesis or altering receptor trafficking. This dysregulation is not a bystander phenomenon but an active driver of pathology. Within bone, it fosters a

senescence-associated secretory phenotype (SASP) in stromal cells and promotes osteoclastogenesis, directly linking vascular-derived ET-1 to net bone loss^[168]. Concurrently, in the vasculature, disrupted ET-1/ETB signalling exacerbates endothelial dysfunction, reducing perfusion and creating a hypoxic, pro-inflammatory niche that further undermines skeletal health^[169]. This creates a vicious, self-perpetuating cycle of degeneration between the two organ systems. Therefore, the ET-1/ETB axis emerges not merely as a biomarker but as a central therapeutic target, the disruption of which is essential to halt this crosstalk and promote a return to regenerative homeostasis.

The translation of endothelin receptor antagonists from cardiovascular to musculoskeletal therapeutics presents a significant pharmacological challenge centred on the issue of systemic exposure. While drugs like bosentan and macitentan are effective in pulmonary arterial hypertension, their use is associated with dose-dependent adverse effects, including peripheral oedema, hepatic enzyme elevation, and teratogenicity^[170; 171]. These limitations are particularly problematic for treating chronic, non-fatal conditions like osteoporosis or localised tissue degeneration, where long-term tolerability is paramount. Systemic administration necessitates lower doses to avoid complications, which may be sub-therapeutic for the intended skeletal or vascular regenerative effects. Furthermore, the ubiquitous expression of

endothelin receptors means systemic blockade affects renal, pulmonary, and cardiac function, creating an unacceptable risk-benefit profile for a localised musculoskeletal indication. A localised delivery strategy elegantly circumvents these hurdles. By encapsulating therapeutic agents within an implantable hydrogel depot, high local concentrations can be achieved and sustained directly within the pathological tissue microenvironment—be it a fracture site, a segment of osteoporotic bone, or a critical bone defect. This approach maximises therapeutic impact on the target cells (osteoblasts, osteoclasts, endothelial cells) while minimising systemic circulation and off-target organ exposure. It transforms the therapeutic window, allowing the use of potent pathway modulators like BQ788 or macitentan at effective concentrations without incurring their systemic liabilities, thereby enabling precise, long-term modulation of the bone-vascular axis where it is most needed.

4.1.2 Alginate hydrogel as a versatile local delivery platform

Alginate-based hydrogels demonstrate distinct advantages over other biomaterial bases used in wound healing applications, stemming from their unique combination of natural origin, physicochemical properties, and functional capabilities^[172]. Derived from brown seaweed, alginate is a naturally occurring polysaccharide that offers inherent biocompatibility and

biodegradability, ensuring the hydrogel matrix breaks down safely within the physiological environment without requiring surgical removal – a significant advantage over many synthetic polymer bases that may degrade into potentially inflammatory by-products or necessitate extraction. This natural origin also contributes to alginate's excellent non-toxicity profile, minimising adverse reactions and promoting better tissue integration compared to some synthetic alternatives or even other natural polymers like collagen or gelatin^[173], which can exhibit batch-to-batch variability or carry risks of immunogenicity or pathogen transmission if not rigorously processed.

A particularly defining feature of alginate is its ability to form stable hydrogels rapidly and efficiently under exceptionally mild, physiologically relevant conditions via simple ionic crosslinking with divalent cations like calcium (Ca^{2+}) or strontium (Sr^{2+})^[174]. This gentle gelation mechanism stands in stark contrast to hydrogels formed from synthetic polymers like polyethylene glycol (PEG) or polyvinyl alcohol (PVA), which often require potentially cytotoxic chemical crosslinkers, UV light initiation, or harsh conditions that could damage sensitive therapeutic agents or surrounding tissue^[175]. Similarly, while chitosan, another popular natural polysaccharide, offers inherent antimicrobial properties, its gelation often requires pH adjustments or chemical crosslinking agents, whereas alginate's ionic crosslinking is simpler and more versatile for *in*

situ formation. Furthermore, alginate hydrogels excel in maintaining a moist wound environment crucial for healing, comparable to other hydrophilic bases, but their high swelling capacity and tunable degradation kinetics can be more readily controlled than those of some alternatives. The incorporation of specific ions like Sr^{2+} in Alg@Sr adds another layer of functionality; strontium is known for its osteogenic and potentially angiogenic properties, offering therapeutic benefits beyond mere structural support – a feature not inherently present in neutral synthetic polymers or even other ionically crosslinked systems like simple calcium alginate. While decellularized extracellular matrix (dECM) hydrogels provide excellent biomimicry, their complex composition, variable sourcing, and intricate processing present challenges that alginate, with its well-defined chemistry and ease of modification, largely avoids.

Therefore, alginate-based hydrogels represent a uniquely advantageous platform within the spectrum of tissue regeneration biomaterials, combining natural biocompatibility and safety, mild and versatile fabrication, excellent moisture management, tunable properties, and the potential for enhanced bioactivity through ion incorporation, positioning them favourably against both synthetic polymers and other natural alternatives for promoting effective tissue regeneration.

4.1.3 Targeting skeletal regeneration

The pathophysiology of skeletal ageing is multifaceted, driven not by a single deficit but by the concurrent rise of catabolic processes and the decline of anabolic ones^[176]. Therefore, an effective therapeutic strategy must be equally multifaceted, employing a dual-pronged approach to simultaneously dismantle detrimental pathways and actively promote regeneration. On one front, the accumulation of senescent cells and the associated SASP create a pro-inflammatory, pro-resorptive local milieu that actively inhibits endogenous repair mechanisms and disrupts normal bone remodelling. Our work positions ET-1/ETB signalling as a contributor to this senescent burden. Thus, the first therapeutic arm aims to disrupt this pathological niche using the ETB antagonist BQ788. By blocking this receptor, we hypothesise a reduction in SASP factor production and a potential shift in the tissue environment away from senescence. However, merely removing inhibitory signals is insufficient to restore lost bone mass. Hence, the second, complementary arm is direct anabolic stimulation. This is achieved through the sustained local release of calcium ions, a fundamental osteogenic signal. Calcium acts as a potent inducer of osteoblast proliferation, differentiation, and mineralising activity^[177]. This combination strategy is designed to break the cycle of degeneration by pharmacologically silencing a key driver of the aged phenotype (via BQ788)

while concurrently providing the raw molecular signal (via Ca^{2+}) to catalyse the formation of new, healthy bone tissue, thereby addressing both sides of the imbalanced remodelling equation.

4.1.3.1 BQ788 – a senolytic and anti-resorptive agent via ETB blockade

The selection of BQ788 as the lead pharmacological agent is a direct translational consequence of this thesis's core mechanistic findings. While classical pharmacology indicates that ETB blockade should elevate circulating ET-1 by inhibiting its clearance, our *in vivo* data in aged models revealed a more complex, adaptive response: a significant reduction in plasma ET-1 following chronic administration. This suggests BQ788 is not a passive blocker but an active modulator of the systemic and local endothelin system, potentially triggering a feedback downregulation of ET-1 production. Within the skeletal microenvironment, this modulation is theorised to yield two key benefits. First, by interfering with ET-1/ETB signalling, BQ788 may disrupt the communication network that sustains cellular senescence, potentially exerting a senomorphic effect that dampens the SASP or a senolytic effect that selectively removes senescent cells. Second, ET-1 is a known stimulator of osteoclast differentiation and activity^[178], an effect mediated through both direct and indirect pathways. Therefore, ETB blockade with BQ788 could attenuate this

pro-resorptive signal, leading to a reduction in bone breakdown. Its local delivery from the hydrogel ensures these potentially beneficial effects are concentrated within the bone tissue, maximising the impact on the senescent niche and bone turnover while insulating the wider system from the paradoxical hypertensive effects that can accompany systemic ETB blockade.

4.1.3.2 Calcium – an osteostimulative cation

The incorporation of calcium within the hydrogel system serves a critical dual function, operating as both a structural component and a potent therapeutic agent. Primarily, calcium ions (Ca^{2+}) are the classic cross-linking agent for alginate, instantly forming the stable, three-dimensional "egg-box" junctions between guluronic acid chains that give the hydrogel its structural integrity^[179]. This role is fundamental to the material's immediate mechanical performance and its ability to be delivered as a cohesive implant. Therapeutically, the subsequent controlled release of Ca^{2+} ions during the hydrogel's gradual biodegradation is central to the anabolic strategy. Calcium is the principal mineral constituent of bone hydroxyapatite and a ubiquitous intracellular secondary messenger^[180]. Locally elevated Ca^{2+} concentrations act as a powerful chemotactic and proliferative signal for osteoprogenitor cells and mature osteoblasts. It activates the calcium-sensing receptor (CaSR) on these

cells, triggering downstream signalling cascades that promote osteogenic gene expression, collagen synthesis, and matrix mineralisation^[181]. Furthermore, the sustained local release helps maintain a supersaturated microenvironment conducive to *de novo* mineral nucleation and growth. By leveraging Ca²⁺ in this way, the hydrogel transcends being a passive drug carrier; it becomes an active participant in regeneration, providing the essential ionic building blocks and signalling cues to directly orchestrate the deposition of new bone mineral within the defect site, synergising with the pharmacological action of BQ788.

4.1.4 Targeting Vascular regeneration

Effective regeneration of mineralised tissues like bone is critically dependent on concomitant revascularisation to deliver oxygen, nutrients, and progenitor cells^[182]. The aged and diabetic microenvironments targeted in this work are characterised by endothelial dysfunction and impaired angiogenesis. ET-1, elevated in these states, exacerbates this via ETA-mediated vasoconstriction and endothelial cell dysfunction. Furthermore, as discussed, ET-1 signalling is intertwined with cellular senescence, creating a barrier to new vessel formation. Normalising endothelin tone is therefore a logical strategy to promote a pro-angiogenic environment.

4.1.4.1 Macitentan –dual ETA/ETB antagonist to normalise endothelin tone

Macitentan, clinically established as an endothelin receptor antagonist, is primarily licensed for the management of pulmonary arterial hypertension, targeting the dysregulated endothelin pathway central to that condition^[183]. However, emerging preclinical and clinical evidence suggests its therapeutic potential may extend significantly into the field of wound repair and regeneration^[184]. This potential stems from its documented anti-inflammatory and pro-angiogenic properties. The rationale lies in the recognised role of ET-1, the primary ligand for these receptors, in the pathogenesis of chronic, non-healing wounds. Elevated ET-1 levels within the wound microenvironment contribute detrimentally by driving persistent inflammatory cascades and promoting excessive fibrosis (scar tissue formation), both of which impede the normal progression of healing^[185]. Macitentan exerts its beneficial effects in this context by functioning as a potent blocker of endothelin signalling. By antagonising the binding of ET-1 to its receptors (ETA and ETB), Macitentan disrupts these deleterious pathways. Consequently, it acts to dampen chronic inflammation, improve local tissue perfusion by mitigating ET-1 induced vasoconstriction and endothelial dysfunction, and critically, stimulate the formation of new blood vessels (angiogenesis). These combined actions – reducing inflammation, enhancing microcirculation, and promoting

neovascularisation – address fundamental barriers to healing, positioning Macitentan as a promising pharmacological agent for enhancing outcomes in challenging wound scenarios.

4.1.4.2 Strontium – a pro-angiogenic and osteogenic agent

Strontium (Sr^{2+}), a divalent cation sharing significant chemical similarities with calcium (Ca^{2+}), exhibits a notable affinity for biological mineralisation processes and has garnered considerable interest for its beneficial effects on tissue regeneration, particularly within bone and skin^[186]. Its biological activity stems partly from its ability to mimic calcium ions, allowing it to interact with calcium-sensing receptors and potentially influence intracellular signalling pathways relevant to cell growth and differentiation. Within the context of skin repair, strontium ions demonstrate a capacity to actively stimulate the proliferation of key cellular players: fibroblasts, responsible for synthesising the extracellular matrix scaffold, and keratinocytes, essential for re-epithelialisation and restoring the skin barrier. Furthermore, strontium enhances the synthesis and deposition of collagen, a fundamental structural protein crucial for providing tensile strength to the nascent tissue and facilitating proper wound remodelling. Beyond its direct effects on matrix production and cell proliferation, strontium also promotes angiogenesis – the formation of new

blood vessels – which is vital for delivering oxygen and nutrients to the healing wound site, thereby supporting the metabolic demands of regenerating tissue. Complementing these pro-regenerative actions, strontium possesses documented anti-inflammatory properties, helping to modulate the often dysregulated inflammatory response characteristic of chronic wounds and creating a more conducive environment for healing progression. This multifaceted profile – stimulating essential cell types, boosting collagen production, encouraging vascularisation, and dampening detrimental inflammation – positions strontium as a highly valuable functional component within advanced wound healing biomaterials.

4.1.5 Significance of hydrogel in tissue regeneration

Reduces Inflammation and Senescence: Anti-inflammatory and anti-senescence effects of BQ788 and macitentan help resolve the prolonged inflammatory phase seen in chronic wounds.

Promotes Angiogenesis: Strontium enhance blood vessel formation, addressing the inadequate blood supply often observed in chronic wounds. Supports ECM

Remodeling: Strontium ions and alginate work together to promote collagen deposition and tissue organization, facilitating proper wound closure.

Moist Wound Environment: The hydrogel maintains a moist environment, which is crucial for cell migration, nutrient exchange, and preventing scab formation.

Sustained and Controlled Drug Delivery: The hydrogel formulation functions as a reservoir facilitating the controlled, prolonged liberation of Macitentan.

This sustained release profile ensures extended pharmacological activity directly at the wound site, maintaining therapeutic concentrations over time. Such prolonged delivery is critically advantageous, particularly for managing persistent non-healing lesions where consistent therapeutic intervention is essential over extended periods. Furthermore, the kinetics governing the release of Macitentan from the hydrogel matrix are not fixed but can be precisely engineered. This tunability is achieved through deliberate modifications to the hydrogel's composition, such as altering polymer concentration, crosslinking density, or incorporating specific functional groups. This capacity for adjustment allows for meticulous control over the drug delivery profile, enabling optimisation tailored to specific wound healing requirements and timelines.

Enhanced Mechanical and Biological Properties: The inclusion of strontium ions (Sr^{2+}) within the hydrogel matrix confers significant advantages beyond

mere structural support. Firstly, Sr^{2+} integration enhances the material's structural integrity and robustness. This reinforcement improves the hydrogel's ability to withstand the dynamic physiological stresses encountered within a healing wound site, making it a more reliable scaffold for facilitating tissue regeneration under such conditions. Furthermore, Sr^{2+} acts as a potent bioactive agent, actively contributing to the regenerative process. Its presence stimulates key cellular processes essential for repair, such as promoting fibroblast activity and collagen production. Concurrently, Sr^{2+} contributes to the modulation of inflammatory pathways, helping to create a more favourable microenvironment for healing progression. Thus, strontium serves a dual purpose: fortifying the hydrogel physically while simultaneously delivering beneficial biological effects that actively foster regenerative processes.

Potential for Personalized Medicine: The hydrogel's composition can be easily modified to accommodate different wound types or patient-specific needs. For example, the concentration of Macitentan or strontium can be adjusted based on the severity of the wound or the underlying condition (e.g., diabetic wounds, burns).

Novelty in Combining Drug and Ion Therapy: The strategic combination of alginate hydrogel, acting as a biocompatible delivery vehicle and moist wound

environment manager, with the dual endothelin receptor antagonist Macitentan, targeting pathological inflammation and promoting angiogenesis via ET-1 blockade, and the bioactive strontium ions, offers a synergistic therapeutic approach. This triad aims to comprehensively address the complex pathophysiology of impaired healing by simultaneously targeting inflammation, enhancing vascular supply, stimulating cellular activity, and providing structural support for accelerated and improved skin regeneration. While alginate-based hydrogels, Macitentan, and strontium have individually shown promise in wound healing, their combined use has not been extensively explored. Beyond wound healing, this hydrogel could be adapted for other tissue regeneration applications, such as bone repair or cartilage regeneration, given the known osteogenic and chondrogenic properties of strontium and the anti-fibrotic effects of Macitentan. Due to time constraints, we only managed to test our hydrogels on a mice wound healing model in this chapter. The hydrogel's effect on bone tissue regeneration is yet to be validated.

4.2 Methodology

4.2.1 Materials

The primary polymeric component, sodium alginate (AR; S278630-500g), along with the crosslinking agents calcium chloride (ACS; C110768-100g), strontium

chloride (S189488-100g) were procured from Aladdin Biochemical Technology Co., Ltd. Endothelin-1 (HY-P0202) and the endothelin receptor antagonist, BQ788 (HY-15894A) and Macitentan (ACT-064992), were sourced from MedChemExpress. Essential reagents for cell culture work included Dulbecco's Modified Eagle Medium (DMEM; C11995500BT), foetal bovine serum (FBS; 10270106), trypsin-EDTA solution, and penicillin-streptomycin solution (PS; 15140122), all supplied by Gibco (ThermoFisher). Specific assay kits were acquired for various biological evaluations: the Cell Counting Kit-8 (CCK-8; C0038) for quantifying cellular proliferation and viability; the Calcein AM/PI Double Stain Kit (C2015M) for performing live/dead cell viability and cytotoxicity assays; and the Senescence β -Galactosidase Staining Kit (C0602) for detecting cellular senescence, all obtained from Beyotime Institute of Biotechnology. Molecular biology reagents comprised the Total RNA Kit I for RNA extraction, supplied by Omega Bio-Tek (R6834-02), the RT Master Mix for qPCR II (HY-K0510A), and SYBR Green qPCR Master Mix (HY-K0501), both procured from MedChemExpress.

4.2.2 Fabrication of drug-loaded alginate strontium hydrogels

A hydrogel precursor solution was prepared by dissolving sodium alginate powder in deionised distilled water (ddH₂O) to achieve a final concentration of

4% (w/v). This dissolution process was facilitated by heating the mixture to 70°C under continuous agitation. Subsequently, the homogeneous alginate solution was subjected to overnight stirring at room temperature using an electric mixer operating at 300 revolutions per minute (rpm). Concurrently, Macitentan was solubilised in phosphate-buffered saline (PBS) adjusted to pH 7.4. The dissolved sodium alginate solution was then divided equally into four separate beakers. To these aliquots, Macitentan was introduced at distinct final concentrations: 0nM (Alg@Sr), 100nM, 1µM, and 10µM. Each of these four mixtures underwent thorough blending for a period of two hours at room temperature, employing the electric mixer set at 200 rpm to ensure uniform distribution of the therapeutic agent within the alginate matrix. Following this incorporation phase, the drug-loaded alginate solutions were individually dispensed, dropwise, into a bath containing a 5% (w/v) aqueous solution of strontium chloride (SrCl₂). This ionic crosslinking step resulted in the formation of discrete hydrogel beads, designated as Alg@Sr (for the control without drug) and Mac/Alg@Sr (for the drug-loaded variants). The freshly formed hydrogel beads were subsequently retrieved from the crosslinking solution. To eliminate any unincorporated polymers and residual strontium salts adhering to the bead surfaces, the collected beads underwent a rigorous washing protocol, involving three successive rinses with copious amounts of ddH₂O.

