The Role of the Osteocyte in Orchestrating Ageing-related Bone Loss and Alzheimer's Disease

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Bachelor of Medicine

Master of Clinical Medicine (Surgery)

This thesis is presented for the degree of *Doctor of Philosophy* of

The University of Western Australia

Medical School, Faculty of Health and Medical Sciences

2022

THESIS DECLARATION

I, Jun Yuan, certify that:

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The following approvals were obtained prior to commencing the relevant work described in this thesis:

Human data was from the Kerr Anglican Retirement Village Initiative in Aging Health (KARVIAH) cohort, the Bellberry Human Research Ethics Committee, Australia (reference number 2012-09-1086) and the Macquarie University Human Research Ethics Committee (reference number 5201701078) provided approval for the study. Written patient consent has been received and archived for the research involving patient data reported in this thesis.

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ABSTRACT

Osteocytes are a major constituent of bone, accounting for approximately 90% of the total cells present in this organ. Over the past decades, the function of osteocytes has not been extensively studied in part due to their location in lacunae, which are structures situated deep within bone tissue. However, recent advancements in molecular and cellular technologies, have unveiled new insight into the function of osteocytes including their unexpected contribution to ageing-related diseases.

Osteoporosis and Alzheimer's Disease (AD) are two major and often co-existing disorders mainly affecting older individuals. Owing to the high morbidity and mortality related to these two diseases, an understanding of their pathogenesis and any association is paramount. Current evidence indicates that osteocytes, like endocrine cells, can also affect tissues outside bone by secreting a variety of molecules. This thesis investigated the potential role of an endocrine-like function of osteocytes in osteoporosis and AD.

Also contributing to bone mineral density decline are the glucocorticoids (GC) steroids hormones, which are increasingly secreted during ageing and frequently used as an anti-inflammatory therapeutic. Therefore, to investigate the role of osteocytes in age-related osteoporosis, an *ex vivo* primary osteocyte culture system and an osteocyte-like cell line (MLO-Y4) were exposed to GC. It was demonstrated that GC induced primary osteocytes to produce cathepsin K, which is a proteinase responsible for the degradation of type I collagen in the extracellular matrix of bone, thereby contributes to bone loss. It was also demonstrated that GC increased mitochondrial fission and membrane depolarization in osteocytes, indicating the augmented metabolic activity towards dysfunctional mitochondria. In addition, the activation of PTEN-induced putative kinase 1 (PINK1)-mediated mitophagy in osteocytes was demonstrated to have an involvement in glucocorticoid-induced mitochondrial dysfunction.

Examination of the relationship between mitophagy and cathepsin K expression revealed that inhibition of mitophagy, but not canonical autophagy, abolished GC-induced osteocyte production of cathepsin K. Together, this study has identified a potentially novel pathogenic pathway, whereby GC induces osteocytes to secrete cathepsin K through a process involving PINK1-mediated mitophagy, resulting in osteoporosis associated with increasing age and or steroid treatments.

Although osteoporosis and AD are two seemingly unrelated ageing-related disorders affecting two separate organs, intriguingly, they share several epidemiological features including the observation that osteoporosis patients are prone to develop AD. To explore a biological association between osteoporosis and AD, this thesis focussed on sclerostin, a protein primarily secreted by osteocytes, and whose levels increase in the body with increasing age. Sclerostin negatively regulates bone formation by inhibiting Wnt signalling, which is a pathway that also contributes to AD pathogenesis by modulating several aspects of brain development and function.

To investigate a possible association between sclerostin and AD, plasma sclerostin levels were compared with brain amyloid-β (Aβ) load in cognitively healthy older individuals (n = 100, male = 32, female = 68; age range 66-89). The participants were divided into Aβ- (n = 65) and Aβ+ (n = 35) groups based on their Aβ load status. Analysis revealed a positive correlation between increasing plasma sclerostin levels and ageing ($r = 0.309$, $p = 0.002$), which is in line with previous studies. Importantly, plasma sclerostin levels were significantly higher in Aβ+ individuals compared with Aβ- individuals ($p = 0.003$, $p^* = 0.008$), before and after adjusting for covariates of age, gender and apolipoprotein E ε4 (APOE ε4) status. Furthermore, it was demonstrated that combining SOST with a base model built with AD risk factors including age, gender and APOE ε4 status had a higher diagnostic accuracy (AUC = 0.818, 95% CI =

0.733 – 0.903) than using the base model alone (AUC = 0.787, 95% CI = 0.693 – 0.882). Together, these findings highlight a potential pathogenic association between bone, specifically osteocyte-derived sclerotin, and the development of AD in aging individuals.

To ascertain whether osteocytes produce more sclerostin with ageing, we examined sclerostin gene expression (*Sost)* in the osteocyte-like cell line, MLO-Y4, following exposure to advanced glycation end products (AGEs), which are biomarkers implicated in ageing and worsening of degenerative diseases (e.g., diabetes and AD). It was demonstrated that exposure of MLO-Y4 cells to AGEs, increased *Sost* expression in a time- and dose-dependent manner. To further investigate how sclerostin may contribute to AD pathogenesis, its impact on neurogenesis in the neuron stem cell line, NE-4C, was examined. It was demonstrated that exposure of NE-4C cells to sclerostin inhibited cell proliferation and viability in a time- and dose-dependent manner. Moreover, sclerostin slowed NE-4C cell maturation as measured by microtubule-associated protein 2 (MAP2) mRNA expression and had an adverse effect on neurosphere formation. In addition, sclerostin impacted dendritic spine density development in differentiated NE-4C cells and the expression of postsynaptic scaffolding protein 95 (PSD95). Taken together these findings provide a potential mechanism whereby osteocytes through the secretion of sclerostin, promote the onset of AD during ageing.

Overall, this thesis has revealed evidence of crosstalk between bone and brain, and highlights the potential of osteocytes in orchestrating two ageing-related diseases, osteoporosis and AD. In brief, osteocytes may contribute to bone loss by secreting cathepsin K and may also promote pathological alterations in AD through secretion of sclerostin. These findings further advance the knowledge of osteocytes as a multifunctional and dynamic cell, and importantly, represent a novel therapeutic target in osteoporosis and AD.

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ACKNOWLEDGEMENTS

This research was supported by an Australian Government Research Training Program (RTP) Scholarship and the UWA Safety Net PhD Top-Up Scholarship.

Completing PhD study is a long and hard journey, especially with the interruption of COVID-19 pandemic. I could never have made it without the support and help from my supervisors, colleagues and friends, most importantly, my family. I am so grateful every single day for the people in my life who have encouraged me and helped me to achieve my dreams.

To all my supervisors, Prof. Minghao Zheng, Adj. A/Prof. Bruno Meloni and Dr. Junjie Gao, thank you for your wisdom, guidance in the composition and conduction of my project. Your work ensured that my project could continue and finally be finished. To Prof. Frank Mastaglia, you are always right there to provide me with precious comments to my projects based on your decades' expertise. I would also like to thank you for your revision for this thesis which makes the writing more scientific. To Mr Vince Clark and Mrs Aline Domingos, thanks for your patience in helping me applying animal ethics approval and teaching me how to operate with animals. Your professional knowledge is invaluable to this project.

To my colleagues and peers, thanks for sharing your stories and experience which really make the life of my PhD a lot easier, especially when I encountered difficulties. Thanks Mrs Euphemie Landao-Bassonga for placing all the orders of the reagents and consumables for my projects, your work saves me plenty of time.

To my mother and all the other family members living in China, thanks for the endless sacrifices you have made for me and the unconditional love you have shown me, in the past 30 years of my life. Look forward to seeing you soon after the COVID.

To my loving wife, I am so blessed to meet you from the moment of entering university. Your company through the past 5-year undergraduate, 3-year master study and now 4-year PhD study, makes me feel the hope of life and keep going on every time when I was about to give up. And thank you for bringing me a precious gift, my lovely baby son, during such a tough time. There are no words eloquent to convey my deep love and appreciation to you. Let's embrace every wonderful tomorrow for the rest of our life together, forever.

AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

This thesis contains work that has been published and prepared for publication.

Details of the work: Literature review published in the Journal of Alzheimer's Disease: Yuan J, Meloni BP, Shi T, Bonser A, Papadimitriou JM, Mastaglia FL, Zhang C, Zheng M, Gao J. The Potential Influence of Bone-Derived Modulators on the Progression of Alzheimer's Disease. J Alzheimers Dis. 2019;69(1):59-70. doi: 10.3233/JAD-181249.
Location in thesis: Chapter 2
Student contribution to work: manuscript drafting 90%, manuscript revision 80%

Details of the work: Research article accepted by the *Journal of Orthopaedic Translation*: Yuan J, Gao YS, Liu DL, Tai A, Zhou Hong, Papadimitriou JM, Zhang CQ, Zheng MH, Gao JJ. 2022. PINK1-mediated Mitophagy Contributes to Glucocorticoid-induced Cathepsin K Production in Osteocytes.
Location in thesis: Chapter 4
Student contribution to work: project design 50%, experimental procedures 70%, data collection 70%, data analysis 80%, manuscript drafting 80%

Details of the work: Research article has been prepared and formatted for submission: Yuan J, Pedrini S, Chatterjee P, Meloni B, Mitchell C, Tai A, Gao JJ, Mastaglia F, Martins RN, Zheng MH. 2022. Elevated Bone-derived Sclerostin in Plasma is Associated with High Brain Amyloid-β Load in Older Adults.
Location in thesis: Chapter 5
Student contribution to work: project design 50%, data analysis 90%, manuscript drafting 90%

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As all co-authors' signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis.

Coordinating supervisor signature: 

Date: 15/11/2022

PUBLICATIONS ASSOCATED WITH THIS PROJECT

Yuan J, Meloni BP, Shi T, Bonser A, Papadimitriou JM, Mastaglia FL, Zhang C, Zheng M, Gao J. The Potential Influence of Bone-Derived Modulators on the Progression of Alzheimer's Disease. J Alzheimers Dis. 2019;69(1):59-70. doi: 10.3233/JAD-181249.

Yuan J, Gao YS, Liu DL, Tai A, Zhou Hong, Papadimitriou JM, Zhang CQ, Zheng MH, Gao JJ. PINK1-mediated Mitophagy Contributes to Glucocorticoid-induced Cathepsin K Production in Osteocytes. J Orthop Translat. 2022. (Accepted)

PRESENTATIONS ASSOCIATED WITH THIS PROJECT

National Meetings

Podium Presentation:

2022 "*Elevated bone-derived sclerostin in plasma is associated with high brain amyloid-β load in cognitively normal older adults*", the ANZBMS Christopher and Margie Nordin Young Investigator Award Symposium, Gold Coast, QLD, Australia.

2021 "*PINK1-mediated mitophagy in osteocyte contributes to glucocorticoid-induced bone loss*", the 26th annual ANZORS (Australian & New Zealand Orthopaedic Research Society) Conference, online.

2019 "*Osteocyte mitophagy contributes to glucocorticoids-induced bone loss*", the 25th annual ANZORS (Australian & New Zealand Orthopaedic Research Society) Conference, Canberra, ACT, Australia.

Poster:

2022 "*Elevated bone-derived sclerostin in plasma is associated with high brain amyloid-β load in cognitively normal older adults*", Poster Session of the first joint meeting of the Australia and New Zealand Bone and Mineral Society (ANZBMS), the Molecular & Experimental Pathology Society of Australasia (MEPSA) and the Australian & New Zealand Orthopaedic Research Society (ANZORS), Gold Coast, QLD, Australia.

AWARDS

Scholarships

2018-2021 International Research Training Program (RTP) Fees Offset Scholarship.

2018-2021 University Postgraduate Award.

2018-2021 UWA Safety-Net Top Up Scholarship.

Travel Awards

2022 The first joint meeting of the Australia and New Zealand Bone and Mineral Society (ANZBMS), the Molecular & Experimental Pathology Society of Australasia (MEPSA) and the Australian & New Zealand Orthopaedic Research Society (ANZORS), AMZBMS Travel Grant.

2019 The 25th annual ANZORS (Australian & New Zealand Orthopaedic Research Society) Conference Travel Grant.

3-Minute Thesis Competition joint second place prize winner

2022 The Perron Institute for Neurological and Translational Science; Nedlands, Australia.

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LIST OF ABBREVIATIONS

Aβ	amyloid beta
AD	Alzheimer's disease
ADHR	autosomal dominant hypophosphatemic rickets
AGEs	advanced glycation end products
APOE	apolipoprotein E
APP	amyloid precursor protein
BBB	blood-brain barrier
BMD	bone mineral density
BMP	bone morphogenetic protein
CCCP	carbonyl cyanide chlorophenylhydrazone
CKD	chronic kidney disease
CNS	central nervous system
CPP	cell penetrating peptide
CSF	cerebrospinal fluid
CTSK	cathepsin K
CTX	carboxyterminal telopeptide of type I collagen
Dex	dexamethasone
DG	hippocampal dentate gyrus
Dkk1	dickkopf-1
DMP1	dentin matrix protein 1
Drp1	dynamin-related protein 1
EBM	extracellular bone matrix
EGF	epidermal growth factor
FBB	18F-florbetaben
FGF	fibroblast growth factor
FGF23	fibroblast growth factor 23
GABA	gamma-aminobutyric acid
GC	glucocorticoid
GC	glucocorticoid
GFAP	glial fibrillary acidic protein
HMGB1	high mobility group box protein 1
HSC	hematopoietic stem cell

IGF	insulin like growth factor
LCN2	lipocalin-2
LRP	low-density lipoprotein receptor-related protein
MCSF	macrophage colony-stimulating factor
MEPE	matrix extracellular phosphoglycoprotein
Mfn2	mitofusin 2
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
MSC	mesenchymal stem cell
NFTs	neurofibrillary tangle
NSC	neural stem cell
OD	optical density
OP	osteoporosis
OPG	osteoprotegerin
PET	positron emission tomography
PGE 2	prostaglandin E2
PHEX	phosphate-regulating neutral endopeptidase
PINK1	PTEN-induced putative kinase 1
PSD95	postsynaptic scaffolding protein 95
PTH	parathyroid hormone
RA	retinoid acid
RANKL	receptor activator of nuclear factor kappa-B ligand
sFRP3	secreted frizzled-related protein 3
SUVR	standard uptake value ratio
SVZ	subventricular zone
TFEB	transcription factor EB
TGF-b	tumor growth factor-beta
TRAP	tartrate-resistant acid phosphatase
WNT	translated products of WNT gene
ΔΨm	mitochondria membrane potential

CHAPTER 1

General Bone and Osteocyte Biology

1.1 Introduction

Bone is a rigid tissue that constitutes part of the skeletal system comprising the central framework of the human body. Bone itself is made up of bone cells namely osteocytes, osteoblasts and osteoclasts and bone matrix including collagen and bone mineral (Buck and Dumanian 2012). Interestingly, contrary to the commonly held view, bone is a highly dynamic organ undergoing constant self-remodeling throughout life (Datta, Ng et al. 2008). However, with ageing, the imbalance in the bone remodeling process leads to bone loss, which is known as osteoporosis. It was previously thought that osteoblasts and osteoclasts were the major cells modulating bone turnover, however, osteocytes, which account for up to 90% of the total bone cells, are now also considered major players in the pathogenesis of bone loss. Importantly, it is now known that bone can also influence the function of other organs to regulate various metabolic processes through the secretion of hormone-like proteins (Guntur and Rosen 2012, Han, You et al. 2018). In this chapter, information about bone biology and metabolism will be discussed, with a particular focus on the role of osteocytes in ageing-related bone loss and on their endocrine-like functions.

1.2 Bone biology and metabolism

1.2.1 Bone anatomy

Bone is a connective tissue that supports skeletal structure and facilitates movement of the human body. Since bones of the body come in a variety of sizes and shapes, multiple systems have been created for bone classification (Buckwalter, Glimcher et al. 1996). According to its structure, bone can be classified as cortical bone and trabecular bone (Figure 1.1) (Buck and Dumanian 2012). Cortical bone is composed of abundant osteons which make bone stiff and rigid. Osteons are cylindrical-like structures, consisting of concentric layers of compact bone tissue surrounded by a central canal called harversian canal, which contains blood vessels. The membrane covering the external surface of cortical bone is called periosteum, which provides

attachments for ligaments, the vascular supply for the bone and the niche for osteoprogenitor cells that are crucial in the initial formation of fracture callus (i.e., connective bridge of a bone fracture). Trabecular bone is composed of spongy cancellous component, which endows it with the feature of maximized surface area for nutrient diffusion and exposure to cytokines and hormones for bone homeostasis.

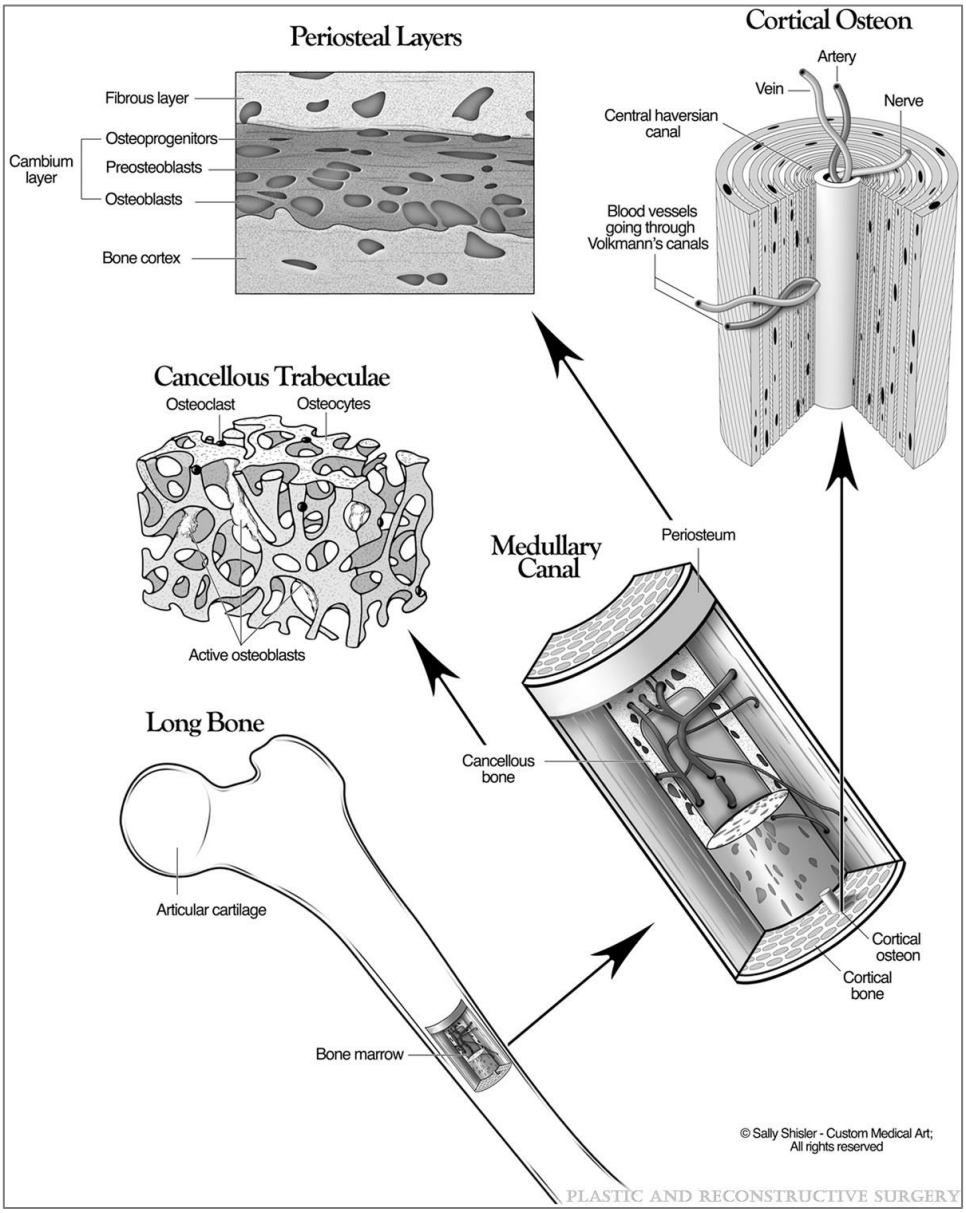

Figure 1.1 General structure of bone. Cortical and cancellous bone are two main structural types of bone. Cortical bone is mainly composited by osteon which contributes to its strength. Cancellous trabeculae are spongy components which functions to maximize surface area for the diffusion of nutrient and crucial circulating

cytokines and hormones. External surface of cortical bone is covered by periosteum. (Adapted from Buck and Dumanian 2012)

1.2.2 Bone blood supply

In addition to the components described above, the normal structure and function of bone cannot be divorced from the blood vessels which are responsible for delivering nutrients and oxygen. Although the blood flow to different organs differs, between 10 to 20 percent of resting cardiac output, which is a similar volume received by the kidney, supplies the skeletal system (Buck and Dumanian 2012). In most situations, the blood flow pattern in bone is primarily centrifugal (Tomlinson and Silva 2013). For example, the main blood supply to healthy long bone comes from the principal nutrient arteries in the marrow cavity. After penetrating into the medulla, nutrient arteries connect to the smaller periosteal arterial supply to enable perfusion of cortical bone (Marenzana and Arnett 2013). However, blood flow patterns can vary significantly in different skeletal sites. For instance, the blood supply in the greater trochanter comes from the arteries entering from the medial, lateral and superior surfaces (Churchill, Brookes et al. 1992).

1.2.3 Bone composition

1.2.3.1 Bone cells

Approximately 10 percent of total bone volume is made up of four cellular elements: osteoclasts, osteoblasts, bone lining cells and osteocytes (Downey and Siegel 2006). The osteoblasts, osteocytes and bone lining cells originate from bone mesenchymal stem cells which are a type of osteoprogenitor cell, whereas osteoclasts originate from hemopoietic stem cells (Buckwalter, Glimcher et al. 1996, Buck and Dumanian 2012). Osteoblasts are cuboidal shaped cells, usually present as tightly packed cells located on the bone surface. As the bone forming cells, osteoblasts synthesize and secret organic bone matrix, which consist of dense collagen layers. Osteoclasts are characterized as large multinucleated phagocytic cells (Walker

1973), which migrate from the bone surface to deep resorption cavities during bone remodeling and healing (Buck and Dumanian 2012). Further details regarding the function of osteoclasts and osteoblasts during bone remodeling will be reviewed in Section 1.2.4 (bone remodeling). Osteocytes are terminally differentiated osteoblasts, represent the most abundant and long-lived cells in bone and play an important role in bone metabolism (Franz-Odendaal, Hall et al. 2006). Additional information regarding osteocytes structure, location, development, function and novel endocrine-like roles will be introduced in Section 1.3 (osteocyte biology). Bone lining cells cover inactive bone surface where neither bone resorption nor bone formation occurs. They have a flat slightly ovoid nuclei and connect to other bone lining cells and osteocyte processes through gap junctions (Miller, de Saint-Georges et al. 1989).

1.2.3.2 Extracellular bone matrix

About 90 percent of overall bone volume is made up by extracellular bone matrix (EBM), which comprises 40 percent organic and 60 percent inorganic compounds (Lin, Patil et al. 2020). The organic part of bone matrix is primarily synthesized by osteoblasts before mineralization and consists mainly of type I collage (90%) and non-collagenous proteins including proteoglycans, glycoproteins and growth factors (10%). The inorganic part of bone matrix is the main storage site for a variety of mineral substances in the body, such as calcium, phosphorous and magnesium (Buck and Dumanian 2012). The bone matrix also provides bone tissue with integrity and elasticity, and is critical in regulating bone remodeling through dynamically interactions with osteoblast-lineage cells and osteoclasts (Lin, Patil et al. 2020).

1.2.4 Bone metabolism

1.2.4.1 Bone modeling and remodeling

Throughout human life, bone is constantly undergoing a continual cycle of bone growth and resorption, orchestrated by the dynamic relationship between osteoclasts and osteoblasts and regulatory influences from diverse hormonal factors. Bone metabolism varies in different life

stages, generally, longitudinal and radial growth and bone modeling are prominent during childhood and adolescence, whereas bone remodeling is activated in adults (Clarke 2008).

Bone modeling is a process of bone formation or bone resorption occurring on certain bone surfaces, it allows for bone growth and shape changes in response to physiological influences and biomechanical forces (Langdahl, Ferrari et al. 2016). The pattern of bone growth variers with bone types and bone location. Long bones grow in a longitudinal way through endochondral ossification at the physis (Hunziker 1994), whereas radial bones grow in a pattern of appositional growth. During bone modeling, in contrast to bone remodeling, osteoblasts and osteoclasts are not activated in anatomically or temporally coupled fashion (Langdahl, Ferrari et al. 2016). Regulation of bone modeling relies on local forces, in brief, the formation modeling is initiated when the local strain on bone exceeds a certain threshold, whereas the resorptive modeling is stimulated when the strain on bone is low (Allen and Burr 2014).

The bone remodeling process involves continuous removal of old bone and formation of new bone, which enables the maintenance of bone strength and mineral homeostasis (Clarke 2008). The remodeling cycle consists of four sequential phases, namely, activation, resorption, reversal and formation (Clarke 2008, Langdahl, Ferrari et al. 2016). The accomplishment of physiological bone remodeling requires communication among different bone cells, especially the interaction between osteoblasts and osteoclasts (Rucci 2008). Briefly, bone-forming osteoblasts produce the organic components of bone matrix and aid its mineralization (Karsenty, Kronenberg et al. 2009). The bone-degrading osteoclasts dissolve bone mineral and degrade extracellular matrix proteins through an enzymatic mechanism (Teitelbaum 2007). Osteocytes sense mechanical stress and regulate the activity of osteoblasts and osteoclasts through an endocrine-like manner (Bonewald and Johnson 2008). While the function of bone lining cells in remodeling is not fully understood, it is thought that they play a role in coupling

bone resorption and bone formation by physically defining bone remodeling compartments (Andersen, Sondergaard et al. 2009).

1.2.4.2 Regulation of bone remodeling

As aforementioned, osteoblasts and osteoclasts are the two principal bone cells involved in bone remodeling. Therefore, any factors that influence their differentiation and function will compromise bone mass, and lead to skeletal pathologies. To maintain normal bone remodeling processes, various local and systemic mediators are involved.

Four major systematic hormonal regulators have been demonstrated to control osteoclastic bone resorption, namely calcitonin, parathyroid hormone (PTH), calcitriol (also known as 1,25 vitamin D3) and estrogen. The production of calcitonin, PTH, calcitriol is driven by the requirement of maintaining serum calcium levels within precise physiological limits. Calcitonin promotes osteoblast proliferation and suppresses bone resorption through directly inhibiting osteoclast activity (Zaidi, Inzerillo et al. 2002). PTH promotes the proliferation and differentiation of osteoprogenitors into mature osteoblasts via stimulating the synthesis of insulin-like growth factor-1 (IGF-1) (Abraham 1990). Meanwhile, PTH can induce osteoclastogenesis through inducing secretion of receptor activator of nuclear factor kappa-B ligand (RNAKL) and macrophage colony-stimulating factor (MCSF) from mature osteoblasts (Boyce and Xing 2007, Ben-awadh, Delgado-Calle et al. 2014). In addition, PTH also stimulates the production of calcitriol, which in turn facilitates the absorption of calcium in the gut and the kidney. Calcitriol can also trigger osteoblastogenesis through promoting the differentiation of bone mesenchymal stem cells (MSCs) to osteoblasts (Chen, Adhikari et al. 2021). The fourth component, estrogen, mainly acts as a bone-sparing hormone due to its action on receptors expressed by both osteoclasts and osteoblasts. Estrogen can inhibit bone resorption through directly inducing apoptosis of osteoclasts and indirectly by suppressing osteoclast bone resorption via influencing osteoblasts/osteocytes and the RNAKL/RANK/osteoprotegerin

system, which is the central local regulatory system for bone remodeling (Hadjidakis and Androulakis 2006). In addition, there is growing awareness that systematic growth factors such as IGFs, tumor growth factor-beta (TGF-β), fibroblast growth factors (FGFs), epidermal growth factor (EGF), WNT proteins and bone morphogenetic proteins (BMPs) also play significant roles in regulating physiological bone remodeling (Siddiqui and Partridge 2016).

1.3 Osteocyte biology

1.3.1 Morphology of osteocytes and the lacunocanalicular system

The osteocyte is an oblate shaped cell which is approximately 7 μm in depth and 15 μm in length (Sugawara, Kamioka et al. 2005). Each osteocyte has 40-60 dendritic processes (Tanaka-Kamioka, Kamioka et al. 1998). As the most abundant and long-lived cell type in mature bone tissue, osteocytes constitute over 90% of all bone cells (Knothe Tate, Adamson et al. 2004). Osteocytes reside in small chambers known as lacunae, which are contained in the mineralized collagen matrix of bone. The connected processes between osteocytes are contained within channels termed canaliculi, and together with lacunae, make up the lacunar-canalicular network (Crockett, Rogers et al. 2011) (Figure 1.2). The lacunar-canalicular network enables osteocyte to conduct cell-to-cell communication and to coordinate their activity, as well as allowing the diffusion of substances through the bone (Mohamed 2008).

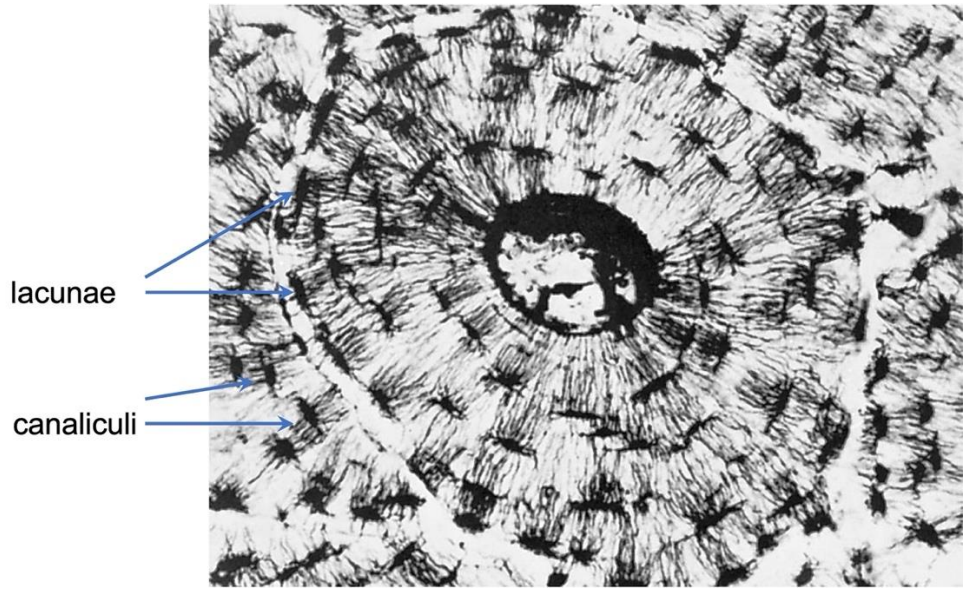

Figure 1.2 Lacunar-canalicular network. This image shows a ground section of bone (Magnified about 125 x). The osteocytes occupy flat lacunae which are connected by the processes contained within the canaliculi. (Adapted and modified from *Britannica, T. Editors of Encyclopaedia (2020, February 28). osteocyte. Encyclopedia Britannica. https://www.britannica.com/science/osteocyte*)

1.3.2 Development of osteocyte

Osteocytes are terminally differentiated osteoblasts. According to Manolagas' theory, the fate of the osteoblast is either to differentiate into an osteocyte or undergo apoptosis (Manolagas 2000). At the end stage of bone formation, osteoblasts that do not undergo apoptosis incorporate into the bone matrix. During their entombment within the bone matrix, osteoblasts experience significant changes in morphology, with over 70% of cell organelles and cytoplasm lost while acquiring the osteocyte specific stellar shape and processes. The newly formed osteocytes connect with other osteocytes, as well as osteoblasts located on the bone surface through their processes (Rochefort, Pallu et al. 2010). Although osteocytes have a long lifespan, the presence of empty lacunae in ageing bone, which are possibly caused by disruption of intercellular gap junctions or cell-matrix interactions, indicate the loss of osteocytes (Xing and Boyce 2005). Osteocyte apoptosis increases during ageing, especially in situations where estrogen levels decrease and when bone loading is reduced (Manolagas and Parfitt 2010).

1.3.3 Function of osteocytes

It is now recognized that osteocytes play an important role not only in bone physiology but also in maintaining the function of other organs (Bonewald 2011, Han, You et al. 2018). The functions of osteocytes within and beyond bone are described below.