BQ788/Alg@Ca was prepared in a similar manner with formulation of 4% (w/v) sodium alginate and 1% (w/v) calcium chloride.

4.2.3 Hydrogel characterisation: morphology and structure

To facilitate structural analysis, the hydrogel underwent lyophilisation for a period of 48 hours, utilising a vacuum freeze-drier (Scientz-10N/A). The resultant freeze-dried samples were subsequently subjected to morphological examination via scanning electron microscopy (SEM) using a Nova NanoSEM450 instrument. Sample preparation for SEM involved sectioning the lyophilised hydrogels with a Lycra blade to achieve specimens of appropriate dimensions. These sections were then mounted onto the SEM sample stage using conductive adhesive tape, ensuring the cut surface was oriented upwards to expose the internal structure. To ensure adequate conductivity and prevent charging artefacts, the mounted samples received a coating of platinum applied via sputtering for a duration of 60 seconds. Imaging of the hydrogel's internal microstructure was then performed under an accelerating voltage of 5 kV.

Complementary chemical characterisation was conducted using Fourier Transform Infrared Spectroscopy (FTIR). Spectra were acquired on a Vector33 spectrometer (Bruker). Preparation of the hydrogel samples for FTIR analysis involved grinding the lyophilised material into a fine powder. This powder was

then thoroughly mixed with potassium bromide (KBr) powder and compressed under high pressure to form translucent solid pellets suitable for transmission-mode measurement. The FTIR analysis identifies characteristic chemical functional groups within the samples by detecting specific absorption bands across the infrared spectrum. Spectral data were systematically collected over the wavenumber range of 4000 to 400 cm^{-1} , capturing the fingerprint region essential for identifying molecular bonds and structural features within the alginate-based hydrogels and their incorporated components.

4.2.4 Thermal properties

The thermal stability characteristics of the synthesised hydrogels were evaluated using thermogravimetric analysis (TGA). This characterisation was conducted employing a Mettler TGA2 instrument. Measurements were acquired across a defined temperature spectrum, commencing at ambient temperature (25°C) and extending to 400°C. The temperature was increased at a controlled ramp rate of 10 °C per minute throughout the analysis. To ensure an inert atmosphere and prevent oxidative degradation during heating, the sample environment was maintained under a continuous purge of dry nitrogen gas flowing at a rate of 50 mL per minute.

4.2.5 Mechanical performance

The bulk mechanical characteristics of the composite hydrogels were assessed using a Rheometer HAAKE MARS III (ThermoScientific) operating at ambient temperature. Hydrogel specimens were fabricated within cylindrical moulds possessing dimensions of 20 mm in diameter and 2 mm in height. To investigate their rheological behaviour under deformation, oscillatory strain amplitude sweeps were conducted. This methodology involved systematically varying the applied strain amplitude, either incrementally increasing or decreasing it, while simultaneously monitoring the resultant stress response generated by the material. Through this analysis, the key viscoelastic parameters characterising the hydrogels – specifically, the storage modulus (G') and the loss modulus (G'') – were quantified. The storage modulus (G') reflects the elastic, solid-like component of the material's response, indicative of its ability to store energy, while the loss modulus (G'') represents the viscous, liquid-like component, signifying energy dissipation.

4.2.6 Swelling ratio

Following preparation, the hydrogel samples underwent lyophilisation (freeze-drying) to remove all aqueous content. The resulting dry mass of each sample, denoted as W_0 , was accurately determined using a precision analytical balance

(Sartorius, Germany). Subsequently, the lyophilised samples were individually immersed in 10 mL of Dulbecco's Modified Eagle Medium (DMEM), maintained at a physiological temperature of 37°C to initiate the swelling process. To characterise the swelling kinetics, measurements of the swollen mass (W_t) were taken at predetermined intervals: specifically at 2, 4, 6, 8, 10, 24, 36, 48, and 60 hours post-immersion. At each designated time point, a sample was carefully retrieved from the medium. Any excess liquid adhering to the hydrogel surface was meticulously removed by gentle blotting with filter paper, ensuring minimal disturbance to the absorbed fluid within the polymer network, before the swollen weight was recorded. This procedure was performed in triplicate ($n=3$) for each hydrogel formulation and time point to ensure statistical robustness and minimise the impact of random experimental variation. The mean value of these triplicate measurements, expressed alongside the standard deviation ($\pm$ SD), was calculated and used as the representative water uptake value for each specific sample at that time. The swelling ratio (S_t), quantifying the extent of fluid absorption relative to the dry state, was then determined for each time point using the following equation:

$$S_t = (W_t - W_0)/W_0 \quad (4-1)$$

4.2.7 In vitro BQ788 / Macitentan drug release study

In *vitro* drug release behaviours of hydrogels were performed. Briefly, 0.3 g of hydrogel samples were placed into a dialysis bag in 100 mL (V_0) PBS medium (PH=7.4) with 0.1% w/v Tween 80, and then the systems were transferred to a shaking incubator set at a shaking speed of 100 rpm at 37°C. The release solutions (1mL, V_e) were collected and the same volume of preheated PBS was replenished at predetermined time points. Subsequently, the Macitentan concentration of the obtained solutions (C_t) was determined using HPLC (Agilent 1260 Infinity) at 220 nm. Additionally, to calculate the cumulative drug release percentage at set time intervals, the total amount of Macitentan (M_0) in different hydrogels was measured according to the following steps: first, all hydrogels were crushed using a pestle in an agate mortar, and placed into a conical flask, respectively; then, ethanol absolute was poured into the flasks, and the mixtures were sonicated using an ultrasound instrument (SB-4200D, Scientz) for 30 min; after that, the mixtures were centrifuged, and the obtained supernatant fluids were used for HPLC analysis. The cumulative Macitentan release percentage in different hydrogels was calculated following the equation below:

$$Cumulative\ percentage\ (\%) = (Ve \sum_{1}^{n-1} C_t + V_0 C_n) / M_0 \times 100\% \quad (4-2)$$

4.2.8 Ca^{2+} / Sr^{2+} ions release study

The release profile of calcium ions (Ca^{2+}) from the Alg@Sr hydrogel and strontium ions (Sr^{2+}) from the Alg@Sr hydrogel formulation was quantitatively determined using inductively coupled plasma optical emission spectrometry (ICP-OES; Spectro Arcos II MV instrument). To conduct this analysis, precisely weighed hydrogel samples (0.1 g) were individually encapsulated within dialysis membranes. These prepared samples were subsequently immersed in 100 mL of phosphate-buffered saline (PBS) release medium, maintained at physiological pH (7.4) and temperature (37°C), within an orbital shaking incubator operating at 100 revolutions per minute. Following encapsulation, the release study proceeded by periodically withdrawing 1 mL aliquots of the surrounding medium at predetermined time intervals. Immediately after each aliquot was removed, the release vessel was replenished with an equivalent volume of fresh, pre-warmed PBS to maintain a constant total volume and sink conditions throughout the experiment. The aliquots collected at each time point were then subjected to Sr^{2+} concentration analysis via ICP-OES, employing appropriate calibration standards and previously established instrumental protocols. This methodology allowed for the

systematic characterisation of the sustained release behaviour of strontium ions from the hydrogel matrix over time.

4.2.9 Cell culture

Murine mesenchymal stem cells (MSC), human umbilical vein endothelial cells (HUVEC) and mouse embryonic fibroblasts (NIH3T3) were maintained under standard cell culture conditions (37°C, 5% CO₂ humidified atmosphere). These cell lines were propagated in Dulbecco's Modified Eagle Medium (DMEM 1x, Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco) and 1% (v/v) Penicillin Streptomycin solution (PS, Gibco), using 25 cm² culture flasks. Mouse macrophage cells (RAW264.7) were cultivated under identical environmental conditions but using DMEM supplemented with 5% FBS and 1% PS. The culture medium for all cell types was refreshed every 48 hours. Upon reaching approximately 90% confluency, adherent cells were detached using Trypsin-EDTA solution (Gibco) for routine subculturing.

To model cellular senescence *in vitro*, distinct treatments were applied to MSC, HUVEC, NIH3T3, and RAW264.7 cells. Cells at approximately 80% confluency were exposed for a 24-hour period to either hydrogen peroxide (H₂O₂) or Endothelin-1 (ET-1, sourced from MCE) dissolved directly into their respective culture media. Untreated cells served as the negative control group.

Hydrogen peroxide, administered at a concentration of 100 μ M, functioned as a positive control for oxidative stress-induced senescence, a well-established model system. Parallel cultures were treated with 100nM ET-1 to investigate its specific impact on senescence induction.

For inflammation studies, cells at approximately 80% confluency were stimulated by adding Lipopolysaccharide (LPS) to their culture medium at a final concentration of 2 μ g/ml. Following a 24-hour incubation period under standard conditions, cells were processed for subsequent analysis to assess the inflammatory response.

Hydrogel extract preparation involved incubating specific hydrogel formulations in complete culture medium. Aliquots of 3g lyophilised Alg@Sr hydrogel, along with Alg@Sr hydrogels loaded with Macitentan at concentrations of 100nM, 1 μ M, and 10 μ M (designated Mac/Alg@Sr), were individually submerged in 30 mL of DMEM. This incubation was carried out for 24 hours at 37°C. The resulting supernatant solutions, representing the extracted components released from the hydrogels, were then collected and utilised for subsequent experimental treatments on the cultured cells.

4.2.10 Biocompatibility of hydrogels

Hydrogel Extract Exposure: A suspension containing 2×10^3 cells per well was seeded into a 96-well plate and allowed to adhere for a 24-hour incubation period. Following this initial culture phase, the existing medium was carefully aspirated and replaced with 100 μ L of hydrogel extracts derived from the various experimental formulations. The control group received Dulbecco's Modified Eagle Medium (DMEM) without hydrogel extracts as the treatment medium.

Cell Proliferation Assessment (CCK-8 Assay): Cell proliferation kinetics were evaluated using the Cell Counting Kit-8 (Beyotime) at designated time points (days 1, 3, and 5). At each interval, the culture medium containing hydrogel extracts was removed from all wells and substituted with fresh, standard culture medium. Subsequently, 10 μ L of the CCK-8 reagent was introduced into each well. The plate was then returned to the cell culture incubator for a 1-hour incubation period. Following incubation, the absorbance of the solution in each well was quantified at a wavelength of 450 nm using a microplate reader.

Cell Viability/Cytotoxicity Analysis (Calcein AM/PI Staining): Concurrent with the proliferation assessment, cell viability and cytotoxicity were

determined using the Calcein AM/PI Live/Dead Viability/Cytotoxicity Assay Kit (Beyotime) on days 1, 3, and 5. After removing the culture medium, the cell monolayers were gently washed twice with phosphate-buffered saline (PBS). A working solution of the Calcein AM and propidium iodide (PI) dyes was prepared according to the manufacturer's protocol. 100 μ L of this working solution was then added to each well. The plate was incubated at 37°C for 30 minutes in the dark to prevent photobleaching. Post-incubation, cellular fluorescence was visualised and imaged using a NIKON Ti2 fluorescence microscope. Live cells, stained by Calcein AM, were detected using excitation/emission (Ex/Em) wavelengths of 494 nm and 517 nm, respectively. Dead or compromised cells, stained by PI, were detected using Ex/Em wavelengths of 535 nm and 617 nm.

4.2.11 SA- β - Galactosidase Staining

The presence of cellular senescence was evaluated using a commercially available Senescence β -Galactosidase Staining Kit (Beyotime Biotechnology). Cells were initially cultured until reaching approximately 70% confluency within multi-well plates. At this stage, distinct treatment regimens were applied for a duration of 24 hours: exposure to hydrogen peroxide (H₂O₂), endothelin-1 (ET-1), or ET-1 concurrently administered with an extract derived from the

hydrogel formulation. Following the treatment period, the culture medium was carefully aspirated and discarded. The cell monolayers were then gently rinsed with PBS to remove residual medium and treatment agents.

After fixation, the staining procedure commenced with the preparation of the β -galactosidase substrate working solution, meticulously following the instructions supplied by the kit manufacturer. To prevent evaporation and ensure consistent staining conditions throughout the extended incubation, the entire plate was securely sealed using Parafilm® laboratory film. The sealed plate was then transferred to a standard cell culture incubator set at 37°C. After the overnight incubation, the staining solution was aspirated. Cellular morphology and the development of a characteristic blue chromogenic precipitate indicative of senescence-associated β -galactosidase (SA- β -gal) activity were subsequently visualised and documented using brightfield microscopy.

4.2.12 Real-time PCR analysis

For quantitative polymerase chain reaction (qPCR) analysis, the initial step involved cellular lysis followed by RNA extraction. This extraction was performed utilising the Total RNA Kit I (Omega Bio-Tek), strictly adhering to the manufacturer's prescribed protocol. The purity of the isolated RNA was

subsequently assessed spectrophotometrically. Only RNA samples demonstrating absorbance ratios (A260/280 and A260/230) approximating 2.0 were deemed suitable for further processing. Complementary DNA (cDNA) synthesis was then carried out via reverse transcription of the qualified RNA samples. This reaction employed the RT master mix for qPCR II (MCE) according to the kit instructions. Finally, quantitative PCR amplification was performed on the generated cDNA templates. Amplification reactions incorporated primers specific to the reference gene GAPDH and the target genes of interest: p16^{INK4a}, p21^{Cip1}, p53, ET-1, IL-1 β , IL-6, and TNF- α . All primer sequences were sourced from Integrated DNA Technologies (details provided in Table 2). Relative quantification of target gene mRNA expression levels was determined using the comparative cycle threshold ($\Delta\Delta C_t$) method. Expression values for each target gene were normalised to the endogenous reference gene and calculated relative to the appropriate control group.

Table 2 List of qPCR primers

Primer	Sequence (5' – 3')
Mouse GAPDH	F: AGGTCGGTGTGAACGGATTTG R: GGGGTCGTTGATGGCAACA

Mouse TNF- α	F: CAGGCGGTGCCTATGTCTC R: CGATCACCCCGAAGTTCAGTAG
Mouse IL-1 β	F: GAAATGCCACCTTTTGACAGTG R: TGGATGCTCTCATCAGGACAG
Mouse IL-6	F: TCCATCCAGTTGCCTTCT R: TAAGCCTCCGACTTGTGA
Mouse COL1A1	F: GCTCCTCTTAGGGGCCACT R: CCACGTCTCACCATTGGGG
Mouse p16	F: CGCAGGTTCTTGGTCACTGT R: TGTTCACGAAAGCCAGAGCG
Mouse p21	F: CCTGGTGATGTCCGACCTG R: CCATGAGCGCATCGCAATC
Mouse p53	F: CCCCTGTCATCTTTTGTCCCT R: AGCTGGCAGAATAGCTTATTGAG

Human GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG
Human bFGF	F: GCGACCCACACGTCAAATA R: TCCCTTGATAGACACAACCTCCTC
Human EGF-L6	F: ACTTCGGTGTTCTGCTATC R: GCTTCGCTCCTCTATGTC
Human VEGF	F: GGAGGAAGAAGAGAAGGAAG R: CAGGATGGCTTGAAGATGT
Human TNF- α	F: CCTCTCTCTAATCAGCCCTCTG R: GAGGACCTGGGAGTAGATGAG
Human IL-1 β	F: ATGATGGCTTATTACAGTGGCAA R: GTCGGAGATTCGTAGCTGGA
Human IL-6	F: ACTCACCTCTTCAGAACGAATTG R: CCATCTTTGGAAGGTTTCAGGTTG

Human p16	F: GATCCAGGTGGGTAGAAGGTC R: CCCCTGCAAACCTTCGTCCT
Human p21	F: TGTCCGTCAGAACCCATGC R: AAAGTCGAAGTTCCATCGCTC
Human p53	F: CAGCACATGACGGAGGTTGT R: TCATCCAAATACTCCACACGC

4.2.13 Immunofluorescence staining of ROS

For immunofluorescence detection of specific proteins, tissue sections underwent antigen retrieval followed by quenching of endogenous peroxidase activity. After blocking non-specific binding sites, sections were incubated with primary antibodies overnight at 4°C. The primary antibody was incubated: 4-Hydroxynonenal (4HNE) antibody (1:200; Invitrogen, MA5-27570). Positive signals were visualised using fluorescent secondary antibodies: Goat anti-Mouse IgG (H+L) (Alexa Fluor® 488). The cell nucleus was stained with Hoechst 33342 (Beyotime, C1022). Negative control sections underwent identical processing steps with the omission of primary antibodies. Quantitative analysis,

assessing either the percentage of positive cells or fluorescence intensity, was performed where applicable. Fluorescence images were captured using a Nikon ECLIPSE Ti2-E microscope at 20x magnification.

4.2.14 Animal wound healing model

Female Balb/c mice were purchased from GemPharmatech Co., Ltd and raised in Southern University of Science and Technology. Animal procedures were approved by the Southern University of Science and Technology (SUSTech-JY202504001) and performed according to the laboratory animal guideline for ethical review of animal welfare (GB/T 35892-2018), and licenced (Larc-2022-155).

Animals were randomly allocated into five distinct groups: one blank control group treated with sterile saline only, one group treated with a commercially available 3M™ wound dressing, one group treated with strontium alginate hydrogel (Alg@Sr), and two groups treated with different drug-loaded Mac/Alg@Sr hydrogels. On the day of surgery, the dorsal surgical site on each animal was shaved to remove hair. The exposed skin area was then cleansed with iodine, 75% ethanol, and sterile saline. Under anaesthesia, a circular excisional wound (d=8mm) was created on the prepared dorsal skin using a sterile biopsy punch. The wounds were subsequently treated according to group

assignment. To maintain sterility and prevent bacterial contamination, each wound was covered with a sterile Tegaderm™ semi-permeable membrane after treatment application. Furthermore, subcutaneous injection of penicillin (80,000 units per mouse) was given for 3 days. Treatments were reapplied every two days. Wound healing progression was monitored and documented daily throughout the postoperative period. 7 days after surgery, animals were sacrificed and the wound tissue, including the surrounding area, was harvested for histological analysis. Tissue samples were processed, embedded, sectioned, and stained with haematoxylin and eosin (H&E) for general morphological assessment. Immunohistochemical CD31 and Masson Trichrome staining were performed on tissue sections to evaluate specific molecular markers relevant to the healing process. The efficacy of the different treatments in promoting wound healing was ultimately determined by comparing parameters such as wound closure rates, histological features of re-epithelialisation, granulation tissue formation, neovascularisation, and the expression patterns of key markers across the experimental groups.

4.2.15 Statistical analysis

Quantitative data are expressed as the arithmetic mean accompanied by the standard deviation (mean $\pm$ SD). Statistical comparisons between experimental

groups were conducted employing either one-way analysis of variance (ANOVA) or the unpaired, two-tailed Student's t-test, with the selection guided by the specific experimental design and data structure. Where ANOVA yielded a statistically significant overall difference ($p < 0.05$), appropriate post-hoc analyses were subsequently performed to identify pairwise differences between specific groups. The threshold for determining statistical significance was established at a probability value (p) less than 0.05 for all inferential tests. Statistical computations and the generation of corresponding graphical representations were executed utilising Prism version 10 software (GraphPad Software, Inc.). Visualisation of Fourier-transform infrared spectroscopy (FTIR) data was achieved using OriginPro software (Version 2022).

4.3 Results

4.3.1 BQ788/Calcium-loaded alginate hydrogel for skeletal regeneration

4.3.1.1 Morphology characterisation and structure

Fig. 16 presents the structural and physicochemical characterisation of calcium-crosslinked alginate hydrogels (Alg@Ca) and their BQ788-loaded counterparts (BQ788/Alg@Ca), utilising scanning electron microscopy (SEM) and Fourier-transform infrared (FTIR) spectroscopy. Representative SEM micrographs of

freeze-dried Alg@Ca and BQ788/Alg@Ca hydrogels loaded with 200 nM, 2 μ M, and 20 μ M BQ788 was displayed in Fig. 16a. All formulations exhibited a highly porous, three-dimensional network architecture characteristic of ionically crosslinked alginate, with interconnected pores of irregular shape and micron-scale dimensions. Critically, the incorporation of BQ788, even at the highest concentration of 20 μ M, did not induce any observable alteration to the fundamental microstructure. The average pore size, pore wall thickness, and overall porosity appeared consistent across all samples, indicating that the physical entrapment of the peptide antagonist within the alginate matrix does not disrupt the gelation process or the resultant scaffold morphology. This preservation of structure confirms that BQ788 loading is a physically composite process, maintaining the essential porous network required for cell infiltration, nutrient diffusion, and drug release.

The FTIR spectral analysis, comparing pure alginate (Alg), pure BQ788, crosslinked Alg@Ca, and the loaded BQ788/Alg@Ca hydrogels was shown in Fig 16b. The spectrum for pure alginate showed its characteristic vibrations: the asymmetric stretch of the carboxylate group ($-\text{COO}^-$) at approximately 1597 cm^{-1} , the symmetric $-\text{COO}^-$ stretch near 1403 cm^{-1} , and the C-O-C stretching vibration of the glycosidic linkage at around 1027 cm^{-1} . Successful ionic crosslinking with calcium (Ca^{2+}) was confirmed by distinct shifts in the

Alg@Ca spectrum. The asymmetric -COO^- stretch experienced a redshift to 1584 cm^{-1} , indicating a decrease in bond vibration energy due to electrostatic interaction between the carboxylate groups and Ca^{2+} ions. Concurrently, the C-O-C stretch underwent a blueshift to 1033 cm^{-1} , suggesting an increase in bond rigidity and restricted polymer chain mobility resulting from the crosslinked "egg-box" structure. The spectra for the BQ788-loaded hydrogels at all concentrations (200 nM, 2 μM , 20 μM) faithfully reproduced these key peaks at 1584 cm^{-1} and 1033 cm^{-1} . The absence of any shift in these diagnostic bands confirms that BQ788 does not interfere with the primary ionic crosslinking between alginate and calcium. No new peaks attributable to BQ788 emerged in the composite hydrogels, indicating the absence of covalent bonding. However, a subtle shift in a broad spectral region between approximately $2300\text{--}2470\text{ cm}^{-1}$ was noted specifically in the hydrogel with the highest BQ788 concentration (20 μM). This shift may arise from weak physical interactions, such as $\pi\text{-}\pi$ stacking or altered hydrogen bonding networks induced by the high local concentration of the entrapped drug, which subtly perturbs the microenvironment without affecting the primary chemical structure of the crosslinked matrix. Collectively, the data demonstrate that BQ788 is successfully and physically encapsulated within the Alg@Ca hydrogel without

compromising its structural integrity or its fundamental ionic crosslinking mechanism.

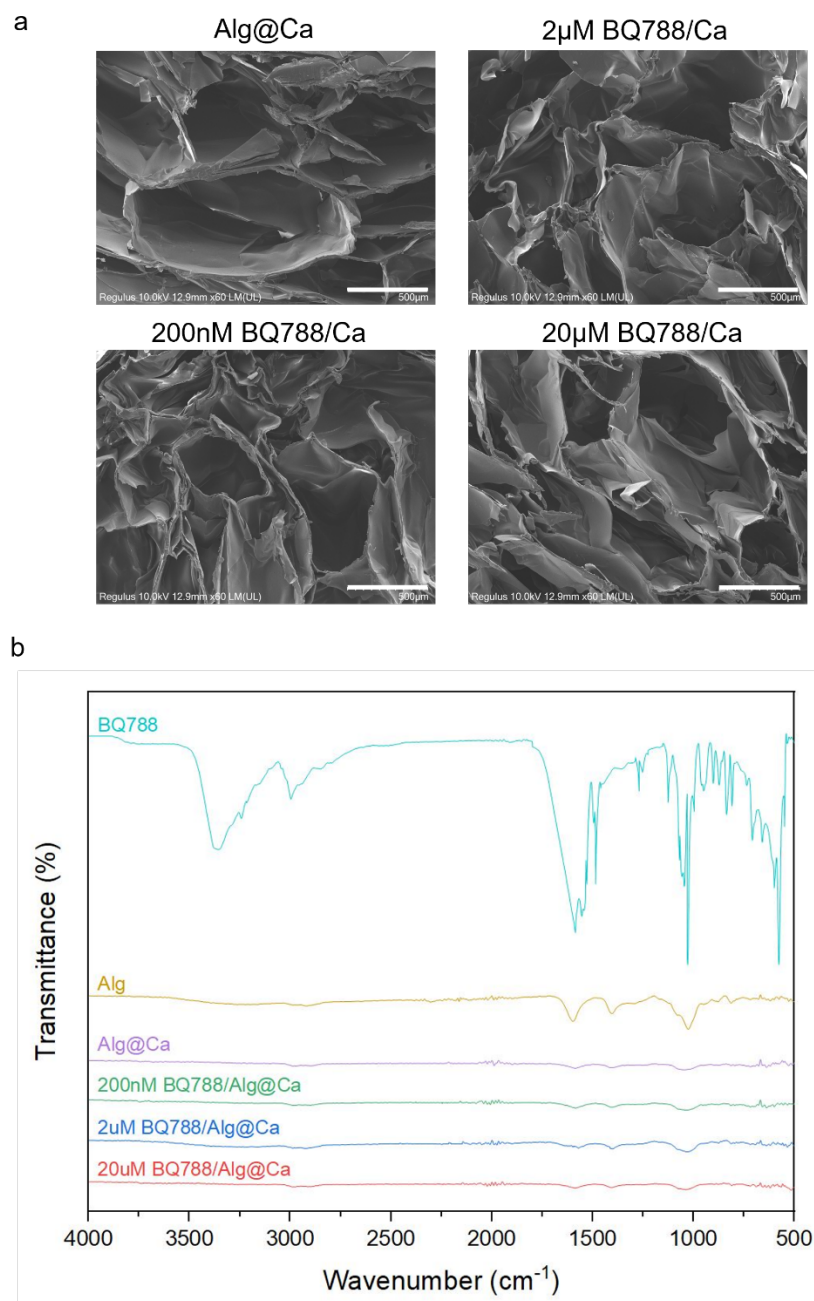


Figure 16 Morphology of BQ788/Calcium hydrogel. (a) SEM images. (b) FTIR analysis.

4.3.1.2 Swelling behaviour

The swelling kinetics of calcium ion (Ca^{2+})-crosslinked alginate hydrogels (Alg@Ca), both unmodified and loaded with the endothelin receptor antagonist BQ788 at concentrations of 200 nM and 2 μM , were quantitatively assessed over a 30-hour period (Figure 17). This analysis is critical for understanding the water uptake capacity and hydration dynamics of the formulations, which directly influence drug release profiles, mechanical integrity in a hydrated state, and suitability for maintaining a moist wound environment.

All investigated formulations—pristine Alg@Ca, 200 nM BQ788/Alg@Ca, and 2 μM BQ788/Alg@Ca—exhibited a characteristic biphasic swelling profile. An initial phase of rapid fluid uptake was observed within the first 10 hours of immersion in phosphate-buffered saline (PBS) at 37°C. During this period, the swelling ratio increased sharply as the aqueous medium penetrated the porous hydrogel network via capillary action, hydrating the hydrophilic alginate polymer chains and leading to significant matrix expansion. This process is driven by the substantial osmotic pressure difference between the dry, ionic polymer network and the surrounding physiological solution. Subsequently, the swelling kinetics transitioned to a plateau phase between approximately 10 and 30 hours, indicating the approach of an equilibrium swelling state where the

expansive force of the hydrated polymer chains is balanced by the restorative force of the ionic crosslinks formed by calcium ions.

A comparative analysis of the equilibrium swelling values reveals a subtle yet consistent trend: hydrogels incorporated with BQ788 demonstrate a marginally reduced swelling ratio compared to the unmodified Alg@Ca hydrogel.

Furthermore, this reduction appears slightly more pronounced in the hydrogel loaded with the higher concentration of BQ788 (2 μ M). This phenomenon can be attributed to the physical presence of the BQ788 molecules within the interstitial spaces of the polymer network. The incorporation of the drug effectively increases the physical crosslinking density by restricting the mobility of the alginate chains and occupying volume that would otherwise be available for water penetration. Consequently, this leads to a modest decrease in the ultimate water uptake capacity of the drug-loaded hydrogels, a factor that must be considered in the context of their intended application and release kinetics.

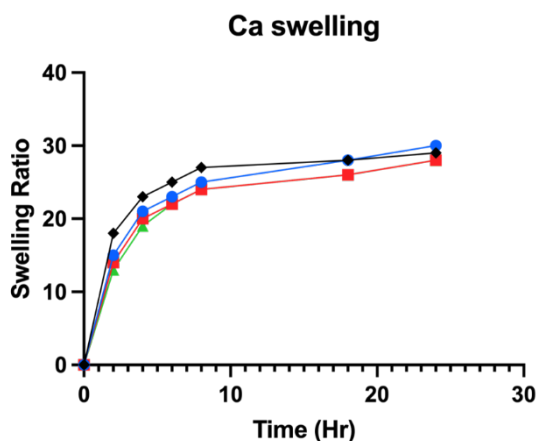


Figure 17 Swelling ratio of BQ788/Calcium hydrogels over time.

4.3.1.3 Thermal property

The thermal stability and decomposition characteristics of the BQ788-loaded calcium alginate system were evaluated using thermogravimetric analysis (TGA), with the results for key components and composites presented in Figure 18. The samples analysed included pure BQ788, pure sodium alginate (NaAlg), the unmodified calcium-crosslinked hydrogel (Alg@Ca), and the drug-loaded composites (200 nM and 2 μ M BQ788/Alg@Ca).

The TGA profiles for all hydrogel-based samples (Alg@Ca and both BQ788/Alg@Ca formulations) exhibited a multi-stage mass loss. The first event, occurring between ambient temperature and approximately 220°C, corresponds to the loss of physically adsorbed and bound water from the hydrophilic polymer matrix. Following this dehydration step, a major, sharp

mass loss event was observed initiating around 220°C and continuing to approximately 300°C. This primary decomposition step is directly associated with the thermal breakdown of the ionic crosslinks formed between the guluronic acid blocks of the alginate chains and the calcium (Ca^{2+}) ions, followed by the subsequent depolymerisation and combustion of the alginate backbone.

A critical comparison can be made between the pure sodium alginate and the ionically crosslinked hydrogels. Pure NaAlg demonstrated a significantly delayed onset for its main decomposition event, commencing only after 250°C. This indicates a higher inherent thermal stability of the non-crosslinked alginate polymer. In contrast, the introduction of Ca^{2+} ions as crosslinking agents reduces the thermal stability of the network, as evidenced by the earlier onset of major degradation in the Alg@Ca sample. The Ca^{2+} -O coordination bonds within the "egg-box" structure possess lower thermal resilience than the native interactions in pure alginate, making the crosslinked matrix more susceptible to thermal breakdown.

Importantly, the incorporation of BQ788 into the Alg@Ca matrix at both tested concentrations (200 nM and 2 μM) resulted in no significant alteration of the thermal degradation profile. The TGA curves for the drug-loaded hydrogels are

largely superimposable with that of the unmodified Alg@Ca hydrogel. This demonstrates that BQ788, when physically entrapped within the hydrogel network, does not participate in or catalyse any additional thermal decomposition pathways. The thermal behaviour of the composite material remains governed by the calcium-alginate framework, confirming that the drug acts as a thermally stable guest component without compromising the intrinsic thermal properties of the delivery system.

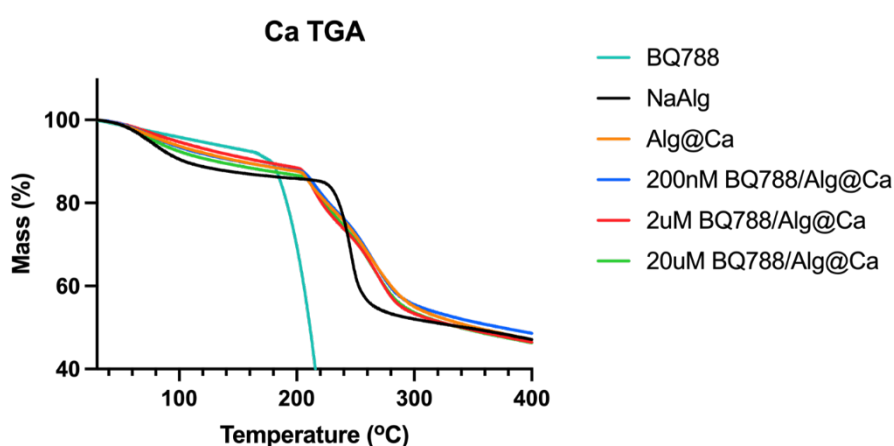


Figure 18 TGA analysis of BQ788/Calcium hydrogels' thermal property.

4.3.1.4 *In vitro* BQ788 and Calcium ions release

The cumulative release profiles of the endothelin receptor antagonist BQ788 and calcium ions (Ca^{2+}) from the calcium-crosslinked alginate hydrogel (Ca-Alg-BQ) were evaluated, as shown in Figure 19. The release of BQ788 exhibited a distinct kinetic pattern (Fig. 19a). An initial lag phase of minimal

drug release was observed, lasting for the first several hours. Following this lag period, the cumulative release entered a steady, prolonged phase characterised by a near-linear increase over time. This pattern indicates a controlled, diffusion-mediated release mechanism rather than a rapid burst. The release profiles for the different loaded concentrations of BQ788 (200 nM, 2 μ M, and 20 μ M) were highly consistent, demonstrating that the release kinetics are governed principally by the hydrogel matrix's properties and not by the initial drug loading concentration.

The concomitant release profile of calcium ions presented a complementary, though distinct, pattern (Fig. 19b). Measurements of the Ca^{2+} concentration in the release medium at 24, 36, and 48 hours revealed a consistent concentration of approximately 30 mg/L at each time point. This indicates that the crosslinking Ca^{2+} ions were released from the hydrogel matrix in a controlled, non-instantaneous manner, reaching a stable equilibrium concentration in the surrounding medium by 24 hours. The maintenance of this stable concentration over the subsequent 24-hour monitoring period suggests a steady-state release, likely driven by the gradual, surface-erosion degradation of the alginate network and the ion-exchange dynamics between the hydrogel and the bulk medium.

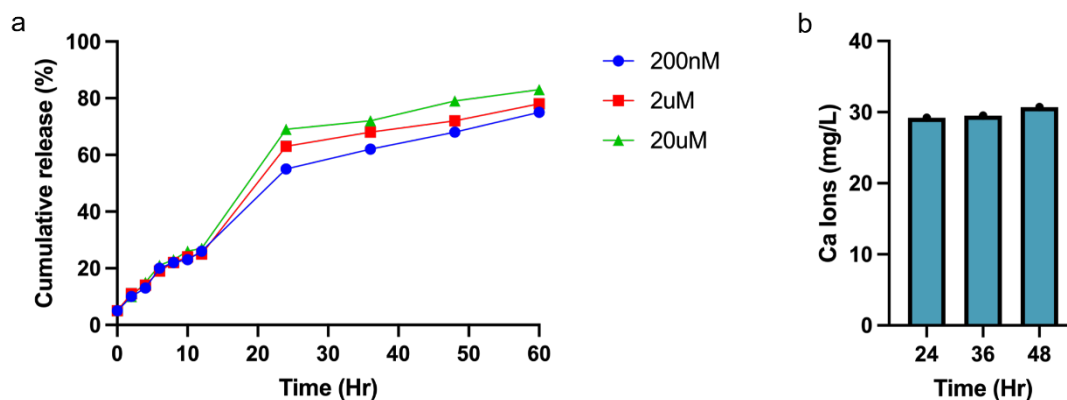


Figure 19 BQ788 and Calcium ions release profile. (a) Cumulative BQ788 release. (b)

Calcium ions release.

4.3.1.5 Biocompatibility of hydrogels in MSCs

The biocompatibility of a bone-regenerative hydrogel is paramount, particularly its interaction with mesenchymal stem cells (MSCs) as the key progenitors for osteogenic differentiation and new bone formation. While calcium-crosslinked alginate (Alg@Ca) is recognised for its favourable biocompatibility, the localised and sustained release of the potent ETB receptor antagonist BQ788 necessitated a thorough assessment of its impact on MSC viability and proliferation. MSCs were cultured in the presence of Alg@Ca hydrogels loaded with varying concentrations of BQ788 (100 nM, 2 μ M, 20 μ M, and 200 μ M) over a period of 5 days, with cell proliferation quantified and membrane integrity visualised via live/dead staining.

The proliferation data, presented in Figure 20a, demonstrate that the unmodified Alg@Ca hydrogel and all BQ788-loaded variants supported MSC growth effectively over the 5-day culture period. No statistically significant difference in proliferation rate was observed at any time point (Day 1, 3, or 5) between the control group, the Alg@Ca group, and any of the BQ788-loaded groups, including the highest concentration of 200 μ M. This indicates that neither the hydrogel matrix itself nor the eluted BQ788, across the full range of concentrations tested, exerted an inhibitory effect on the proliferative capacity of MSCs under these conditions.