1.3.3.1 Osteocytes as mechanosensory cells

The appropriate frequency, intensity and timing of mechanical stress are crucial for bone remodeling and bone architecture (Rubin 1984, Turner, Forwood et al. 1994, Robling, Hinant et al. 2002), and can affect bone metabolism both locally (e.g., professional tennis player have bigger bones in their serving arm) and systemically (e.g., astronauts living in zero gravity and immobilized patients experience bone loss) (Jacobs, Temiyasathit et al. 2010, Crockett, Rogers et al. 2011). The strategic location of osteocytes within the bone matrix together with their

interconnecting network of dendritic processes enable them to detect mechanical stress. Osteocytes convert stress signals originated from bone bending or stretching into signals that affect the biological activity of bone. External forces on bone changes the flow of canalicular fluid and stimulates various responses in osteocytes, while the rapid fluxes of bone calcium across filopodia gap junctions are believed to induce biological information transmission between osteoblasts on the bone surface and osteocytes within bone (Jorgensen, Teilmann et al. 2003). Changes in mechanical loading also influence osteocyte gene expression, and thereby alter the production of secondary messenger molecules such as ATP, nitric oxide, Ca^{2+} and prostaglandin E2 (PGE2) that are important for bone physiology (Clarke 2008, Bonewald 2011).

1.3.3.2 Osteocytes and perilacunar remodeling - Osteocytic osteolysis

Osteocyte-bone matrix interactions occur in the lacuna and canalicular wall through where osteocyte process networks interconnect (McNamara, Majeska et al. 2009). These interactions between osteocyte and bone matrix are crucial for the mechanosensitive function of osteocytes, through which, the stress signals induced by bone deformation are generated and amplified (Wang, McNamara et al. 2007). Given that the surface area of the osteocyte lacuna-canalicular system is much larger than the area of the bone surface, even a few angstroms of mineral removal by each osteocyte can cause significant effects on the circulating and systemic levels of ions, such as calcium (Marotti, Ferretti et al. 1995). Integrins, which are transmembrane receptors that facilitate cell to cell and cell to extracellular matrix adhesion, are thought to play a role in osteocyte-bone matrix interactions. Although it is unclear which kind of integrins are involved in this interaction, the roles of β1 and β3 integrins are implicated (McNamara, Majeska et al. 2009, Litzenberger, Kim et al. 2010).

The interaction between osteocytes and bone matrix is also important in regulating perilacunar remodeling and formation of new bone. In 1983, Zambonin Zallone et al observed that at least 20% of the osteocytes were active during bone formation in the egg-laying hen using autoradiography and tetracycline labeling (Zambonin Zallone, Teti et al. 1983). Earlier, Baylink and Wergedal showed the binding of tetracycline within the osteocyte perilacunar matrix (Baylink and Wergedal 1971). These findings suggest that osteocytes have the potential to form new perilacunar matrix. On the other hand, the term "osteocytic osteolysis" which refers to the resorbing effects of osteocytes on lacunae was coined in 1969 (Belanger 1969). The finding that osteocyte lacunae are significantly enlarged in both cortical and trabecular bone during lactation, compared with virgin and postweaned animals, further evidenced the capability of healthy osteocytes in removing and replacing perilacunar matrix (Qing, Ardeshirpour et al. 2012).

It has been demonstrated that the activation of osteocytic osteolysis is associated with the expression of an "osteoclast-like" gene profile, involving genes that are responsible for acidification, degrading and/or dissolving the extracellular matrix and mineral (e.g., tartrate-resistant acid phosphatase, cathepsin K) (Bonewald 2011, Delgado-Calle and Bellido 2022). PTH and its receptor parathyroid hormone receptor 1 (PTH1R) are important mediators for the maintenance of extracellular calcium and phosphate levels in bone. Conditional deletion of PTH1R in osteocytes leads to an increase of osteocyte lacunar size and bone loss in lactating animals (Qing, Ardeshirpour et al. 2012). In contrast, constitutive expression of PTH1R in osteocytes increases bone mass and remodeling. More recently, a study using $Rankl^{-/-}$ mice, which lack functional osteoclasts, revealed that PTH administration still led to the widening of osteocytic lacunae and calcium deposition, independently of osteoclastic activity. Therefore, PTH signalling may play a crucial part in the process of osteocytic osteolysis.

In addition, it is speculated that osteocytic osteolysis can occur in some other pathological situations (Tsourdi, Jahn et al. 2018). Glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis. Previous studies attributed the effects of glucocorticoid usage on bone loss mainly due to its effects on osteoblasts and osteoclasts. However, glucocorticoids are now known to induce changes in osteocytes morphology (Haggerty and Burg 1992) and upregulate the expression of cathepsin K, which is one of the most potent proteases involved in bone resorption through its ability to degrade type I collagen (Jia, Yao et al. 2011). Another example is related to periprosthetic bone loss, which is a complication after joint replacement surgery. Polyethylene particles originating from the wear of joint prostheses, can cause the up-regulation of osteoclastic genes (e.g., RANKL) in the osteocyte-like MLO-Y4 cell line (Atkins, Welldon et al. 2009). However, the exact mechanisms by which the genes above are stimulated and whether the overexpression of these genes in the osteocytes could lead to the osteoclastic-like degradation of bone are still unclear.

1.3.3.3 Osteocyte autophagy and ageing-related bone loss

Autophagy refers to the removal of damaged proteins, organelles as well as intracellular components by lysosomes (Yang and Klionsky 2010). According to the cellular components that are targeted to the lysosome for degradation, autophagy is classified into multiple types (Levine and Kroemer 2008). Macroautophagy (hereafter referred to simply as autophagy), representing the major form of autophagy and involves the engulfment of large portions of the cytoplasm including protein aggregates and organelles by the autophagosome. Given that the major function of autophagy is to degrade cellular components into substrates for energy production and recycle damaged proteins and organelles, suppression of autophagy is associated with the increased production reactive oxygen species (ROS) (Scherz-Shouval and Elazar 2011), which are major contributors to the ageing process (Piotrowska and Bartnik 2014). For this reason, reduced autophagic processes within cells is proposed as an underlying

cause of cellular and organismal ageing (Yen and Klionsky 2008, Salminen and Kaarniranta 2009, Vellai, Takacs-Vellai et al. 2009). Furthermore, autophagy plays an important role in maintaining bone homeostasis. Since osteocytes are long-lived and involved in many aspects of bone remodeling, autophagy in osteocytes is critical for bone health and contributes to skeletal ageing (Jilka and O'Brien 2016). For example, the deletion of an essential gene for autophagy (e.g., Atg7) in osteocytes of young adult mice (6-month-old), mimics the impact of ageing on the skeleton including decreasing cancellous bone volume and cortical thickness and increasing cortical porosity (Onal, Piemontese et al. 2013).

Increasing systemic glucocorticoid levels is a natural change during ageing, and is known to suppress bone formation, increase bone resorption and cause the apoptotic cell death of osteoblasts and osteocytes (Weinstein 2012). Moreover, glucocorticoids have been shown to trigger autophagy in osteocytes and osteocyte-like cell lines (Xia, Kar et al. 2010), suggesting a possible protective role of autophagy in glucocorticoid-induced bone loss. However, a study that suppressed autophagy in osteocytes did not appear to inhibit the adverse effects of glucocorticoids in cortical bone (Piemontese, Onal et al. 2015). Together these findings suggest that while glucocorticoids can induce autophagy which can promote new bone formation, other biochemical and cellular signals overriding the beneficial effects of osteocyte autophagy are involved (Jilka and O'Brien 2016). These findings highlight the need to further investigate the role of autophagy and other biological processes in osteocytes in bone during ageing.

1.3.4 Novel functions of osteocytes

1.3.4.1 Paracrine effects of osteocytes within bone

As mentioned in Section 1.2.4.2 (regulation of bone remodeling), osteoblasts and osteoclasts are the major cells in bone that are responsible for bone remodeling. However, it is now evident

that osteocytes are also involved in modulating bone metabolism through coordinating the function of osteoblasts and osteoclasts in paracrine manner.

Osteocyte apoptosis and RANKL

The death of osteocytes usually occurs in pathological conditions associated with a decrease in bone mass such as osteoporosis and osteoarthritis (Weinstein, Nicholas et al. 2000). The apoptotic osteocytes are engulfed by osteoclasts, a process that can aggravate osteoclast bone resorption (Chen, Senda et al. 2015). In addition, factors released by apoptotic osteocytes promote neighboring healthy osteocytes to produce RNAKL, which is a key regulatory protein for osteoclast formation and activation (Kennedy, Herman et al. 2012). RANKL binds to its receptor RANK expressed by osteoclasts, and this interaction is modulated by osteoprotegerin which functions as a decoy receptor (Kobayashi, Udagawa et al. 2009). Loss of osteoprotegerin-producing osteocytes due to cell death and increased RANKL from neighboring viable osteocytes together induce increasing numbers of local osteoclasts. Additionally, apoptotic osteocytes can release high mobility group box protein 1 (HMGB1), which can signal to nearby osteocytes to further increase RANKL levels resulting in an increase in the RANKL/osteoprotegerin ratio to promote osteoclast recruitment (Yang, Shah et al. 2008).

Osteocyte-derived sclerostin and bone formation

Sclerostin is a glycoprotein selectively secreted by mature osteocytes, importantly, it antagonizes Wnt/β-catenin signalling by binding to receptors LRP5/6 (low-density lipoprotein receptor-related protein 5/6) (Li, Zhang et al. 2005). Gene expression of sclerostin is influenced by many factors including mechanical loading, sex hormones, parathyroid hormone and inflammation (Chen, Senda et al. 2015). Elevation of mechanical loading on bone suppresses the production of sclerostin, thereby increasing bone mass and strength. In contrast, lack of

mechanical loading increases sclerostin levels in bone and leads to bone loss (van Oers, van Rietbergen et al. 2011, Moustafa, Sugiyama et al. 2012). In agreement with this, it was observed that expression was significantly increased in immobilized patients (Gaudio, Pennisi et al. 2010) and deleting or inhibiting sclerostin gene expression protected mice from bone loss associated with mechanical unloading (Tian, Jee et al. 2011). The level of sclerostin was observed to be negatively correlated with systemic parathyroid hormone concentrations and free estrogen index in postmenopausal women (Calvi, Sims et al. 2001, Mirza, Padhi et al. 2010). In addition, the inflammatory mediator prostaglandin E2 down-regulates *Sost* expression, whereas TNF-α has an opposite effect (Findlay and Atkins 2011, Genetos, Yellowley et al. 2011).

Wnt/β-catenin signalling has been demonstrated to regulate osteogenic differentiation of mesenchymal stem cells and bone formation (Houschyar, Tapking et al. 2018). Disruption of Wnt signalling causes a variety of pathological conditions including bone healing suppression, autoimmune diseases and malignant degeneration. In line with the inhibiting effects of sclerostin on Wnt signalling during bone formation, sclerostin gene mutation or sclerostin deficiency in humans causes sclerosteosis and Van Buchem disease, disorders characterized by a high bone mass (Dixon, Cull et al. 1982). A similar bone overgrowth disease phenotype also occurs in *Sost* knock-out mice (Li, Ominsky et al. 2008). In contrast, mice that overexpress sclerostin exhibit features of suppressed bone formation, which results in the reduction of bone mass and volume (Rhee, Allen et al. 2011).

In addition, sclerostin has been demonstrated to promote osteoclastic bone resorption through regulating RNAKL production. In an *in vitro* study investigating the impacts of exogenous recombinant human sclerostin on MLO-Y4 cells and the human primary cells at the pre-osteocyte stage, the expression of RANKL mRNA was found to be up-regulated while

expression of osteoprotegerin was down-regulated following sclerostin administration (Wijenayaka, Kogawa et al. 2011). These findings indicate that sclerostin can promote the formation and activity of osteoclasts in a RANKL-dependent manner.

Osteocyte-derived FGF23 and bone mineralization

Fibroblast growth factor (FGF) 23 is a member of the FGF family and is primarily secreted by osteocytes. It was first discovered as the cause of autosomal dominant hypophosphataemic rickets, which is characterized by a low phosphate concentration (Consortium 2000). In patients with elevated FGF23 serum levels such as autosomal dominant hypophosphataemic rickets, X-linked hypophosphataemia, oncogenic osteomalacia, autosomal recessive hypophosphataemia and autosomal recessive hyphosphatemic rickets plasma phosphate concentration are decreased (Staudenmayer, Smith et al. 1976, Francis, Hennig et al. 1995, Consortium 2000). Multiple factors are known to regulate the production and/or function of FGF23, including systemic phosphate levels, calcitriol concentrations and phosphate-regulating genes (Lu and Feng 2011, Guo and Yuan 2015).

The main biological functions of FGF23 are to regulate systemic phosphate homeostasis and vitamin D metabolism (Shimada, Mizutani et al. 2001, Shimada, Kakitani et al. 2004). In addition, it has been demonstrated that FGF23 is involved in inhibiting bone mineralization through both direct and indirect mechanisms. For example, overexpression of human FGF23 in fetal rat calvarian cells significantly inhibited their differentiation and mineralization capabilities (Wang, Yoshiko et al. 2008). It was also observed that FGF23 overexpression enhanced phosphorylation of FGF receptors (FGFRs), and that repressing the effects of excessive FGF23 on matrix de-mineralization was reversed by an inhibitor of FGFR1 tyrosine kinase activity. In agreement with these findings, FGF23 null mice administrated with a phosphate-deficient diet demonstrated persistent bone mineralization defects, which was not

completely reversed by hyperphosphatemia (Stubbs, Liu et al. 2007). Together, the above studies support a direct role of FGF23 in bone modeling/remodeling process independent of systemic phosphate homeostasis.

Osteocyte-derived DMP1

Dentin matrix protein 1 (DMP1) is an extracellular matrix pro-peptide with high levels also present in osteocytes. Like FGF23, one of the main functions of DMP1 is to regulate bone mineralization. Dmp1-null mice display low phosphate and high FGF23 serum levels, which results in the impairment of bone mineralization and an osteomalacic phenotype (Feng, Ward et al. 2006). In addition, DMP1 is found to be a major negative regulator of FGF23, which is evidenced by the fact that deficiency DMP1 mutations in autosomal dominant hypophosphataemic ricket patients demonstrate a feature of FGF23 overproduction by osteocytes, leading to osteomalacia (Liu, Zhou et al. 2008, Martin, Liu et al. 2011). Moreover, with respect to bone modelling, DMP1 can nucleate the formation of hydroxyapatite through binding to calcium ions, and promote bone mineralization (He, Dahl et al. 2003).

PHEX and MEPE

Phosphate-regulating neutral endopeptidase (PHEX) and matrix extracellular phosphoglycoprotein (MEPE) are two proteins that are predominantly expressed by osteocytes, and are involved in bone mineralization through acting on FGF23. Both DMP1 and FGF23 were found to be overexpressed in PHEX-associated hypophophatemic rickets cases patients. Mice with PHEX gene mutations, also have elevated serum levels of FGF23 (Liu, Tang et al. 2007). However, MEPE increases FGF23 levels indirectly by inhibiting PHEX enzymatic activity (Liu, Rowe et al. 2007).

1.3.4.2 Endocrine actions of osteocytes beyond bone

Besides acting on other bone cells and the extracellular matrix by paracrine mechanisms in bone, osteocytes also have functions outside of the skeletal system. By an endocrine-like mechanisms, osteocytes can secrete soluble factors into the vasculature, which influence the function of cells in tissue beyond bone (Agoro, Ni et al. 2020). FGF23 and sclerostin are two hormone-like proteins that are predominantly produced by osteocytes and have been shown to influence the function of other organs.

FGF23 and systematic phosphate homeostasis

The initial discovery that elevated levels of FGF23 are associated with hypophosphatemia implicated a role for FGF23 in phosphate homeostasis. This was supported by the observation that FGF23-null mice exhibited hyperphosphataemia, hypercalcaemia, high serum calcitriol levels and decreased serum parathyroid hormone levels (Larsson, Yu et al. 2005, Razzaque, Sitara et al. 2006, Sitara, Razzaque et al. 2006). Additional studies in genetically modified mice revealed that the kidney is the major target of FGF23, whereby the protein stimulates phosphaturia through inhibiting the luminal expression of sodium-phosphate cotransporters in proximal tubules (Shimada, Urakawa et al. 2004). FGF23 also suppresses the production of $1,25(OH)_2D$ by inhibiting 25-hydroxyvitamin D-1α-hydroxylase and stimulating 24-hydroxylase (Shimada, Hasegawa et al. 2004). Recent studies indicate that elevated FGF23 is associated with the progression of chronic kidney disease (Wahl and Wolf 2012). Data from a cohort study involving 227 nondiabetic patients with chronic kidney disease suggest that FGF23 can be employed as a biomarker to predict the progression of renal disease in patients with nondiabetic kidney disease (Fliser, Kollerits et al. 2007).

Sclerostin-related disorders

Wnt signalling is involved in the process of nephrogenesis, renal repair and fibrosis (He, Kang et al. 2011, Vivante, Mark-Danieli et al. 2013). Given the important role of Wnt signalling in

renal function, it is not surprising that sclerostin as a Wnt signalling inhibitor, is associated with chronic kidney disease progression. In mouse models of moderate renal failure, osseous *Sost* expression was upregulated in early chronic kidney disease (Sabbagh, Graciolli et al. 2012). In addition, circulating levels of sclerostin are higher in patients with advanced or end-stage renal disease, compared with individuals with normal renal function. Furthermore, sclerostin levels gradually increase with deterioration in patients with calcifying arteriosclerosis and vascular calcification (Brandenburg, Kramann et al. 2013). In addition to this, sclerostin may also be involved in the development of aortic valve and vascular calcifications, since these patients have elevated serum sclerostin levels compared with healthy individuals. Moreover, up-regulated sclerostin expression was found to parallel prototypic markers of osteogenic trans-differentiation, implying a role of sclerostin in the valvular calcification process (Koos, Brandenburg et al. 2013). In agreement with this, clinical data revealed that increased levels of sclerostin are associated with an increased risk of coronary artery calcification (Kuipers, Miljkovic et al. 2015).

Sclerostin may also have a role in regulating peripheral fat and glucose metabolism. Mice overproducing sclerostin have an increased peripheral fat mass and an impairment of glucose metabolism. In contrast, *Sost* knock-out mice have a reduced peripheral fat mass (Kim, Frey et al. 2017, Kim, Da et al. 2019), as do mice with LRP5 mutations, which blocking the binding of sclerostin to Wnt receptors (Loh, Neville et al. 2015). In addition, abnormal peripheral fat deposition and glucose metabolism in mice can be reversed by sclerostin neutralizing antibodies (Kim, Frey et al. 2017). Clinical studies have also demonstrated increased sclerostin levels in patients with type 2 diabetes, and a positive correlation between serum sclerostin levels and body and fat mass in both healthy and diabetic individuals (Amrein, Amrein et al. 2012, Garcia-Martin, Rozas-Moreno et al. 2012, Urano, Shiraki et al. 2012).

1.4 Summary

In this Chapter, we have introduced the general biology of bone, and in particular, highlighted the role of osteocytes in bone and their endocrine-like functions on other organs by secreting FGF23 and sclerostin.

Ageing is one of the major factors that influence the metabolism of human body and is also closely associated with the biological changes occurring in osteocytes. Given the functions of osteocytes described in this Chapter, any alteration in the production of hormone-like molecules secreted by osteocytes could be potential candidates contributing the pathogenesis of ageing-related disorders.

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CHAPTER 2

Osteoporosis and Alzheimer's Disease: Potential Crosstalk between Bone and Brain

2.1 Preamble

As highlighted in Chapter 1, bone has recently been found to interact with several other organ systems through the actions of secretory proteins produced from bone-derived cells. Interestingly, the brain is one organ that appears to fall into this interconnected network. Furthermore, the fact that osteoporosis (OP) and Alzheimer's disease (AD) are two common age-related disorders strengthens a possible link between bone and brain with respect to the pathogenesis of these two disorders. In this chapter, we review the latest evidence demonstrating the association between bone health and brain function and the impact of bone-derived cells and bone-derived proteins on the central nervous system, and in doing so shed light on the potential of osteocyte-derived sclerostin to contribute to the development of AD.

2.2 Introduction

Alzheimer's disease (AD) is the most common cause of dementia, constituting more than half of all dementia cases, and predominantly affects individuals older than 65 years of age (Querfurth and LaFerla 2010, Livingston, Sommerlad et al. 2017). Osteoporosis (OP), is a bone degenerative disorder that also occurs mainly in the elder population (Nayak and Greenspan 2018). Due to an ageing human population and increased life expectancy, AD and OP pose a significant social burden worldwide (Rachner, Khosla et al. 2011, Haines 2018). AD ranks as the sixth most common cause of death (Haines 2018), while the 12-month mortality rate from hip and spine fractures caused by OP is in excess of 20% (Center, Nguyen et al. 1999).

Although AD and OP appear as seemingly two independent diseases, they share several epidemiological features, especially among women over 60 years old (Downey, Young et al. 2017). A meta-analysis study revealed that AD patients are at a higher risk for hip fracture and have a lower hip bone mineral density than healthy controls (Zhao, Shen et al. 2012). Similarly, OP is implicated as a risk factor for AD, as low bone mineral density correlates with both cognitive impairment and its severity in adults aged over 50 years (Kang, Park et al. 2018). Moreover, low bone mineral density and the rate of decreasing bone density were associated with a higher risk of AD (Zhou, Deng et al. 2011). Interestingly, other studies have shown that patients with a high risk of bone fracture also have a high probability of developing AD (Sato, Kanoko et al. 2004, Chen and Lo 2017).

If AD and OP are inter-related diseases, what are the driving mechanisms involved? One mechanism may be a lack of physical activity, and that when one disorder manifests, it decreases physical activity and increases the morbidity associated with the other disorder (Reitz and Mayeux 2014, Solomon, Mangialasche et al. 2014, Otero, Esain et al. 2017, Yuede, Timson et al. 2018). Genetic factors may also be involved. The major genetic risk factor for AD, the

apolipoprotein E 4 allele (APOE 4), also has a crucial role in maintaining bone mass (Raber, Huang et al. 2004, Noguchi, Ebina et al. 2018). Furthermore, the central nervous system (CNS) can regulate bone mass by producing specific neurotransmitters and by processing peripheral hormonal signals that affect bone homeostasis (Rousseaud, Moriceau et al. 2016). This raises the possibility that with the onset of AD, the ability of the brain to modulate bone is compromised and consequently bone mass decreases. Moreover, recently bone has been viewed as an endocrine organ with the potential to influence the function of other organs through a range of secretory proteins such as osteocalcin (Greenhill 2013). The fact that bone has important endocrine-like functions raises the hypothesis as to whether bone can also influence brain function. To elucidate the inter-relationship between bone and brain and address the possibility of a common pathophysiological mechanisms in OP and AD, this chapter focuses on how bone can influence the function of the CNS, and in particular highlight key bone-derived cells and bone secretory proteins that may be involved in both AD and OP. Also discussed, is how bone-brain interactions can be targeted to develop therapeutic strategies for AD.

2.3 AD and OP pathophysiology

2.3.1 AD pathophysiology

AD is characterized by a range of symptoms including memory loss, problems with language, executive dysfunction, disorientation, mood swings, loss of motivation, inability to manage self-care, and other behavioral issues (Burns and Iliffe 2009). Various potential risk factors contribute to AD pathogenesis, including genetics (Tasaki, Gaiteri et al. 2018), depression (Burke, Cadet et al. 2017), female gender (Cavedo, Chiesa et al. 2018), abnormal interactions with metal ions (Rana and Sharma 2019), brain injury (Sundstrom, Nilsson et al. 2007), lack of physical activity (Burke, Cadet et al. 2017) and periodontal pathogens (Cerajewska, Davies et al. 2015). At present there is no effective treatment that can prevent AD, and current

approaches to slow disease progression have only modest effects. As a consequence, most treatments aim to minimize the symptoms of AD (Morley, Farr et al. 2018).

The molecular mechanisms in the pathogenesis of AD have not been fully elucidated. However, senile plaques composed of amyloid-β (Aβ) peptide aggregation, neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau, and neuronal and synaptic loss are together considered the pathologic hallmarks of this disorder (Selkoe 2001, Duara, Barker et al. 2009). While the pathogenic mechanisms causing AD are constantly being questioned, some consider that the abnormal processing of the amyloidogenic amyloid precursor protein (APP), which generates Aβ, is thought to play an important role in deregulating kinases, phosphatases, and several signalling pathways promoting tau phosphorylation, synaptic failure and neurodegeneration (Golde and Miller 2009, Selkoe and Hardy 2016). Neuroinflammation is also recognized in playing a role in accelerating the progression of AD. For example, any cell stress and degeneration that occur as a consequence of the accumulation of Aβ and NFTs, can activate the immune cells of the CNS, namely microglia and astrocytes which produce toxic cytokines and proinflammatory mediators that contribute to further neurodegeneration (Ferreira, Clarke et al. 2014).

2.3.2 OP pathophysiology

The skeleton and musculature together provide vital internal organ protection as well as maintaining stature and weight-bearing locomotion (Han, You et al. 2018). In addition, adult bone is the major source of minerals, including calcium, phosphorus and magnesium, for the body (Feng and McDonald 2011, de Baaij, Hoenderop et al. 2015). In order to maintain the integrity of the skeleton, bone is constantly being remodelled by a process involving homeostatic adjustments to bone architecture and composition (Raggatt and Partridge 2010, Sadowski KJ. Roberts F 2017). Bone remodeling is regulated by three key bone cells:

osteoclasts, osteoblasts and osteocytes (Becker 2006, Sims and Martin 2014). Osteoclasts originate from hematopoietic stem cells (HSCs) and have the unique capability of absorbing bone, and thus are responsible for removing old or damaged bone (Holtrop and King 1977, Ross 2011). Osteoblasts, on the other hand, differentiate from mesenchymal stem cells (MSCs), and are recruited to the osteoclast-excavated sites, where they differentiate into osteocytes, which synthesize new bone tissue (Crockett, Rogers et al. 2011, Florencio-Silva, Sasso et al. 2015). Osteocytes are a predominant cell in bone, comprising 90% of the cells in the skeleton. The resorption and formation of bone occurs throughout life, and is of major importance for maintaining bone strength (Becker 2006). Unfortunately, with increasing age, the ability of osteoblasts to differentiate into osteocytes diminishes, hence the rate of bone formation does not match the rate of bone resorption. The imbalance in bone formation and resorption is a hallmark of OP, which is characterized by low bone mineral density and an increased risk of bone fractures (Harada and Rodan 2003, Coughlan and Dockery 2014).

2.4 Bone-derived modulators in AD

2.4.1 Bone secretory proteins

Besides maintaining the homeostasis of bone, it is well established that osteoclasts, osteoblasts and osteocytes secrete multiple bone-specific molecules that influence the function of other tissues and organs. For instance, fibroblast growth factor 23 (FGF23) is secreted by osteoblasts and osteocytes, and can regulate phosphate metabolism by inhibiting phosphate reabsorption and calcitriol production in the kidney, as well as decrease circulating phosphate levels by reducing parathyroid hormone (PTH) synthesis in the parathyroid gland (Quarles 2012, Rousseaud, Moriceau et al. 2016). Osteocalcin (OCN) is produced by osteoblasts and can regulate various metabolic and bodily functions including energy expenditure, glucose homeostasis and male fertility, by acting on adipose cells, liver, striated muscle, pancreas and testis (Lee, Sowa et al. 2007, Ferron, Wei et al. 2010, Oury, Sumara et al. 2011). Osteoblast-

secreted lipocalin-2 (LCN2) can suppress food intake and regulate glucose homeostasis (Mosialou, Shikhel et al. 2017). In addition, osteocalcin and two other proteins secreted by bone, osteopontin and sclerostin, have been shown to influence CNS function, and possibly play a role in the development of AD (Figure 2a).

2.4.1.1 Osteocalcin

Osteocalcin is a bone-specific protein secreted by osteoblasts (Hauschka, Lian et al. 1975, Price, Otsuka et al. 1976), and for this reason is often used as a biomarker for bone formation (Ducy, Desbois et al. 1996, Ducy 2011). In recent years, osteocalcin has been regarded as a bone-derived hormone that functions in several physiological processes (see above). Among these, its regulation of brain development and function is receiving increasing attention. The effect of osteocalcin in the brain was highlighted by the extreme passivity observed in OCN $^{-/-}$ mice, which could not be explained by other factors (Oury, Khrimian et al. 2013). A subsequent study confirmed the necessity of osteocalcin in mice for brain development and cognition (Oury, Khrimian et al. 2013). It was demonstrated that osteocalcin crossed the blood-brain barrier and enhanced the synthesis of monoamine neurotransmitters, which include serotonin, dopamine and noradrenaline, and inhibited the synthesis of the major inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). It was also demonstrated that osteocalcin binds to neurons in the brainstem, midbrain and hippocampus.

In addition, administration of osteocalcin to OCN $^{-/-}$ mice alleviated anxiety and depression, and improved their learning and memory capabilities (Oury, Khrimian et al. 2013); this landmark study highlighted the importance of a bone-specific protein in brain function. A study by Villeda et al. (2014) demonstrated that the cognitive function of 16-month-old mice improved after repeated intravenous injections of plasma obtained from 3-month-old mice (Villeda, Plambeck et al. 2014). A subsequent study, which reproduced the findings of Villeda

et al. in wild-type but not $OCN^{-/-}$ mice, provided strong evidence that osteocalcin was the critical factor for preventing age-related cognitive decline (Khrimian, Obri et al. 2017). Moreover, the G protein-coupled receptor 158 (GPR158) located on pyramidal neurons of the CA3 region of the hippocampus was identified as a receptor for osteocalcin in the brain (Khrimian, Obri et al. 2017). Given that cognitive function and circulating levels of osteocalcin inversely correlate with age, osteocalcin may be regarded as an anti-gerontic hormone that mitigates age-related cognitive decline (Obri, Khrimian et al. 2018).

2.4.1.2 Osteopontin

Osteopontin is a matricellular glycoprotein with multiple functions (Ardawi, Qari et al. 2011), it is secreted by bone marrow-derived myelomonocytic cells and inversely modulates their migration, inter-cellular communication and immunological responses (Lund, Giachelli et al. 2009, Uede 2011). It is thought that osteopontin plays a crucial role in bone remodeling by anchoring osteoclasts to the mineral matrix of bones, thereby enhancing the bone resorption process (Reinholt, Hultenby et al. 1990). In postmenopausal women, high serum osteopontin levels have been associated with low bone mineral density (Cho, Cho et al. 2013, Fodor, Bondor et al. 2013).

On the other hand, osteopontin expression in the brain is associated with protecting neurons and promoting tissue repair in acute brain injuries including stroke, cerebral ischemia and traumatic brain injury (Brown 2012, Chan, Reeves et al. 2014, Gliem, Krammes et al. 2015). Furthermore, elevated levels of osteopontin have been observed in the cerebrospinal fluid (CSF) and plasma of patients with mild cognitive impairment and AD (Sun, Yin Xs Fau - Guo et al. 2013) with the levels in plasma providing a prognostic marker for AD (Jongbloed, van Dijk et al. 2015). A study exploring the potential role of osteopontin in mediating macrophage clearance of Aβ demonstrated that the expression of osteopontin is upregulated by glatiramer

acetate, an immunomodulatory drug used for the treatment of multiple sclerosis. Furthermore, in an AD mouse model, glatiramer acetate treatment increased plasma levels of osteopontin and reduced Aβ levels in the cerebral cortex and hippocampus (Rentsendorj, Sheyn et al. 2018). Osteopontin is believed to bind CD44, a polymorphic hyaluronate receptor, which can exert anti-apoptotic functions (Lin and Yang-Yen 2001) and this may be another potential mechanism whereby this protein may reduce neuronal loss in AD (Carecchio and Comi 2011).

Therefore, when examining the functions of osteopontin, it appears that levels of this protein increase with age, but there are opposing functions in bone and brain. On the one hand, osteopontin accelerates the progression of bone demineralization, while potentially slows the progression of AD. However, whether bone-derived osteopontin could effectively participate in neuronal protective mechanisms remains to be determined.

2.4.1.3 Sclerostin

Sclerostin is a predominantly osteocyte-specific secreted glycoprotein (Shah, Shoback et al. 2015). Mutations in the sclerostin gene, or in its gene regulatory elements cause bone disorders such as sclerosteosis and Van Buchem disease. Patients with sclerostin mutations exhibit very high bone mass in the appendicular and axial skeleton (Morrell, Brown et al. 2018). Sclerostin was originally characterized as a bone morphogenetic protein antagonist because of its sequence homology to other members of the DAN (differential screening selected gene aberrative in neuroblastoma) family of cysteine knot proteins. However, more recently it has been shown to bind to low-density lipoprotein-receptor-related protein-5 or -6 (LRP5/6) with high affinity to inhibit the Wnt (wingless-type murine-mammary-tumour virus integration site) signalling pathway (Roser-Page, Vikulina et al. 2018). By binding to LRP5/6, sclerostin prevents Wnt ligands from binding to LRP5/6 and its co-receptor, Frizzled. As a result, bone formation decreases and bone resorption increases through the mechanisms outlined above.