This quantitative finding was corroborated by qualitative live/dead staining assays (Figure 20b). Representative images from Days 1, 3, and 5 for the Alg@Ca, 20 μ M BQ788/Alg@Ca, and 200 μ M BQ788/Alg@Ca groups showed a consistent and dominant presence of viable cells, fluorescing green with Calcein AM. Notably, red fluorescence from propidium iodide, indicative of compromised membrane integrity, was negligible across all samples and time points. The images for the Alg@Ca and 20 μ M BQ788/Alg@Ca groups showed confluent layers of healthy, adherent cells by Day 5. While the 200 μ M BQ788/Alg@Ca group also displayed excellent viability with minimal cell death, a modestly lower cell density was observed by Day 5 compared to the other groups, aligning with the slight, though non-significant, numerical trend

in the proliferation assay. Collectively, these results confirm the excellent cytocompatibility of the BQ788-loaded calcium-alginate hydrogel system with MSCs, establishing that the local release of BQ788 does not induce acute cytotoxicity and supports a cellular environment conducive to subsequent osteogenic studies.

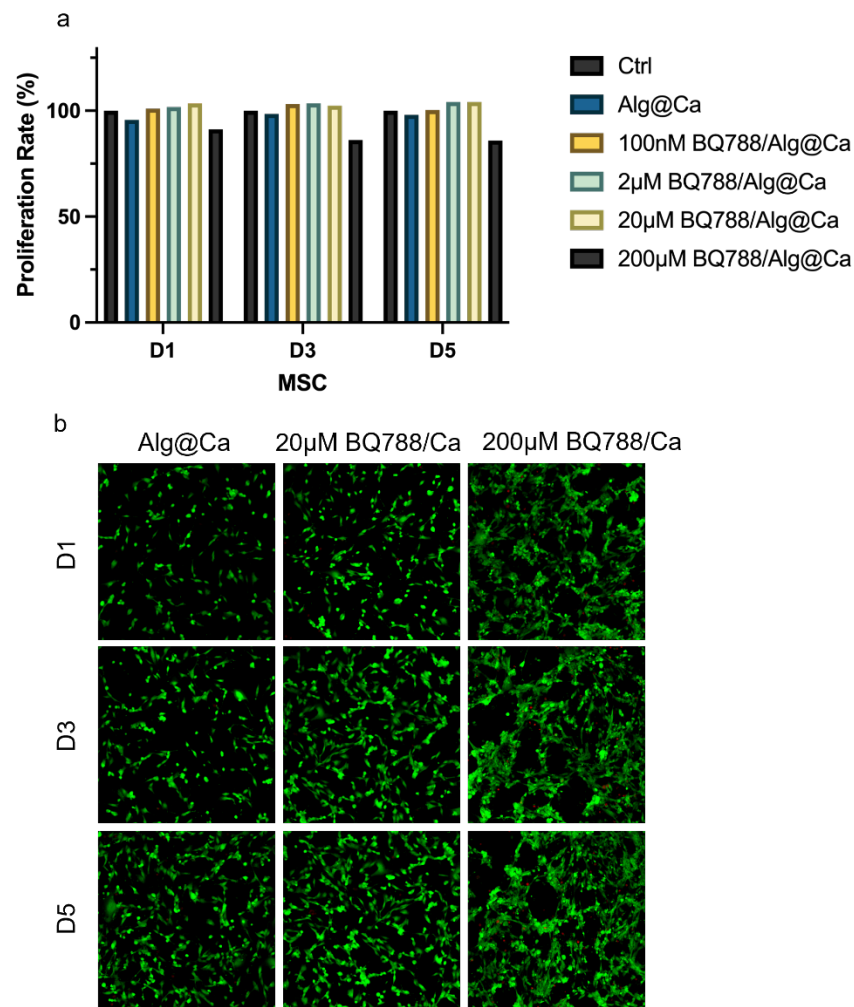


Figure 20 Biocompatibility of hydrogel in MSCs. (a) CCK-8 analysis. (b) Live (green) and dead (red) staining. Scale bar, 100µm.

4.3.1.6 Reducing senescence in MSCs

Reducing Senescence in Mesenchymal Stem Cells

The persistence of senescent cells within the microenvironment is a major barrier to regeneration, as these cells not only lose their proliferative and functional capacity but also secrete deleterious factors that disrupt normal healing. Mesenchymal stem cells (MSCs) are crucial for tissue repair, and their premature senescence severely compromises this regenerative potential. To investigate the protective capacity of the developed hydrogel against this dysfunction, MSCs were subjected to a senescence-inducing challenge with ET-1, with H₂O₂ treatment serving as a positive control. The efficacy of the calcium-crosslinked alginate hydrogel (Alg@Ca) and its BQ788-loaded formulation (BQ788/Alg@Ca) in mitigating this induced senescence was assessed through qPCR analysis of key molecular markers and confirmatory β -galactosidase staining.

Analysis of senescence-associated gene expression via qPCR revealed a clear, concentration-dependent protective effect of the BQ788-loaded hydrogel (Fig. 21a and c). Treatment of MSCs with extract from the Alg@Ca hydrogel alone resulted in a modest reduction in the expression of the canonical senescence markers *p16^{CDKN2A}*, *p21^{CDKN1A}*, and *p53* compared to the ET-1 and H₂O₂ control

groups. This suggests that the calcium ions released from the base hydrogel matrix may provide a mildly supportive microenvironment, potentially aiding basic cellular homeostasis. In contrast, treatment with extract from the BQ788/Alg@Ca hydrogel elicited a more pronounced response. The formulation loaded with a lower concentration of BQ788 (200 nM) induced a noticeable decrease in senescence marker expression, while the hydrogel loaded with a higher concentration (2 μ M BQ788) triggered a substantial and statistically significant downregulation of all assessed markers. This potent, dose-dependent anti-senescence effect was visually confirmed by β -galactosidase staining, which demonstrated a marked reduction in the number of SA- β -gal-positive senescent cells in the 2 μ M BQ/Alg@Ca treatment group compared to both the positive controls and the drug-free Alg@Ca group.

The potent efficacy of the high-concentration BQ788 hydrogel is mechanistically coherent with BQ788's role as a selective ETB receptor antagonist. In MSCs, ET-1 signalling through the ETB receptor can activate pathways leading to oxidative stress and cell cycle arrest. By competitively inhibiting this receptor, BQ788 directly blocks the primary molecular trigger of ET-1-induced senescence in this cell type. This intervention prevents the downstream cascade involving DNA damage response and the sustained upregulation of cyclin-dependent kinase inhibitors like p16 and p21, thereby

preserving the proliferative and regenerative capacity of MSCs within a challenging pathological milieu.

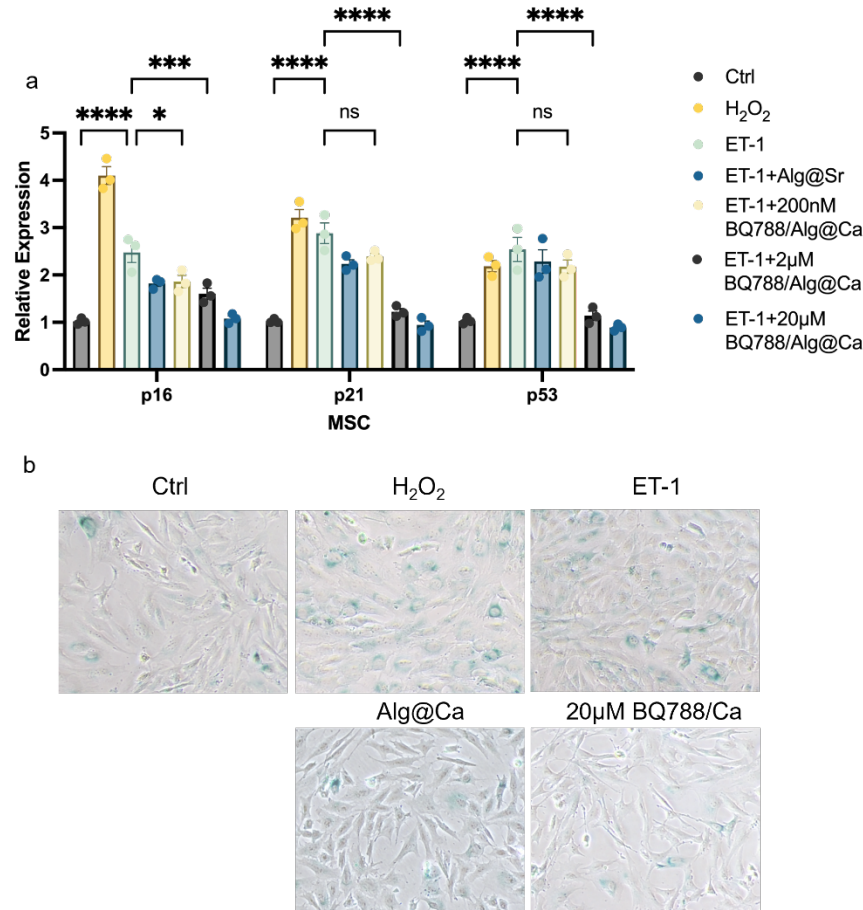


Figure 21 Reducing senescence in MSCs. Cells were treated with 100 μM H₂O₂, 100 nM ET-1 or hydrogel together with ET-1. (a) qPCR of senescence markers (b) Representative images of β-gal staining (blue). Scale bar, 100 μm.

4.3.2 Macitentan/Strontium-loaded alginate hydrogel for vascular regeneration

4.3.2.1 Morphology characterisation and structure

Structural characterization of the fabricated hydrogels, namely the strontium-crosslinked alginate hydrogel (Alg@Sr) and the macitentan-loaded counterpart (Mac/Alg@Sr), was performed using scanning electron microscopy (SEM) to elucidate their internal morphology and pore architecture, critical factors influencing drug diffusion, cell infiltration, nutrient exchange, and overall performance in a wound environment. Representative SEM micrographs obtained from freeze-dried samples, prepared under controlled conditions to preserve the hydrated gel structure as faithfully as possible, revealed a characteristic and well-defined three-dimensional microporous network for both hydrogel formulations in Fig. 22a. This interconnected porous structure is a hallmark feature of ionically crosslinked alginate hydrogels and is essential for facilitating the necessary biological and physicochemical processes required for tissue regeneration. The pores displayed a generally irregular shape but a consistent size distribution within the micron-scale range for both Alg@Sr and Mac/Alg@Sr. Images demonstrated that the incorporation of macitentan into the Alg@Sr matrix did not induce any significant alterations in the

morphological characteristics of the porous network. Specifically, the addition of the drug did not result in statistically significant changes in the average pore diameter or the overall porosity (pore density) compared to the unloaded Alg@Sr hydrogel. Furthermore, the micrographs showed no evidence of the pore walls becoming noticeably thicker or thinner, nor did the network exhibit a visibly more compact or looser arrangement upon drug loading. The fundamental microporous architecture remained remarkably consistent regardless of the presence of macitentan. This observed invariance in morphology upon macitentan incorporation can be attributed to the physical composite nature of the drug loading process and the specific physicochemical properties of the components, rather than a chemical reaction altering the gelation mechanism.

Fig. 22b shows the FTIR spectrums of the hydrogel fabrication components alginate and drug Macitentan, as well as Alg@Sr and Mac/Alg@Sr hydrogel. Alginate's main peaks appear around 1600 cm^{-1} (asymmetric -COO^- stretch), 1400 cm^{-1} (symmetric -COO^- stretch), and around 1030 cm^{-1} (C-O-C stretching). When strontium (Sr^{2+}) is added to alginate, the peaks shift from 1597 to 1584 cm^{-1} and 1027 to 1033 cm^{-1} . Alginate is known to crosslink with divalent cations like Ca^{2+} or Sr^{2+} . The carboxylate groups (-COO^-) in alginate bind with Sr^{2+} . The peak around 1597 cm^{-1} is attributed to the asymmetric

stretching of the carboxylate group. The shift to a lower wavenumber, from 1597 to 1584 cm^{-1} , indicates a decrease in bond stiffness due to Sr^{2+} binding. Sr^{2+} interacts electrostatically with the negatively charged ($-\text{COO}^-$) groups, redistributing electron density and weakening the vibrational frequency. The shift from 1027 to 1033 cm^{-1} may be related to the (C-O) stretching in the glycosidic linkages or other oxygen-containing groups in alginate. The shift to a higher wavenumber (blueshift) suggests increased bond rigidity caused by Sr^{2+} crosslinking, which restricts polymer chain mobility and alters the local environment of oxygen-containing groups.

The absence of changes at 1584 cm^{-1} and 1033 cm^{-1} confirms that macitentan does not interfere with alginate's carboxylate or glycosidic bond environments. Macitentan is a drug with poor water solubility. Even though we used 5% DMSO was used to enhance its solubility ($>4.25\text{mM}$), it likely did not engaged in covalent or strong ionic interactions with alginate or Sr^{2+} . It is likely physically entrapped within the hydrogel matrix without altering the primary chemical bonds of alginate and its crosslinks with Sr^{2+} , or only weak interactions occurred which do not significantly affect vibrational modes detectable by FTIR in the studied regions. A peak shift at ~ 2300 to 2470 cm^{-1} was observed for hydrogel with higher macitentan concentration. The shift may arise from: (1) Aggregation-induced changes: Increased drug concentration

could lead to π - π stacking of aromatic rings or dipole interactions, altering vibrational environments. (2) Hydrogen bonding or microenvironment effects: Enhanced intermolecular interactions might perturb overtone bands. (3) Physical strain: Higher drug loading may stress the hydrogel matrix, modifying vibrational modes indirectly.

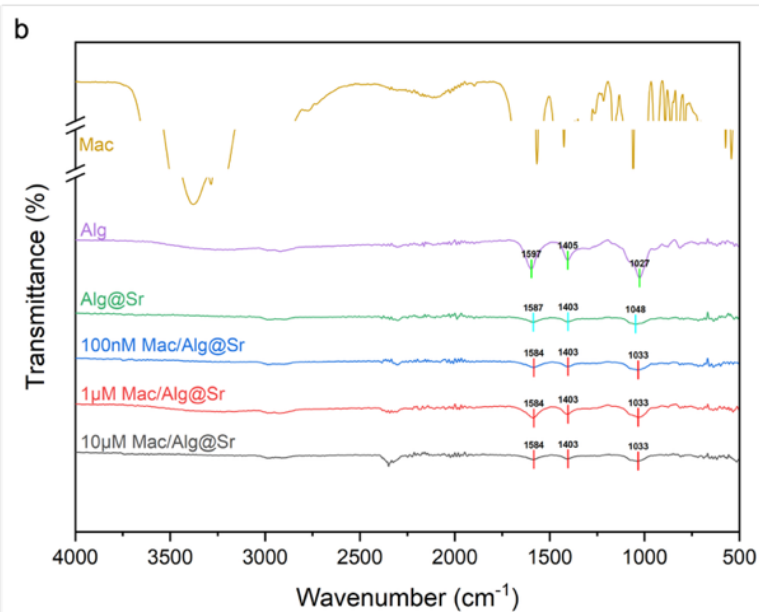
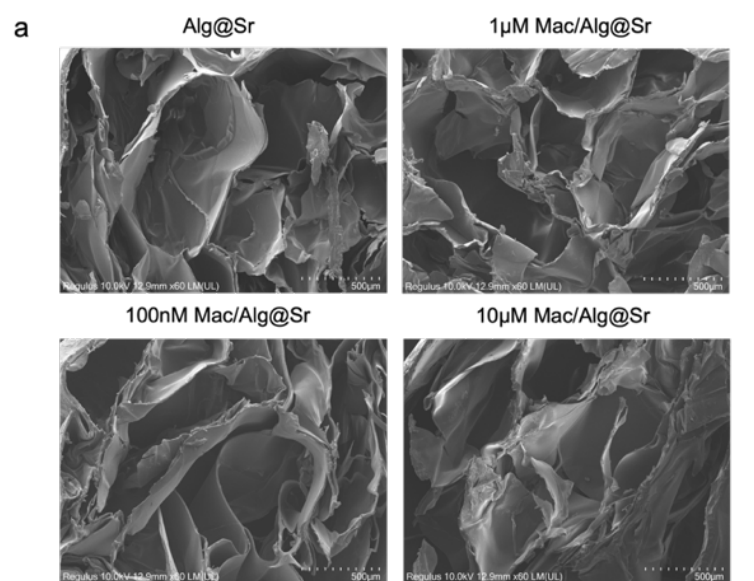


Figure 22 Morphology of Macitentan/Strontium hydrogel. (a) SEM images. (b) FTIR analysis.

4.3.2.2 Swelling behaviour

The swelling behaviour of the strontium-crosslinked alginate hydrogels (Alg@Sr), both unmodified and loaded with macitentan at concentrations of 100nM, 1 μ M, and 10 μ M, was quantitatively assessed over a 30-hour period to understand their water uptake capacity and kinetics, critical parameters influencing drug diffusion, physical stability, and performance in a moist wound environment. Representative data illustrating the percentage swelling ratio as a function of immersion time in phosphate-buffered saline (PBS) at 37°C is presented in Fig.23 demonstrating clear trends related to both time and drug concentration.

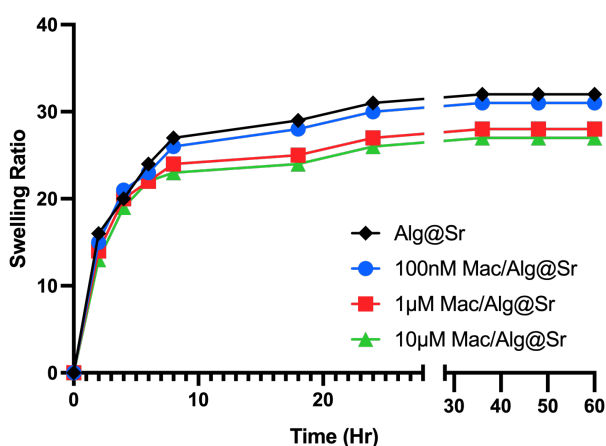


Figure 23 Swelling ratio of Macitentan/Strontium hydrogels over time.

All hydrogel formulations exhibited a characteristic biphasic swelling profile, irrespective of the macitentan concentration. An initial rapid water uptake phase dominated the first 10 hours of immersion. During this period, the swelling ratio increased sharply as PBS penetrated the porous hydrogel matrix, hydrating the hydrophilic alginate polymer chains and causing significant network expansion. This phase is primarily driven by the high osmotic pressure gradient between the dry polymer network and the surrounding aqueous medium, coupled with capillary forces drawing fluid into the interconnected pores. Following this initial surge, the swelling curves entered a plateau phase between approximately 10 and 30 hours, indicating that the hydrogels approached their maximum equilibrium swelling capacity. The attainment of equilibrium signifies a balance between the swelling force exerted by the hydrated polymer chains and the restraining force provided by the strontium ion crosslinks within the network. All samples exhibited good swelling behaviour, and the swelling ratio slightly decreased when a higher Macitentan concentration was introduced into the hydrogel. Incorporating molecules like Macitentan into the hydrogel matrix increases crosslinking density by occupying interstitial spaces within the polymer network. This restricts chain mobility and reduces water uptake, leading to slightly reduced swelling rate.

4.3.2.3 Thermal property

Thermogravimetric analysis (TGA) was employed to assess the thermal stability and decomposition profiles of the fabricated hydrogels and their key components: pure macitentan (Mac), pure sodium alginate (NaAlg), strontium-crosslinked alginate hydrogel (Alg@Sr), and macitentan-loaded Alg@Sr hydrogels (Mac/Alg@Sr) at concentrations of 100nM, 1 μ M, and 10 μ M. The TGA curves, depicting mass loss as a function of increasing temperature, revealed distinct degradation patterns and provide critical insights into the influence of ionic crosslinking and drug incorporation on thermal behaviour.

All analysed materials exhibited characteristic multi-stage mass loss profiles (Fig.24). The initial mass loss phase, observed from room temperature up to approximately 200-240°C across all samples, is unequivocally ascribed to the evaporation of bound and adsorbed water within the hydrogels or hydrated components. While this dehydration process was universal, subtle variations in the temperature range were noted between different materials. Pure Mac exhibited a broad dehydration profile spanning from approximately 60°C to 240°C. In contrast, both the unmodified Alg@Sr hydrogel and its macitentan-loaded counterparts (100nM, 1 μ M, 10 μ M Mac/Alg@Sr) displayed a slightly shifted dehydration range, commencing around 70°C and concluding near

220°C. This minor shift likely reflects subtle differences in water binding characteristics and hydrophilicity inherent to the pure drug compared to the hydrogel matrices. Following dehydration, a second, more pronounced mass loss event occurred specifically for the Alg@Sr and all Mac/Alg@Sr hydrogels, sharply evident within the temperature window of 220°C to 290°C. This primary decomposition event is directly attributed to the thermal breakdown of the ionic crosslinks formed between the alginate polymer chains and strontium (Sr^{2+}) ions. The disintegration of these Sr^{2+} -mediated crosslinks initiates the main polymer degradation cascade.

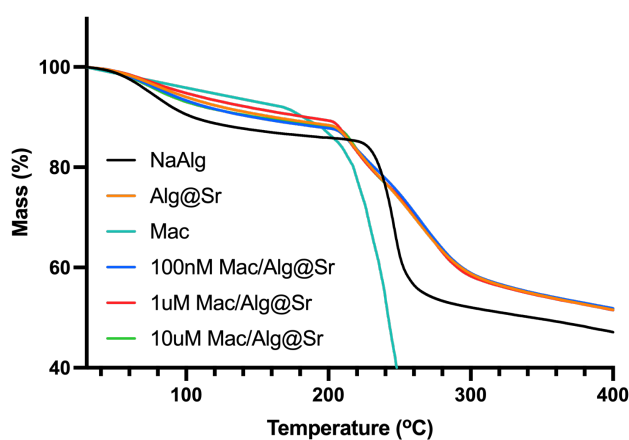


Figure 24 TGA analysis of Macitentan/Strontium hydrogels' thermal property.

A critical observation emerges when comparing the thermal stability of pure sodium alginate (NaAlg) to the strontium-crosslinked hydrogels (Alg@Sr and Mac/Alg@Sr). Pure NaAlg demonstrated a significantly delayed onset of its

main decomposition phase, commencing only beyond temperatures of approximately 250°C. This highlights its superior inherent thermal stability compared to the ionic crosslinked systems. The introduction of Sr²⁺ ions as a crosslinking agent clearly reduces the overall thermal stability of the alginate matrix. The Sr-O coordination bonds within the structure possess lower thermal resilience than the associations within pure NaAlg, potentially due to a combination of bond energy differences and the disruption of native chain packing by the divalent cation bridges. Consequently, the Alg@Sr network begins major degradation at a substantially lower temperature (220°C) than pure alginate, confirming that ionic crosslinking introduces structural vulnerabilities to thermal stress.

The addition of Macitentan into the hydrogel did not affect the overall thermal stability as the crosslinked drug-polymer ratio was negligible and the alginate's thermal degradation remained the governing process. Further increases in Macitentan concentration also did not significantly increase the degradation rate. The TGA result indicated that the Mac/Alg@Sr was thermally stable, and the concentration of Macitentan had little influence on the thermal stability. TGA conclusively demonstrates that the integration of macitentan, when physically entrapped component, has no discernible detrimental impact on the

thermal stability or degradation profile of the hydrogel. The Sr-Alg framework retains its thermal integrity.

4.3.2.4 Mechanical performance

Rheological characterization, encompassing strain amplitude and frequency sweep tests, was performed to evaluate the fundamental mechanical and viscoelastic properties of the strontium-crosslinked alginate hydrogel (Alg@Sr) and its macitentan-loaded counterpart (Mac/Alg@Sr). These properties are critical determinants of hydrogel performance in wound healing applications, influencing injectability, shape retention, resistance to deformation from exudate or movement, and interaction with cells/tissues.

The strain amplitude sweep results unequivocally demonstrated that both Alg@Sr and Mac/Alg@Sr exhibit classic hydrogel behavior characterized by dominant elastic solid-like properties within a defined low-strain regime, followed by yielding at higher strains (Fig. 25a). Specifically, both hydrogels showed a distinct viscoelastic region where the storage modulus (G' , representative of elastic, solid-like behavior) remained substantially higher than the loss modulus (G'' , representative of viscous, liquid-like behavior) and largely constant. Beyond a critical yield strain, G'' surpassed G' , indicating network breakdown and transition to viscous flow. Alg@Sr exhibited a

significantly higher G' compared to Mac/Alg@Sr, signifying a stiffer, more elastic network structure in the absence of the drug. Furthermore, Alg@Sr yielded at a markedly lower critical strain (1.48%) compared to Mac/Alg@Sr (2.39%). This indicates that while the unloaded hydrogel possesses greater strength/stiffness initially, its structure is inherently less resistant to large deformations and yields sooner than the drug-loaded version. These results suggests a more brittle network structure in Alg@Sr, whereas a greater resilience and ability to withstand larger deformations before yielding is present in Mac/Alg@Sr.

The frequency sweep results provided deeper insight into the network dynamics and time-dependent mechanical responses of both hydrogels at low strain (Fig. 25b). For both formulations, G' consistently exceeded G'' across the entire frequency range tested (0.1 Hz to 10 Hz), confirming their predominant elastic character over a broad spectrum of timescales relevant to handling and biological interactions. However, significant differences in the frequency dependence of G' were observed between the two hydrogels. Alg@Sr demonstrated a slight but discernible increasing trend in G' with increasing frequency, particularly evident as frequency rose from 0.1 Hz to approximately 1 Hz. Beyond 1 Hz, this rising trend plateaued. Conversely, Mac/Alg@Sr

exhibited a flatter G' response across the entire frequency range, with G' values consistently lower than those of Alg@Sr and minimal variation with frequency.

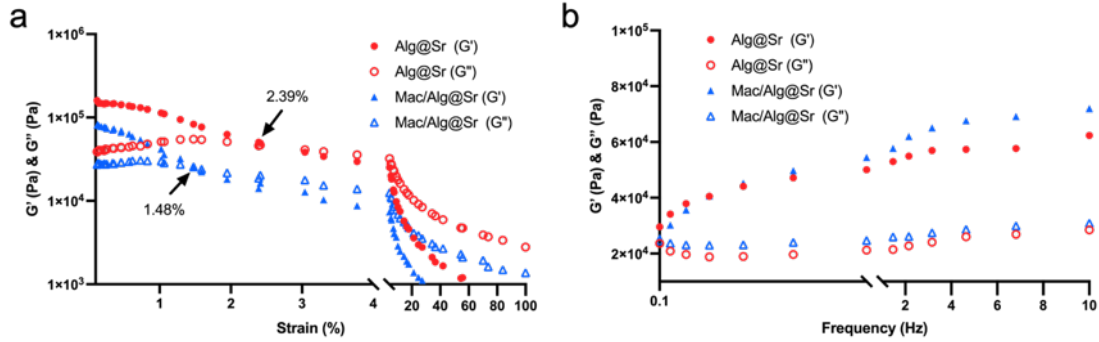


Figure 25 Rheology analysis of hydrogels. (a) Strain amplitude test. (b) Frequency sweep test.

4.3.2.5 *In vitro* Macitentan and Strontium ions release

The cumulative release profiles of both macitentan and strontium ions (Sr^{2+}) from the strontium-crosslinked alginate hydrogel (Sr-Alg-Mac) were quantitatively assessed. The macitentan release profile demonstrates a controlled and sustained release behaviour over 60 hours (Fig. 26a). Irrespective of the initial loaded concentration (100 nM, 1 μM , and 10 μM), all release curves exhibit a consistent biphasic pattern: a rapid initial release phase within the first 20 hours, followed by a significantly slower, sustained release phase leading towards a plateau. Specifically, by 20 hours, a substantial portion of the drug (>60%) was released from the hydrogel across all concentrations.

Following this initial phase, the release rate markedly decreased, resulting in near-complete cumulative release (>80%) by 60 hours for all concentrations tested. Crucially, the release profiles for all three concentrations were essentially superimposable throughout the entire time course, indicating concentration-independent release kinetics dominated by the hydrogel matrix properties rather than concentration gradients or drug-drug interactions.

Strontium ion release presented a different profile (Fig. 26b). Measurements taken at 24, 36, and 48 hours revealed a high and remarkably stable concentration of approximately 100 mg/L in the release medium at each time point. This indicates that the majority of the crosslinking Sr^{2+} ions were released from the hydrogel matrix very rapidly, reaching equilibrium concentration within the first 24 hours and maintaining this stable concentration level throughout the subsequent 24 hours of monitoring.

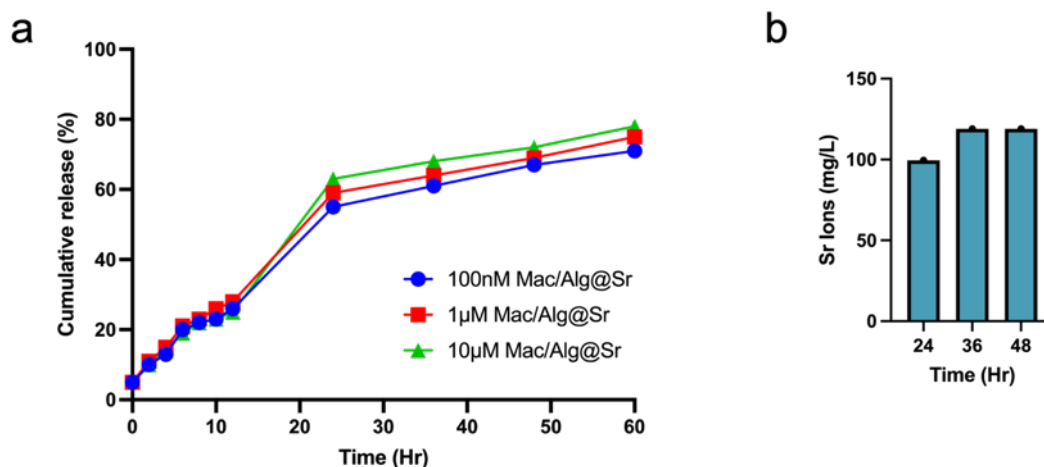


Figure 26 Macitentan and Strontium ions release profile. (a) Cumulative Macitentan release. (b) Strontium ions release.

4.3.2.6 Biocompatibility of hydrogels in various cells

The biocompatibility of biomaterials represents a fundamental prerequisite for their successful translation into clinical wound healing applications. Sodium alginate possesses well-established characteristics of biocompatibility, biodegradability, and low immunogenicity, underpinning its extensive use in biomedical contexts. While strontium-crosslinked alginate hydrogels have generally demonstrated favourable cytocompatibility in prior studies, consideration must be given to the potential for excessive strontium ion release to disrupt local ionic homeostasis within physiological microenvironments. Although macitentan is an FDA-approved drug for systemic administration in pulmonary arterial hypertension, its localized delivery via a hydrogel matrix necessitates careful evaluation of its impact on cellular viability and function within the wound bed, particularly given its potent pharmacological activity.

To comprehensively assess the biocompatibility profile of the developed macitentan-loaded strontium-alginate hydrogels (Mac/Alg@Sr), we evaluated cell proliferation and apoptosis across four distinct and clinically relevant cell types: human umbilical vein endothelial cells (HUVECs), murine macrophage-

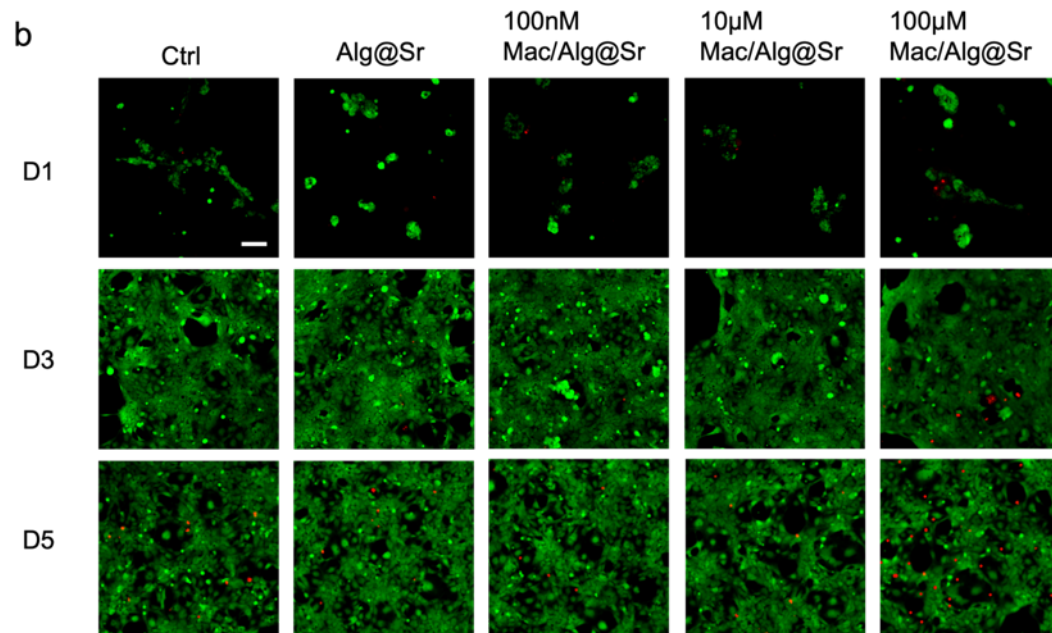
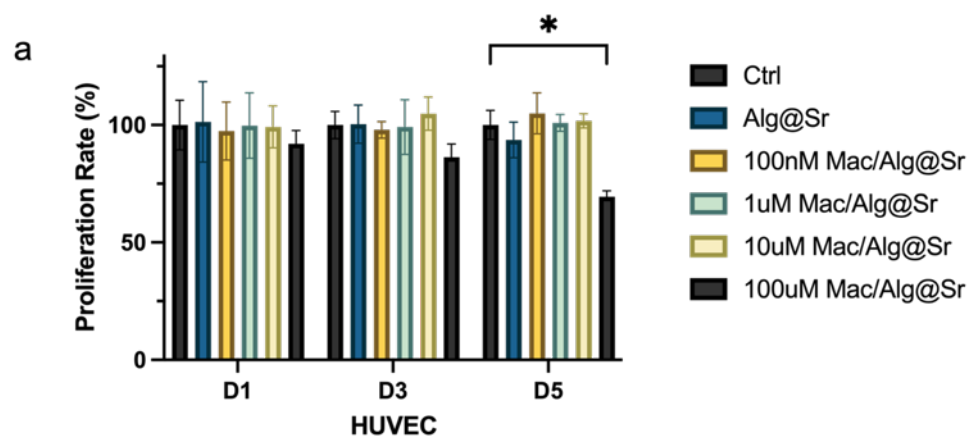
like cells (RAW264.7) and murine embryonic fibroblasts (NIH3T3). These cell types were selected for their critical roles in the sequential phases of wound healing: HUVECs model endothelial function crucial for angiogenesis; RAW264.7 macrophages represent key regulators of inflammation, phagocytosis, cytokine signalling, and tissue remodelling; NIH3T3 fibroblasts are instrumental in wound contraction, collagen synthesis, and extracellular matrix (ECM) deposition, essential for restoring skin integrity. Cells were cultured in direct or indirect contact with hydrogel extracts or the materials themselves for periods of 1, 3, and 5 days. Cell viability and proliferation were quantified using the CCK-8 assay, while live/dead staining (Calcein AM/PI) provided visual confirmation of membrane integrity and apoptosis/necrosis.