Wnt signalling is initiated by Wnt ligands (Wnts) that are encoded by 19 genes and can activate several intracellular signalling pathways, including the canonical Wnt/β-catenin pathway and the non-canonical Wnt pathways. The non-canonical Wnt pathways include the Wnt/planar cell polarity pathway, the Wnt/Ca^{2+} pathway and the β-catenin-independent Wnt/LRP6 pathway, which are related to Wnt/stabilization of protein signalling and Wnt/target of rapamycin signalling (Baron and Kneissel 2013, Nusse and Clevers 2017). Once Wnt ligands bind to the co-receptors Frizzled and LRP5/6, the Wnt-Fz-LRP5/6 complex is dissociated through phosphorylation of LRP5/6, and β-catenin is then be released from the complex and enters the nucleus. In the nucleus β-catenin binds to the transcription factors, DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF), and finally activates downstream target genes (MacDonald, Tamai et al. 2009). The importance of Wnt signalling in many developmental and adult processes, such as gastrulation, axis formation, cell polarity, organ development and maintenance of stem cell pluripotency, has been widely acknowledged (Logan and Nusse 2004).

In the mammalian CNS, Wnt signalling transduction is involved in neural induction and patterning during early embryogenesis. Previous studies (Inestrosa, De Ferrari et al. 2002) have linked altered Wnt signalling to neurodegenerative disorders such as AD. For example, there is evidence that loss of Wnt function is implicated in the pathophysiology of neuronal degeneration in AD, since this pathway regulates the processes of synaptic plasticity and memory (Inestrosa and Varela-Nallar 2014). Moreover, Wnt signalling is impaired in different AD mouse models (Scali, Caraci et al. 2006, Toledo and Inestrosa 2010).

Given the effects of sclerostin on Wnt signalling, it seems that sclerostin might impact on the degeneration of neurons through inhibition of the Wnt pathway, and contribute to the development of AD.

2.4.2 Bone marrow-derived cells and AD

Bone marrow is a soft viscous tissue enclosed by bone (Yin and Li 2006). It is composed of blood vessels and various populations of cells, some of which are multipotent and exhibit potent self-renewal abilities, especially the mesenchymal stem cells (MSCs) (Themistocleous, Chloros et al. 2018). It has been proposed that MSCs can migrate into the CNS and alter the local tissue environment that induce the progression of AD (Figure 2b).

2.4.2.1 Microglia-like cells

Microglia make up approximately 10% of all cells found within the brain, and act as the immune cells of the CNS (Lawson, Perry et al. 1992). They are involved in CNS development as well as assisting in maintaining the steady-state functioning of the brain (Deczkowska, Keren-Shaul et al. 2018). Resident microglia are involved in the 'reactive microgliosis' that occurs in neurodegenerative diseases, during which microglia proliferate and change their morphology and functions (Song and Colonna 2018). Reactive microglia can be found in the periphery of Aβ aggregates in the AD brain, as well as in increased numbers in the brains from people with other degenerative diseases (McGeer, Itagaki et al. 1988, Itagaki, McGeer et al. 1989, Sapp, Kegel et al. 2001). It is proposed that individuals with AD have a reduced natural protective mechanism that enables microglia, together with other cells of the innate immune system, to effectively clear Aβ (Lampron, Gosselin et al. 2011). Moreover, microglia degenerate with ageing resulting in their reduced capacity to phagocytose Aβ aggregates (Streit, Sammons et al. 2004, Njie, Boelen et al. 2012).

Apart from resident microglia, another type of microglia has been identified in the brain that originate from precursors cells from the bone marrow (Soulet and Rivest 2008). To distinguish externally derived brain microglia cells from the resident microglia cells, they are referred to as 'microglia-like cells' (Han, Arlian et al. 2018). In AD, bone marrow-derived microglia-like

cells cross the blood-brain barrier and migrate into the brain in a chemokine-dependent manner (El Khoury and Luster 2008). There is evidence that bone marrow-derived microglia have the ability to prevent the formation or rapidly clear Aβ deposits in AD mouse models. In one study, bone marrow-derived microglia-like cells expressing green fluorescent protein were transplanted into transgenic AD mice. By comparing young and old animals with age-matched wild-type mice, it was observed that the number of engrafted cells was higher in both young and old AD mice compared with wild-type animals. This result suggests that bone marrow-derived cells infiltrate into the brain and contribute to the 'reactive microgliosis' in AD (Malm, Koistinaho et al. 2005). In another study using an AD transgenic mouse model expressing the thymidine kinase protein controlled by the microglia-like specific CD11b promoter, it was established that bone marrow-derived microglia, but not resident microglia were responsible for removing Aβ deposits (Simard, Soulet et al. 2006). The AD mouse studies suggest that in the early stage of neuronal injury, microglia originating from bone marrow migrate into the CNS in an attempt to reduce neurodegeneration. Importantly, these bone marrow-derived microglia may provide potential therapeutic tools for the development of treatments in AD (Lampron, Gosselin et al. 2011).

2.4.2.2 Bone marrow-derived stem cells

Stem cells have the potential to differentiate into multiple cell types. One of the three known accessible sources of autologous adult stem cells in humans is bone marrow. Bone marrow-derived stem cells include hematopoietic stem cells (HSCs), bone marrow mesenchymal stem cells (BM-MSCs), and endothelial progenitor cells (EPCs) (Wu, Wang et al. 2016). Bone marrow-derived stem cells can migrate into the peripheral circulation and enter the injured brain and exert positive homeostatic effects (Borlongan, Glover et al. 2011). For example, hematopoietic stem cells can enter the injured brain and through the secretion of hematopoietic growth factors (HGFS) and cytokines, promote brain repair by increasing neurogenesis,

inhibiting apoptosis and augmenting the migration of bone marrow-derived microglia into affected tissue (Sanchez-Ramos, Song et al. 2008).

Bone marrow mesenchymal stem cells are important components in regulating the bone marrow microenvironment. They give rise to hematopoietic-supporting stromal cells and provide a suitable cellular environment for hematopoietic stem cell development (Wu, Zhang et al. 2018). Additionally, they have the ability of self-renewal and can differentiate into various mesodermal cell types (Infante and Rodriguez 2018). Given their multipotent cellular properties the impact of BM-MSCs in AD mouse models have been investigated. In a recent study, BM-MSCs intravenously injected into AD mice reduced Aβ plaque size and triggered the activation of microglia in the cerebral cortex and hippocampus (Naaldijk, Jager et al. 2017). Another study transplanted BM-MSCs overexpressing vascular endothelial growth factor (VEGF) into AD mice, the cells promoted neovascularization and diminished senile plaques formation in specific hippocampal layers, possibly by reducing toxicity caused by Aβ (Garcia, Ornellas et al. 2014). Moreover, BM-MSCs may be involved in the replenishment of neural lineages and therefore provide the potential to replace neurons, astrocytes and microglia that have degenerated as a result of AD (Wu, Wang et al. 2016).

2.4.2.3 Potential therapeutic application of bone marrow-derived cells in AD

A common view in the pathogenesis of AD centers around the toxicity of Aβ, and therefore reducing Aβ levels and deposition in the brain is regarded as a critical therapeutic target (Grill and Cummings 2010, Godyn, Jonczyk et al. 2016). Given the interactions between bone and brain, exploring novel cell-based treatments for AD by targeting bone-derived cells and proteins released from bone-derived cells that may impact Aβ has also gained attention in recent years.

One such experimental therapeutic strategy explored the use of bone marrow-derived microglia (Cartier, Lewis et al. 2014). The study, transplanted bone marrow-derived microglia into the lateral ventricles of rats after an intra-hippocampal Aβ injection. The transplanted microglia migrated to the site of injected Aβ and increased Aβ clearance from the brain (Takata, Kitamura et al. 2007). An *in vitro* study, using mouse differentiated bone marrow-derived microglia-like cells demonstrated that the cells were capable of effectively phagocytosing Aβ, whereas bone marrow-derived microglia-like cells injected into AD mice, ameliorated cognitive impairment. These finding raise the possibility that bone marrow-derived microglia-like cells could be applied as a cell-based disease-modifying therapy for AD, however it needs to be keep in mind that cell-based therapies present a number of challenges such as quality control and availability of cells, survival of transplanted cells, dosage, neuro-inflammation and tumour formation (Kawanishi, Takata et al. 2018).

Another approach is to use bone marrow-derived stem cells, especially BM-MSCs which have the capacity to differentiate into several neural cell types (Infante and Rodriguez 2018). At present, 10 clinical trials utilizing various delivery routes to administer BM-MSCs have been completed or are in progress. Among the various routes examined, the intracerebroventricular route seems to be the most effective for stimulating the activation of neurogenesis (Park, Lee et al. 2018). Although BM-MSCs have emerged as a promising therapeutic in the treatment of AD, the risks of transplant rejection or transplant-induced toxicity in the CNS are of particular concern (Wu, Wang et al. 2016).

Given the risk factors associated with cell-based therapies, an alternative approach is to use growth-stimulating factors to activate the proliferation of a patient's own hematopoietic stem cells. Granulocyte-colony stimulating factor (G-CSF) is a potent growth factor, which can reduce neurological defects associated with AD in mouse models by mobilizing hematopoietic

stem cells as well as by its direct neurotrophic effects (Tsai, Tsai et al. 2007, Diederich, Sevimli et al. 2009, Shin, Lee et al. 2011). Macrophage-colony stimulating factor (M-CSF), another hematopoietic growth factor, is involved in the proliferation, differentiation and survival of mononuclear phagocytes, other immune cells and bone marrow progenitor cells (Stanley, Berg et al. 1997). In an AD model, mice treated with M-CSF on a weekly basis prior to the appearance of learning and memory deficits, prevented cognitive decline, increased the number of microglia in the cerebral parenchyma, and reduced Aβ brain deposits (Boissonneault, Filali et al. 2009).

2.5 Conclusions and future directions

We have highlighted the crosstalk between bone and brain, which appears to be reliant on the migration of bone marrow-derived cells and the passage of bone-derived cellular secretory proteins into cerebral tissues (Table 2). Importantly, there is evidence that the crosstalk between bone and brain may impact on the development and progression of AD. For example, bone marrow-derived stems cells have the potential to migrate to the brain, differentiate into microglia-like cells and promote Aβ clearance. The bone-derived protein, osteocalcin can affect the production of neurotransmitters and may thus play a role in cognitive function. Likewise, sclerostin may also contribute to AD pathogenesis due to its negative impact on Wnt signalling in the brain. However, the explicit role of sclerostin in the progression of AD is still unknown and additional studies are required to determine if circulating sclerostin is biologically active in the brain.

Taken together, this review indicates that bone is emerging as a novel and important regulator of cerebral function and potential modifier of disease progression in AD. It is possible that bone is a “secondary brain modulating system”, and more specifically, osteocyte-derived sclerostin being an important factor. Sclerostin has the potential to orchestrate a variety of

pathological pathways in the brain, and further attention to these processes need to be investigated, as it may lead to the development of new therapeutic strategies for AD and other dementias.

Figure title and legend section

Figure 2a, b. Regulation of bone on AD.

a. Bone cells influence the progression of AD via secretory proteins including osteocalcin (OCN), osteopontin (OPN), sclerostin (SOST); **b.** Bone marrow-derived cells including stem cells and microglia-like cells can affect AD.

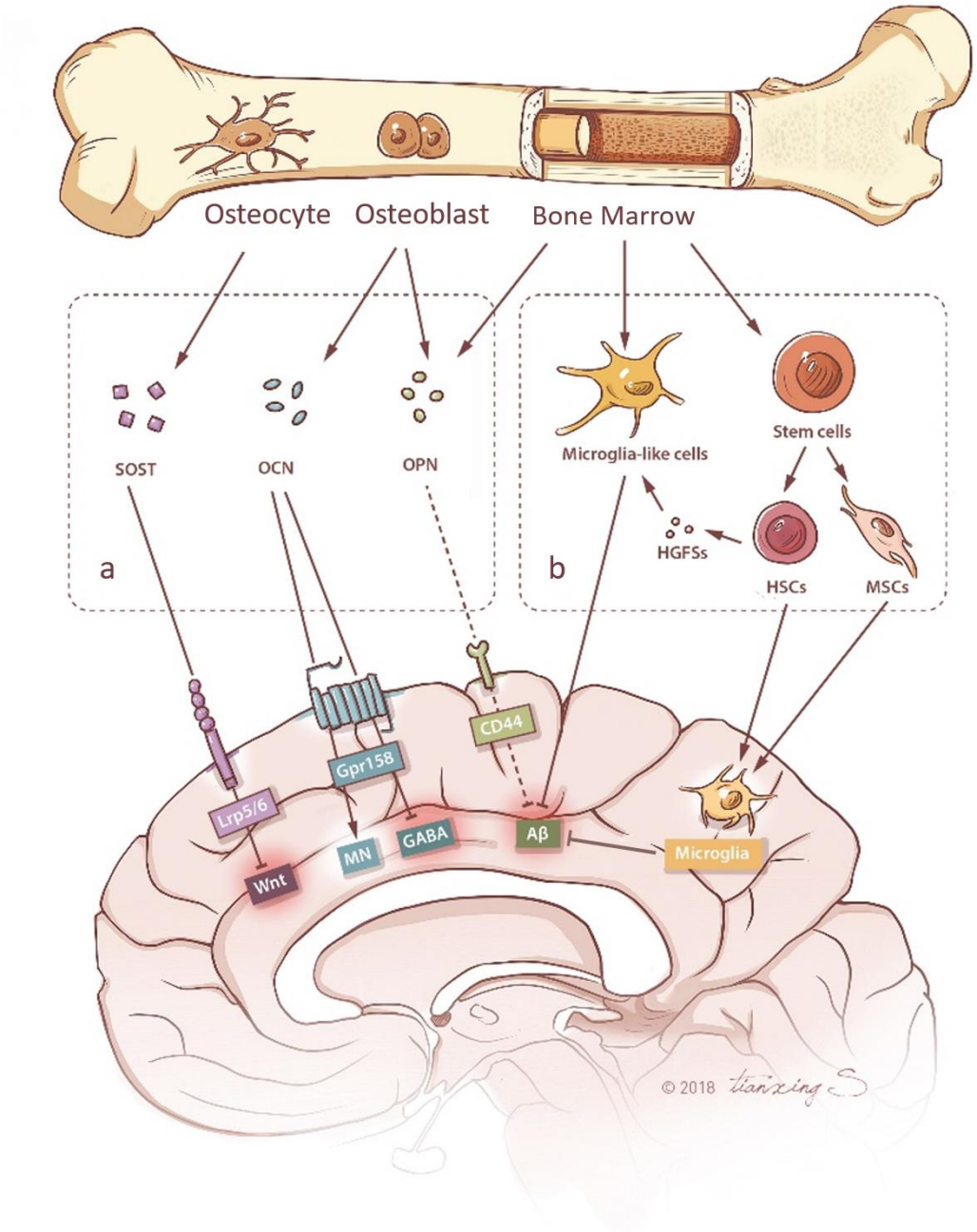

Table title and legend section

Table 2 Bone-derived Modulators on the Progression of Alzheimer's Disease

Types	Regulator	Origin	Potential effects	Reference
Bone secretory proteins	Osteocalcin	Osteoblast	↑monoamine neurotransmitters ↓inhibitory neurotransmitter	(Oury, Khrimian et al. 2013)
	Osteopontin	Osteoblast	↓Aβ	(Rentsendorj, Sheyn et al. 2018)
	Sclerostin	Osteocyte	Aggravate neurons degeneration	(Inestrosa and Varela-Nallar 2014, Roser-Page, Vikulina et al. 2018)
Bone marrow-derived cells	Microglia-like cell	Bone marrow	↓Aβ	(Simard, Soulet et al. 2006, Cartier, Lewis et al. 2014)
	Hematopoietic stem cells		Promoting brain repair	(Sanchez-Ramos, Song et al. 2008)
	Mesenchymal stem cells		Reducing toxicity caused by Aβ	(Garcia, Ornellas et al. 2014)

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CHAPTER 3

Hypothesis and Aims

3.1 Rationale

The conventional recognition of the osteocyte, as a passive cell residing within the lacunae of bone has changed in recent times. It is now accepted that osteocytes are actively involved in various biological processes maintaining the homeostasis of bone. Due to the expanding knowledge of osteocyte function in bone, it is now evident that osteocytes can affect other organs through the secretion of a number hormone-like proteins. For this reason, the osteocyte is considered an endocrine cell. With ageing, osteocytes are exposed to diverse and changing cellular environmental factors that can significantly affect their function and ability to secrete hormone-like proteins. As a consequence, osteocytes have the potential to impact a number of age-related diseases.

Osteoporosis is an age-related disorder that develops gradually over a period of many years. Although a good understanding of the roles of osteoclasts and osteoblasts in osteoporosis have been established, the role of osteocytes in the disease are only beginning to emerge. Elevation in glucocorticoid levels in the body is one change that accompanies ageing, and glucocorticoid-induced bone loss is one of the most common drug-induced adverse events affecting bone. Therefore, investigating the role of osteocytes in glucocorticoid-induced bone loss may provide a mechanism whereby osteocytes contribute to osteoporosis. The concept of 'osteocytic osteolysis' and the finding of cathepsin K, a protease capable of degrading bone matrix (see Section 1.3.3.3), in osteocytes suggests that osteocytes contribute to glucocorticoid-induced bone loss.

Alzheimer's disease (AD) is a degenerative disease affecting older individuals, and notably, has a higher incidence among patients in an osteopenic or osteoporotic state. The clinical association of AD and bone deterioration indicates a possible underlying functional metabolic relationship between brain and bone. Given that osteocytes secrete sclerostin and the ability of

this protein to inhibit Wnt signalling, a crucial pathway in regulating neuronal cell function (see Section 2.4.1.3), it is proposed that osteocytes have the potential to contribute to the development of AD during ageing.

Taken together there is an urgent need to undertake studies to determine the role of osteocyte-derived cathepsin K in the pathogenesis of ageing-related bone loss, and to confirm the association between sclerostin and AD pathogenesis.

3.2 Hypothesis and Aims

This project aims to examine the role of osteocytes in two common and potentially related age-associated diseases, namely osteoporosis and AD.

It is hypothesized that:

1) Osteocyte-derived cathepsin K contributes to glucocorticoid-induced bone loss by degrading type I collagen present in bone extracellular matrix.

2) Osteocyte-derived sclerostin is associated with AD pathological alterations and contributes to the onset of AD.

The specific aims of this project are:

1. To examine the expression of cathepsin K in cultured primary osteocytes following exposure to glucocorticoid.
2. To investigate the effects of osteocyte-derived cathepsin K on extracellular bone matrix.
3. To explore the molecular mechanism by which the production of cathepsin K is regulated in osteocytes under glucocorticoid stress.
4. To determine the relationship between plasma sclerostin concentrations and AD-associated changes in brain amyloid-β load, as determined by PET scanning in older adults.

5. To examine whether sclerostin contributes to early onset AD-relevant neuronal dysfunction by inhibiting neurogenesis and suppressing neuronal dendritic spine formation using an in vitro neuronal differentiation model.

It is anticipated that findings from this research will provide a better understanding of the cellular and endocrine-like function of osteocytes in the age-related and potentially inter-related diseases, osteoporosis and AD.

CHAPTER 4

Osteocytes Participant in Glucocorticoid-induced Bone Loss by Secreting Cathepsin K

4.1 Preamble

Since GC level is elevated with ageing and GC treatment is a major risk factor for bone loss in later life, hereinafter primary osteocytes and osteocyte-like cell lines exposed to GC are employed for relevant experiments. Although sporadic studies have indicated that osteocytes are subject to a series of pathological changes under GC stress, the definitive role of osteocytes in GC-triggered bone loss remains largely unclear. Osteocytic osteolysis is an overlooked bone metabolism, in which osteocytes act to remove perilacunar matrix. In this chapter, we aim to explore the role of osteocyte-derived cathepsin K in the pathogenesis of GC-induced bone loss, which will help to complete the knowledge of osteocytic osteolysis. Furthermore, the molecular mechanism regulating the production of cathepsin K is investigated.

4.2 Introduction

Glucocorticoid (GC), as an anti-inflammatory pharmacological agent, has been widely used for the treatment of a variety of inflammatory diseases, allergic and autoimmune disorders (Overman, Yeh et al. 2013). Despite its therapeutic efficacy, GC administration is often accompanied with bone loss, which is the most common cause of medication-induced osteoporosis (Lane 2019). Previous evidence suggests that GC induces bone loss by means of enhancing osteoclastic bone resorption (Jia, O'Brien et al. 2006) and inhibiting osteoblastic bone formation (Weinstein, Jilka et al. 1998, Weinstein 2012). Even though osteoclasts and osteoblasts are recognized to be responsible for the pathological mechanism of GC-induced bone loss, the role of osteocytes in this pathological change still remains unknown. Osteocytes as the most abundant cells in bone, are embedded in the lacuna-canalicular system. Although overlooked in the past decades, recently, the osteocyte has been recognized as a signaling cell with the ability to communicate with other effector cells in bone/bone marrow through its extensive long cytoplasmic extensions (Delgado-Calle and Bellido 2021). The concept of “osteocytic osteolysis” was firstly suggested in 1962, evidenced by transmission electron

microscopy showing the irregular bordered lacunar walls (Baud 1962). Notably, the finding in GC-treated mice that the size of lacunae enlarged while the elastic modulus around lacunae were reduced, makes “osteocytic osteolysis” an appealing candidate that also accounts for GC-induced bone loss (Lane, Yao et al. 2006).

Cathepsin K (CTSK) is a cysteine protease mainly produced by osteoclasts and has the unique capacity to degrade type I collagen in an acidic environment (Dai, Wu et al. 2020). Type I collagen is the most abundant collagen species contributing to the mechanical scaffold of bone matrix, and degradation of type I collagen has been recognized as the essential event in bone resorption (Garnero, Ferreras et al. 2003). Measurement of the serum carboxyterminal telopeptide of type I collagen (CTX), which is released by cathepsin K cleavage of type I collagen, is employed to assess bone resorption in patients with metabolic bone disease. Studies reporting an increasing concentration of serum CTX both in patients on short-term GC therapy (Dovio, Perazzolo et al. 2004) and ovariectomized sheep exposed to GC administration (Cabrera, Wolber et al. 2018), indicate a crucial role of cathepsin K in GC-induced bone loss. Although osteocytes have been demonstrated to utilize mechanisms similar to those in osteoclasts to remove mineralized matrix by overexpressing tartrate-resistant acid phosphatase (TRAP) and cathepsin K during lactation (Qing, Ardeshirpour et al. 2012), whether osteocyte-derived cathepsin K give rise to type I collagen damage under GC stress has not been investigated.

Emerging evidence suggests that macroautophagy (hereafter referred to as autophagy), which is a recognized delivery process of cytoplasmic cargos to lysosomes for degradation (Mizushima, Levine et al. 2008, Levine and Kroemer 2019), appears to be involved in maintaining bone homeostasis. For instance, autophagy is shown to participate in the differentiation of osteoblasts and osteoclasts, and dysregulation of autophagic activity mediates

the onset and progression of multiple bone diseases (Wang, Niu et al. 2011, Liu, Fang et al. 2013, Nollet, Santucci-Darmanin et al. 2014, Yin, Zhou et al. 2019). The study from Jia *et al.* reported the existence of autophagy and increased production of cathepsin K in osteocytes after GC administration (Jia, Yao et al. 2011), raising the possibility that autophagy activation and osteocytic cathepsin K overproduction could be coupled events. Intriguingly, osteocytes are suggested to respond to stressful conditions including GC treatment by means of autophagy (Xia, Kar et al. 2010, Jia, Yao et al. 2011), suggesting that autophagy in osteocytes could be a new target for preventing GC-induced osteoporosis. However, later research reported that suppression of autophagy in osteocytes does not reverse the negative impact of GC on bone mass (Piemontese, Onal et al. 2015), implying that some other biological processes, besides autophagy, also participate in the pathogenesis of osteocytic osteolysis. Mitophagy is a type of non-canonical autophagy that selectively removes damaged mitochondria *via* an autophagic mechanism (Hirota, Yamashita et al. 2015, Pickles, Vigie et al. 2018). Mitochondria as the so-called "powerhouse" of cells provide essential energy for cell metabolism. GC as a stress factor has been reported to have the potential to interfere with mitochondrial homeostasis and dysfunction of mitochondria has been associated with various diseases (Sanderson, Reynolds et al. 2013, Gorman, Chinnery et al. 2016). A previous study has shown that GC can influence neuronal function and survival by directly regulating mitochondrial functions (Du, Wang et al. 2009). Given the fact that inhibition of autophagy cannot stop GC-induced bone loss (Piemontese, Onal et al. 2015), investigating the role of mitophagy in this pathological process and its relationship with cathepsin K will be of high significance.

Herein, we demonstrated a novel mechanism whereby mitophagy in osteocytes contributes to GC-induced bone loss. Using an *ex vivo* cell culture system, we observed that GC-induced production of cathepsin K in osteocytes caused the degradation of extracellular type I collagen. Meanwhile, we revealed that GC stress led to increasing mitochondrial fission as well as

impaired mitochondria membrane potential (ΔΨm), and subsequent activation of PINK1-mediated mitophagy in osteocytes (Chen, Kroemer et al. 2020, Kobayashi, Zhao et al. 2020). Additionally, we showed that GC-induced mitophagy, rather than canonical autophagy, promoted the production of cathepsin K in osteocytes. Our results therefore indicate a possible role of osteocytes in ageing-related bone loss and elucidate the underlying molecular mechanisms.

4.3 Methods

4.3.1 Animals

C57BL/6 mice were maintained in-house with a 12-h light/dark cycle and normal chow diet. The use of animals was approved by Animal Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (NO. 2019-0312). All animal operative procedures and animal housing were conducted under Permission to Use Animals (PUA).

4.3.2 Cell culture

MLO-Y4 cells were maintained in α-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (P/S; Sigma-Aldrich). For primary osteocyte *ex vivo* culture, fresh calvaria with intact pericranium were extracted from young mice (1-week old). Following triple washing with phosphate buffered solution (PBS), and three washes with α-MEM containing 1% P/S, the pericranium were then gently stripped off from both sides of calvaria through scraping and extensive washing using microsurgical instruments under the dissecting microscope. The calvaria were then directly cultured with complete α-MEM and incubated in a 5% CO_2 incubator at 37 °C. Cells or calvaria pieces were treated with dexamethasone (Dex, D-2915, Sigma-Aldrich) for different time courses (0, 12 and 24 hours) and concentration courses (0, 10^{-8} M and 10^{-6} M).

4.3.3 Cell transfections

For siRNA transfection, cells were transfected with PINK1 siRNA (s206144, ThermoFisher), Atg5 siRNA (MSS247019, ThermoFisher) *or* negative control siRNA (AM4611, ThermoFisher) using Lipofectamine 3000 transfection reagents (L3000008, Invitrogen) following the manufacturer's protocol. In brief, when cells reached an 80% confluency at transfection, Lipofectamine 3000 reagent was diluted in Opti-MEM and mixed well. Master mix of siRNA was prepared by diluting siRNA in Opti-MEM medium (111058021, Gibco) and mixed thoroughly, which was then added to each tube of diluted Lipofectamine 3000 reagent (1:1 ratio) and incubated at room temperature for 15 min. Subsequently, siRNA transfection was performed for 48 h followed with further analysis. Gene knock-down efficiency was evaluated by analyzing corresponding gene and protein levels through real-time PCR and western blotting, respectively.

4.3.4 Western Blotting

Cellular proteins were extracted by incubating in RIPA lysis buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich) for 20 min at 4 °C. Clarified cell lysate (centrifuge at 12,000 g for 20 min at 4 °C) were diluted and boiled at 95 °C with 4 × SDS sampling buffer for 5 min. Proteins were loaded in each lane and fractionated on 10%-17.5% SDS-PAGE gel and resolved protein transferred to nitrocellulose membrane (Millipore). Membranes were then blocked and incubated with primary antibodies including β-actin JLA20 (1:5000, Developmental Studies Hybridoma Bank), VDAC1 (1:1000, ab15895, Abcam), TOM20 (1:500, sc-1774, Santa Cruz Biotechnology), COX IV (1:1000, #4844, Cell Signaling Technology), cathepsin K (1:500, sc-48353, Santa Cruz Biotechnology), PINK1 (1:500, ab23707, Abcam), and the corresponding HRP-conjugated secondary antibodies including HRP-conjugated goat anti-mouse IgG (1:5000, A9917, Sigma-Aldrich), HRP-conjugated goat anti-rabbit IgG (1:5000, A0545, Sigma-Aldrich). Proteins were ultimately visualized by enhanced chemiluminescence reagent (Perkin Elmer) and

autoradiography (ChemiDoc MP Imaging Systems, Bio-Rad). Immunoblotting images presented are representatives of at least three independent experiments.

4.3.5 Immunofluorescence

MLO-Y4 cells seeded on 5 mm or 8 mm coverslips (ProSciTech) were fixed in 4% PFA at room temperature for 20 min, and *ex-vivo* cultured mice calvaria were fixed for 2 h, washed 3 times in PBS and permeabilized with using 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells or mice calvaria were again washed in PBS 3 times and non-specific antibody binding was blocked by 3% BSA-PBS for 30 min at room temperature. Cells were incubated with PINK1 antibody (1:250, ab23707, Abcam) in 0.2% BSA-PBS overnight at 4 °C. Calvaria were incubated with collagen I antibody (1:100, ab34710, Abcam), cathepsin K antibody (1:100, abcam19207, Abcam), Alexa Fluor 647 Phalloidin (1:500, A22287, ThermoFisher), Alexa Fluor 488 Phalloidin (1:500, A12379, ThermoFisher) for 45 min, or Hoechst dye (1:5000; 33342, Perkin Elmer) for 15 min at room temperature. Cells and calvaria were then washed 3 times in PBS and incubated with Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (1:1000; A-21244, ThermoFisher), Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568, (1:1000; A-11011, ThermoFisher), Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:1000; A-11001, ThermoFisher). After 3 times 0.2% BSA-PBS wash and 3 times PBS wash, cells were incubated with Hoechst dye (1:5000; 33342, Perkin Elmer) for 15 min at room temperature. The cells were then rinsed three times in PBS and mounted in ProLong Diamond Anti-fade medium (Invitrogen).

For fluorescent dye staining, cells were placed on 35 mm glass-bottom petri dishes (P35g-1.5-14-C, MatTek), and were transfected with MitoTracker Red CMXRos (M7512,

ThermoFisher), CYTO-ID Autophagy detection kit (ENZ-5131-K200) or OsteoSense (1:1000, NEV10020EX, Perkin Elmer) according to the manufacturer's instructions.

4.3.6 RNA isolation and quantitative real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, 15596-026) and PureLink RNA Mini Kit (Invitrogen, ThermoFisher) following the manufacturer's instructions. RNA (1 mg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed using 2 × SYBR Green Master Mix (Bio-Rad) in a CFX Connect Real-Time PCR detection system (Bio-Rad). Each sample was run in triplicate and gene expression levels were normalized to housekeeping gene *Gapdh*. All measurements were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used for real-time PCR are listed as follows (5'-3'): TGGCCTTCCGTGTTCCTAC (forward), GAGTTGCTGTTGAAGTCGCA (reverse) for *Gapdh*; AGCAACCAGCATGGCTCATA (forward), GGAGACTGTCTTTAATGCTCGC (reverse) for *Pink1*; GGGAGAAAAACCTGAAGC (forward), ATTCTGGGGACTCAGAGG (reverse) for *Ctsk*; GTGATCCCGGCAGACAGAAC (forward), CAACCAAAGCCAAACCGAGG (reverse) for *Atg5*.

4.3.7 Mitochondria morphology quantification

MLO-Y4 cells were incubated with 50 nM MitoTracker Red for 30 min at 37°C in the dark after GC or Carbonyl cyanide chlorophenylhydrazone (CCCP) administration and were fixed to visualize their mitochondrial morphology by using a confocal microscope. Images were captured at the excitation wavelength of 550 nm, 11 image slices were collected through the Z stack encompassing the top and bottom of the cells.

To determine mitochondrial morphology, more than 250 cells were randomly selected from 3 independent experiments for quantitative analysis using the NIH ImageJ software. Mitochondrial morphology were visually scored into one of three classifications: tubular,

where the majority of the mitochondrial network was interconnected and had a reticular appearance; intermediate, where the percentages of the tubular and fragmented network were close; and fragmented, where the majority of the network was highly fragmented.

4.3.8 Mitochondrial membrane potential (△Ψm)

Cells were treated with 10^{-6} M Dex for 24h. Following washes, cells were stained with 33 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, ThermoFisher) in complete α-MEM and incubated for 30 min at 37°C. Cells were then washed with PBS. Fluorescence readings were taken using a CLARIOstar (BMG Labtech) by the emission of 590 nm and 520 nm.

4.3.9 Cell imaging and analysis

A Nikon A1 microscope was employed to acquire the confocal images using the 60X/1.4 immersion objective, and digital images were acquired using NIS Elements Software. All images were assembled and analyzed by ImageJ (NIH).

4.3.10 Statistical Analysis

GraphPad Prism 8 software was used to conduct statistical analyses. To determine the differences between two groups, Student's *t* test was performed. For multiple groups, one-way ANOVA was conducted to analyse group differences for each experiment. Data were presented as the mean ± SD; n.s: non-significant; p values < 0.05 were considered statistically significant, **p*<0.05, ***p*<0.01, ****p*<0.001.