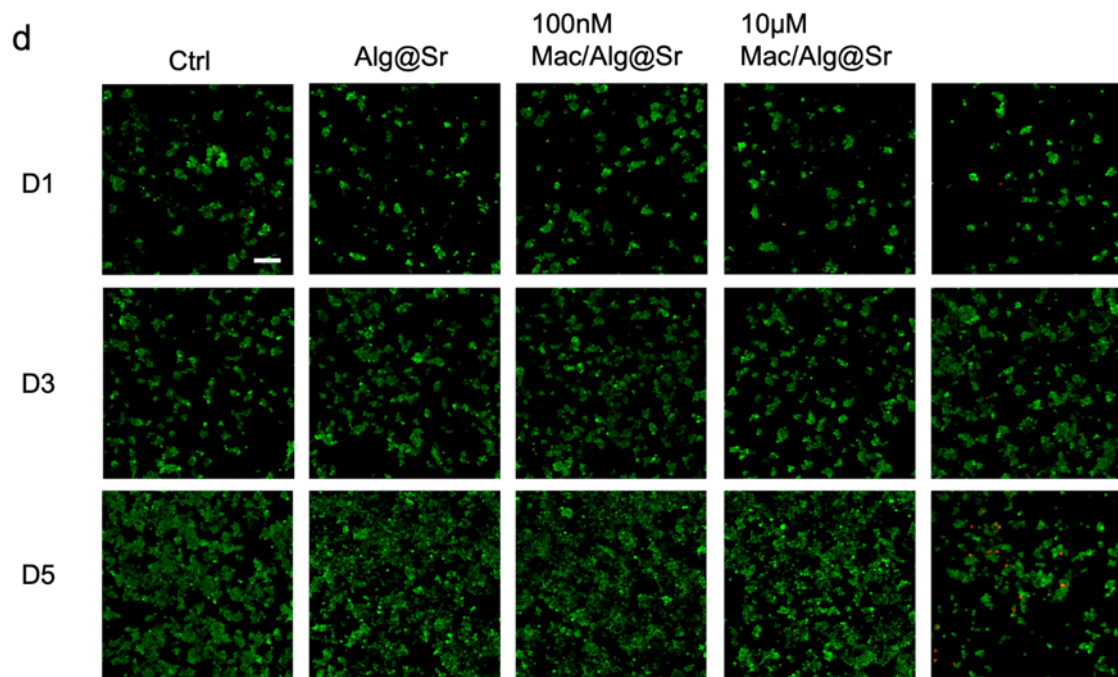
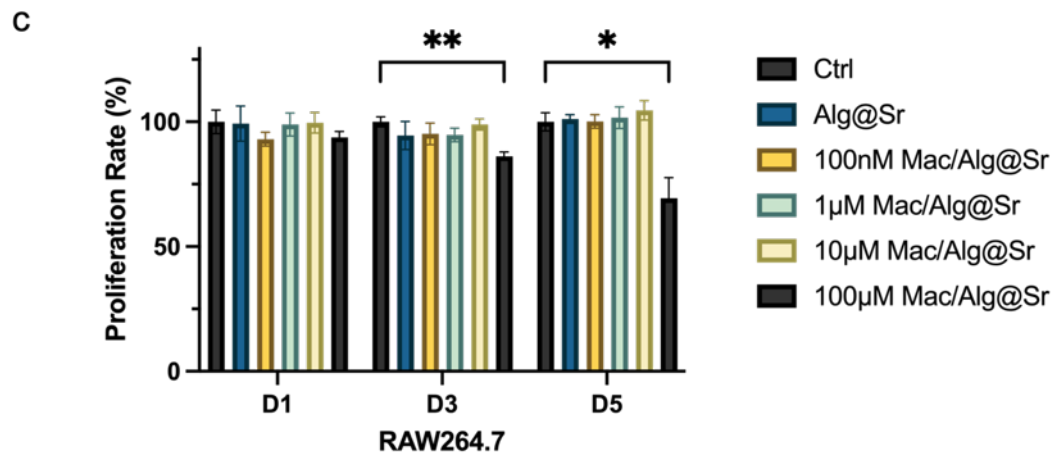
The results demonstrated excellent biocompatibility for the unmodified strontium-crosslinked alginate hydrogel (Alg@Sr) and its macitentan-loaded counterparts at concentrations of 100nM, 1 μ M, and 10 μ M (Fig. 27). Across all four cell types and throughout the 5-day culture period, cell proliferation rates in the presence of these hydrogels showed no statistically significant differences compared to control groups cultured in standard medium. The CCK-8 data revealed consistent metabolic activity and growth kinetics, indicating that neither the alginate polymer, the strontium ions released at physiological levels, nor the macitentan eluted from the hydrogels at these concentrations

exerted any inhibitory effect on cellular expansion. This finding was corroborated by the live/dead staining, which consistently displayed vibrant green fluorescence (indicative of live cells with intact esterase activity and membranes) and minimal red fluorescence (indicative of dead or late apoptotic cells with compromised membranes) across all samples containing Alg@Sr, 100nM Mac/Alg@Sr, 1µM Mac/Alg@Sr, and 10µM Mac/Alg@Sr. The near absence of apoptotic signals confirmed that these formulations did not induce significant cellular stress or cytotoxicity under the tested conditions.

However, a concentration-dependent effect on biocompatibility emerged at the highest tested macitentan loading of 100µM (100µM Mac/Alg@Sr). While initial viability on days 1 and 3 remained comparable to controls for most cell types, a significant reduction in proliferation became evident by day 5 for HUVECs, RAW264.7, and NIH3T3. This was accompanied by a noticeable increase in the proportion of apoptotic and dead cells visualised via live/dead staining at the same time point. This observed toxicity at 100µM macitentan loading is likely attributable to the significantly higher local concentration of the drug released into the cellular microenvironment. Macitentan, as a potent dual endothelin receptor antagonist, may induce cellular stress or disrupt critical signalling pathways essential for sustained proliferation and survival when present at excessively high levels. This finding underscores the

importance of optimizing drug loading concentrations to remain within a therapeutic window that maximizes efficacy while avoiding cytotoxic effects.





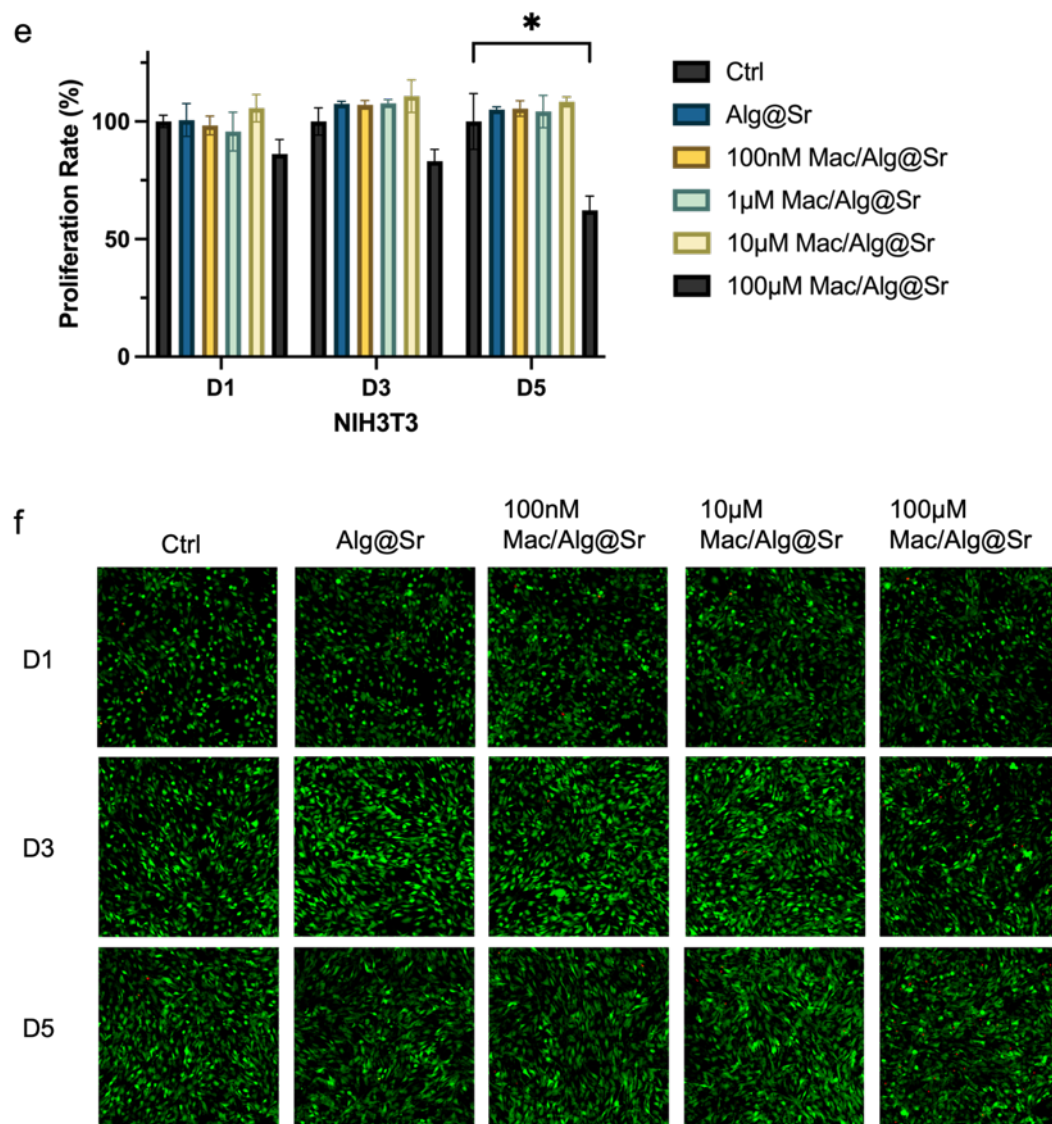


Figure 27 Biocompatibility of hydrogel in various cells. (a, c, e) CCK-8 analysis. (b, d, f) Live (green) and dead (red) staining. Scale bar, 100μm. (a-b) In HUVEC. (c-d) In RAW264.7. (e-f) In NIH3T3.

The demonstrated biocompatibility of Alg@Sr and Mac/Alg@Sr (up to 10μM macitentan) across HUVECs, RAW264.7, and NIH3T3 is highly encouraging. It suggests that the hydrogel platform itself, the released strontium ions within

physiological ranges, and therapeutically relevant doses of macitentan are well-tolerated by the key cellular players involved in wound repair. This favorable cellular response supports the potential of the Mac/Alg@Sr hydrogel for topical application. The therapeutic synergy anticipated from this platform stems from the complementary actions of its components: macitentan effectively targets pathological pathways by antagonizing endothelin receptors, thereby mitigating vasoconstriction, reducing oxidative stress-induced inflammation, and cellular senescence, and preventing excessive fibrosis. Concurrently, the released strontium ions contribute beneficially by promoting angiogenesis, stimulating fibroblast activity and collagen production for enhanced ECM deposition, and further modulating the inflammatory response towards a pro-regenerative state. This combined action fosters a conducive microenvironment within the wound bed that actively suppresses detrimental chronic inflammation, accelerates the formation of new blood vessels to improve perfusion, and supports robust matrix synthesis necessary for effective tissue contraction and structural restoration. Consequently, the Mac/Alg@Sr hydrogel, with its established biocompatibility at appropriate drug loadings and its multifaceted therapeutic mechanisms, presents a highly promising advanced biomaterial strategy for promoting healing in challenging wounds, particularly those associated with diabetes and other conditions impairing normal tissue regeneration. The

observed toxicity at high macitentan concentration emphasizes the need for careful dose selection in future translational studies.

4.3.2.7 Effects of hydrogels on cellular senescence and inflammation

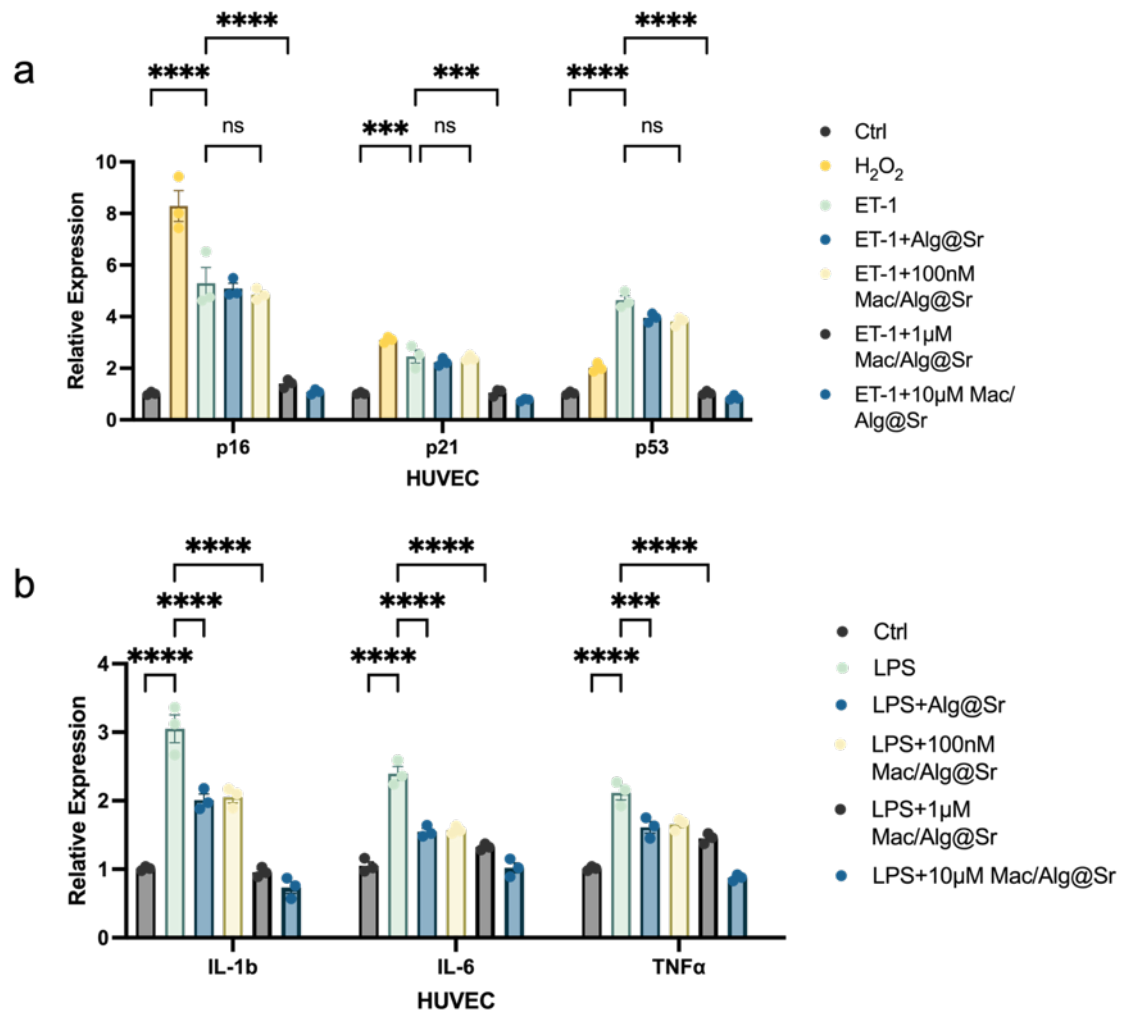
Chronic wounds, particularly in diabetic conditions, are characterized by a pathological microenvironment featuring excessive cellular senescence and unresolved inflammation, which critically impede the healing process.

Endothelin-1 (ET-1), a potent vasoconstrictor and profibrotic peptide upregulated at wound sites, contributes significantly to this pathology. Previous research has demonstrated that ET-1 overexpression activates receptor-mediated pathways that elevate oxidative stress, cause DNA damage, and increase the expression of key senescence markers p16/p21, thereby driving cells into a senescent state. To model this clinically relevant mechanism of senescence induction, HUVEC cells were exposed to ET-1. H₂O₂, as a known oxidative stressor that induces senescence, acted as the positive control. Subsequently, the potential of the strontium-crosslinked alginate hydrogel (Alg@Sr) and its macitentan-loaded counterpart (Mac/Alg@Sr) to mitigate these detrimental processes was evaluated using qPCR for key molecular markers and β -galactosidase staining as a functional indicator of senescence.

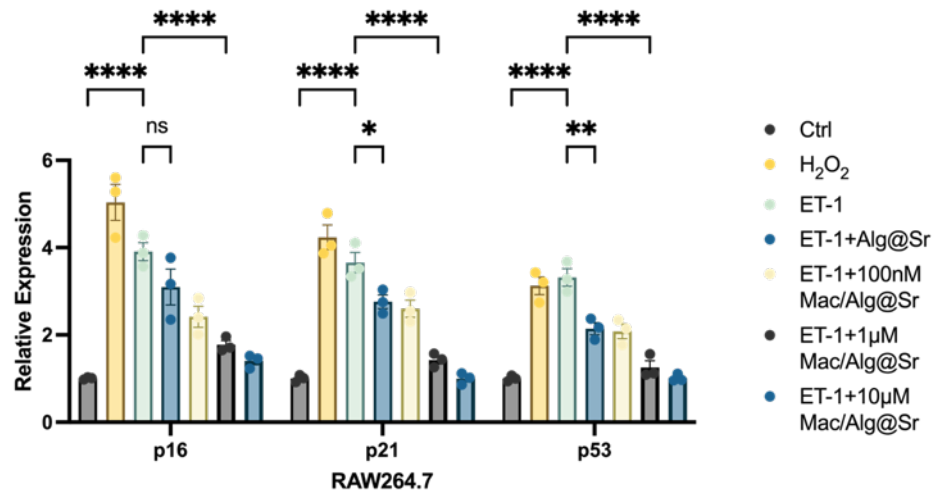
Analysis of senescence markers via qPCR revealed distinct responses to the hydrogel treatments following ET-1 challenge (Fig. 28a and c). HUVEC and RAW264.7 cells treated with Alg@Sr hydrogel extract showed a slight reduction in the expression levels of senescence-associated genes p16^{CDKN2A}, p21^{CDKN1A} and p53 compared to the ET-1 or H₂O₂ treated positive controls. This modest effect suggests that the bioactive strontium ions released from the hydrogel may exert a limited protective influence against senescence induction, potentially through indirect mechanisms such as mild antioxidant effects or subtle modulation of stress response pathways. In contrast, treatment with extract from the Mac/Alg@Sr hydrogel loaded with 10 μ M macitentan resulted in a statistically significant and pronounced decrease in the expression of senescence markers. This robust anti-senescence effect was corroborated by β -galactosidase staining, which visually demonstrated a significant reduction in the number of senescent cells specifically in the 10 μ M Mac/Alg@Sr group compared to both the positive controls and the Alg@Sr group. The potent anti-senescence activity of the high-concentration macitentan hydrogel is mechanistically aligned with macitentan's function as a dual endothelin receptor antagonist. By blocking ET-1 binding to ETA and ETB receptors on endothelial cells, macitentan directly interrupts the downstream signaling cascade responsible for ET-1-induced oxidative stress, DNA damage, and the

subsequent upregulation of p16/p21 that drives cellular senescence. This targeted pharmacological intervention effectively shields cells from a primary driver of senescence in the wound context.

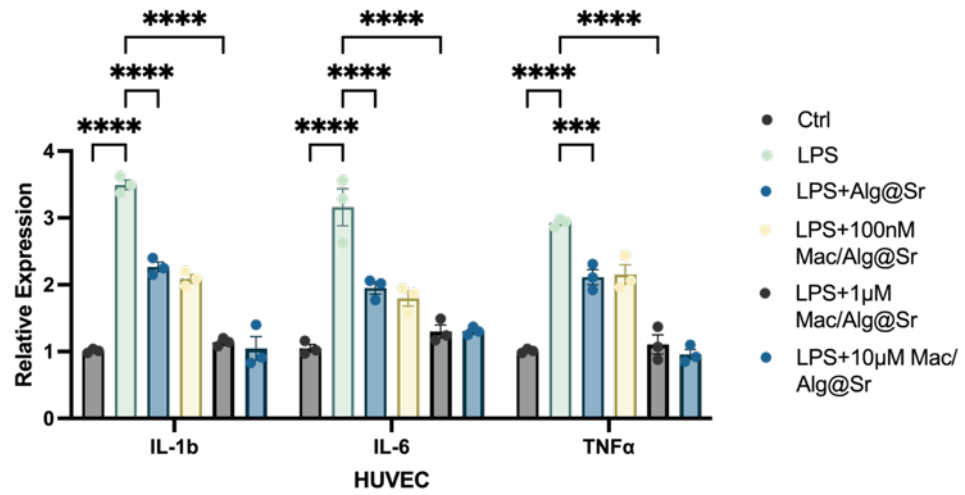
Evaluation of inflammation markers (IL-1 β , IL-6, TNF- α) by qPCR in HUVEC and RAW264.7 exposed to inflammatory stimuli (LPS) yielded a similar pattern (Fig. 28b and d). Treatment with Alg@Sr extract induced a slight downregulation of these pro-inflammatory cytokines. The released strontium ions are known to possess some immunomodulatory properties, potentially contributing to this mild anti-inflammatory trend. However, the extract from the 10 μ M Mac/Alg@Sr hydrogel induced a significant and substantial reduction in the expression of IL-1 β , IL-6, and TNF- α . This potent anti-inflammatory effect underscores the capability of the delivered macitentan to suppress the production of key inflammatory mediators. The mechanism likely involves the mitigation of ET-1 signalling, which is known to stimulate pro-inflammatory cytokine synthesis, combined with the intrinsic anti-inflammatory properties of macitentan itself. The reduction in inflammation complements the decrease in senescence, as chronic inflammation is a major contributor to the senescence-associated secretory phenotype (SASP) and vice versa.



c



d



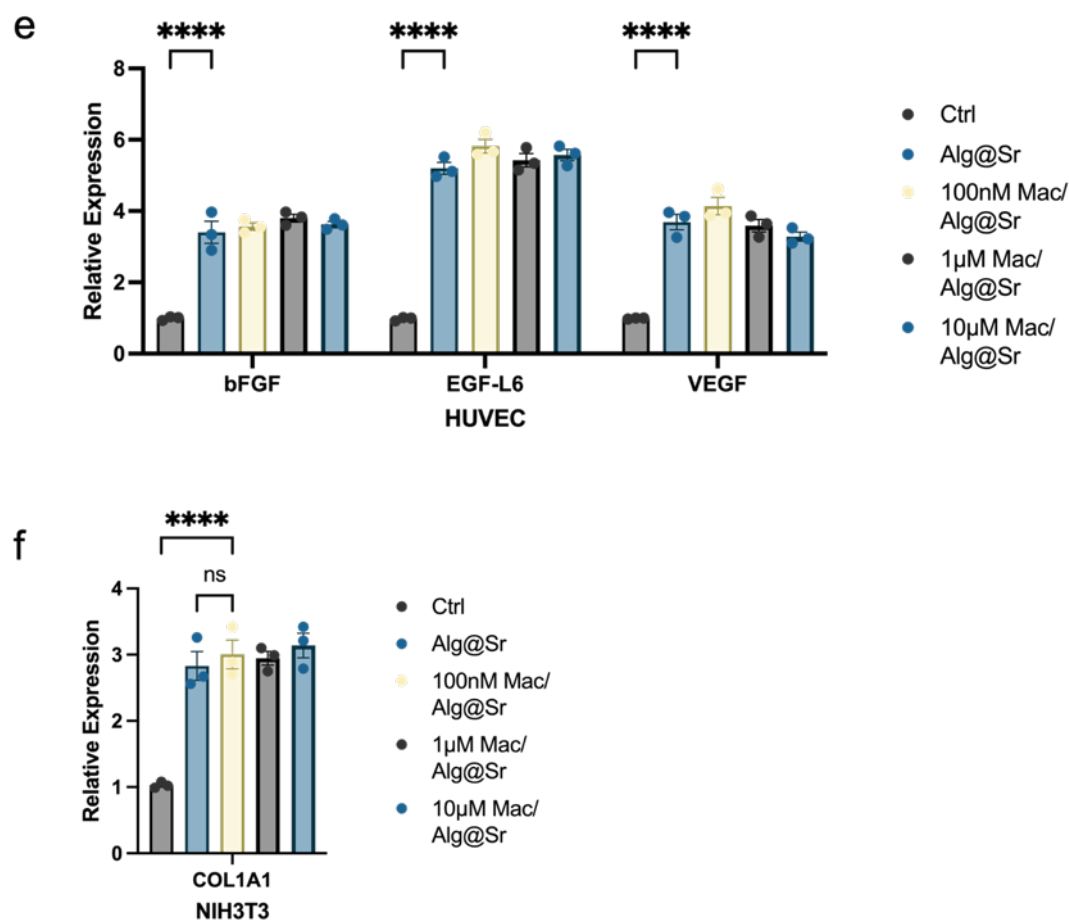


Figure 28 Anti-senescence and anti-inflammatory effects in cells. (a) qPCR of senescence markers in HUVEC. (b) qPCR of inflammatory markers in HUVEC. (c) qPCR of senescence markers in RAW264.7. (d) qPCR of inflammatory markers in RAW264.7. (e) qPCR of angiogenesis markers in HUVEC. (f) qPCR of collagen marker in NIH3T3.

The impact of the hydrogels on pro-angiogenic factors in HUVECs was also assessed (Fig. 28e). qPCR analysis for markers bFGF, EGF-L6, and VEGF demonstrated a significant upregulation in cells treated with Alg@Sr extract compared to controls. This finding confirms the well-established pro-

angiogenic bioactivity of strontium ions, which stimulate endothelial cell function and promote the expression of factors critical for new blood vessel formation. However, the addition of macitentan within the Mac/Alg@Sr hydrogel did not induce a further change in the expression of these angiogenic markers beyond the level achieved by Alg@Sr alone. This suggests that the pro-angiogenic effect is primarily driven by the strontium component, and macitentan, while beneficial for mitigating senescence and inflammation, does not provide an additive stimulus nor inhibition for angiogenesis marker expression under these specific *in vitro* conditions.

Furthermore, the effect on collagen production, a critical process for wound strength and closure mediated by fibroblasts, was evaluated in NIH3T3 cells (Fig. 28f). qPCR for COL1A1 revealed a significant increase in collagen gene expression in cells exposed to Alg@Sr extract, consistent with the known ability of strontium ions to stimulate fibroblast activity and matrix synthesis. Treatment with Mac/Alg@Sr extract (10 μ M macitentan) resulted in a further increase in COL1A1 expression compared to Alg@Sr alone. This suggests a potential additive or synergistic effect in which macitentan may modestly enhance the pro-fibrogenic stimulus provided by strontium.

4.3.2.8 Reduction of reactive oxygen species

Fig. 22 illustrates the efficacy of Alg@Sr and Mac/Alg@Sr hydrogels in mitigating reactive oxygen species (ROS) accumulation within HUVEC and RAW264.7 cells, as visualised through 4-hydroxynonenal (4HNE) immunostaining. In HUVEC cells (Fig. 29a), Ctrl cells exhibited faint cytoplasmic 4HNE fluorescence alongside prominent nuclear counterstaining. Exposure to H₂O₂ induced substantial ROS elevation, manifesting as intense green fluorescence throughout the cytoplasm. ET-1 treatment similarly increased 4HNE signal intensity, confirming its role in oxidative stress induction. Treatment with Alg@Sr hydrogel extract markedly attenuated 4HNE staining, indicating ROS reduction. Critically, the 10µM Mac/Alg@Sr hydrogel elicited the most pronounced suppression of 4HNE fluorescence, restoring signal intensity to near baseline control levels.

Parallel observations were documented in RAW264.7 macrophages (Fig. 29b). Control cells displayed minimal 4HNE staining with distinct nuclei. H₂O₂ and ET-1 treatments both provoked robust ROS generation, evidenced by intensified cytoplasmic green fluorescence. Alg@Sr hydrogel treatment substantially diminished 4HNE signal intensity. However, the 10µM Mac/Alg@Sr hydrogel achieved the greatest ROS reduction, yielding the weakest 4HNE fluorescence

among all treatment groups. Nuclear counterstaining remained consistently vivid across all conditions for both cell types, confirming assay integrity.

These findings demonstrate that both Alg@Sr and Mac/Alg@Sr hydrogels effectively counteract oxidative stress in endothelial cells and macrophages.

The 10μM Mac/Alg@Sr formulation exhibits superior ROS-scavenging capability, consistent with its therapeutic potential for ameliorating redox imbalance during impaired wound healing.

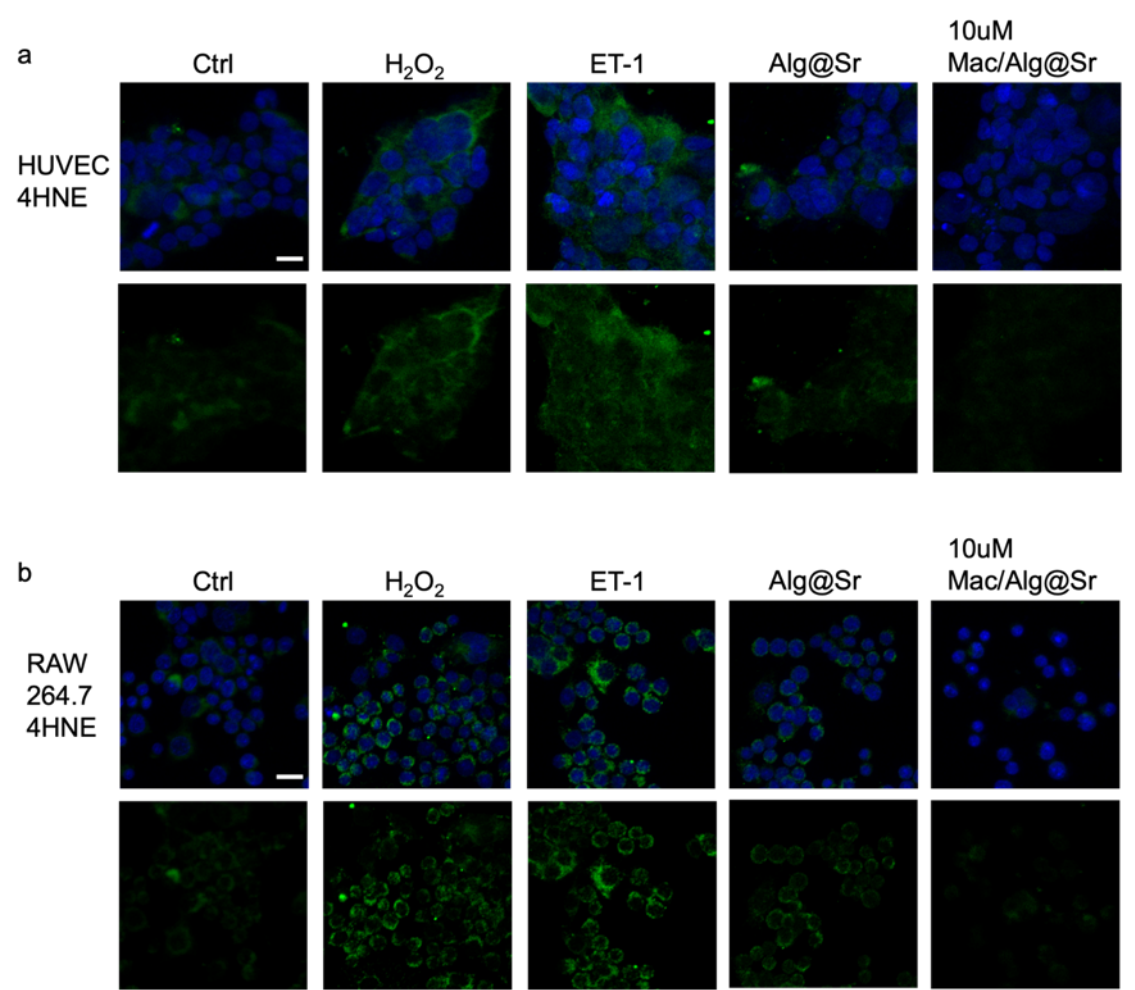


Figure 29 Reduction of reactive oxygen species in cells. (a) Representative images of 4HNE staining in HUVEC. (b) Representative images of 4HNE staining in RAW264.7.

4.3.2.9 *In vivo* wound healing

An *in vivo* wound healing assessment was conducted using Balb/c mice to longitudinally evaluate the efficacy of several treatment modalities. The experimental design comprised five groups: a negative control group receiving no active treatment, a group treated with a commercially available 3M™ wound dressing, a group treated with strontium alginate hydrogel (Alg@Sr), and two groups treated with Macitentan-loaded strontium alginate hydrogel (Mac/Alg@Sr) at distinct concentrations (100nM and 10μM). Wound progression was visually monitored and documented over a seven-day period (Days 0, 3, 5, and 7 post-injury), with representative images compiled in Fig.30. Initial wounds were standardised at 8mm in diameter (D0). All groups exhibited a progressive reduction in wound area over time, yet clear variations in healing kinetics became apparent during follow-up assessments. Mice treated with the Alg@Sr hydrogel and both concentrations of Mac/Alg@Sr hydrogel consistently demonstrated accelerated rates of wound closure relative to both the untreated control group and the group managed with the commercial 3M dressing. Significantly, the group receiving the 10μM Mac/Alg@Sr hydrogel

consistently displayed the most substantial reduction in wound dimensions throughout the observation period. By Day 7, this group exhibited the smallest residual wound area, indicative of its superior capacity to facilitate dermal regeneration. This enhanced macroscopic healing observed with the 10 μ M Mac/Alg@Sr formulation corresponded directly with the statistically significant pro-healing effects previously quantified in *in vitro* analyses.

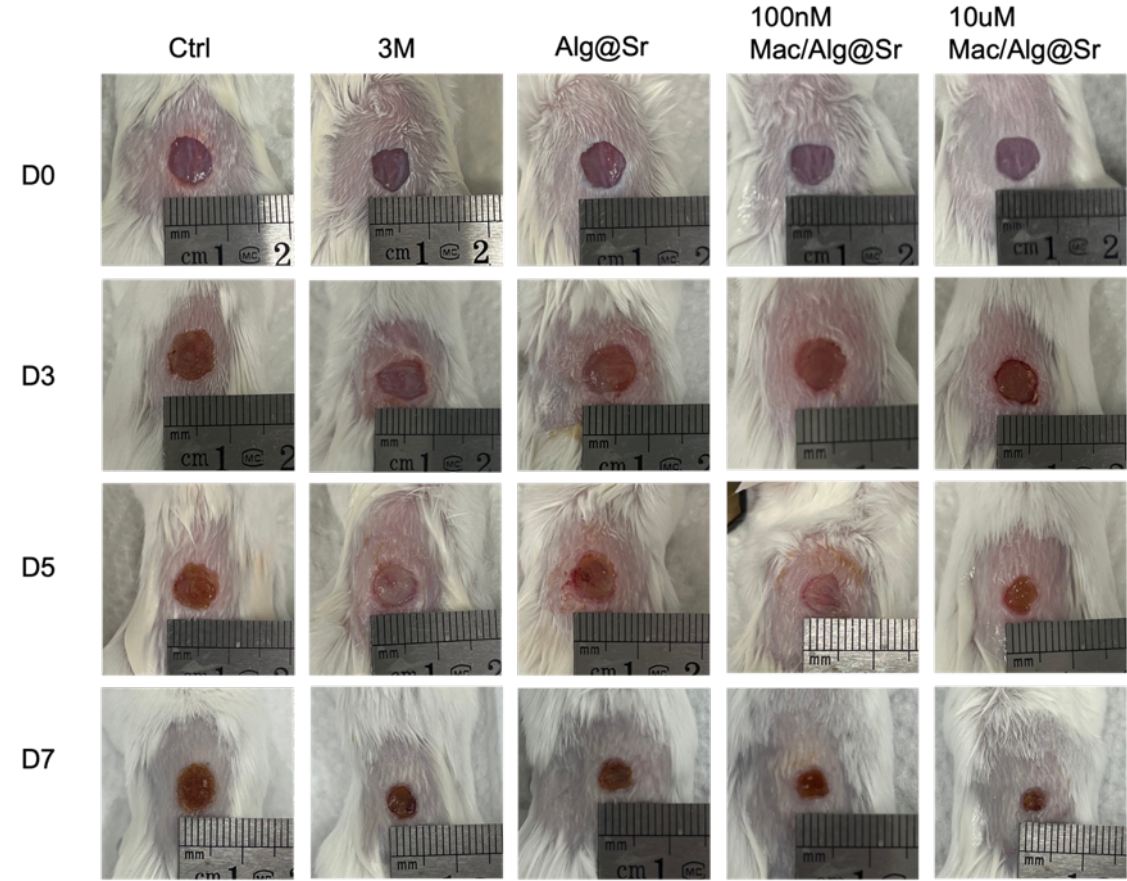


Figure 30 Skin wound healing in mice. Wound treated with different hydrogels was observed at different time points (n=6).

To elucidate the underlying therapeutic mechanisms, comprehensive histological and immunohistochemical examinations were performed on wound tissues harvested at Day 7. Haematoxylin and eosin (HE) staining (Fig.31a) revealed enhanced vascularisation and robust granulation tissue formation across all treatment cohorts, consistent with active repair. Notably, wounds treated with Mac/Alg@Sr hydrogels exhibited granulation tissue characterised by markedly improved organisation and structural density compared to other groups.

Immunohistochemical staining for CD31, a specific endothelial cell marker, was employed to quantify neovascularisation (Fig.31b). This analysis revealed significant disparities in microvessel density between groups. Both the Alg@Sr and Mac/Alg@Sr hydrogel-treated groups demonstrated substantially higher densities of CD31-positive microvessels than the untreated control (Ctrl) or 3M-treated groups. This finding confirms the potent pro-angiogenic activity inherent to the strontium-crosslinked hydrogel platform, facilitating improved perfusion critical for healing.

Masson's trichrome staining provided further insight into collagen deposition and extracellular matrix organisation (Fig.31c). The Ctrl and 3M groups displayed sparse and disorganised collagen fibres, indicative of immature

matrix remodelling. Conversely, wounds treated with Alg@Sr hydrogels showed improved collagen density and alignment. Most significantly, the 10 μ M Mac/Alg@Sr group demonstrated exceptionally mature collagen architecture, featuring densely packed, parallel-aligned fibres within a characteristic pink-blue stromal matrix, signifying advanced tissue maturation essential for mechanical integrity and barrier function restoration.