4.4 Results

4.4.1 GC enhances overproduction of CTSK in osteocytes to promote type I collagen degradation

Cathepsin K is the protease responsible for type I collagen degradation. To examine whether osteocytes participate in GC-induced bone loss, we first analyzed the gene expression of *Ctsk*

in the osteocyte-like cell line MLO-Y4. Quantitative PCR results showed a significant increasing cathepsin K gene level in Dex (10^{-6} M) stimulated MLO-Y4 cells (Figure 4.1A). Consistently, analysis of protein level by western blotting also demonstrated a higher production of cathepsin K following Dex (10^{-6} M) administration (Figure 4.1B, C). To further investigate whether osteocyte-derived cathepsin K has the ability to degrade type I collagen, we immunostained cathepsin K and type I collagen in *ex vivo* cultured osteocytes following GC treatment of different concentrations. Interestingly, we observed that osteocytes exposed to high dose Dex (10^{-6} M) contained type I collagen fragments that were found to colocalize with cathepsin K in their cytoplasm. Conversely, such damaged fragments were absent after vehicle or low dose Dex (10^{-8} M) administration (Figure 4.1D and E), implying a digestion process of cathepsin K towards to type I collagen induced by GC stress.

Next, we analyzed the morphological change of extracellular type I collagen matrix surrounding primary osteocyte lacunae *ex vivo*. We labelled matrix collagen with anti-type I collagen antibody and bone mineral with OsteoSense probe for visualizing the edge of lacunae. Confocal imaging showed that in vehicle-treated primary osteocyte group, there is a positive immunostained type I collagen layer surrounding the edge of lacunae (Figure 4.1F). This pattern of collagen layer is very similar to mineralized components, reflecting the typical constitution of bone matrix. However, when the *ex vivo* calvarial bone was treated with Dex (10^{-6} M), there was a distinct change of collagen structure. The collagen layer became a punctate structure along the edge of lacunae, suggesting the disruption of type I collagen by secreted cathepsin K. Quantitative analysis showed that there were increased numbers of type I collagen puncta-like staining in the edge of lacunae when exposed to high dose Dex (Figure 4.1G) indicating an increasing degradation of type I collagen in bone matrix following GC treatment. Collectively, these data suggested that GC induces cathepsin K production in osteocytes, which could be responsible for the degradation of type I collagen within lacunae.

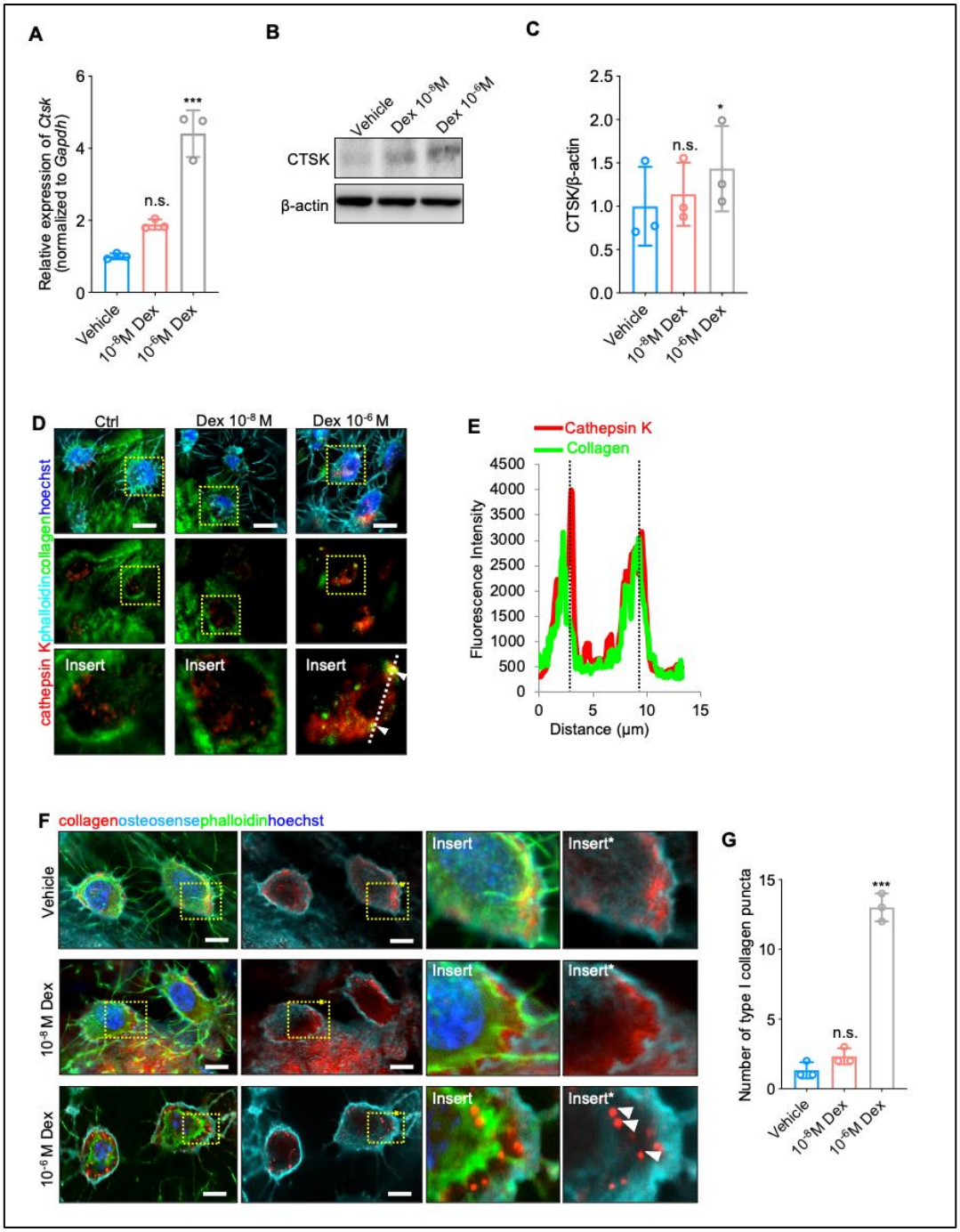

Figure 4.1 GC induces type I collagen degradation by enhancing cathepsin K production in osteocytes. (A) Evaluation of *Ctsk* gene expression in MLO-Y4 treated with vehicle or Dex for 24 hours by RT-qPCR. Relative expression was normalized to *Gapdh*. Data are presented as mean ± SD; n =3, n.s., not significant; ***p<0.001. (B, C) Evaluation of cathepsin K protein (MW = 39 kDa) levels in MLO-Y4 cells treated with vehicle or GC for 24 hours by western blotting. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD, n=3, n.s., not significant, *p<0.05. (D, E) Representative confocal images of *ex vivo* cultured primary osteocytes following 24 hours vehicle or Dex treatment, cathepsin K (red) and type I collagen (green) are stained

with corresponding antibodies (n=3). Intensity profiles were obtained using ImageJ software, along the dashed line. Triangles and dashed lines refer to the typical colocalization points. Scale bar = 10 μm. (F) Representative confocal images of type I collagen puncta (marked by white triangles) detected in *ex vivo* cultured primary osteocytes following 24 hours vehicle or Dex treatment, bone mineral was labelled with OsteoSense (turquoise) (n=3). Scale bar = 5 μm. (G) Quantitative analysis of type I collagen puncta number in primary osteocytes with ImageJ (n =3). Data are presented as mean ± SD; n.s., not significant; ***$p<0.001$.

4.4.2 GC causes increased mitochondrial fission and membrane potential impairment in osteocytes

The balance between mitochondrial fission and fusion is known to be the key for maintaining healthy mitochondrial dynamics and normal mitochondrial function (Chen, Ren et al. 2015). Increasing fission rate is indicated as a universal stress response and is able to induce the depolarization of the mitochondrial membrane potential ($\Delta\Psi m$), which drives mitochondrial ATP synthesis (Twig and Shirihai 2011). In addition, mitochondrial membrane potential is claimed as one of the key mediators to stabilize PINK1 and therefore initiate mitophagy (Jin, Lazarou et al. 2010, Kondapalli, Kazlauskaite et al. 2012). Mitochondrial fission and depolarization are thereby recognized as the prerequisites for clearance of damaged mitochondrial through mitophagy (Giacomello, Pyakurel et al. 2020). To explore the effects of GC on osteocyte mitochondria, we first examined mitochondrial morphology and membrane potential using a osteocyte-like cell line, MLO-Y4 (Kato, Windle et al. 1997). Observations on confocal imaging revealed that the majority of mitochondria in the control group are tubular-like. However, increased mitochondrial fragmentations were observed in Dex treated groups, in a dose-dependent manner, which is similar to the oxidative phosphorylation uncoupler (Carbonyl cyanide chlorophenylhydrazone, CCCP) (Miyazono, Hirashima et al. 2018) treated group (Figure 4.2A). Quantitative analysis showed that MLO-Y4 cells had a larger population of fragmented mitochondria after the addition of Dex (10^{-6} M) with a lower proportion of tubular-like mitochondria (Figure 4.2B). Further, we assessed the proteins involved in shaping mitochondria, including dynamin-related protein 1 (Drp1) and mitofusin 2 (Mfn2). It has been demonstrated that Drp1 is essential for mitochondria fission while Mfn2 plays a crucial role in promoting fusion (Chen, Detmer et al. 2003, Fonseca, Sánchez-Guerrero et al. 2019). The immunoblotting results revealed an increased expression of Drp1, while a decreased expression

of Mfn2 in high dose Dex administration group compared to that in control cells (Figure 4.2C), which further suggested that mitochondrial fission was induced by Dex.

To explore whether GC can cause mitochondrial membrane depolarization, we performed the JC-1 assay to evaluate ΔΨm after Dex (10^{-6} M) treatment. Confocal imaging showed that, compared to the control group, accumulated in mitochondria indicated by a fluorescence emission shift from red to green in GC-treated MLO-Y4 cells (Figure 4.2D). Further, quantification by spectrophotometer verified a significant decreased ratio of red/green fluorescence intensity in GC-treated group, compared to those untreated cells (Figure 4.2E). These data suggested that mitochondrial membrane potential in MLO-Y4 was impaired after GC administration. Together these findings indicate that GC treatment induced mitochondrial fission and mitochondrial membrane depolarization, which both strengthen the possibility of mitophagy induction.

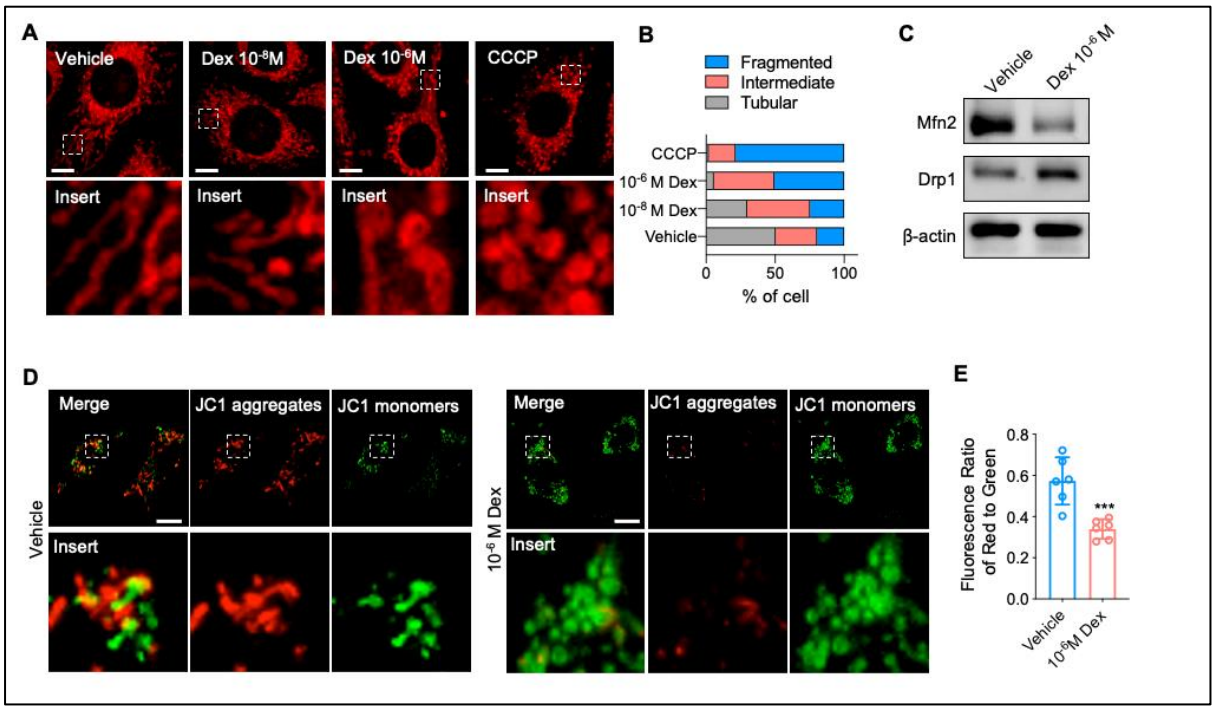

Figure 4.2 GC causes increased mitochondrial fission and membrane potential impairment in osteocytes. (A) Representative images of mitochondria morphology in MLO-Y4 cells stained with MTR (50 nM, 30 min) and visualized by fluorescence microscopy. Cells were exposed to vehicle or Dex (10^{-8} M and 10^{-6} M) for 24 hours, CCCP (20 nM, 24 hours) was used as positive control (n=3). (B) Quantitative scoring of mitochondrial morphology graded as tubular, intermediate and fragmented, with > 250 cells counted from three independent experiments in each group. (C) Western blotting analysis for protein level of Mfn2 and Drp1 in MLO-Y4 cells treated with vehicle or 10^{-6} M Dex for 24 hours. (D, E) Evaluation of mitochondria membrane potential in MLO-Y4 treated vehicle or 10^{-6} M Dex for 24 hours by JC-1 assay. Data are presented as a ratio of 590 nm (red):520 nm (green) fluorescence values. Data are represented as mean ± SD, n=5, ***p<0.001.

4.4.3 GC causes degradation of dysfunctional mitochondria in osteocytes

Given that previous studies have demonstrated the induction of autophagy (Xia, Kar et al. 2010, Jia, Yao et al. 2011), together with our finding of increased mitochondrial fission in GC-treated osteocytes, we then explored whether autophagic degradation was also activated for the removal of accumulated stressed mitochondria. As the engulfment of dysfunctional mitochondria by autophagosome is a central process of mitophagy, we first labelled autophagosomes and mitochondria in MLO-Y4 with CytoID probe and MitoTracker Red (MTR), respectively. CytoID probe is a cationic amphilic trace dye that can incorporate into pre-autophagosomes, autophagosomes, and autolysosomes while minimally stain the lysosomes. Following the stimulation of Dex (10^{-6} M), confocal imaging showed colocalizations between CytoID positive autophagosomes and the MTR positive mitochondria which are fragmented, while such kind of colocalization was absent in control group (Figure 4.3A and B). This indicated that dysfunctional mitochondria are likely to be removed by an autophagic process under GC stress. To further confirm mitochondrial degradation, we analyzed the level of mitochondrial proteins including TOM20, VADC1 and COX IV by western blot assay. Quantification of the protein band intensity showed a decrease of the mitochondrial proteins level in a GC dose-dependent manner (Figure 4.3C and D).

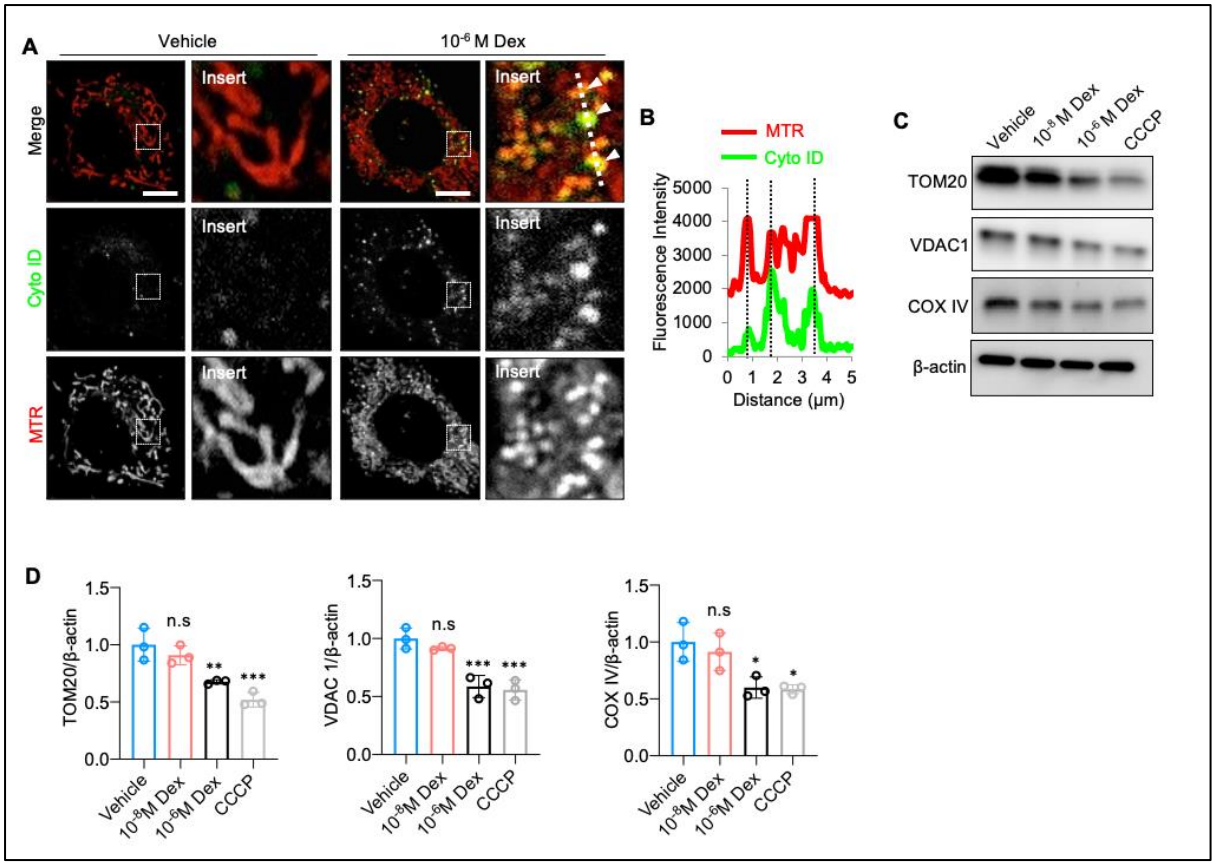

Figure 4.3. GC causes degradation of dysfunctional mitochondria in osteocytes. (A, B) Representative confocal images showed colocalization between autophagosomes and mitochondria labelled with CytoID (green) and MTR (red), respectively. MLO-Y4 cells were treated with vehicle or 10^{-6} M Dex for 24 hours (n=3). Intensity profiles were obtained using ImageJ software, along the white dashed line. White triangles refer to the typical colocalization points. Scale bar = 5 μm. (C, D) Evaluation of mitochondrial proteins including TOM20, VDAC1 and COX IV expression levels in MLO-Y4 cells by western blotting. Cells were exposed to vehicle or Dex (10^{-8} M and 10^{-6} M) for 24 hours, CCCP (20 nM, 24 hours) was used as positive control (n=3). Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD, n=3, n.s., not significant, *p<0.05, **p<0.01, ***p<0.001.

4.4.4 PINK1-mediated mitophagy is responsible for mitochondria degradation in osteocytes

Currently, various proteins are reported to participate in mitochondrial quality control, in particular, PTEN-induced putative kinase 1 (PINK1) plays a central role in maintaining mitochondrial quality. Under naive conditions, PINK1 is imported into mitochondria and rapidly degraded by proteolysis (Geisler, Holmstrom et al. 2010). Nevertheless, in the stressed scenario, stabilized PINK1 proteins are accumulated at the mitochondrial outer membrane (Matsuda, Sato et al. 2010, Narendra, Jin et al. 2010). To examine whether PINK1 is involved in this GC-induced cleavage towards damaged mitochondria, we first stained PINK1 protein with anti-PINK1 antibody and labelled mitochondria with MTR probe. Notably, we observed that more PINK1 puncta accumulated around mitochondria and an increasing fluorescence intensity of PINK1 staining following Dex (10^{-6} M) treatment compared with cells in vehicle-treated group (Figure 4.4A and B). We then analyzed the protein level of PINK1 under high GC stress by western blot, the immunoblotting result showed an increased production of PINK1 protein (Figure 4.4C). To further investigate whether PINK1 positive mitochondria are delivered into autophagosomes for degradation, we analyzed the position association between PINK1 stained protein and Cyto ID positive autophagosomes. As expected, confocal imaging together with fluorescence intensity profile demonstrated an obvious co-localization between PINK1 and CytoID positive autophagosomes during GC administration (Figure 4.4D and E), indicating that increased autophagic degradation towards dysfunctional mitochondria is associated with PINK1. Together, our findings imply that GC induces PINK1-mediated mitophagy in osteocytes.

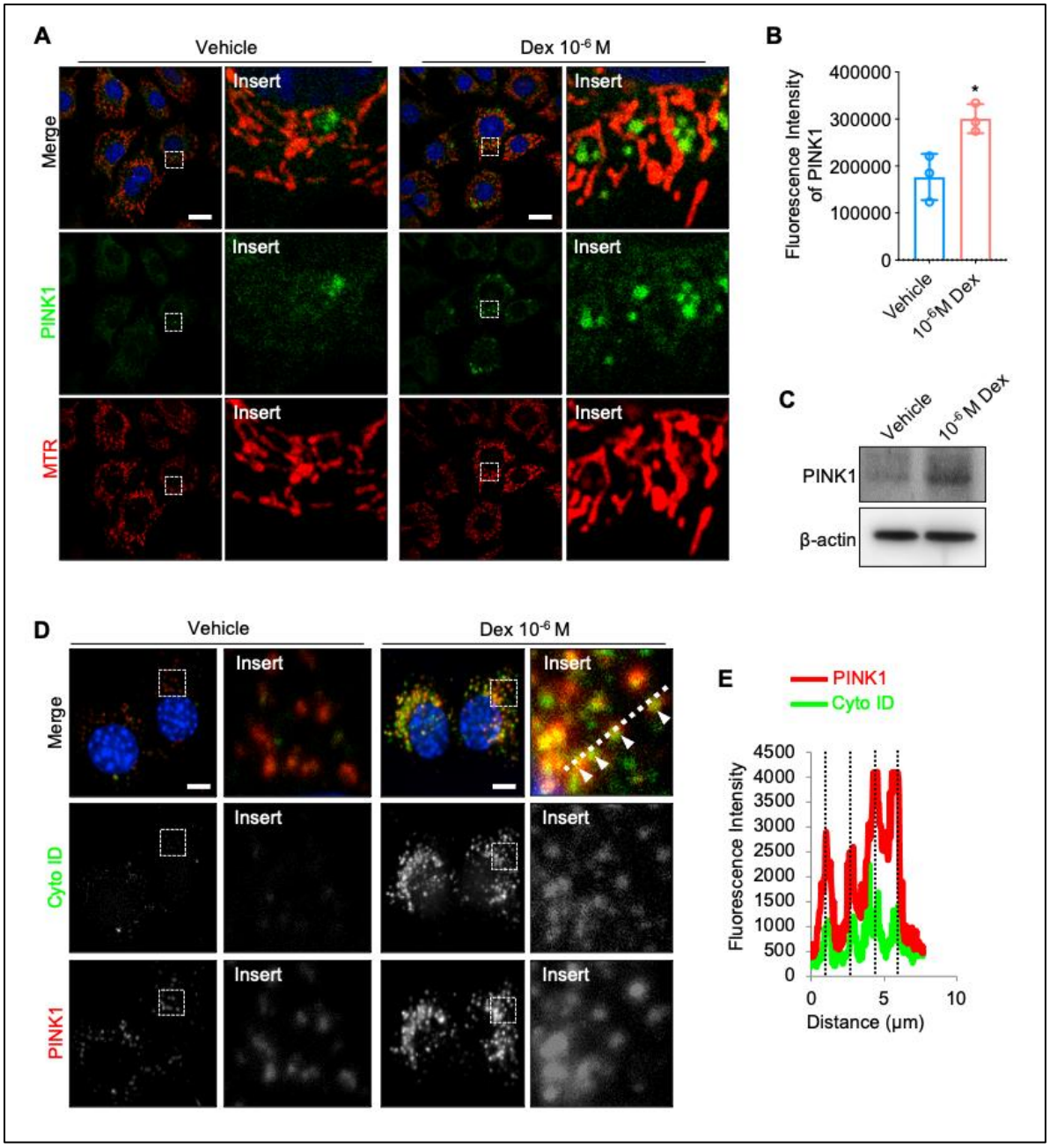

Figure 4.4 PINK1-mediated mitophagy is responsible for mitochondria degradation. (A) Representative confocal images of MLO-Y4 cells after 24 hours treatment with vehicle or 10^{-6} M Dex. Cells were stained for PINK1 (green) and MTR (red) (n=3). Scale bar = 10 μm. (B) Fluorescence intensity of PINK1 (green) was quantified with over 60 cells included in each group. Data are represented as mean ± SD, n=3, *p<0.05. (C) Western blotting analysis of PINK1 protein level in MLO-Y4 cells treated with vehicle or 10^{-6} M Dex for 24 hours. (D, E) Representative confocal images of colocalization between PINK1 (red) and CytoID (green) (n=3). MLO-Y4 cells treated with vehicle or 10^{-6} M Dex for 24 hours. Intensity profiles were obtained using ImageJ software, along the white dashed line. White triangles and white dashed lines refer to the typical colocalization points. Scale bar = 5 μm.

4.4.5 GC-triggered PINK1-mediated mitophagy regulates cathepsin K production in osteocytes

Mitophagy is a selective autophagic degradation, and its activation is accompanied with an increased demand for autophagic and lysosomal proteins, of which the gene expressions are regulated by transcription factor EB (TFEB) (Settembre, Di Malta et al. 2011, Simon, Friis et al. 2017). A study from Catherine et al. demonstrated that the tendency of translocation into the nucleus and transcriptional activity of TFEB are dependent on PINK1 and Parkin (Nezich, Wang et al. 2015). Notably, previous study has identified that cathepsin K is a transcriptional target of the MiT/TFE family (including TFE3, TFEB and TFEC) in osteoclast (Motyckova, Weilbaecher et al. 2001). These lead us to hypothesize that PINK1-mediated mitophagy has the potential to mediate cathepsin K production through promoting TFEB to translocate into nucleus. To verify the translocation of TFEB, we analyzed the protein level of TFEB in both cytoplasmic and nuclear lysates. The immunoblotting results showed an increasing TFEB in cell nuclei following high dose GC administration, indicating that GC induced more TFEB transfer into nucleus in osteocytes (Figure S4.1). To determine whether mitophagy is involved in the effects of GC on osteocyte-derived cathepsin K, we inhibited the activation of mitophagy by knocking down the expression of *Pink1 via* transfecting siRNA (Figure S4.2A and B) and the expression level of cathepsin K was then analyzed. In the scramble group, the induction of cathepsin K occurred at both gene and protein level after GC treatment. On the contrary, a significant decrease of cathepsin K production was observed in the *Pink1* knockdown group, suggesting that PINK1-mediated mitophagy plays a crucial role in the regulation of cathepsin K production in GC-treated osteocyte (Figure 4.5A-C).

Given that GC also induces autophagy in osteocytes, we next verified that mitophagy triggered upregulation of cathepsin K is associated with autophagy. As Atg5 has be characterized as an indispensable gene for canonical autophagy (Mizushima, Yamamoto et al. 2001), and the

elimination of dysfunctional mitochondria can be conducted in an Atg5-independent process (Honda, Arakawa et al. 2014, Ma, Li et al. 2015), we suppressed canonical autophagy by knocking down the expression of *Atg5* (Figure 4.5D, Figure S4.2C, D). Immunoblotting showed the inhibition of canonical autophagy had no influence on cathepsin K production in osteocytes (Figure 4.5E and F). Therefore, it appears that it is GC-induced PINK1-mediated mitophagy that enhances the production of osteocytic cathepsin K, rather than canonical autophagy.

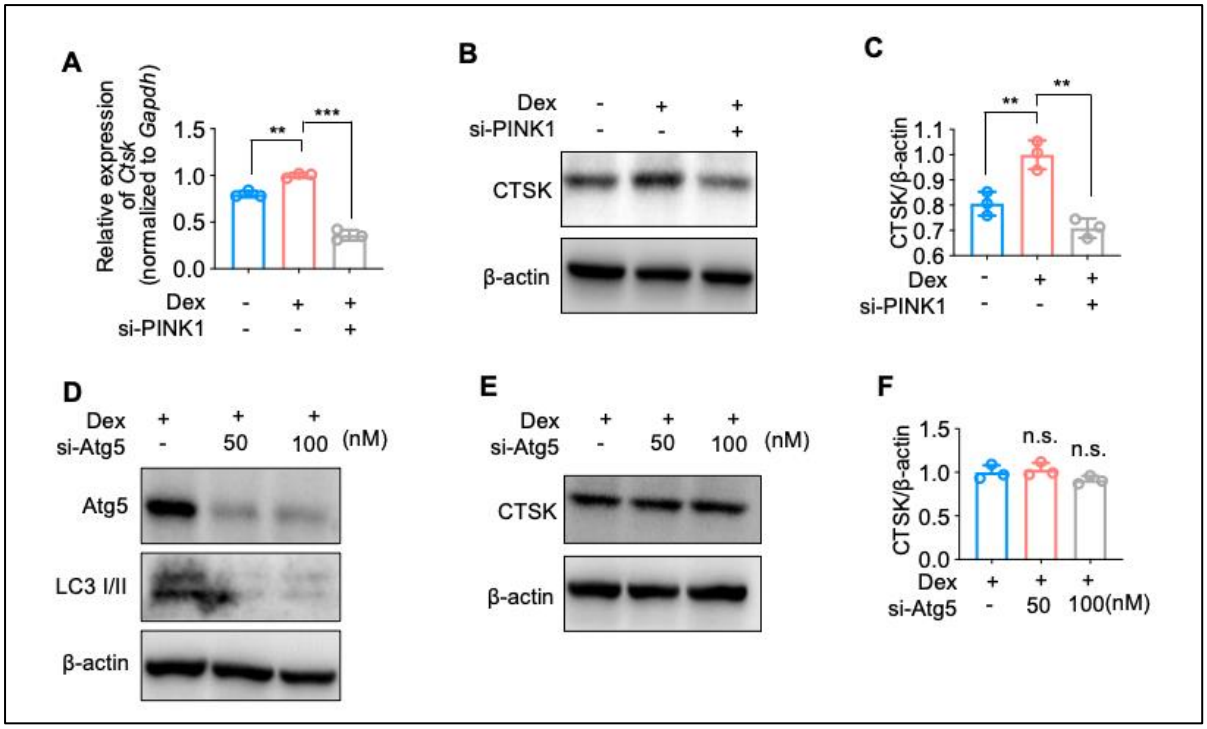

Figure 4.5 GC-triggered PINK1-mediated mitophagy instead of canonical autophagy regulates cathepsin K production in osteocytes. (A) Evaluation of *Ctsk* gene expression in *Pink1* knock-down MLO-Y4 treated with vehicle or 10^{-6} M Dex for 24 hours by RT-qPCR. Relative expression was normalized to *Gapdh*. Data are presented as mean ± SD; n =3, **p<0.01, ***p<0.001. (B, C) Evaluation of cathepsin K protein levels in *Pink1* knock-down MLO-Y4 cells following vehicle or 10^{-6} M Dex treatment for 24 hours by western blotting. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD, n=3, **p<0.01. (D) Evaluation of canonical autophagy in Atg5-knockdown MLO-Y4 cells following 24 hours 10^{-6} M Dex treatment by western blotting assay towards to ATG5 and LC3 protein. (E, F) Evaluation of cathepsin K level in Atg5-knockdown MLO-Y4 cells following 10^{-6} M Dex treatment. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD, n=3, n.s., not significant.

4.5 Discussion

GCs are hormones released by the adrenal glands and play a central role in stress responses and survival. However, prolonged exposure to high GC levels has been found to lead to deleterious consequences, of which, GCs-induced osteoporosis is the notorious one. Although osteocytes are deeply located within mineralized bone matrix within lacunae, the dendritic processes of osteocytes are able to extend through canaliculi to form highly interconnected communication networks between neighboring osteocytes and bone surface cells to orchestrate the bone homeostasis (Sugawara, Kamioka et al. 2005, Bonewald 2007, Bonewald 2007). In the present study, we observed type I collagen degradation as a result of GC-induced cathepsin K overproduction. Meanwhile, we uncovered that GC caused increased mitochondrial fission and mitochondrial membrane depolarization in osteocytes. As a result, mitophagy was activated as a cleavage method to remove the dysfunctional mitochondria which are destined to autophagosomes for degradation. PINK1, as one of the key mitophagy regulators of note, was found to be responsible for the induction of mitophagy in this situation. Importantly, our study has further forged a novel mechanistic association that the lysosome protein cathepsin K, as a downstream target of PINK1, is overproduced and participates in the GC-induced bone loss pathophysiology (Figure 4.6). This is the first demonstration that mitophagy rather than canonical autophagy triggers GC induced osteocytic cathepsin K. This could be a possible explanation of why autophagic inhibition in osteocytes could not attenuate GC induced bone loss (Piemontese, Onal et al. 2015).