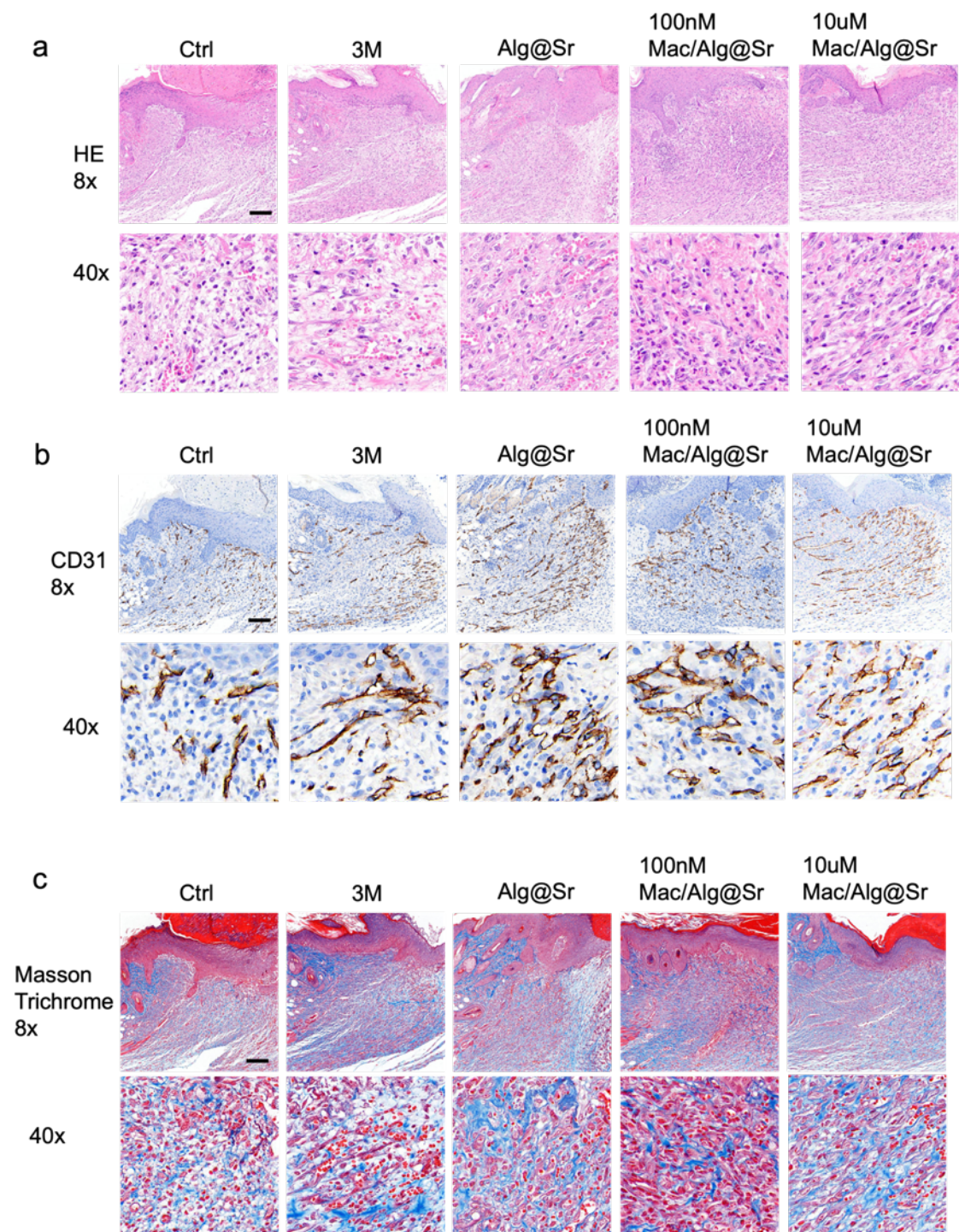


Figure 31 Wound tissue histology analysis. (a) H&E staining. (b) CD31. (c) Masson

trichrome. Scale bar, 100 μ m.

The immunohistochemical analysis of p21 and p53 expression within the wound tissue provided compelling evidence of the treatment's impact on cellular senescence (Fig. 32). In the Ctrl and 3M groups, the signals for both senescence-associated markers were notably strong and widespread, indicating a high burden of senescent cells and a similar, limited capacity for either approach to mitigate this process. A discernible reduction in the intensity and distribution of both p21 and p53 staining was observed in wounds treated with the strontium-cross-linked alginate hydrogel (Alg@Sr), particularly within certain regions of the dermis, suggesting a modest beneficial effect on reducing cellular senescence. The group treated with the macitentan-loaded hydrogel (10Mac/Sr) exhibited a profound and significant attenuation in the signals for both markers. The staining for p21 and p53 was substantially weaker and more focal compared to all other groups, demonstrating that the localised and sustained delivery of macitentan effectively targeted the senescent cell population, thereby markedly reducing the expression of these key regulators of the cell cycle arrest that characterises senescence^[187]. These visual results strongly correlate the superior efficacy of the 10Mac/Sr treatment with a direct reduction in the molecular hallmarks of cellular senescence in the wound bed.

The concordance of these histological findings – enhanced granulation tissue organisation (HE), superior angiogenesis (CD31), advanced collagen maturation

(Masson) and senescence expression (p21 and p53) – collectively underscores the therapeutic superiority of the 10 μ M Mac/Alg@Sr hydrogel. The accelerated macroscopic wound closure observed in this group correlates intrinsically with these definitive microscopic hallmarks of effective regenerative healing, positioning this biomaterial formulation as a highly promising therapeutic strategy for managing challenging wounds.

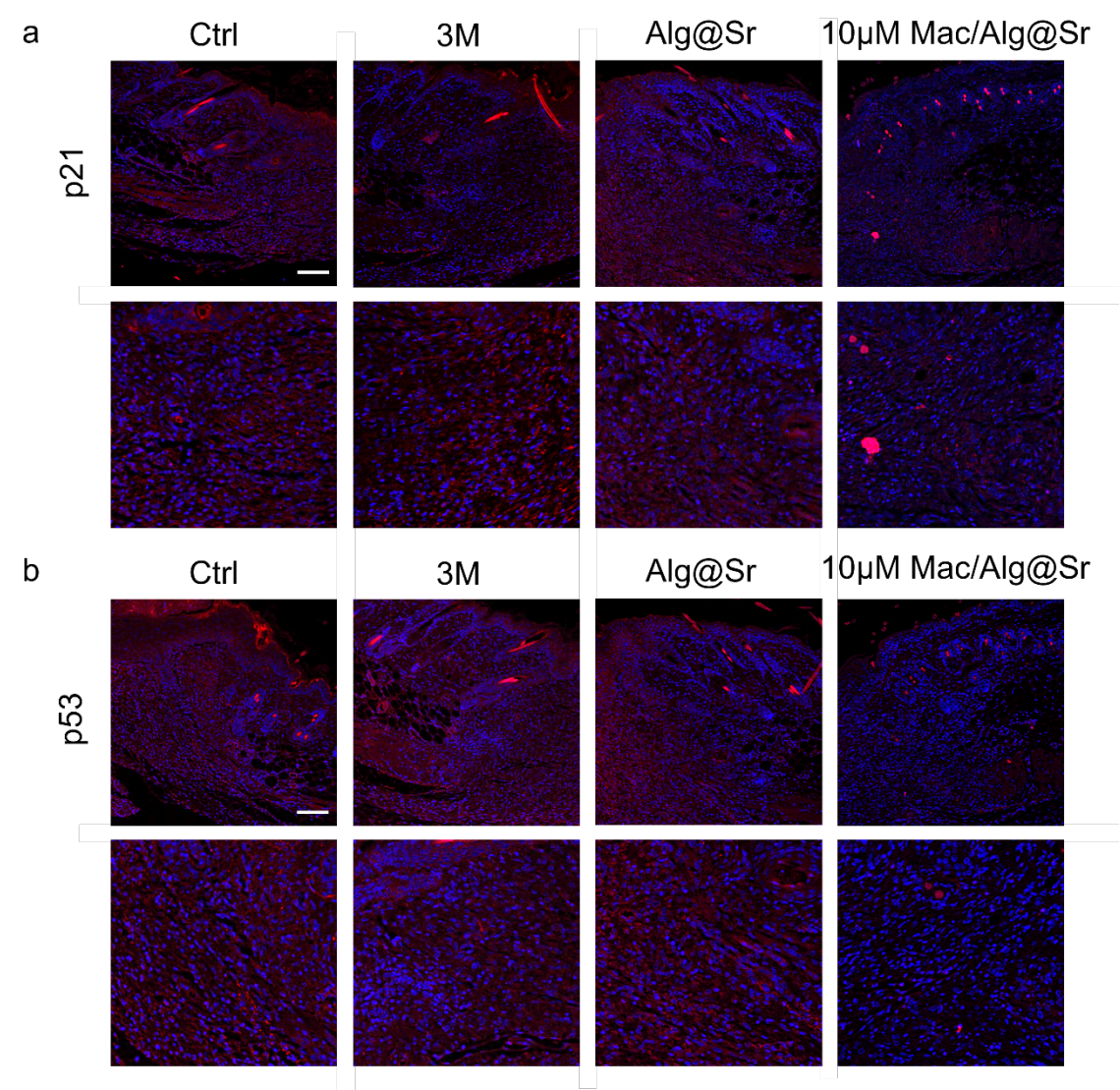


Figure 32 Wound tissue senescence analysis. (a) Representative images of p21 staining. (b) Representative images of p53 staining. Scale bar, 200µm.

4.4 Discussion

The development of the two distinct alginate-based hydrogel systems detailed in this chapter represents a direct translational application of the central thesis findings, moving from the mechanistic understanding of endothelin signalling in ageing to the design of targeted, local therapeutic strategies. Each hydrogel is engineered with specific material and drug release properties tailored to address the unique pathophysiological microenvironment of its target tissue: the Macitentan/Strontium (Mac/Alg@Sr) hydrogel for vascular regeneration in chronic wounds, and the BQ788/Calcium (BQ/Alg@Ca) hydrogel for combating skeletal ageing.

The comprehensive characterisation of both systems confirms their fundamental suitability as local delivery platforms. The invariant morphology upon drug incorporation in both hydrogels indicates that loading is achieved through physical entrapment within the ionically crosslinked network, preserving the gentle gelation mechanism crucial for maintaining biomolecule bioactivity. The distinct release profiles of each system, however, were engineered to fulfil different therapeutic requirements. The Mac/Alg@Sr hydrogel exhibited a

rapid, substantial release of bioactive Sr^{2+} ions, establishing an immediate pro-regenerative milieu, coupled with a sustained, biphasic release of macitentan providing prolonged pharmacological action. This profile is strategically aligned with the dynamic needs of a chronic wound, where immediate modulation of inflammation and stimulation of angiogenesis (via Sr^{2+}) must be followed by continuous suppression of pathological ET-1 signalling (via macitentan) throughout the prolonged healing phases. Conversely, the BQ/Alg@Ca hydrogel demonstrated a more synchronized and gradual release profile for both its components. The near-linear, sustained release of BQ788, paired with the controlled, steady elution of Ca^{2+} ions, reflects a design optimised for the slower, sustained remodelling processes of bone tissue. This ensures that the senolytic signal (BQ788) and the osteogenic signal (Ca^{2+}) are presented concurrently over an extended period, which is essential for coordinately suppressing the senescent niche while actively promoting new bone matrix deposition.

The biological efficacy data powerfully validates the therapeutic rationale behind each combinatorial approach. For the Mac/Alg@Sr hydrogel, the significant reduction in ET-1-induced cellular senescence and pro-inflammatory cytokine expression in endothelial cells is a direct consequence of macitentan's dual receptor antagonism, effectively shielding cells from a key driver of

vascular dysfunction. This specific pharmacological action is complemented by the pro-angiogenic and collagen-stimulating effects of the Sr^{2+} ions, creating a multifaceted strategy that addresses the core barriers to healing in chronic wounds: excessive inflammation, impaired angiogenesis, and poor matrix synthesis.

Similarly, the BQ/Alg@Ca hydrogel demonstrated a potent, dose-dependent protection of mesenchymal stem cells from ET-1-induced senescence. The significant downregulation of senescence markers *p16* and *p21* by the high-concentration BQ788 formulation confirms the successful targeting of the ETB-mediated senescent pathway in a progenitor cell population critical for skeletal maintenance. The concurrent release of Ca^{2+} ions from the same matrix provides the essential anabolic signal to promote osteogenic differentiation, thereby addressing the dual pathology of ageing bone—increased catabolic/senescent burden and declining anabolic function. This dual capability to potentially clear a senescent barrier while stimulating new bone formation exemplifies a sophisticated approach to skeletal regeneration.

In conclusion, the data presented in this chapter confirm that both engineered hydrogel systems successfully fulfil their design criteria as localised, sustained-delivery platforms for modulating the dysregulated endothelin pathway in a

tissue-specific manner. The Mac/Alg@Sr hydrogel emerges as a promising strategy to transform the hostile microenvironment of chronic wounds by concurrently resolving inflammation, reducing senescence, and promoting vascularisation. The BQ/Alg@Ca hydrogel presents a novel therapeutic paradigm for skeletal ageing, aiming to disrupt the deleterious cycle of cellular senescence and impaired osteogenesis. Together, these systems exemplify a rational translational pipeline, leveraging fundamental mechanistic insights into endothelin signalling to develop targeted biomaterial solutions for complex age-related tissue degeneration.

Conclusion

This thesis has systematically investigated the profound and previously underexplored connection between vascular dysfunction and skeletal ageing, establishing endothelial-derived Endothelin-1 (ET-1) signalling through the Endothelin B receptor (ET_BR) as a critical driver of bone loss and osteoporosis pathogenesis. The research presented herein provides compelling evidence that skeletal and vascular ageing are intrinsically linked, with vascular factors playing a decisive regulatory role in tissue-specific ageing processes. While ET-1 is essential for physiological vascular tone regulation, its excessive expression, a hallmark of endothelial dysfunction, emerges as a key pathological mediator. Crucially, this work elucidates that elevated ET-1 secretion from dysfunctional endothelium within the highly vascularised bone marrow niche exerts a direct and deleterious impact on perivascular skeletal stem cells (SSCs), a previously unresolved aspect of skeletal ageing.

The central finding of this research is the identification of the ET-1/ET_BR signalling axis as a primary mechanism governing SSC senescence and subsequent osteoporotic degeneration. Through comprehensive analyses in both natural ageing and ovariectomy-induced osteoporotic mouse models, we demonstrated that increased ETBR expression correlates strongly with bone

loss and is mechanistically linked to the accumulation of reactive oxygen species (ROS) within SSCs, driving them into a senescent state. This causal relationship was further solidified using transgenic models: endothelial-specific ET-1 overexpression (TET-1 mice) accelerated skeletal ageing, recapitulating key features of osteoporosis, while RNA sequencing revealed the ET-1 induction of SSC senescence via the PI3K/Akt/mTOR pathway. Most significantly, targeted interventions provided definitive proof of concept. Conditional genetic knockout of ET_BR specifically within PRX1⁺ SSCs (*Prx1-Cre::ETBR^{fl/fl}*) effectively prevented ET-1-induced senescence, mitigated ROS accumulation, and alleviated osteoporotic degeneration in OVX mice. Mirroring these genetic results, systemic pharmacological inhibition of ET_BR using the selective antagonist BQ788 successfully rejuvenated bone structure and function in both aged and OVX models, demonstrating the therapeutic tractability of this pathway.

These findings collectively reveal a direct ET-1/ET_BR-ROS signalling axis that acts as a master regulator of SSC senescence and skeletal ageing. The identification of this axis positions ET_BR inhibition as a highly promising and novel therapeutic strategy for osteoporosis, moving beyond the limitations of current treatments that primarily target mature osteoclast or osteoblast activity without addressing the fundamental stem cell dysfunction and hostile niche

environment. To bridge the gap between these mechanistic insights and potential clinical application, this research pioneered the development of advanced drug delivery systems. An alginate-based hydrogel – Macitentan/Alg@Sr – was engineered for localised, sustained release of ET-1 antagonists alongside osteogenic ions (Strontium). The hydrogel demonstrated significant efficacy in pre-clinical settings, effectively reducing cellular senescence and inflammation within target tissues, thereby promoting a more regenerative microenvironment for both vascular and bone tissue repair.

By definitively elucidating the mechanistic link between vascular endothelial dysfunction, specifically ET-1 overproduction, and the induction of SSC senescence via ET_BR signalling and ROS generation, this study fundamentally advances our understanding of osteoporosis pathogenesis. It shifts the paradigm from viewing bone loss merely as an imbalance in mature cell activity to recognising it as a consequence of stem cell failure driven by vascular-derived signals within a deteriorating niche. Consequently, this work identifies ET_BR on SSCs as a precision therapeutic target with significant potential for combating age-related and pathological bone loss. The successful development and validation of targeted hydrogel delivery systems further underscore the translational potential of this approach. The implications extend beyond osteoporosis, offering a novel framework and therapeutic rationale for

addressing a range of degenerative skeletal disorders characterised by compromised stem cell function and vascular insufficiency, ultimately paving the way for more effective regenerative strategies aimed at restoring skeletal health in an ageing population.

Limitations and Future Work

While this thesis provides robust evidence for the ET-1/ETBR axis as a key regulator of SSC senescence and skeletal ageing, and demonstrates the therapeutic promise of its modulation, several important limitations define clear avenues for future investigation. Firstly, although we established a critical role for ET-1/ETBR signalling in promoting SSC senescence and impairing osteogenesis, a comprehensive analysis of its effects on tri-lineage differentiation potential—towards osteogenic, chondrogenic, and adipogenic fates—was not conducted. Determining whether ET-1 exposure or ETBR inhibition specifically skews SSC lineage commitment, potentially favouring adipogenesis at the expense of osteogenesis (a hallmark of aged bone marrow), is essential for fully understanding its pathological impact and therapeutic implications.

Secondly, whilst RNA-sequencing data strongly implicated the PI3K/Akt/mTOR pathway in mediating ET-1-induced senescence, this requires

direct biochemical validation. Future work should employ genetic (e.g., siRNA) or pharmacological inhibition of key nodes within this pathway in SSCs challenged with ET-1 to confirm its necessity and sufficiency in driving the senescent phenotype. This would solidify the mechanistic model and potentially identify more downstream targets for intervention.

Thirdly, the promising *in vitro* efficacy of the BQ788/Calcium (BQ/Alg@Ca) hydrogel in mitigating MSC senescence necessitates rigorous *in vivo* validation. Future studies must implant this hydrogel in relevant osteoporotic defect models (e.g., in aged or OVX mice) to assess its capacity to locally reduce senescence burden, promote bone formation, and improve repair outcomes. Comparing its efficacy to systemic BQ788 administration will be crucial to demonstrate the advantages of localised delivery for mitigating skeletal side-effects.

Beyond these specific points, broader considerations remain. The long-term systemic consequences of chronic ETBR inhibition, while not evident in our shorter-term studies, require thorough evaluation in extended models to fully de-risk this therapeutic strategy. Furthermore, whilst murine models are invaluable, the human skeletal stem cell niche and its response to ET-1 may differ; thus, validating these findings in human SSCs and bone marrow samples

from osteoporotic patients is a critical translational step. Finally, it is improbable that ET-1 acts in isolation; other endothelial-derived factors are almost certainly co-contributors to the ageing niche, and their interactions with the ET-1 pathway warrant exploration.

These limitations naturally chart a course for subsequent research. Immediate priorities include the clinical validation of the ET-1/ETBR signalling axis in human bone ageing through analysis of patient biopsies and serum biomarkers. Furthermore, exploring combination strategies that simultaneously target senescence (e.g., via senolytics) and actively promote regeneration (e.g., via anabolic agents) alongside ETBR inhibition could yield synergistic benefits. The optimisation of bone-specific, niche-targeted biomaterials—perhaps with enhanced SSC homing or controlled sequential release profiles—represents a key bioengineering challenge. Finally, progressing to large-animal translational models with bone physiology closer to humans will be an essential step in advancing this therapeutic paradigm towards clinical application.

In conclusion, this thesis establishes a novel vascular-skeletal signalling axis and provides a compelling proof-of-concept for its therapeutic targeting. By candidly addressing its limitations and pursuing the outlined future work, this research can evolve from a fundamental discovery into a viable strategy for

combating osteoporosis and other age-related degenerative conditions,
ultimately aiming to restore skeletal health and resilience.

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