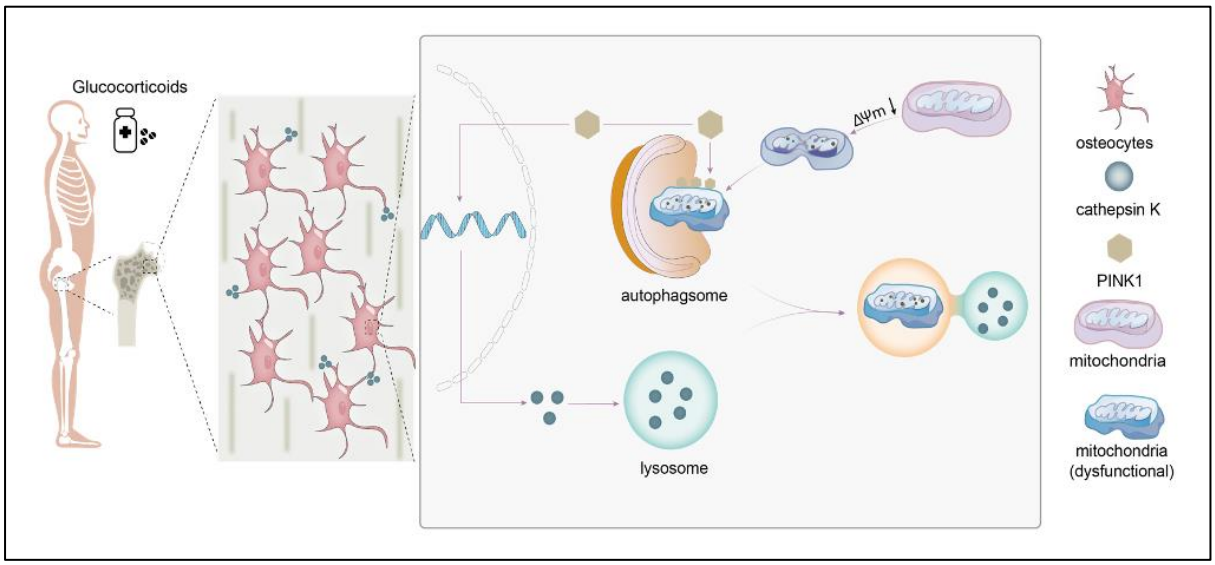

Figure 4.6 Diagram for PINK1-mediated mitophagy in osteocytes contributes to GC induced osteocytic osteolysis. During GC administration, PINK1 accumulate on dysfunctional mitochondria membrane and initiates mitophagy to remove dysfunctional mitochondria. At the same time, the lysosome protein cathepsin K are over produced and causes type I collagen degradation, which contributes to GC-related osteocytic osteolysis.

PINK1-mediated mitophagy is the most common type of mitophagy. Knock-down of *Pink1* abolished GC induced *Ctsk* expression suggests its major role in regulating cathepsin K production. However, there are several other types of mitophagy reported. One is the ubiquitin-independent mitophagy, in which various mitochondrial proteins including NIX/Bnip3, FUNDC1, Bcl2-L-13 and FKBP8 act as receptors while the ubiquitin is not involved (Zhang, Bosch-Marce et al. 2008, Hanna, Quinsay et al. 2012, Ney 2015, Yuan, Zheng et al. 2017). Another one is the ivermectin-induced mitophagy, for which the initiation relies on mitochondrial fragmentation and ubiquitylation via TRAF2/COA[1/CIAP2 (Zachari and Ktistakis 2020). The third uncommon type of mitophagy is one that does not rely on LC3 lipidation but requires mitochondrial fission, ULK1 and Rab9-positive membranes (Saito, Nah et al. 2019). Whether these three types of mitophagy play the same role in osteocytic osteolysis have yet to be explored.

Given this mechanistic model for GC-induced osteocytic osteolysis, a remaining question is whether GC can lead to acidization of the microenvironment surrounding osteocytes which is the prerequisite for the activation of cathepsin K to degrade type I collagen. The cleavage event of cathepsin K requires an acidified microenvironment during osteoclastic osteolysis (Teitelbaum 2000), and V-ATPase complex is a versatile proton pump that is responsible for acidifying the intracellular compartments in eukaryotic cells as well as the extracellular space in some cases (Xu, Cheng et al. 2007, Hinton, Bond et al. 2009). Studies from Katharina et al. revealed that osteocytes can remove calcium in the perilacunar matrix by acidifying their microenvironment through v-ATPase during lactation (Jähn, Kelkar et al. 2017). However, in the situation of GC stress, whether osteocytes or PINK1-mediated mitophagy is able to generate an acidified compartment remains to be determined. Another question is how PINK1-mediated mitophagy regulates cathepsin K secretion. One possibility is that osteocytes secrete cathepsin K via a mechanism similar to the secretory autophagy. Secretory autophagy is an

unconventional protein secretion form that differs from the classical endoplasmic reticulum (ER)-Golgi pathway (Gonzalez, Resnik et al. 2020). Increasing evidence reported that secretory autophagy is relevant to the secretion of a various of cytosolic proteins. In addition, different types of non-canonical autophagy are demonstrated to be involved in pathogen release from infected cells (Gerstenmaier, Pilla et al. 2015) and the protein trafficking into the plasma membrane (Gee, Noh et al. 2011). In this sense, mitophagy as a form of non-canonical autophagy, is likely to take part in the secretion of the lysosome proteins as the secretory autophagy.

In conclusion, our study provides novel evidence that the osteocytic osteolysis responsible for GC-induced bone loss is mediated by mitophagy which challenges the current view of GC-bone loss being mediated by autophagy. Specifically, GC induces the activation PINK1-mediated mitophagy to eliminate damaged mitochondria in osteocytes, which then enhances the production and secretion of cathepsin K, and subsequent degradation of extracellular type I collagen. The mechanism revealed here could also be the one by which osteocytes are involved in ageing-related bone loss. An advanced model representing bone status in ageing and *in vivo* study will be valuable to provide further evidence to reveal the role of osteocytes in osteolytic osteolysis during ageing.

4.6 Supplementary materials

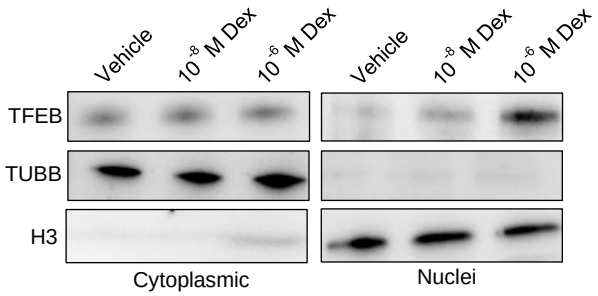

Figure S4.1 Evaluation of TFEB protein level in cytoplasmic and nuclei lysate of MLO-Y4 cells. Western blotting analysis of TFEB protein level in MLO-Y4 cells treated with vehicle or Dex for 24 hours. Histone H3 and TUBB are loading controls for cytoplasmic and nuclei lysate, respectively.

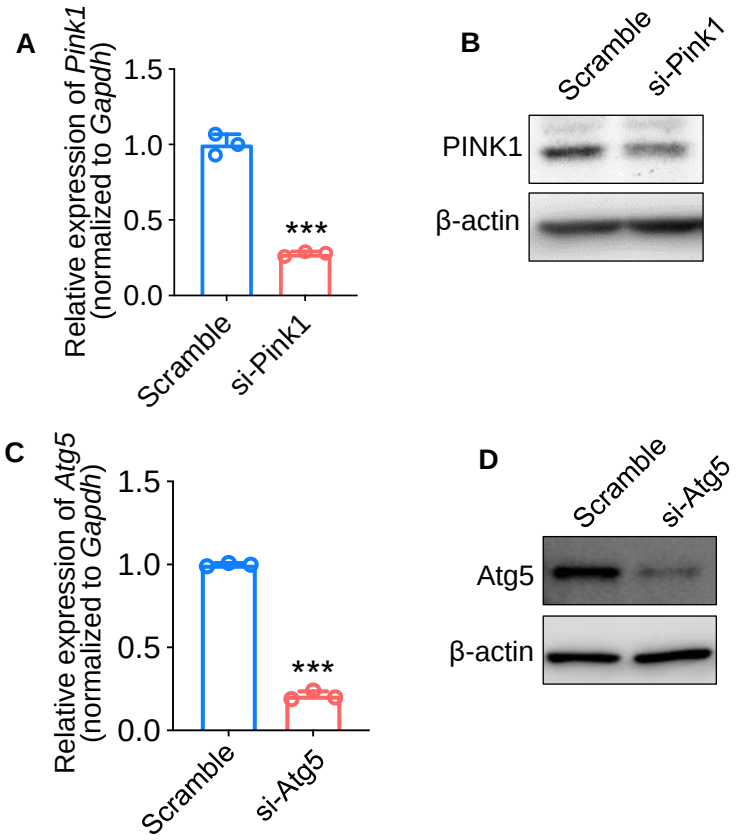

Figure S4.2 Evaluation of *Pink1* and *Atg5* knockdown efficiency by qPCR and western blotting. (A, B) Evaluation of *Pink1* gene and PINK1 protein level by qPCR and western blotting. Relative expression was normalized to house-keeping gene (*Gapdh*) or loading control (β-actin), Data are represented as mean ± SD, n=3, ***p<0.001. (C, D) Evaluation of *Atg5* gene and Atg5 protein level by qPCR and western blotting. Relative expression was normalized house-keeping gene (*Gapdh*) or loading control (β-actin). Data are represented as mean ± SD, n=3, ***p<0.001.

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CHAPTER 5

Elevated Osteocyte-derived Sclerostin in Plasma is Associated with High Brain Amyloid-β Load in Older Adults

5.1 Preamble

The findings in Chapter 4 provide evidence of osteocytes contributing to GC-induced bone loss through its endocrine impacts on nearby tissues *via* secreting cathepsin K. In this chapter, we move forward to explore the possible association between osteocytes and organs beyond bone. As indicated in the literature review in Chapter 2, sclerostin is an osteocyte-derived factor that may link the two common comorbidities, osteoporosis and AD. In this chapter, plasma sclerostin levels in a cohort of older participants were measured and the association of plasma sclerostin with brain amyloid-beta status was analyzed.

5.2 Introduction

Increasing age is a major risk factor for osteoporosis and Alzheimer's disease (AD), two degenerative disorders characterized by low bone mineral density and cognitive dysfunction, respectively. Osteoporosis is the most common bone disease affecting older adults, and it is estimated that 1 in 3 women and 1 in 5 men over the age of 50 will suffer from fractures caused by osteoporosis in their lifetime (Sözen, Özışık et al. 2017). AD, the most common form of dementia, accounts for up to 70% of individuals diagnosed with dementia. It is estimated that in 2021 over 55 million people are living with dementia worldwide, with a predicted increase of nearly 10 million new cases every year (https://www.who.int/news-room/fact-sheets/detail/dementia). Although osteoporosis and AD appear as seemingly two independent disorders, their shared clinical outcomes revealed some associations between each other. For example, the odds ratio for cognitive impairment in osteoporosis patients is significantly higher than in osteopenia patients (Kang, Park et al. 2018). Furthermore, in women, low femoral neck bone mineral density (BMD) is associated with approximately a 2-fold risk of AD (Zhou, Deng et al. 2011) and BMD and lean body mass are significant predictors of impaired episodic memory, indicating the association between BMD and cognitive function (Sohrabi, Bates et al.

2015). Hence, even though osteoporosis and AD affect two different organ systems, epidemiological data suggest an underlying link between bone and brain.

In the past decade, bone has been increasingly recognized as an endocrine organ due to its ability to secrete a range of proteins that can influence the function of other organs. Our review previously shed light on several bone-derived proteins with the potential to be involved in the progression of AD, among which sclerostin is one of the candidates (Yuan, Meloni et al. 2019). Sclerostin is encoded by the *Sost* gene and is mainly produced by osteocytes (van Bezooijen, ten Dijke et al. 2005). It modulates bone formation and bone turnover through inhibiting the Wnt intracellular signaling pathway in osteoblasts and regulating levels of RANKL which acts on osteoclasts (van Bezooijen, Roelen et al. 2004, van Bezooijen, Svensson et al. 2007). Interestingly, circulating sclerostin protein levels increase with increasing age (Modder, Hoey et al. 2011), and inhibition of Wnt signaling may contribute to AD pathogenesis through modulating several aspects of brain development and function, including neurogenesis and synaptic plasticity (McLeod and Salinas 2018, Tapia-Rojas and Inestrosa 2018, Jia, Pina-Crespo et al. 2019). However, a direct involvement of sclerostin in AD progression through inhibition of the Wnt signaling pathway has yet to be established.

A definitive diagnosis of AD can be made at post-mortem and is based primarily on two major pathological hallmarks, extracellular amyloid-β plaques and intraneuronal neurofibrillary tangles comprising the tau protein. Furthermore, despite much effort, there are no treatments that can reverse or cure AD, and it is generally regarded that interventions focussing on preventing or delaying AD onset offer the best chance of a clinically effective therapy. Because many of the pathological processes underlying AD probably begin decades before the first signs of cognitive impairment, there is an increasing need to identify novel biomarkers with the potential to assist in early diagnosis of the disease in ‘at-risk’ individuals. Notably, brain

positron emission tomography (PET) imaging and cerebrospinal fluid (CSF) biomarkers have been used for the detection of Aβ and tau pathology in the brain up to 20 years prior to the symptom onset (Villemagne, Burnham et al. 2013). More recently, specific proteins in blood have also been employed as biomarkers to predict the early onset of AD, such as: Aβ(1-42/1-40), glial fibrillary acidic protein (GFAP), total tau (t-tau), phosphorylated tau (p-tau 181, p-tau 231), and neurofilament light (NfL) (van Oijen, Hofman et al. 2006, Lewczuk, Ermann et al. 2018, Karikari, Pascoal et al. 2020, Verberk, Thijssen et al. 2020, Ashton, Pascoal et al. 2021, Simren, Leuzy et al. 2021). Therefore, given sclerostin levels increase in the blood with age, and that sclerostin can inhibit Wnt signalling, we investigated the possible pathogenic association of sclerostin in AD and the usefulness of plasma sclerostin levels as a predictive biomarker for AD in 'at risk' individuals.

The study consisted of a cohort of cognitively healthy participants divided into Aβ- and Aβ+ groups according to their brain Aβ load based on PET imaging assessments. Plasma sclerostin concentrations were compared between Aβ- and Aβ+ participants before and after stratification by gender and carriage of the apolipoprotein E gene (*APOE*) ε4 allele. In addition, we evaluated the correlation between plasma sclerostin levels and the severity of brain Aβ status reflected by the mean standard uptake value ratio (SUVR). Furthermore, we investigated the potential of plasma sclerostin levels in distinguishing Aβ+ from Aβ- individuals.

5.3 Methods

5.3.1 Study cohort

The study enrolled participants meeting a set of screening inclusion and exclusion criteria from the Kerr Anglican Retirement Village Initiative in Aging Health (KARVIAH) cohort (Goozee, Chatterjee et al. 2018, Chatterjee, Pedrini et al. 2021). Briefly, the inclusion criteria comprised an age range of 65-90 years, good general health, no known significant cerebrovascular disease,

fluent in English, adequate corrected vision and hearing to enable testing, and absence of dementia or pathological cognitive impairment as screened by a Montreal Cognitive Assessment (MoCA) score ≥26. MoCA scores lying between 18-25 were assessed on a case-by-case basis by the study neuropsychologist following stratification of scores according to age and education. The exclusion criteria comprised a previous diagnosis of dementia based on the revised criteria from the National Institute on Aging-Alzheimer's Association, the presence of an acute functional psychiatric disorder (including lifetime history of schizophrenia or bipolar disorder), severe or extremely severe depression (based on the Depression, Anxiety, Stress Scales; DASS), a history of stroke, and uncontrolled hypertension (systolic blood pressure [BP] >170 mm Hg or diastolic BP >100 mm Hg).

In total, 134 volunteers met the inclusion and exclusion criteria; of these, 105 underwent neuroimaging, neuropsychometric evaluation, and blood collection; the remainder declined to undergo neuroimaging or withdrew from the study. Of these 105 participants, 100 were considered to have normal global cognition based on their Mini-Mental State Examination (MMSE; scores can range from 0 to 30, with higher scores indicating better cognitive function) wherein, a cut-off score <26 was employed to screen out individuals with possible early dementia. All volunteers provided written informed consent prior to participation, and the Bellberry and the Macquarie University Human Research Ethics Committees provided approval for the study.

5.3.2 Brain amyloid-β load evaluation via PET

Neuroimaging was conducted within 3 months of blood collection at Macquarie Medical Imaging in Sydney. PET studies were conducted over a 20-minute static scan (4 x 5 minute frames) that was acquired 50 minutes after administration of an intravenous bolus of 18F-florbetaben (FBB) administered slowly over 30 seconds. Brain Aβ load was calculated as the

mean SUVR of the frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions using image processing software, CapAIBL (v2.0). Participants with an FBB-PET SUVR ≥1.35 were considered to have a high brain Aβ load (Aβ+), while those with an FBB-PET SUVR <1.35 were considered to have a low Aβ load (Aβ-).

5.3.3 Blood collection, measurement of plasma sclerostin and APOE genotyping

All study participants fasted for a minimum of 10 h overnight prior to venesection employing standard serological methods and processing. Following blood sample processing, plasma fractions were stored at -80 ˚C until further testing. Plasma sclerostin concentrations were measured using the Human sclerostin ELISA kit (BI-20492, Biomedica, Wien, Austria) according to the manufacturer's instructions. Briefly, assay buffer was added into pre-coated plates followed with addition of plasma samples in duplicate. Samples were then incubated simultaneously with biotinylated antibody overnight (18-24 h) at room temperature (18-24 ˚C) in dark followed by 5x wash steps and incubated with conjugation buffer for 1 hour at room temperature in the dark. The conjugation buffer was then aspirated and washed 5x followed by 30 min incubation with substrate buffer in the dark. At the end of the incubation, stop solution was added into each well and the absorbance were measured immediately at 450 nm. A standard curve was constructed from the optical density (OD) values of the standards. Plasma sclerostin concentrations of the samples were obtained from the standard curve. Apolipoprotein E (*APOE*) genotype was determined from purified genomic DNA extracted from 0.5 mL whole blood. Each sample was genotyped for the presence of the three *APOE* variants (ε2, ε3 and ε4) based on TaqMan SNP genotyping assays for rs7412 (C 904973) and rs429358 (C3084793) as per the manufacturer's instructions (AB Applied Biosystems by Life Technologies, Scoresby, VIC, Australia). Five percent of the samples were genotyped in duplicate and 100% inter- and intra-assay concordance was observed.

5.3.4 Statistical analysis

Student's t tests or Chi-square tests were employed to compare descriptive statistics including means and standard deviations calculated for Aβ- and Aβ+ groups. Continuous variables between Aβ- and Aβ+ groups were compared by employing linear models after correcting for covariates age, sex, and *APOE* ε4 carrier status. To better approximate normality and variance homogeneity, dependent variables were transformed to natural log as required. Correlations between continuous variables were investigated using the Spearman correlation coefficient (r_s). Predictive models and receiver-operating characteristic (ROC) curves constructed from the logistic scores were evaluated using logistic regression with Aβ-/+ as response. All data analyses and visualization were carried out using IBM® SPSS (v27) or GraphPad Prism (v8).

5.4 Results

5.4.1 Cohort characteristics

Brain imaging and blood samples were obtained from 100 participants, with demographic and clinical characteristics summarised in Table 1. No significant difference was observed in terms of age and sex between the Aβ- and Aβ+ groups. As expected, there was a higher ratio of *APOE* ε4 carriage in the Aβ+ group (45.7%) compared with Aβ- group (7.7%; $p < 0.001$). By design, the SUVR values in the Aβ+ group (1.71 ± 0.26) were significantly higher than the Aβ- group (1.16 ± 0.09; $p < 0.001$).

5.4.2 Associations of AD-related risk factors, age, sex and APOE allele status with plasma sclerostin

Plasma sclerostin levels were higher in males (76.66 ± 27.67 pmol/L), compared with females (54.73 ± 18.74 pmol/L; $p < 0.001$; Figure 1A). Plasma sclerostin levels did not differ significantly in *APOE* ε4 non-carriers and *APOE* ε4 carriers (non-carriers = 62.45 ± 25.76 pmol/L; carriers = 59.12 ± 16.98 pmol/L; $p = 0.57$: Figure 1B). Consistent with previous

studies(Modder, Hoey et al. 2011), plasma sclerostin levels were positively correlated with age (r_s = 0.283, p = 0.004; Figure. 1C).

5.4.3 Comparison of plasma sclerostin between Aβ- participants and Aβ+ participants

Plasma sclerostin levels were significantly higher in the Aβ+ group (71.49 ± 25.00 pmol/L) compared with the Aβ- group (56.51 ± 22.14 pmol/L), before and after adjusting for potential AD risk factors including age, sex and *APOE* ε4 status (p < 0.01; Table 2 and Figure 2A). On stratifying study participants by *APOE* ε4 carriage status (ε4 non-carriers: n = 79, and ε4 carriers: n = 21), significantly higher plasma sclerostin levels were observed in the APOE ε4 non-carrier Aβ+ group (n =19, 80.93 ± 27.18 pmol/L) compared with the APOE ε4 non-carrier Aβ- group (n= 60, 55.94 ± 22.63 pmol/L), before and after adjusting for potential risk factors including age and sex (p < 0.001, p* = 0.001; Table 2 and Figure 2B). Within the *APOE* ε4 carriers, no significant difference in plasma sclerostin levels were observed between the Aβ+ group (n = 16, 51.37-69.47 pmol/L) and the Aβ- group (n = 5, 63.42 ±15.06 pmol/L, p = 0.728, p* = 0.368; Table 2 and Figure 2C).

When stratifying study participants by sex (male: n = 32; female: n = 68), there was a trend of higher plasma sclerostin levels in male Aβ+ participants (n= 13, 87.07 ± 24.28 pmol/L) compared with male Aβ- participants (n = 19, 69.55 ± 28.18 pmol/L, p = 0.078, p* = 0.050, Table 2 and Figure 3D). It is likely that an increased sample size of male participants would uncover a statistically significant effect. In females, a significantly higher plasma level of sclerostin was observed in Aβ+ participants (n = 22, 62.28 ± 20.87 pmol/L) compared with Aβ- participants (n = 46, 51.12 ± 16.68 pmol/L), before and after adjusting for potential risk factors including age and sex (p = 0.020, p* = 0.033, Table 2 and Figure 2E).

5.4.4 Evaluation of plasma sclerostin as a predictor of brain Aβ status

Given the higher plasma sclerostin levels in the Aβ+ group compared with the Aβ- group, we investigated the predictive value of plasma sclerostin and brain Aβ status. We first determined the correlation between plasma sclerostin levels and brain SUVR using Spearman's correlation coefficient (r_s). Intriguingly, plasma sclerostin levels were found to have a significant positive correlation with SUVR (r_s = 0. 321, p = 0.001; Supplementary Figure 5.6.1). The predictive accuracy of plasma sclerostin as a potential marker for differentiating Aβ+ from Aβ- participants was evaluated using logistic regression. A 'base model' incorporating the risk factors for AD including age, sex and *APOE* ε4 allele status was generated, and the area under the ROC curve (AUC) calculated. The AUC of the base model was 0.787 (confidence interval [CI] = 0.693 – 0.882, Figure 3A), which outperformed the model using plasma sclerostin levels alone (AUC = 0.695, CI = 0.585 – 0.804, p = 0.205, Figure 3B). However, when combining the base model with the plasma sclerostin model, a higher degree of accuracy (AUC = 0.818, CI = 0.733 – 0.903, p = 0.228, Figure 3C) was obtained compared with the base model alone. In conclusion, the analysis indicates that plasma sclerostin levels when combined with AD risk factors of age, sex and *APOE* ε4 allele status can increase the diagnostic accuracy to detect preclinical AD status.

5.5 Discussion

The present study has demonstrated that plasma levels of the bone-derived protein, sclerostin are elevated with increasing age in adults and positively correlated with brain Aβ load. When combined with the common AD risk factors, namely age, gender and *APOE* ε4 carriage, sclerostin plasma levels increase the accuracy to distinguish Aβ+ from Aβ- individuals. Therefore, the findings of this study have identified plasma sclerostin as an additional novel biomarker to predict the preclinical AD state in older adults who still have normal cognitive function. Importantly, the positive correlation between plasma sclerostin and age also

highlights a possible pathophysiological relationship between osteoporosis and AD. To our knowledge, this is the first study demonstrating the association between a bone-derived protein, and preclinical AD pathological alterations related to Aβ accumulation in the brain.

Sclerostin is a protein primarily secreted by osteocytes and is known to antagonise the canonical Wnt/β-catenin signaling pathway. It has been demonstrated that sclerostin participates in bone turnover, and for this reason has been identified as a therapeutic target for the treatment of osteoporosis (Appelman-Dijkstra and Papapoulos 2016). In addition to the role of sclerostin in bone, the protein can also affect various other organs beyond the skeletal system. For example, blood sclerostin levels correlate inversely with glomerular filtration rate, after correction for age and gender, in patients with chronic kidney disease (Thambiah, Roplekar et al. 2012). In addition, higher sclerostin blood levels have been observed in diabetes mellitus type 2 (DM2) patients compared to healthy individuals (Garcia-Martin, Rozas-Moreno et al. 2012, Gennari, Merlotti et al. 2012), and there is a positive correlation between blood sclerostin levels and insulin resistance (Daniele, Winnier et al. 2015). These findings implicate a crosstalk between bone and other organs mediated by sclerostin.

Senile plaques associated with the accumulation of Aβ in the brain are a key hallmark of AD and considered as an end-stage event caused by pathobiological processes initiating decades before the onset of AD symptoms (Palmqvist, Janelidze et al. 2019). Given that Aβ deposition is a hallmark of AD, abnormal brain Aβ load has been employed as an early diagnostic indicator for preclinical AD (Albert, DeKosky et al. 2011, McKhann, Knopman et al. 2011). In this study, PET imaging using radiotracers with high specificity for Aβ plaques was used to quantify the brain Aβ load in participants (Vlassenko, Benzinger et al. 2012). Currently, PET imaging measuring Aβ deposition using SUVR calculated from selected cerebral cortical areas is being used as a screening tool to detect early Aβ changes in clinical trials examining

therapeutic interventions (Bullich, Roe-Vellve et al. 2021). By employing Aβ brain load status in a cohort of older adults with preserved cognitive function we have demonstrated that participants with a high Aβ load had significantly higher plasma sclerostin levels compared to those with a low Aβ load.

The canonical Wnt/β-catenin signaling pathway plays a crucial role in several aspects of brain function, such as neurogenesis, synaptic plasticity and maintenance of blood-brain barrier integrity (Jia, Pina-Crespo et al. 2019). More recently, Wnt/β-catenin signaling has been implicated in AD pathological mechanisms, including Aβ plaque formation. Specifically, inhibition of the Wnt signaling pathway can promote the amyloidogenic processing of amyloid precursor protein (APP) and thereby lead to an increasing production of the Aβ42 peptide, resulting in a higher Aβ42/Aβ40 ratio and Aβ oligomer levels (Tapia-Rojas, Burgos et al. 2016). While sclerostin can inhibit the Wnt/β-catenin signaling pathway, no study has implicated the protein in promoting amyloidogenic Aβ changes in the brain. If bone-derived sclerostin can have a biological effect in the brain, as suggested by our present findings, an outstanding question which has yet to be addressed relates to its ability to cross the blood-brain barrier (BBB). Interestingly, sclerostin like many cell penetrating peptides (CPPs) is positively charged due to the presence of arginine and lysine residues. For example, the sclerostin protein has an overall net positive charge of +12, whereas the c-terminal half of the protein has a net change of +17. In addition, in silico studies using the BBB penetrating predicting program "B3Pred" (Kumar, Patiyal et al. 2021), identified that in the c-terminal half of the protein, forty-five 30-amino acid length fragments (from a total of 184 fragments for the entire protein) possess properties capable of crossing the BBB (for predicting method and results see Supplementary 5.6.2). Therefore, like other proteins (e.g., the TAT protein) which contain an arginine-rich positively charged region and possess cell penetrating actions and ability to cross the BBB, sclerostin may have similar membrane traversing properties.

This study also investigated the association between blood sclerostin levels and several major AD risk factors by stratifying the participants based on gender and *APOE* ε4 status. We found that in both male and female subgroups, participants with an Aβ+ status had higher blood sclerostin levels compared with participants with an Aβ- status. This indicates that the association between blood sclerostin and brain Aβ load is independent of gender. Interestingly, we also observed high blood levels of sclerostin in Aβ+ individuals who did not carry the *APOE* ε4 allele, whereas there was no difference in *APOE* ε4 carriers. This observation could be attributable to the modest sample size of the *APOE* ε4 carriers, or alternatively, and probably more likely that APOE 4 protein has a greater influence on AD development than sclerostin.

The strengths of this study include the utilization of data from the highly characterized KARVIAH aging cohort which has been reported in previous studies (Goozee, Chatterjee et al. 2018, Chatterjee, Pedrini et al. 2021). However, it is acknowledged that the current study has some limitations, such as the modest sample size and cross-sectional nature of the study design. Further studies are therefore required to validate these observations through longitudinal monitoring of sclerostin levels in an independent cohort. In addition, the comparison of blood sclerostin levels between healthy individuals and those with mild cognitive impairment or established AD would further strengthen the usefulness of sclerostin as an AD biomarker, and for a possible role of the protein in AD progression. Moreover, *in vitro* and *in vivo* studies investigating the mechanisms by which sclerostin may contribute to AD pathogenesis would help to unveil the molecular basis for the findings observed in this study.

In conclusion, this study has demonstrated that blood sclerostin levels are increased in cognitively preserved older adults at high risk of AD. A higher degree of accuracy in

differentiating Aβ- and Aβ+ individuals was achieved when combining sclerostin blood measurements with other major AD risk factors such as age and *APOE* genotype, indicating that sclerostin could be a useful biomarker to assist in the detection of preclinical AD. Importantly, the association between bone-derived sclerostin and brain Aβ load status has drawn further attention to the importance of the 'bone-brain axis' and has identified a possible common link in the pathogenesis of AD and osteoporosis.

Table 1 Cohort demographic characteristics (N=100, Aβ-: n = 65, Aβ+: n = 35).

	Aβ-	Aβ+	*p*
Age (years, mean ± SD)	77.62 ± 5.56	79.23 ± 5.38	0.165
Gender (male/female)	19/46	13/22	0.419
***APOE* ε4 carriers (N (%))**	5 (7.7)	16 (45.7)	**<0.001**
SUVR (mean ± SD)	1.16 ± 0.09	1.71 ± 0.26	**<0.001**

(Continuous data are presented as mean ± SD. Student *t* test or Chi-square tests were employed as appropriate.)

Table 5.1 Cohort demographic characteristics. Baseline characteristics including sex, age, APOE ε4 status, brain Aβ load represented by the stand uptake value ratio (SUVR) of ligand 18F-Florbetaben (FBB) in the neocortical region normalized with that in cerebellum, have been compared between Aβ- (SUVR <1.35, n =65) and Aβ+ (SUVR ≥ 1.35, n =35) participants. Student t test or Chi-square tests were employed as appropriate. Data are presented in mean ± SD, and *p* values in bold font were considered as significant ($p < 0.05$).

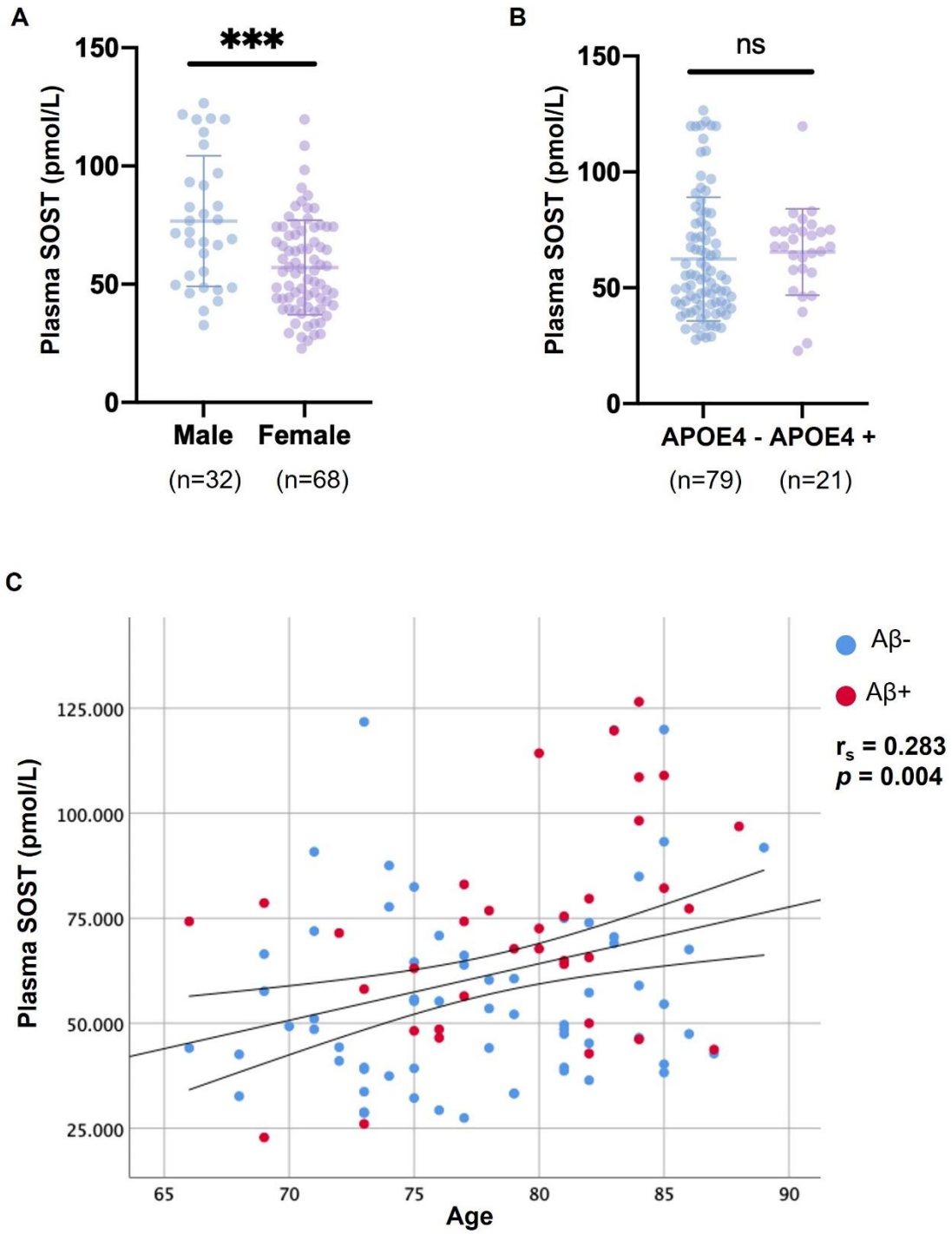


Figure 5.1 Associations of AD-related risk factors, age, sex and APOE allele status with plasma sclerostin. A. Plasma sclerostin levels were compared between male and female participants. B. Plasma sclerostin levels were compared between APOE – and APOE + participants. C. Correlations between plasma sclerostin levels and age. The line segment within each jitter plot represents the median of the data and error bars in the graphs represent the interquartile range for the groups compared. ***$p \leq 0.001$, ns, no significant difference. Correlation coefficients and p values were calculated using Spearman's correlation coefficient (r_s).

Table 2 Comparison of plasma SOST between Aβ- an Aβ+ participants.

	Aβ-	(95% CI)	Aβ+	(95% CI)	*p*	*p**
All participants	65		35			
	56.51 ± 22.14	51.02-61.99	71.49 ± 25.00	62.90-80.07	**0.003**	**0.008**
APOE ε4 non-carriers	60		19			
	55.94 ± 22.63	50.09-61.79	80.93 ± 27.18	67.73-93.93	**<0.001**	**0.001**
APOE ε4 carriers	5		16			
	63.42 ±15.06	44.72-82.12	60.42 ± 16.98	51.37-69.47	0.728	0.368
Male	19		13			
	69.55 ± 28.18	55.97-83.13	87.07 ± 24.28	72.40-101.74	0.078	0.050
Female	46		22			
	51.12 ± 16.68	46.17-56.07	62.28 ± 20.87	53.02-71.53	**0.020**	**0.033**

Table 5.2 Comparison of plasm sclerostin (pmol/L) between Aβ- and Aβ+ participants within all individuals and subgroups stratified by APOE ε4 status and sex, respectively. Student t test or Chi-square tests were employed as appropriate. Data are presented in mean ± SD, and *p* values in bold font were considered as significant ($p < 0.05$). *p** represents p values adjusted for age, sex and APOE ε4 status.

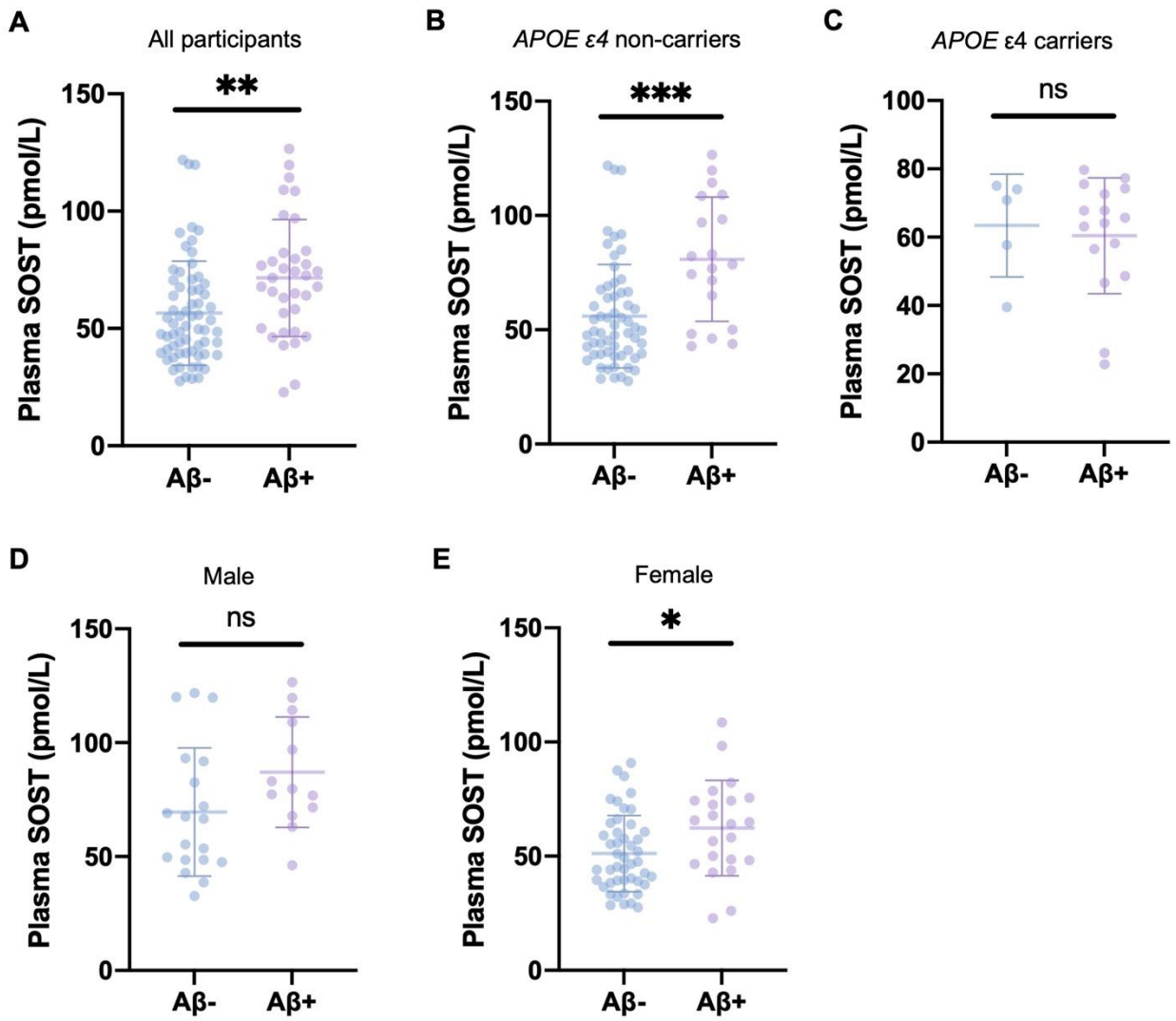


Figure 5.2 Comparison of plasm sclerostin between Aβ- and Aβ+ participants within all individuals (A) and subgroups stratified by APOE ε4 status (B, C) and sex (D, E), respectively. The line segment within each jitter plot represents the median of the data and error bars in the graphs represent the interquartile range for the groups compared. *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns, no significant difference.

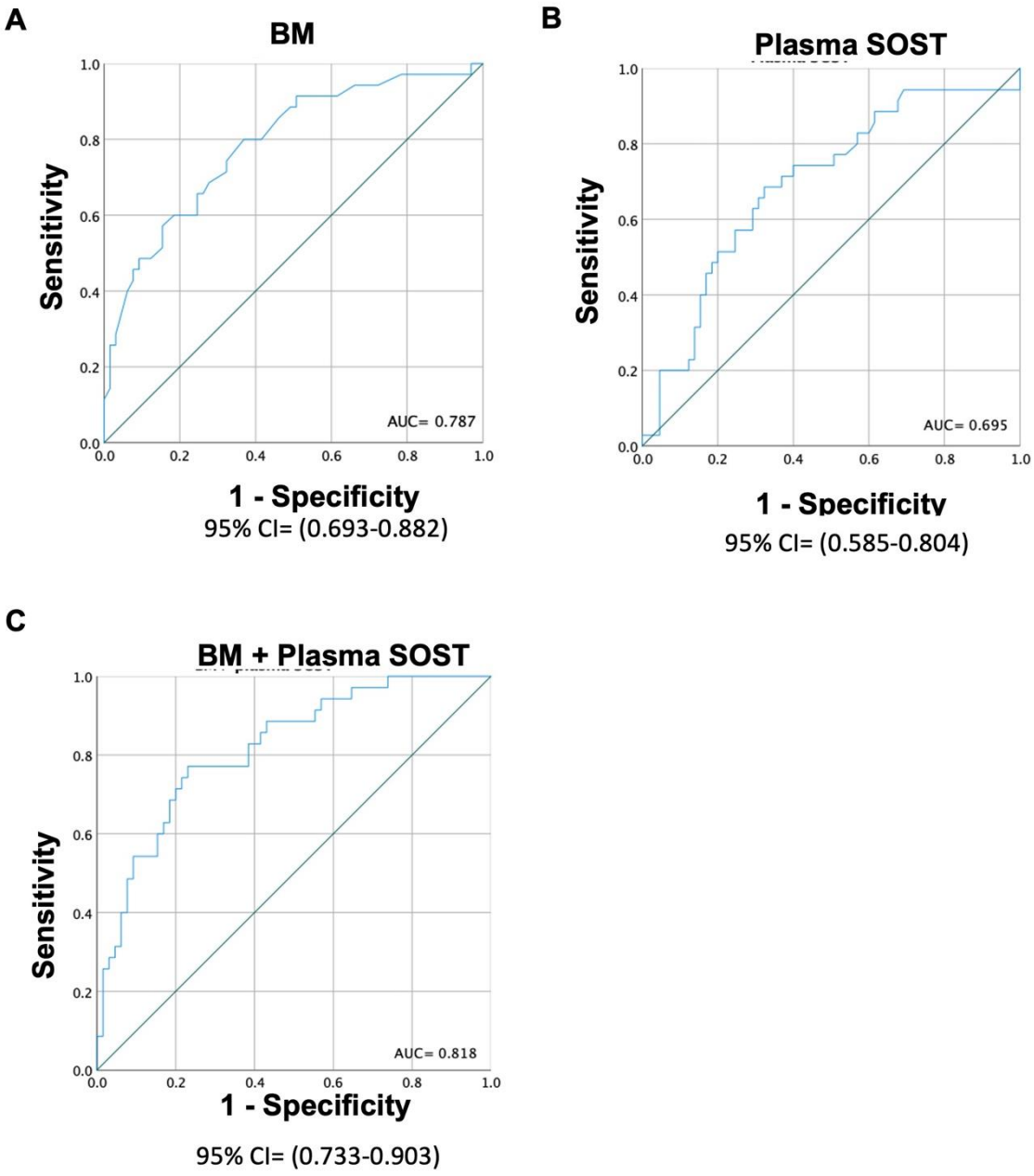
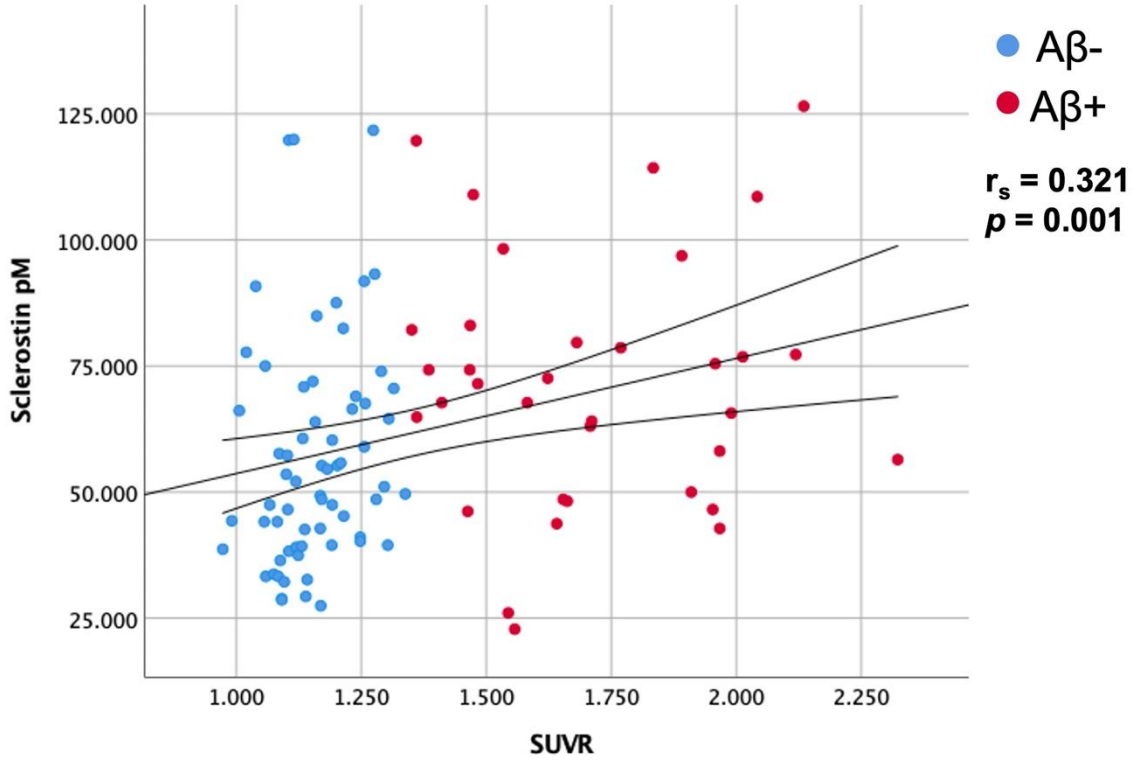


Figure 5.3 Receiver operating characteristic curves for the prediction of Aβ- versus Aβ+ participants. Receiver operating characteristic (ROC) curves are presented for (A) the 'base' model (BM) comprising major risk factors age, sex and APOE ε4 status, (B) plasma sclerostin, (C) BM + sclerostin. Logistic regression models were employed to perform the analyses. AUC, area under the curve. CI, confidence interval.

5.6 Supplementary materials

5.6.1 Supplementary Figure: Association between plasma sclerotin and brain Aβ load measured using PET

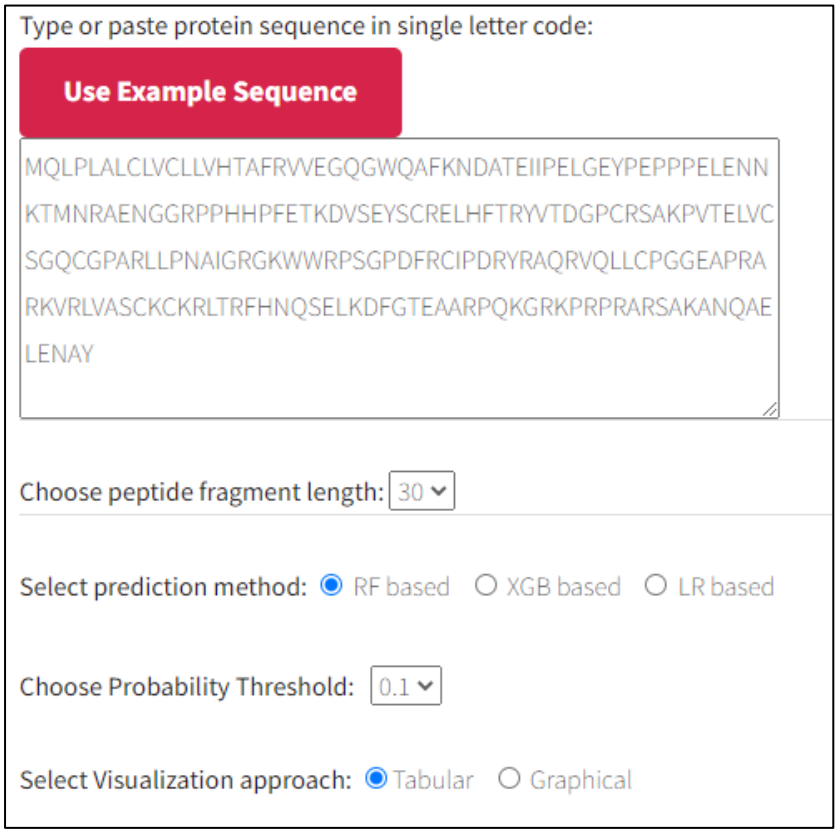


5.6.2 Predicting the BBB penetrating ability of sclerostin

5.6.2.1 Method

The computational tool named “B3Pred” was used to predict the potential of sclerostin to cross the blood brain barrier (https://webs.iiitd.edu.in/raghava/b3pred/) (Kumar, Patiyal et al. 2021). In brief, the “Protein Scan” module was used to identify regions within the sclerostin protein with blood brain penetrating properties. Datasets used for predictions included 269 experimentally validated blood–brain barrier penetrating peptides from the B3Pdb database (https://webs.iiitd.edu.in/raghava/b3pdb/) and negative dataset comprised of 2690 randomly generated peptides. The sclerostin human protein sequence and B3Pred parameters used for analysis provided below.

5.6.2.2 Predicting setting:



5.6.2.3 Prediction results: (BP3red_Protein_scan)

Start	Sequence	Score	Prediction
1	MQLPLALCLVCLLVHTAFRVVEGQGWQAFK	0.02	Non-B3P peptide
2	QLPLALCLVCLLVHTAFRVVEGQGWQAFKN	0.01	Non-B3P peptide
3	LPLALCLVCLLVHTAFRVVEGQGWQAFKND	0	Non-B3P peptide
4	PLALCLVCLLVHTAFRVVEGQGWQAFKNDA	0	Non-B3P peptide
5	LALCLVCLLVHTAFRVVEGQGWQAFKNDAT	0	Non-B3P peptide
6	ALCLVCLLVHTAFRVVEGQGWQAFKNDATE	0	Non-B3P peptide
7	LCLVCLLVHTAFRVVEGQGWQAFKNDATEI	0	Non-B3P peptide
8	CLVCLLVHTAFRVVEGQGWQAFKNDATEII	0	Non-B3P peptide
9	LVCLLVHTAFRVVEGQGWQAFKNDATEIIP	0	Non-B3P peptide
10	VCLLVHTAFRVVEGQGWQAFKNDATEIIPE	0	Non-B3P peptide
11	CLLVHTAFRVVEGQGWQAFKNDATEIIPEL	0	Non-B3P peptide
12	LLVHTAFRVVEGQGWQAFKNDATEIIPELG	0	Non-B3P peptide
13	LVHTAFRVVEGQGWQAFKNDATEIIPELGE	0.01	Non-B3P peptide
14	VHTAFRVVEGQGWQAFKNDATEIIPELGEY	0.02	Non-B3P peptide
15	HTAFRVVEGQGWQAFKNDATEIIPELGEYP	0.04	Non-B3P peptide
16	TAFRVVEGQGWQAFKNDATEIIPELGEYPE	0	Non-B3P peptide
17	AFRVVEGQGWQAFKNDATEIIPELGEYPEP	0	Non-B3P peptide
18	FRVVEGQGWQAFKNDATEIIPELGEYPEPP	0.01	Non-B3P peptide
19	RVVEGQGWQAFKNDATEIIPELGEYPEPPP	0.02	Non-B3P peptide
20	VVEGQGWQAFKNDATEIIPELGEYPEPPPE	0.02	Non-B3P peptide
21	VEGQGWQAFKNDATEIIPELGEYPEPPPEL	0.02	Non-B3P peptide
22	EGQGWQAFKNDATEIIPELGEYPEPPPELE	0.01	Non-B3P peptide
23	GQGWQAFKNDATEIIPELGEYPEPPPELEN	0.01	Non-B3P peptide
24	QGWQAFKNDATEIIPELGEYPEPPPELENN	0.02	Non-B3P peptide
25	GWQAFKNDATEIIPELGEYPEPPPELENNK	0.02	Non-B3P peptide
26	WQAFKNDATEIIPELGEYPEPPPELENNKT	0.02	Non-B3P peptide
27	QAFKNDATEIIPELGEYPEPPPELENNKTM	0.02	Non-B3P peptide
28	AFKNDATEIIPELGEYPEPPPELENNKTMN	0.02	Non-B3P peptide
29	FKNDATEIIPELGEYPEPPPELENNKTMNR	0.03	Non-B3P peptide
30	KNDATEIIPELGEYPEPPPELENNKTMNRA	0.02	Non-B3P peptide
31	NDATEIIPELGEYPEPPPELENNKTMNRAE	0.01	Non-B3P peptide
32	DATEIIPELGEYPEPPPELENNKTMNRAEN	0.01	Non-B3P peptide
33	ATEIIPELGEYPEPPPELENNKTMNRAENG	0.02	Non-B3P peptide
34	TEIIPELGEYPEPPPELENNKTMNRAENGG	0.02	Non-B3P peptide
35	EIIPELGEYPEPPPELENNKTMNRAENGGR	0.02	Non-B3P peptide
36	IIPELGEYPEPPPELENNKTMNRAENGGRP	0.03	Non-B3P peptide
37	IPELGEYPEPPPELENNKTMNRAENGGRPP	0.03	Non-B3P peptide
38	PELGEYPEPPPELENNKTMNRAENGGRPPH	0.06	Non-B3P peptide
39	ELGEYPEPPPELENNKTMNRAENGGRPPHH	0.07	Non-B3P peptide
40	LGEYPEPPPELENNKTMNRAENGGRPPHHP	0.04	Non-B3P peptide
41	GEYPEPPPELENNKTMNRAENGGRPPHHPF	0.06	Non-B3P peptide
42	EYPEPPPELENNKTMNRAENGGRPPHHPFE	0.05	Non-B3P peptide
43	YPEPPPELENNKTMNRAENGGRPPHHPFET	0.05	Non-B3P peptide
44	PEPPPELENNKTMNRAENGGRPPHHPFETK	0.04	Non-B3P peptide

45	EPPPELENNKTMNRAENGGRPPHHPFETKD	0.02	Non-B3P peptide
46	PPPELENNKTMNRAENGGRPPHHPFETKDV	0.04	Non-B3P peptide
47	PPELENNKTMNRAENGGRPPHHPFETKDVS	0.04	Non-B3P peptide
48	PELENNKTMNRAENGGRPPHHPFETKDVSE	0.03	Non-B3P peptide
49	ELENNKTMNRAENGGRPPHHPFETKDVSEY	0.03	Non-B3P peptide
50	LENNKTMNRAENGGRPPHHPFETKDVSEYS	0.04	Non-B3P peptide
51	ENNKTMNRAENGGRPPHHPFETKDVSEYSC	0.03	Non-B3P peptide
52	NNKTMNRAENGGRPPHHPFETKDVSEYSCR	0.04	Non-B3P peptide
53	NKTMNRAENGGRPPHHPFETKDVSEYSCRE	0.03	Non-B3P peptide
54	KTMNRAENGGRPPHHPFETKDVSEYSCREL	0.02	Non-B3P peptide
55	TMNRAENGGRPPHHPFETKDVSEYSCRELH	0.03	Non-B3P peptide
56	MNRAENGGRPPHHPFETKDVSEYSCRELHF	0.03	Non-B3P peptide
57	NRAENGGRPPHHPFETKDVSEYSCRELHFT	0.02	Non-B3P peptide
58	RAENGGRPPHHPFETKDVSEYSCRELHFTR	0.03	Non-B3P peptide
59	AENGGRPPHHPFETKDVSEYSCRELHFTRY	0.03	Non-B3P peptide
60	ENGGRPPHHPFETKDVSEYSCRELHFTRYV	0.03	Non-B3P peptide
61	NGGRPPHHPFETKDVSEYSCRELHFTRYVT	0.04	Non-B3P peptide
62	GGRPPHHPFETKDVSEYSCRELHFTRYVTD	0.04	Non-B3P peptide
63	GRPPHHPFETKDVSEYSCRELHFTRYVTDG	0.04	Non-B3P peptide
64	RPPHHPFETKDVSEYSCRELHFTRYVTDGP	0.04	Non-B3P peptide
65	PPHHPFETKDVSEYSCRELHFTRYVTDGPC	0.03	Non-B3P peptide
66	PHHPFETKDVSEYSCRELHFTRYVTDGPCR	0.04	Non-B3P peptide
67	HHPFETKDVSEYSCRELHFTRYVTDGPCRS	0.04	Non-B3P peptide
68	HPFETKDVSEYSCRELHFTRYVTDGPCRSA	0.03	Non-B3P peptide
69	PFETKDVSEYSCRELHFTRYVTDGPCRSAK	0.02	Non-B3P peptide
70	FETKDVSEYSCRELHFTRYVTDGPCRSAKP	0.02	Non-B3P peptide
71	ETKDVSEYSCRELHFTRYVTDGPCRSAKPV	0.02	Non-B3P peptide
72	TKDVSEYSCRELHFTRYVTDGPCRSAKPVT	0.03	Non-B3P peptide
73	KDVSEYSCRELHFTRYVTDGPCRSAKPVTE	0.02	Non-B3P peptide
74	DVSEYSCRELHFTRYVTDGPCRSAKPVTEL	0.02	Non-B3P peptide
75	VSEYSCRELHFTRYVTDGPCRSAKPVTELV	0.01	Non-B3P peptide
76	SEYSCRELHFTRYVTDGPCRSAKPVTELVC	0.01	Non-B3P peptide
77	EYSCRELHFTRYVTDGPCRSAKPVTELVCS	0.01	Non-B3P peptide
78	YSCRELHFTRYVTDGPCRSAKPVTELVCSG	0.02	Non-B3P peptide
79	SCRELHFTRYVTDGPCRSAKPVTELVCSGQ	0.02	Non-B3P peptide
80	CRELHFTRYVTDGPCRSAKPVTELVCSGQC	0.02	Non-B3P peptide
81	RELHFTRYVTDGPCRSAKPVTELVCSGQCG	0.02	Non-B3P peptide
82	ELHFTRYVTDGPCRSAKPVTELVCSGQCGP	0.04	Non-B3P peptide
83	LHFTRYVTDGPCRSAKPVTELVCSGQCGPA	0.06	Non-B3P peptide
84	HFTRYVTDGPCRSAKPVTELVCSGQCGPAR	0.09	Non-B3P peptide
85	FTRYVTDGPCRSAKPVTELVCSGQCGPARL	0.02	Non-B3P peptide
86	TRYVTDGPCRSAKPVTELVCSGQCGPARLL	0.01	Non-B3P peptide
87	RYVTDGPCRSAKPVTELVCSGQCGPARLLP	0.01	Non-B3P peptide
88	YVTDGPCRSAKPVTELVCSGQCGPARLLPN	0.01	Non-B3P peptide
89	VTDGPCRSAKPVTELVCSGQCGPARLLPNA	0	Non-B3P peptide

90	TDGPCRSAKPVTELVCSGQCGPARLLPNAI	0	Non-B3P peptide
91	DGPCRSAKPVTELVCSGQCGPARLLPNAIG	0	Non-B3P peptide
92	GPCRSAKPVTELVCSGQCGPARLLPNAIGR	0	Non-B3P peptide
93	PCRSAKPVTELVCSGQCGPARLLPNAIGRG	0	Non-B3P peptide
94	CRSAKPVTELVCSGQCGPARLLPNAIGRGK	0	Non-B3P peptide
95	RSAKPVTELVCSGQCGPARLLPNAIGRGKW	0	Non-B3P peptide
96	SAKPVTELVCSGQCGPARLLPNAIGRGKWW	0	Non-B3P peptide
97	AKPVTELVCSGQCGPARLLPNAIGRGKWWR	0	Non-B3P peptide
98	KPVTELVCSGQCGPARLLPNAIGRGKWWRP	0	Non-B3P peptide
99	PVTELVCSGQCGPARLLPNAIGRGKWWRPS	0	Non-B3P peptide
100	VTELVCSGQCGPARLLPNAIGRGKWWRPSG	0.01	Non-B3P peptide
101	TELVCSGQCGPARLLPNAIGRGKWWRPSGP	0.02	Non-B3P peptide
102	ELVCSGQCGPARLLPNAIGRGKWWRPSGPD	0.03	Non-B3P peptide
103	LVCSGQCGPARLLPNAIGRGKWWRPSGPDF	0.05	Non-B3P peptide
104	VCSGQCGPARLLPNAIGRGKWWRPSGPDFR	0.06	Non-B3P peptide
105	CSGQCGPARLLPNAIGRGKWWRPSGPDFRC	0.07	Non-B3P peptide
106	SGQCGPARLLPNAIGRGKWWRPSGPDFRCI	0.07	Non-B3P peptide
107	GQCGPARLLPNAIGRGKWWRPSGPDFRCIP	0.07	Non-B3P peptide
108	QCGPARLLPNAIGRGKWWRPSGPDFRCIPD	0.06	Non-B3P peptide
109	CGPARLLPNAIGRGKWWRPSGPDFRCIPDR	0.08	Non-B3P peptide
110	GPARLLPNAIGRGKWWRPSGPDFRCIPDRY	0.09	Non-B3P peptide
111	**PARLLPNAIGRGKWWRPSGPDFRCIPDRYR**	**0.12**	**B3P peptide**
112	**ARLLPNAIGRGKWWRPSGPDFRCIPDRYRA**	**0.11**	**B3P peptide**
113	**RLLPNAIGRGKWWRPSGPDFRCIPDRYRAQ**	**0.11**	**B3P peptide**
114	**LLPNAIGRGKWWRPSGPDFRCIPDRYRAQR**	**0.11**	**B3P peptide**
115	**LPNAIGRGKWWRPSGPDFRCIPDRYRAQRV**	**0.14**	**B3P peptide**
116	**PNAIGRGKWWRPSGPDFRCIPDRYRAQRVQ**	**0.13**	**B3P peptide**
117	**NAIGRGKWWRPSGPDFRCIPDRYRAQRVQL**	**0.12**	**B3P peptide**
118	**AIGRGKWWRPSGPDFRCIPDRYRAQRVQLL**	**0.1**	**B3P peptide**
119	**IGRGKWWRPSGPDFRCIPDRYRAQRVQLLC**	**0.11**	**B3P peptide**
120	**GRGKWWRPSGPDFRCIPDRYRAQRVQLLCP**	**0.12**	**B3P peptide**
121	**RGKWWRPSGPDFRCIPDRYRAQRVQLLCPG**	**0.12**	**B3P peptide**
122	GKWWRPSGPDFRCIPDRYRAQRVQLLCPGG	0.08	Non-B3P peptide
123	KWWRPSGPDFRCIPDRYRAQRVQLLCPGGE	0.04	Non-B3P peptide
124	WWRPSGPDFRCIPDRYRAQRVQLLCPGGEA	0.04	Non-B3P peptide
125	WRPSGPDFRCIPDRYRAQRVQLLCPGGEAP	0.03	Non-B3P peptide
126	RPSGPDFRCIPDRYRAQRVQLLCPGGEAPR	0.04	Non-B3P peptide
127	PSGPDFRCIPDRYRAQRVQLLCPGGEAPRA	0.04	Non-B3P peptide
128	SGPDFRCIPDRYRAQRVQLLCPGGEAPRAR	0.06	Non-B3P peptide
129	GPDFRCIPDRYRAQRVQLLCPGGEAPRARK	0.07	Non-B3P peptide
130	PDFRCIPDRYRAQRVQLLCPGGEAPRARKV	0.06	Non-B3P peptide
131	**DFRCIPDRYRAQRVQLLCPGGEAPRARKVR**	**0.14**	**B3P peptide**
132	**FRCIPDRYRAQRVQLLCPGGEAPRARKVRL**	**0.15**	**B3P peptide**
133	**RCIPDRYRAQRVQLLCPGGEAPRARKVRLV**	**0.14**	**B3P peptide**
134	CIPDRYRAQRVQLLCPGGEAPRARKVRLVA	0.05	Non-B3P peptide

135	IPDRYRAQRVQLLCPGGEAPRARKVRLVAS	0.04	Non-B3P peptide
136	PDRYRAQRVQLLCPGGEAPRARKVRLVASC	0.05	Non-B3P peptide
137	DRYRAQRVQLLCPGGEAPRARKVRLVASCK	0.09	Non-B3P peptide
138	**RYRAQRVQLLCPGGEAPRARKVRLVASCKC**	**0.12**	**B3P peptide**
139	**YRAQRVQLLCPGGEAPRARKVRLVASCKCK**	**0.12**	**B3P peptide**
140	**RAQRVQLLCPGGEAPRARKVRLVASCKCKR**	**0.13**	**B3P peptide**
141	**AQRVQLLCPGGEAPRARKVRLVASCKCKRL**	**0.1**	**B3P peptide**
142	QRVQLLCPGGEAPRARKVRLVASCKCKRLT	0.09	Non-B3P peptide
143	**RVQLLCPGGEAPRARKVRLVASCKCKRLTR**	**0.14**	**B3P peptide**
144	**VQLLCPGGEAPRARKVRLVASCKCKRLTRF**	**0.12**	**B3P peptide**
145	**QLLCPGGEAPRARKVRLVASCKCKRLTRFH**	**0.16**	**B3P peptide**
146	**LLCPGGEAPRARKVRLVASCKCKRLTRFHN**	**0.17**	**B3P peptide**
147	**LCPGGEAPRARKVRLVASCKCKRLTRFHNQ**	**0.13**	**B3P peptide**
148	**CPGGEAPRARKVRLVASCKCKRLTRFHNQS**	**0.12**	**B3P peptide**
149	**PGGEAPRARKVRLVASCKCKRLTRFHNQSE**	**0.1**	**B3P peptide**
150	**GGEAPRARKVRLVASCKCKRLTRFHNQSEL**	**0.13**	**B3P peptide**
151	**GEAPRARKVRLVASCKCKRLTRFHNQSELK**	**0.13**	**B3P peptide**
152	EAPRARKVRLVASCKCKRLTRFHNQSELKD	0.08	Non-B3P peptide
153	**APRARKVRLVASCKCKRLTRFHNQSELKDF**	**0.1**	**B3P peptide**
154	**PRARKVRLVASCKCKRLTRFHNQSELKDFG**	**0.12**	**B3P peptide**
155	**RARKVRLVASCKCKRLTRFHNQSELKDFGT**	**0.12**	**B3P peptide**
156	ARKVRLVASCKCKRLTRFHNQSELKDFGTE	0.08	Non-B3P peptide
157	RKVRLVASCKCKRLTRFHNQSELKDFGTEA	0.09	Non-B3P peptide
158	KVRLVASCKCKRLTRFHNQSELKDFGTEAA	0.04	Non-B3P peptide
159	VRLVASCKCKRLTRFHNQSELKDFGTEAAR	0.05	Non-B3P peptide
160	RLVASCKCKRLTRFHNQSELKDFGTEAARP	0.06	Non-B3P peptide
161	LVASCKCKRLTRFHNQSELKDFGTEAARPQ	0.06	Non-B3P peptide
162	VASCKCKRLTRFHNQSELKDFGTEAARPQK	0.05	Non-B3P peptide
163	ASCKCKRLTRFHNQSELKDFGTEAARPQKG	0.03	Non-B3P peptide
164	SCKCKRLTRFHNQSELKDFGTEAARPQKGR	0.08	Non-B3P peptide
165	**CKCKRLTRFHNQSELKDFGTEAARPQKGRK**	**0.1**	**B3P peptide**
166	KCKRLTRFHNQSELKDFGTEAARPQKGRKP	0.09	Non-B3P peptide
167	CKRLTRFHNQSELKDFGTEAARPQKGRKPR	0.09	Non-B3P peptide
168	**KRLTRFHNQSELKDFGTEAARPQKGRKPRP**	**0.11**	**B3P peptide**
169	**RLTRFHNQSELKDFGTEAARPQKGRKPRPR**	**0.11**	**B3P peptide**
170	LTRFHNQSELKDFGTEAARPQKGRKPRPRA	0.09	Non-B3P peptide
171	**TRFHNQSELKDFGTEAARPQKGRKPRPRAR**	**0.1**	**B3P peptide**
172	**RFHNQSELKDFGTEAARPQKGRKPRPRARS**	**0.12**	**B3P peptide**
173	**FHNQSELKDFGTEAARPQKGRKPRPRARSA**	**0.11**	**B3P peptide**
174	**HNQSELKDFGTEAARPQKGRKPRPRARSAK**	**0.12**	**B3P peptide**
175	**NQSELKDFGTEAARPQKGRKPRPRARSAKA**	**0.1**	**B3P peptide**
176	QSELKDFGTEAARPQKGRKPRPRARSAKAN	0.09	Non-B3P peptide
177	SELKDFGTEAARPQKGRKPRPRARSAKANQ	0.09	Non-B3P peptide
178	**ELKDFGTEAARPQKGRKPRPRARSAKANQA**	**0.11**	**B3P peptide**
179	**LKDFGTEAARPQKGRKPRPRARSAKANQAE**	**0.11**	**B3P peptide**

180	**KDFGTEAARPQKGRKPRPRARSAKANQAEL**	**0.11**	**B3P peptide**
181	**DFGTEAARPQKGRKPRPRARSAKANQAELE**	**0.1**	**B3P peptide**
182	**FGTEAARPQKGRKPRPRARSAKANQAELEN**	**0.12**	**B3P peptide**
183	GTEAARPQKGRKPRPRARSAKANQAELENA	0.09	Non-B3P peptide
184	**TEAARPQKGRKPRPRARSAKANQAELENAY**	**0.12**	**B3P peptide**

(note: peptide sequences with the feature of BBB penetrating are bolded in the table)

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CHAPTER 6

Osteocyte-derived Sclerostin Inhibits Neurogenesis: A Potential Mechanism Contributing to AD Onset

6.1 Preamble

The results of Chapter 5 showed that plasma sclerostin levels are elevated in older adults with positive brain Aβ status. This is a novel and exciting finding as it is the first cohort study demonstrating an association between osteocyte-derived sclerostin and a preclinical AD pathological indicator. However, a key question remains regarding whether sclerostin plays a direct role in the AD pathobiology, and disease progression. In this chapter, we verified that osteocyte-like MLO-Y4 cells upregulate the expression of sclerostin during exposure to a treatment that mimics stress during ageing. We subsequently investigated a potential mechanism whereby sclerostin may contribute to AD. Importantly, it is demonstrated that sclerostin inhibits neurogenesis, which is a mechanism proposed to play a role in the early stages of AD.

6.2 Introduction

Specific regions of the adult brain are capable of procceding constant neurogenesis (Eriksson, Perfilieva et al. 1998, Spalding, Bergmann et al. 2013, Boldrini, Fulmore et al. 2018). Ongoing neurogensis in the hipocampus is believed to be important in maintaining learning and memory functions during life (Gallardo 2019), and impaired neurogenesis contributes to memory and cognitive decline (Mu and Gage 2011). Remarkably, recent studies have confirmed the waning of neurogenesis in AD, as evidenced by the reduced expression of neurogenesis markers in both the hippocampal dentate gyrus and subventricular zone in postmortem human AD brains (Moreno-Jimenez, Flor-Garcia et al. 2019, Tobin, Musaraca et al. 2019). It has also been demonstrated that adult neurogenesis in the subventricular zone is impaired in presymptomatic stages of AD (Scopa, Marrocco et al. 2020). In addition, in 3XTg AD mice, changes in neurogenesis occur prior to the development of amyloid plaques and neurofibrillary tangle formation (Hamilton, Aumont et al. 2010). Together, these findings indicate that a decline in neurogenesis may represent an early pathological mechanisn contributing to AD.

The canonical Wnt pathway has been elucidated to be a pivotal regulator in neurogenesis in different physiological conditions (Arredondo, Valenzuela-Bezanilla et al. 2020). Furthermore, it appears that reduced Wnt/β-catenin signalling occurs in the ageing brain and contributes to decreased neurogenesis and cognitive impairment. Research involving two Wnt inhibitors, Dickkopf 1 (Dkk1) and secreted frizzled-related protein 3 (sFRP3), indicated that Wnt antagonists had a negative influence on neurogenesis. In addition, Seib et al. observed that loss of Dkk1 led to recovery of neurogenesis and increased dendritic complexity of newborn neurons in old mice (Seib, Corsini et al. 2013). Similarly, it was shown that sFRP3 knock-down resulted in an increased self-renewal of neural progenitors in the hippocampal dentate gyrus (Cho, Yoo et al. 2019). However, the impact of sclerostin in Wnt signalling inhibition and neurogenesis has not been fully investigated.

In Chapter 5, it was observed that the concentrations of sclerostin in the plasma increase during ageing. While osteocytes are the major source of sclerostin, it is unknown whether the increased plasma levels of sclerostin are attributable to these cells. We hypothesize that osteocytes produce more sclerostin during ageing, resulting in elevated plasma levels, and that increased levels of sclerostin in the brain contribute to the onset of AD by inhibiting neurogenesis. To verify this hypothesis, we first examined sclerostin gene and protein expression levels in the osteocyte-like cell line, MLO-Y4, following a treatment to mimic ageing, namely exposure to advanced glycation end products (AGEs). Next, recombinant sclerostin protein was employed to investigate its impact on neurogenesis using a neuronal differentiation system established with the NE-4C neural stem cell line. The influence of sclerostin on the viability of NE-4C cells before initiating differentiation and neuronal gene expression during differentiation were analyzed. In addition, quantitative analysis of the number and size of NE-4C neurospheres was conducted to examine any negative effects of sclerostin on neurosphere formation. Finally, we examined the impact of sclerostin on the

morphological development of newborn neurons by analyzing their dendritic spine density and expression of the postsynaptic scaffolding protein 95 (PSD95) protein, which is an important regulator of spine formation (Mardones, Jorquera et al. 2019).

The results in this Chapter demonstrated that AGEs promote sclerostin gene and protein expression in MLO-Y4 cells in a time- and dose-dependent manner. Sclerostin also inhibited the proliferation and viability of NE-4C cells, and the expression of genes associated with neuronal differentiation. In addition, exposure of neurospheres to sclerostin reduced their size and number compared with the untreated control neurospheres. Moreover, sclerostin reduced the expression of PSD95 and decreased dendritic spine density in mature neurons. Together, these data provide evidence that sclerostin has adverse effects on neurogenesis, which could serve as a mechanism contributing to AD onset and progression. Importantly, the findings in this Chapter also provide further evidence that osteocytes can regulate the function of neurons in an endocrine-like manner.

6.3 Methods

6.3.1 AGEs preparation

The AGEs were prepared and characterized according to a modified protocol described in previous studies (Li, Liu et al. 2012, Li, Li et al. 2016). In brief, 50 mg/mL fatty acid-free BSA (Sigma-Aldrich, A8806-1G) was dissolved in PBS with 600 mM D-ribose (endotoxin free, Sigma-Aldrich, R7500-5G) and incubated under sterile dark condition for 1 week at 37 °C. Free ribose was removed by dialysis against PBS with using Slide-A-Lyzer^{TM} Dialysis Cassette Kit (3.5K MWCO, 0.5mL, #66335). Non-glycated control BSA was incubated in the same conditions without ribose. The formation of AGEs was confirmed based on the characteristic fluorescence acquired with using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek). The

concentration of AGEs was quantified using the BioRad protein assay system and dissolved in PBS to reach a stock concentration of 50 mg/mL.

6.3.2 Cell culture and treatment

MLO-Y4 cells were maintained in α-MEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin/streptomycin (P/S; Sigma-Aldrich). Cells were maintained in an CO_2 incubator (5% CO_2, 37°C and 95% humidity). When MLO-Y4 cells reached 60-70% confluency, AGEs were added to the medium at a final concentration of 250 µg/mL for 8, 16 or 24 hours. For concentration response studies, MLO-Y4 cells were incubated with AGEs for 24 hours at 100, 250 and 500 µg/mL. The control consisted of exposing MLO-Y4 cells to normal BSA (250 µg/mL) for 24 hours.

NE-4C cells (CRL-2925; ATCC) were cultured in α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin and maintained in a CO_2 incubator. Flasks or plates used for NE-4C cell culture were pre-coated with 50 µg/mL poly-D-lysine (70,000 – 150,000 MW; Sigma-Aldrich). Recombinant mouse sclerostin protein (1589 – ST – 025, R & D systems) was reconstituted at 200 µg/mL in sterile PBS containing 0.1% BSA, and stored at -80°C prior to use.. For time courses treatment studies, NE-4C cells were exposed to sclerostin (200 ng/mL) for 12, 24 or 36 hours. For concentration response studies, NE-4C cells were exposed to sclerostin at 50, 200 or 400 ng/mL for 24 hours. When replacing media during NE-4C cell neural differentiation, sclerostin was added to the medium at a final concentration of 200 ng/mL.

6.3.3 Neural differentiation of NE-4C cells

The differentiation procedure was modified from the protocols described in previous studies (PMID 27265882). NE-4C cells were seeded in poly-D-lysine-precoated plates at a density of 5 x 10^4 cells/cm^2 in α-MEM/10% FBS. When cells reached 80% confluency, differentiation

was induced by replacing the culture medium with differentiation medium composed of Neurobasal media (21103049, Gibco) with 2% B27 Serum-Free Supplement (17504044, Gibco) and GlutaMax-I (2mM) and 1% P/S. In addition, during the first two days of NE-4C cell differentiation, retinoid acid (R2625, Sigma-Aldrich) was added to the medium at a concentration of 1 μM. On the third day of differentiation, and every second day thereafter medium was replaced with normal differentiation medium. On cultivation day seven, neuronspheres were digested with 0.25% trysin/EDTA for 1 minute at 37°C and dissociated into single cells using a fire-poslished pipette by slowly pipetteing up and down. The dissociated cells were then reseeded onto poly-D-lysine-precoated plates and cultured in differentiation medium for a further 5 days (day 12) after commencement of differentiation (refer to Figure 6. 3A for the diagram demonstrating the differentiation procedure).

6.3.4 NE-4C cell counting and doubling time calculation

NE-4C cells (undifferentiated) were seeded onto poly-D-lysine-precoated 6-well plates at a density of 10,000 cells/cm^2. Following the plating, sclerostin or vehicle solution (0.1% BSA/PBS) were added to the culture medium in wells (refer to 6.3.2 for specific treatment doses and time courses). At the end of administration, undifferentiated NE-4C cells were collected and diluted with equal volume of 4% trypan blue. Average cell counts were taken from four hemacytometer squares to calculate the number of cells per treatment group. Cell doubling time after 36 h growing were calculated with the formula below (Roth V. 2006 Doubling Time Computing, Available from: http://www.doubling-time.com/compute.php):

$$Doubling\ time = \frac{duration * \log(2)}{\log(final\ concentration) - \log(inital\ concentration)}$$

6.3.5 MTS cell viability assay

The MTS (3-(4,5-dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) assay is a metabolic assay that measures the ability of viable cells to bio-

reduce the MTS compound into a coloured soluble formazan product that is measured spectrophotometrically. The quantity of formazan product formed is directly proportional to the number of viable cells in culture.

NE-4C cells (undifferentiated) were seeded onto poly-D-lysine-precoated 96-well plates at a density of 5,500 cells/well. Twenty-fours after plating, sclerostin or vehicle solution (0.1% BSA/PBS) were added to the culture medium in wells (refer to 6.3.2 for specific treatment doses and time courses). The MTS assay (Cell-Titer 96 aqueous non-radioactive cell proliferation assay; Promega, Australia) was performed by adding 10 µL of MTS solution to 100 µL of medium in wells and incubating plates in a CO_2 incubator for 1-4 hours, followed by measuring absorbance at 490 nm (Biochrom Asys UVM 340). Absorbance data were converted to reflect proportional cell viability relative to the untreated control (100% viability).

6.3.6 Immunostaining and confocal imaging

For immunostaining, neurospheres or neuronal differentiated NE-4C cells (here afer referred to as NE-4C neurons; day 12 after differentiation) were cultured in 24-well plates on poly-D-lysine-precoated glass cover slips. Immunostaining procedures were performed according to an established protocol from our laboratory. In brief, neurospheres or NE-4C neurons on the cover-slips were fixed in 4% paraformaldehyde for 15 minutes room temperature (RT). After three washes with PBS, cells were permeabilized using 0.2% Triton X-100 for 5 minutes, followed by blocking in 3% BSA in PBS for 30 minutes at RT. Cells were incubated with primary antibodies (Tuj1, 1:250, ab18207, Abcam; PSD95, 1:250, ab18258, Abcam; vGlut1, 1:250, ab242204, Abcam) diluted in 0.2% BSA in PBS overnight at 4°C. Cells were washed three times with PBS and incubated with the secondary antibody (Alxe Flour 488 anti-mouse) for 1 hour at RT. After the incubation, cells were washed three times in PBS and incubated in Hoechst33342 diluted in 0.2% BSA (1:5000) for 15 minutes at RT. Following the incubation,

cells were washed three times for 5 minutes with PBS at RT, before mounting coverslips onto glass slides using Diamond Anti-fade Mountant media. To avoid neurospheres detaching from the cover slip, all the wash procedures were performed very gently.

For confocal imaging, a Nikon A1Si confocal microscope was employed to acquire the confocal images, using the 20X/0.75 objective or 60X/1.4 oil immersion objective. Digital images were acquired using NIS Elements Software. All images were assembled and analyzed by ImageJ (NIH).

6.3.7 RNA extraction and real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, 15596-026) and PureLink RNA Mini Kit (Invitrogen, ThermoFisher) following the manufacturer's instructions. RNA (1 mg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed using 2 × SYBR Green Master Mix (Bio-Rad) in a CFX Connect Real-Time PCR detection system (Bio-Rad). Each sample was run in triplicate and gene expression levels were normalized to a housekeeping gene *Gapdh*. All measurements were analyzed using the 2-ΔΔCt method. The primers used for real-time PCR are listed as follows (5'-3'): TGGCCTTCCGTGTTCCTAC (forward), GAGTTGCTGTTGAAGTCGCA (reverse) for *Gapdh*; CTGGCTAAGCTTCCAAGGGC (forward), CCAGGGTCTCCGATTTGCAT (reverse) for *Oct4*; AGCAGCTACTTCGTGGAGTG (forward), GGGCTTCCGATTCCTCGTCA (reverse) for *Tuj1; GGTCACAGGGCACCTATTCA* (forward), TGTTCACCTTTCAGGACTGC (reverse) for *Map2*; AGACGGTGCAGCGCATCAAGAA (forward), AGCGTCTCGATCTTCGTGAGCT (reverse) for *Ngn 2*.

6.3.8 Polymerase Chain Reaction (PCR) and agarose gel electrophoresis

Master mix for PCR reaction contained: 0.5 µL of forward primer (20 µM), 0.5 µL of reverse primer (20 µM), 12.5 µL of 2X Go Taq Master Mix, 10.5 µL of ddH2O, 1 µL of cDNA template (approximately 5 ng). The standard PCR program including the initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 40 sec and 72°C for 40 sec, ended with 72 °C for 10 min. Primers designed for PCR are listed as follows (5'-3'):

GCGAAGAAAACCGCATCACC (forward), AAGGGAGAGCTGGCAGG (reverse) for *Gfap*; GTCCTCGCCTTCTGTCGATT (forward), GCTGTGGGGGAGACTCTTTT (reverse) for *Mapt*; CTGGCTAAGCTTCCAAGGGC (forward), CCAGGGTCTCCGATTTGCAT (reverse) for *Oct4*; GTGACGTTGACATCCGTAAAGA (forward), GCCGGACTCATCGTACTCC (reverse) for *β-actin*. 1.5% agarose gel was used for the visualization of the presence or absence of these genes.

6.3.9 Protein extraction and western blotting

Cellular proteins were extracted by incubating in RIPA lysis buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich) for 20 minutes at 4°C. Clarified cell lysate (centrifuge at 12,000 g for 20 min at 4 °C) were diluted and boiled at 95°C with 4X SDS sampling buffer for 5 minutes. Proteins were loaded in each lane and separated on a 10%-17.5% SDS-PAGE gel, before transferring proteins onto nitrocellulose membrane (Millipore). Membranes were then blocked and incubated with primary antibodies including β-actin JLA20 (1:5000, Developmental Studies Hybridoma Bank), β-catenin (1:1000, ab15895, Abcam), PSD95 (1:1000, ab18258, Abcam), and the corresponding HRP-conjugated secondary antibodies including HRP-conjugated goat anti-mouse IgG (1:5000, A9917, Sigma-Aldrich), HRP-conjugated goat anti-rabbit IgG (1:5000, A0545, Sigma-Aldrich). Proteins were visualized by enhanced chemiluminescence reagent (Perkin Elmer) and images of stained protein bands in blots captured digitally (ChemiDoc MP Imaging Systems,

Bio-Rad). Immunoblotting images presented are representatives of at least three independent experiments.

6.3.10 Neurosphere quantification and size measurement

One week after differentiation of NE-4C cells (day 7 after plating), the number of neurospheres were counted in 10 different sites within wells for each treatmemt from low magnification (X10) bright field microscope images. In the same fields used for counting neurospheres, the diameter of neurospheres was measured using phase-contrast microscope images. Only neurospheres > 50 µm in diameter were considerded; note mature neurosphers are typical > 50 µm in diameter. Bright field and phase contrast images of neurospheres were captured using an Olympus Inverted Fluoro microscope (Olympus IX70). (Data are expressed as mean ± SEM.)

6.3.11 Neuron spine density quantification

On day 13 of NE-4C cell differentiation, cells were transfected with CMV-GFP lentivirus (kindly provided by Dr. Ryan S. Anderton, Notre Dame University). Forty-eight hours after transfection, confocal images of cells were acquired using a Nikon A1Si confocal microscope fitted with a 60X/1.4 oil immersion objective. Two-dimensional maximum projection reconstructions of images were generated, and morphometric analysis was performed on spines present on NE-4C neuronal processes. Analysis of spines on at least 10-15 NE-4C neurons from three independent experiments were examined for each treatment.

6.3.12 Statistic analysis

GraphPad Prism 8 software was used to conduct statistical analyses. To determine the differences between two groups, Student's *t* test was performed. For multiple groups, one-way ANOVA and subsequent Tukey post-hoc test was conducted to analyse differences among each experimental group. Data were presented as the mean ± SEM; n.s: non-significant; P values <

0.05 were considered statistically significant, *p<0.05, **p<0.01, ***p<0.001. Three independent experiments were performed for each treatment condition.

6.4 Results

6.4.1 Increasing sclerostin production in osteocytes following ageing associated stress

Advanced glycation end products (AGEs) are proteins or lipids that are glycated as a result of exposure to sugars (Kim, Park et al. 2017). Formation of AGEs during ageing is regarded as a physiological and essential process *in vivo*, and excessive generation and accumulation of AGEs are found in bone with ageing (Franke, Siggelkow et al. 2007)). In view of this, AGEs are considered to be ideal stressors that can be used *in vitro* to mimic the effects of ageing situation.

To investigate whether the increasing plasma sclerostin concentration during ageing is caused by overproduction of sclerostin in osteocytes, the *Sost* gene expression and protein secretion of sclerostin were compared in the osteocyte-like cell line, MLO-Y4, with or without AGEs administration. Preparation and characterization of AGEs was based on the previous protocol as described in the Methods part. Following 1 week of incubation, the fluorescent emission characteristic of the modified BSA solution was detected, an emission maximum at 445 nm evidenced the formation of AGEs (Galler, Muller et al. 2003)(Figure 6.1A). QPCR analysis results revealed that exposure of MLO-Y4 cells to AGEs increased sclerostin mRNA expression in a time and dose dependent manner (Figure 6.1 B, C). To confirm mRNA expression results in increased sclerostin protein expression and secretion, an ELISA assay was performed to quantify the levels of the protein in conditioned medium from MLO-Y4 cell cultures treated with AGEs. The results demonstrated that MLO-Y4 cells exposed to AGEs secrete increased amounts of sclerostin protein in a time and dose-dependent manner (Figure 6.1D, E). These results indicate that osteocytes produce increasing levels of sclerostin when

exposed to stress associated with ageing, which substantially contributes to the increasing plasma SOST concentration in vivo.

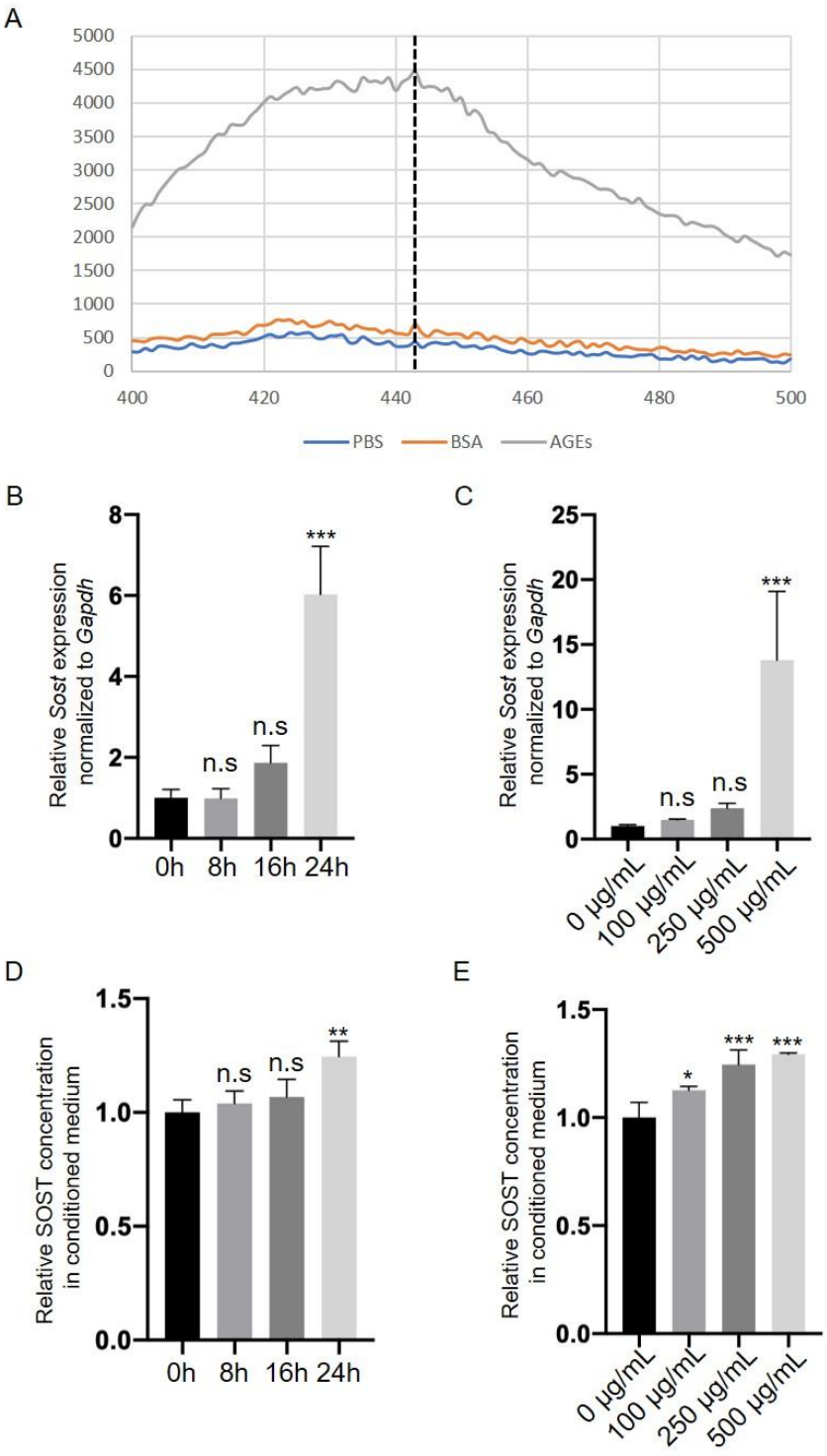


Figure 6.1 AGEs promote sclerostin production in MLO-Y4. A. Relative fluorescence spectra of AGEs. **B, C.** qPCR results for the sclerostin expression in MLO-Y4 treated with AGEs of different time- (250 μg/mL) and dose- (24 h) courses. **D, E.** ELISA assay for the sclerostin concentrations in the medium of MLO-Y4 treated with AGEs of different time- (250 μg/mL) and dose- (24 h) courses. Data are presented as mean ± SEM. n.s, no significance; *p <0.05; **p <0.01; ***p <0.001, one-way ANOVA of three culture triplicates.

6.4.2 Sclerostin inhibits proliferation and viability of NE-4C

Primary neural stem cells (NSCs) are commonly used for investigating neural cell differentiation, neuroactive drug efficiency, and neural disease modeling. However, direct isolation of NSCs from the animal brain tissue involves complex procedures and is not ideal to provide sufficient homogeneous NSCs population. For this reason, neural stem cell lines provide an excellent alternative due to their easy in accessibility, scale-up and homogeneity. NE-4C cells were cloned from the anterior brain vesicles of 9-day-old p53-/- mouse embryo (Schlett, Herberth et al. 1997) and have the capacity to differentiate into mature neurons that display neuronal morphological, biochemical and bioelectric characteristics (Varga, Hadinger et al. 2008, Orsolits, Borsy et al. 2013, Davidson, Wong et al. 2016). Therefore, NE-4C stem cells were employed as an *in vitro* model for neurogenesis in this study.

Prior to establishing the neural differentiation system, we first determined the impacts of sclerostin on the proliferation and viability of NE-4C cells. The cells were treated with vehicle or recombinant sclerostin at various concentrations for different time courses followed by cell number counting and MTS assay as described in the methodology part. As the intracellular function of sclerostin mainly relies on its inhibition of Wnt signalling, we validated the function of sclerostin on the NE-4C cells by examining the protein level of β-catenin, which is crucial for the activation of Wnt pathway. Western blotting analysis confirmed that the recombinant sclerostin was able to cause decreasing expression of β-catenin in a time and dose-dependent manner (Supplementary Figure 6.1A, B), which indicates that sclerostin treatment inhibits Wnt signalling activity in NE-4C. Under such circumstances, cell counts obtained from NE-4c cultures treated with sclerostin for 24 hours revealed that at high concentrations (200 and 400 ng/mL), but not low concentration (20 ng/mL) the protein reduced cell number compared with vehicle treated cultures (Figure 6.2A-D). In addition, the high concentrations of sclerostin prolonged NE-4C doubling time as determined over a 36-hour period, commencing after the

seeding of culture wells with cells (Figure 6.2E). Similarly, the high concentrations of sclerostin reduced NE-4C cell viability in time and dose-dependent manner as measured using the MTS assay (Figure 6.2F, G). Taken together, these results indicate that sclerostin has the capacity to inhibited cell proliferation and affect the cell viability.

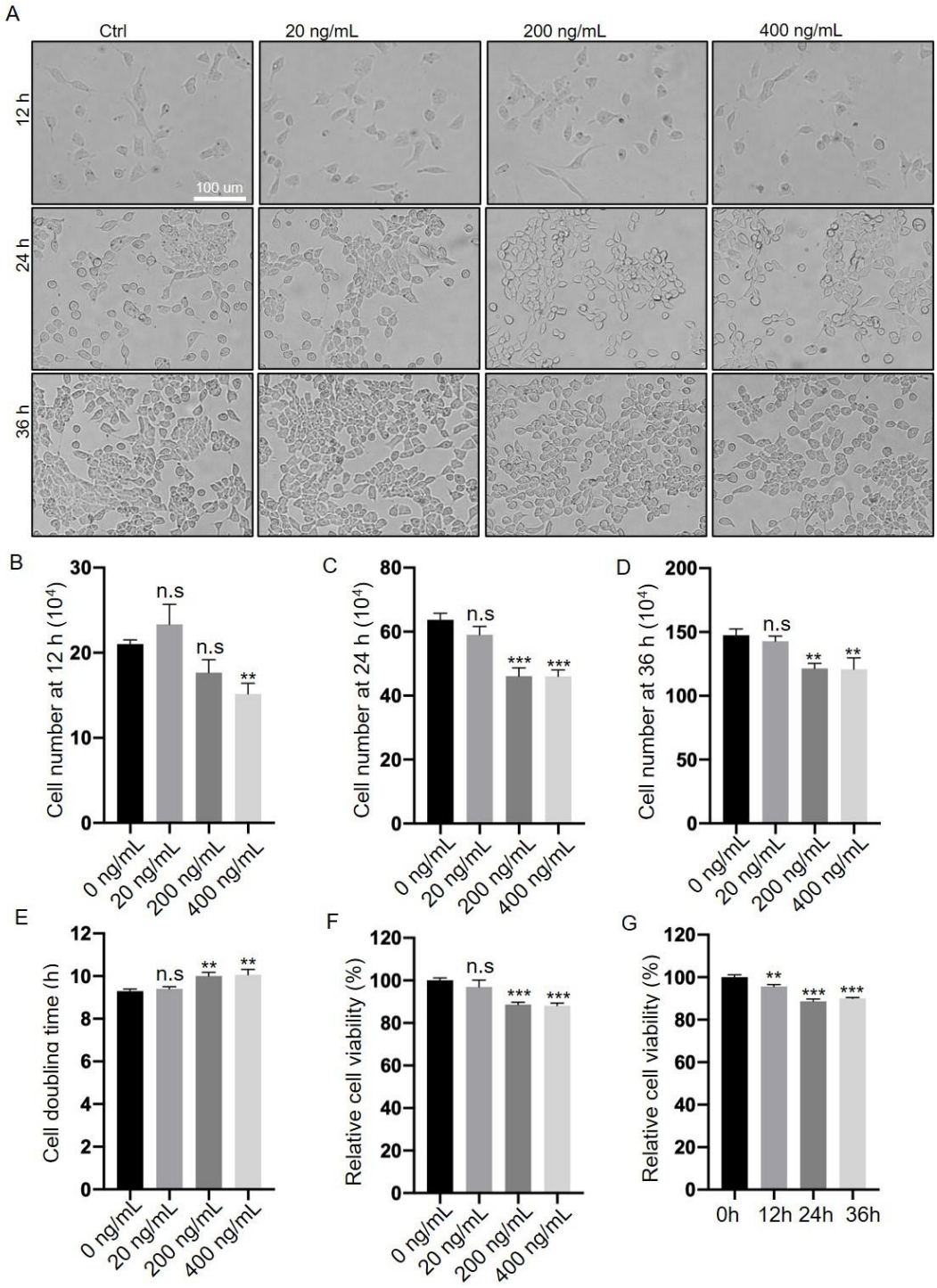


Figure 6.2 Sclerostin inhibits proliferation and viability of NE-4C. A. Representative brightfield images (x 10) of NE-4C treated with sclerostin for different time- and dose- courses. **B-C.** Cell number counting of NE-4C in different time points following sclerostin treatment. **E.** Cell doubling time calculated after sclerostin treatment for 36 hours. **F, G.** MTS assay for cell viability of NE-4C with sclerostin for different dose- (24 h) and time- (200 ng/mL) courses. Data are presented as mean ± SEM. n.s, no significance; **p* <0.05; ***p* <0.01; ****p* <0.001, one-way ANOVA of three culture triplicates.

6.4.3 Establishment and characterization of NE-4C neuronal differentiation system

To explore the impacts of sclerostin on neurogenesis, we first established and characterized an *in vitro* neuronal differentiation model using NE-4C cells (see Methods, Section 6.3.3). A schematic of the NE-4C neuronal differentiation system is provided in Figure 6.3A. Phase contrast images in Figure 6.3B show NE-4C stem cells forming neurospheres by day 6, which following dissociation and replating of cells differentiated into neuronal-like cells by day 12 (Figure 6.3 B). To confirm neuronal differentiation, gene expression analysis of the Oct 4 stem cell marker and Tuj1 neuron marker (*Tuj1*) was performed on NE-4C cells from day 0 to day 12 of the differentiation process. Gene expression analysis using PCR amplification revealed high *Oct4* mRNA levels on day 0, and low-level expression on day 12. In contrast, PCR amplification revealed low *Tuj1* mRNA levels on day 0, and high-level expression on day 12 (Figure 6.3C). Furthermore, immunocytochemistry revealed positive staining for the TUJ1 protein in processes extending from NE-4C neurospheres on day 6 and NE-4C differentiated cells on day 12. This data indicates that NE-4C stem cells could be differentiated into neuronal-like cells using the established neurogenesis induction protocol.

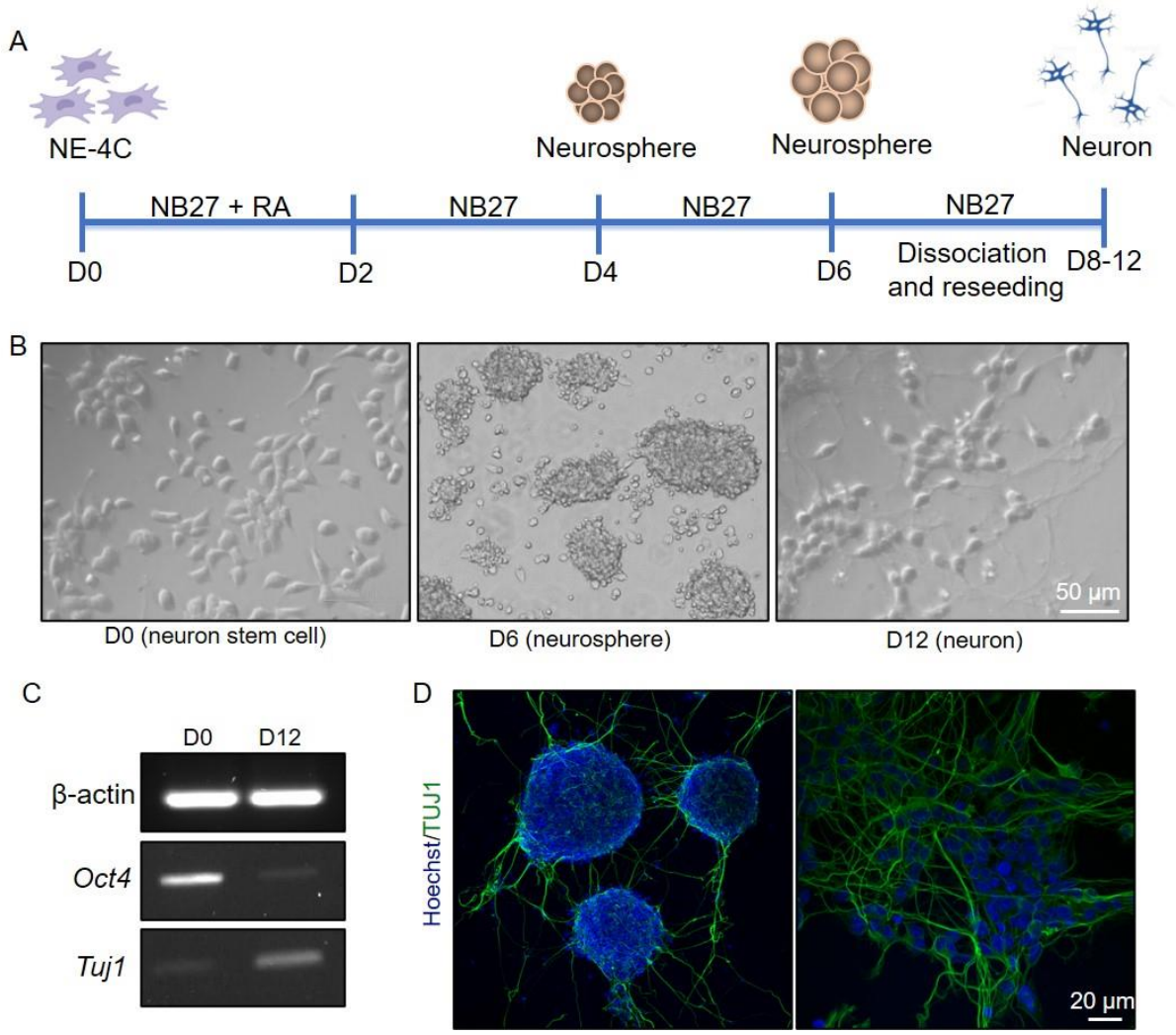


Figure 6.3 Establishment and characterization of neural differentiation with using NE-4C. A. Neural differentiation scheme. **B.** Phase contrast images of cells at different time points during differentiation. **C.** PCR amplification for neural stem cell gene (*Oct4*) and neural gene (*Tuj1*) expression on D0 and D12, β-actin was used as loading control. **D.** Immunostaining of the neurospheres on D6 (left) and neurons on D12 (right) with neuron marker (TUJ1, green) antibody. Cell nuclei are stained with Hoschst (blue).

6.4.4 Sclerostin inhibits NE-4C neurosphere formation

The formation of neurospheres in *in vitro* culture systems is considered a critical step in neurogenesis and neuronal development (Wong, Ussyshkin et al. 2016). To examine the effects of sclerostin on the formation of neurospheres, phase contrast images during the first 6 days of differentiation were analysed for morphological changes in neurospheres. It was observed that NE-4C cells began to aggregate to form neurosphere-like clumps on day 4 of differentiation. From day 4 onwards, the cell clumps slowly enlarged and formed round-like neurospheres by day 6 of differentiation (Figure 6.4A). Quantitative analysis of the neurospheres revealed that sclerostin treatment (200 ng/mL) resulted in the formation of a lower number of neurospheres, compared with the untreated control (Figure 6.4B). Morphometric measurement revealed that neurospheres exposed to sclerostin displayed shorter diameters compared with the untreated control (mean ± SEM: 92.77 ± 36.76 μm vs 101.10 ± 37.24 μm, $p = 0.13$), albeit not to a statistically significant level. These results indicate that sclerostin can inhibit the formation of neurospheres during neurogenesis.

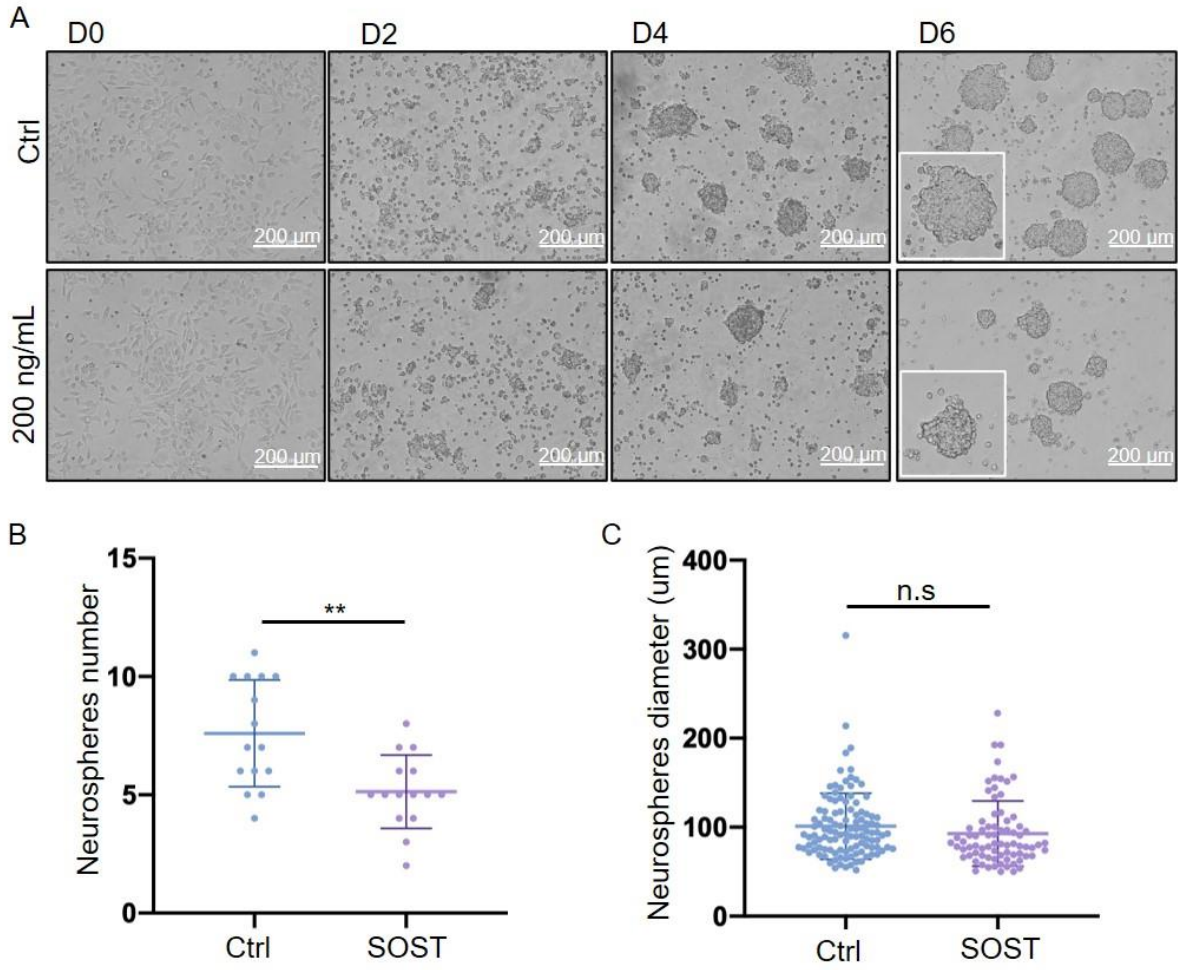


Figure 6.4 Sclerostin inhibits neurosphere formation. A. Representative phase contrast images (x 10) showing the development of neurospheres during differentiation in control and sclerostin-treated (200 ng/mL) group. Quantification of the number of neurospheres (**B**) and of their size (**C**) for each experimental group. Neurospheres form 5 random view fields are included for quantification, n.s, no significance, $^{**}p < 0.01$, student *t* test.

6.4.5 Sclerostin inhibits NE-4C cell differentiation

To confirm the adverse effects of sclerostin on neurogenesis, we studied the gene expression of *Oct4* and *Mapt* (a neural marker) throughout the development of neurospheres. The expression of *Oct4* was observed to decrease while *Mapt* expression was increased on both conditions during neurosphere formation (Figure 6.5A). Notably, we found that there was still a low expression level of *Oct4* detected on day 6 in cells treated with sclerostin, while such gene expression of *Oct4* was not detected in cells in control group, suggesting the existence of NE-4C cells after 6-day differentiation when sclerostin was administrated. In addition, the level of *Mapt* was found to be higher on day 6 in the control condition than the sclerostin-treated condition reflected by a higher band gray value in the PCR gel of the former than the latter, which suggested that a higher percentage of the cells were being differentiated into neurons in the normal condition than sclerostin-treated condition. Consistently, qPCR analysis of the gene expression on day 6 showed significantly higher expression of *Map2* (a neural marker) and lower expression of *Oct4* in the cells without sclerostin treatment compared to those with sclerostin treatment (Figure 6.5B, C). These results indicate that sclerostin was inhibiting NE-4C cell differentiation and neurogenesis.

To confirm the influence of sclerostin on neurogenesis, the expression of *Ngn2*, which is a transcription factor that activates genes required for neuronal differentiation (Hindley, Ali et al. 2012), was examined during the 6-day NE-4C differentiation period. qPCR analysis revealed that *Ngn2* mRNA levels increased during the NE-4C differentiation period, however lower levels were observed in cells treated with sclerostin at day 2 and day 4 compared with untreated cells (Figure 6.5D). These results provide additional evidence indicating that sclerostin inhibits neurogenesis, and that the mechanism involves altering the expression of genes involved in neuronal differentiation.

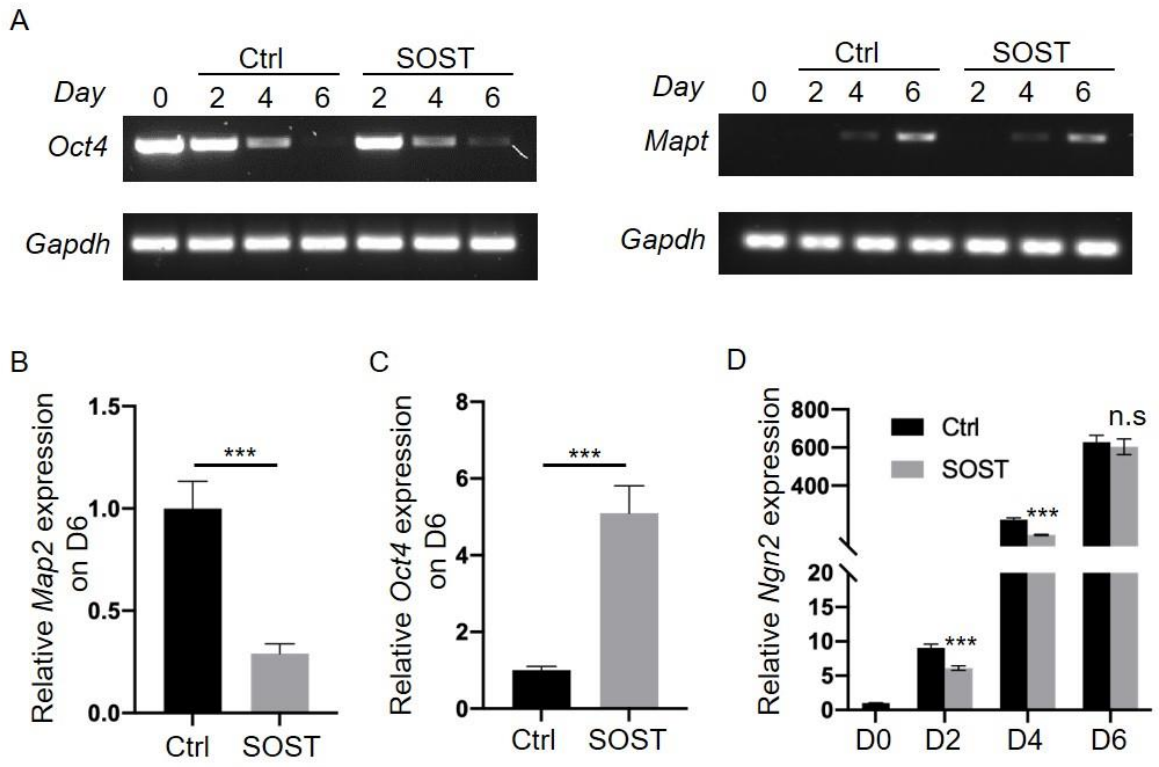


Figure 6.5 Sclerostin slows the expression of neural gene expression during differentiation. A. PCR amplification for gene expression of neural stem cell gene (*Oct4*) and neural gene (*Mapt*) during differentiation. **B, C.** qPCR analysis for gene expression of neural gene (*Map2*) and neural stem cell gene (*Oct4*) on D6. **D.** qPCR analysis for gene expression for *Ngn2* during differentiation. NE-4C cells were exposed to vehicle (control) or sclerostin (200 ng/mL) during differentiation. Data are presented as mean ± SEM, n.s, no significance, ***p*< 0.01, student *t* test.

6.4.6 Sclerostin inhibits neuronal dendritic spine formation

In addition to the neuronal gene expression changes during neurogenesis, dendritic spine formation is a critical process for the structural development of newly formed neurons into mature functioning neurons. Therefore, the impact of sclerostin on dendritic spine formation was examined in mature neurons derived from neurospheres. Immature neurons derived from NE-4C cell neurospheres were exposed to sclerostin for 6 days and maturing neurons were labelled by transfecting cells with a CMV-GFP lentivirus 48 hours prior to dendritic spine and cell morphology assessment (6 days after neurosphere dissociation and cell reseeding). Cell imaging revealed that maturing neurons exposed to sclerostin formed fewer filopodia-like dendritic spines compared with the untreated cells (Figure 6.6A, B). This result indicates that sclerostin inhibits the development of dendritic spines.

Postsynaptic density 95 (PSD95) protein is a regulator for neuron morphological maturation and promotes the formation of dendritic spines (Nikonenko, Boda et al. 2008, Mardones, Jorquera et al. 2019). Importantly, PSD95 is a downstream target of the canonical Wnt signalling pathway (Fortress, Schram et al. 2013, Ramos-Fernandez, Tapia-Rojas et al. 2019). Therefore, I examined the impact of sclerostin on PSD95 expression in maturing neurons derived from NE-C4 cell neurospheres using Immunocytochemistry and western blot analysis. Immunocytochemistry of maturing neurons 6 days after neurosphere dissociation revealed reduced PSD95 staining in cells treated with sclerostin compared with untreated cells (Figure 6.6C, D). Similarly, western analysis demonstrated decreased PSD95 protein expression in sclerostin treated cells (Figure 6.6E). In addition, β-catenin levels, a protein also involved in Wnt signalling were also reduced in cells treated with sclerostin (Figure 6.6E). Together this data indicates that sclerostin inhibits PSD95 expression by inhibiting Wnt signalling and in doing so retards neuronal dendritic spine development.

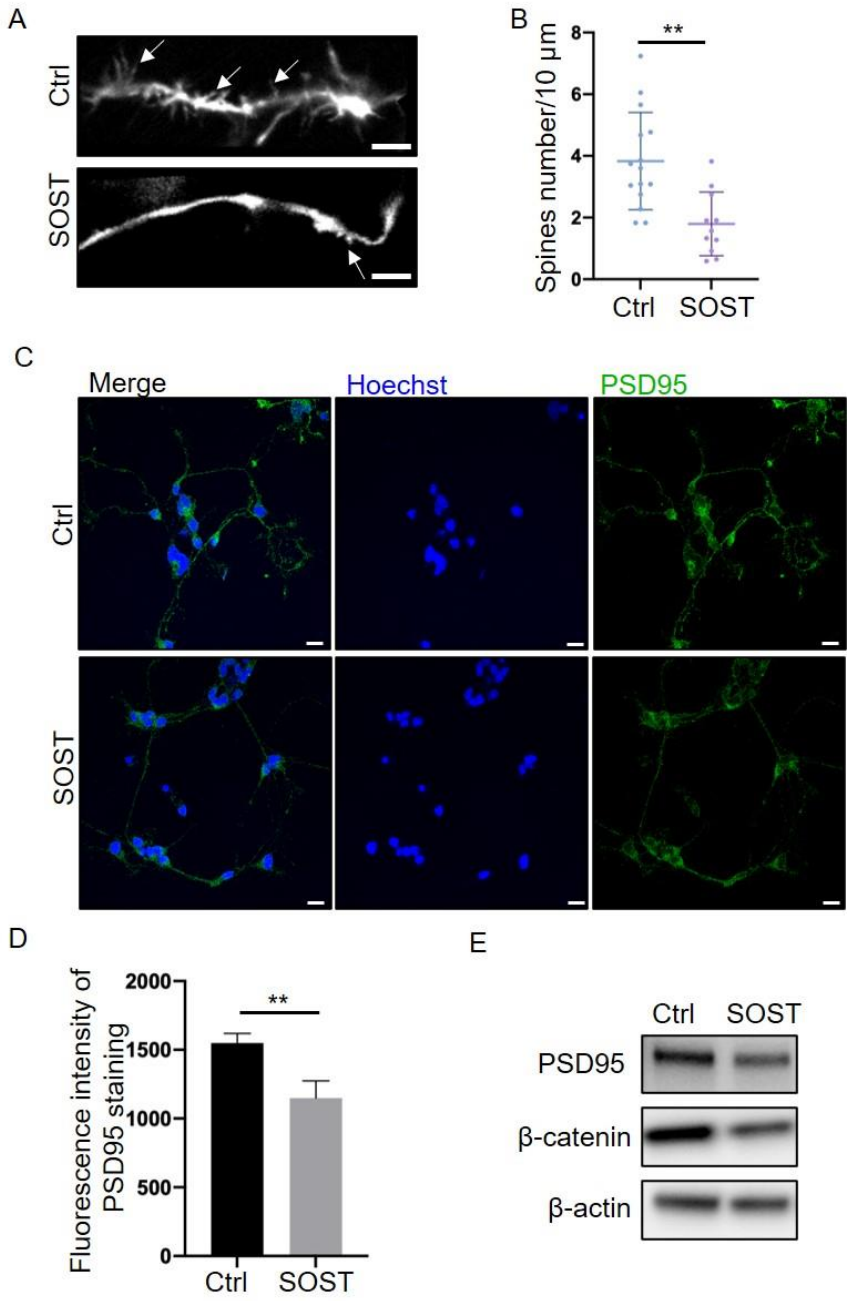


Figure 6.6 Sclerostin inhibits neuron spine formation. A. Representative confocal images of neuron spines. Neurons are differentiated from NE-4C treated with vehicle or sclerostin (200 ng/mL), and immunostained on day 14. White arrow refers to the neuron spines, scale bar = 2 μm. **B.** Quantification for spines number per 10 μm on the dendrites in each experimental group. Over 10 neurons of each group were included for quantification. ***p* <0.01, student *t* test. **C.** Representative confocal images of neurons immunostained with post-synapse protein (PSD95) on day 14. Scare bar = 10 μm. **D.** Quantification of fluorescence intensity of PSD95, data are presented as mean ± SEM. ***p* <0.01, student *t* test. **E.** Western blotting analysis for protein expression of PSD95 and β-catenin of neurons in each group on day 14. β-actin was used as loading control.

6.5 Discussion

Sclerostin is widely regarded as an osteocyte-specific protein (Weivoda, Youssef et al. 2017), despite its mRNA being detected in multiple tissues including cartilage, kidney, heart and liver (Balemans, Ebeling et al. 2001, Brunkow, Gardner et al. 2001, Padhi, Jang et al. 2011). The Wnt signalling pathway is known to be important in neurogenesis, however, the impact of sclerostin, as an inhibitor of Wnt signalling, on the formation and development of neurons has not been fully investigated. In this Chapter, we hypothesized that sclerostin contributes to AD onset development due to its negative impact on neurogenesis as a result of its inhibitory effects on Wnt signalling.

Using an *in vitro* NE-4C stem cell culture system, it was demonstrated that sclerostin inhibited neurogenesis. Sclerostin reduced the expression of genes associated with neuronal differentiation and decreased neurosphere formation, as well as adversely impacting the development of dendritic spines on maturing neurons. Importantly, it was also demonstrated that osteocyte-like MLO-Y4 cells overexpress sclerostin when exposed to AGEs, which is an ageing associated stress. Given that osteocytes are the main producers of sclerostin *in vivo*, these findings provide evidence that increasing plasma sclerostin levels that occur with ageing, as demonstrated in Chapter 5, originate from bone. Furthermore, increasing age is also a major risk factor for AD and neurogenesis is considered to be an early pathological feature in AD (Moreno-Jimenez, Flor-Garcia et al. 2019, Tobin, Musaraca et al. 2019). Therefore, taken together, these findings support a pathophysiological mechanism whereby bone-derived sclerostin contributes to AD onset and progression by inhibiting neurogenesis and thereby further establishes a link between bone and brain.

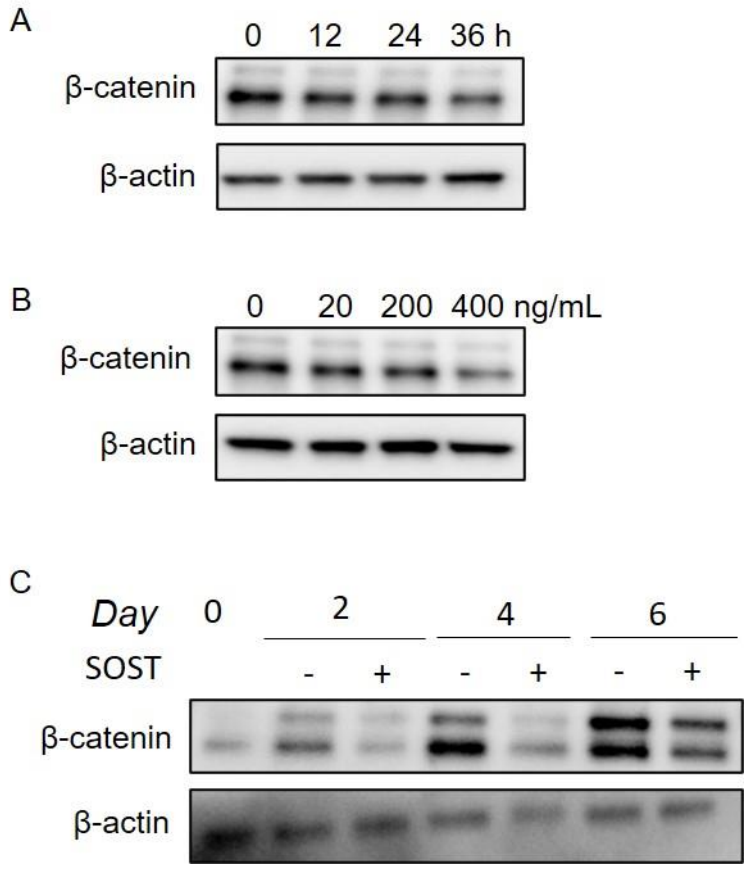
Results obtained in this study for sclerostin share some similarities with the bone-derived protein, osteocalcin. Osteocalcin regulates many physiological functions and developmental

processes, including testosterone secretion and glucose metabolism (Lee, Sowa et al. 2007, Oury, Sumara et al. 2011). Importantly, osteocalcin was the first bone-derived hormone reported to be linked to the functions involving the brain (Karsenty and Olson 2016, Obri, Khrimian et al. 2018). For example, osteocalcin knockout mice display altered brain development and function (Oury, Khrimian et al. 2013). Therefore, given the results in this Chapter, it is likely sclerostin is another bone-derived protein involved in crosstalk between brain and bone. Further studies in genetically modified mouse models will be needed to support the findings in the present study, and confirm the link between increasing sclerostin levels and AD onset. For example, assessing the cognitive function of transgenic mouse that over-express sclerostin can provide clues on whether there is causal relationship between bone-derived sclerostin and early onset of AD. Furthermore, the effects of sclerostin over-expression in AD mouse models would provide additional evidence to confirm a potential role of the bone-derived protein in AD pathobiology.

In summary, the results obtained in this Chapter support a pathobiological mechanism whereby osteocyte-secreted sclerostin contributes to the development and progression of AD. The results obtained in this study, along with the association of increasing plasma sclerostin levels and brain Aβ load in humans, further supports a critical role of osteocyte in the ageing-related brain disorder, AD.

6.6 Supplementary

Figure S6.1 Validation of the inhibiting impacts of SOST on NE-4C before (A, B) and during neural differentiation (C).



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CHAPTER 7

General Discussion

7.1 Overview

Due to the recent advancements in molecular, biochemical and cellular technologies, the diverse biological roles of osteocytes in bone have become increasing apparent. Besides their role in helping to maintain bone homeostasis, osteocytes also possess the ability to secrete an array of hormone-like molecules, which can influence the function of not only bone, but also remote organs. In this thesis, a major aim was to investigate the involvement of osteocyte endocrine-like functions in orchestrating ageing-related diseases involving bone and the brain, focusing on osteoporosis and Alzheimer's disease (AD).

Osteoporosis is the most common disorder affecting bone in older adults. A variety of factors contribute to the decline of bone mineral density with ageing, including clinical situations involving the long-term intake of glucocorticoids. Prolonged glucocorticoids exposure enhances bone resorption by increasing osteoclastogenesis and inhibits bone formation by decreasing osteoblastogenesis (Canalis 2004), two processes that involve osteoclasts and osteoblasts, respectively. In addition, glucocorticoid usage can also stimulate the apoptotic death of osteocytes during bone loss (Canalis, Mazziotti et al. 2007). Notably, a previous study has reported that the production of cathepsin K, which is a protease highly expressed in osteoclasts, is responsible for bone matrix degradation under glucocorticoid stress (Jia, Yao et al. 2011). However, a role for cathepsin K secreted by osteocytes in bone loss associated with glucocorticoid stress has not been previously investigated. Therefore, to study the function of osteocyte-derived cathepsin K in glucocorticoid-induced bone loss, we examined the relationship between cathepsin K and the morphological changes of extracellular type I collagen in an *ex vivo* primary osteocyte culture system. Furthermore, we also investigated the molecular mechanisms regulating glucocorticoid-induced overproduction of cathepsin K in osteocytes (Chapter 4).

Beyond affecting bone itself, several bone-derived hormones, namely osteocalcin, FGF23 and leptin can influence the function of other organs, including the kidney and brain. Given the higher prevalence of AD in individuals with low bone mineral density, we hypothesized that certain hormone-like molecules derived from bone may participate in the onset and progression of AD (Chapter 2). To this end, this thesis examined a role for the osteocyte secreted protein, sclerostin, in contributing to the development of AD in individuals with decreased bone mineral density. To demonstrate the association between sclerostin and AD, plasma sclerostin concentrations were analyzed in a cohort of older adults with increased risk of developing AD (Chapter 5). Positive correlations between plasma sclerostin levels and AD risk led to *in vitro* studies examining the underlying molecular mechanisms whereby sclerostin may be contributing to AD (Chapter 6).

Together, this thesis has uncovered several key and novel findings which broadened the spectrum of osteocyte functions in orchestrating osteoporosis and AD. The findings of this thesis and their significance are discussed below.

7.2 Significance of findings arising from this thesis

7.2.1 Osteocyte-derived cathepsin K and bone loss

7.2.1.1 *Glucocorticoids induce osteocytes to secrete cathepsin K which degrades type I collagen*

Osteocytes are known to express cathepsin K, and deletion of the cathepsin K gene in osteocytes prevents lacunar enlargement and bone resorption during lactation in mice (Qing, Ardeshirpour et al. 2012, Lotinun, Ishihara et al. 2019). Consistent with this, we demonstrated that *ex vivo* cultured primary osteocytes increased their expression of cathepsin K mRNA and protein when exposed to glucocorticoids. Importantly, the increased expression of cathepsin K in osteocytes augmented degradation of type I collagen in bone matrix.

This is a significant and novel finding as it provides strong evidence that like osteoclasts, osteocytes also have the capacity to remove bone matrix by secreting cathepsin K. While these findings indicates that osteocyte-derived cathepsin K may contribute to glucocorticoid-induced bone loss, other mechanisms involving the protease may also be involved. For example, cathepsin K can degrade insulin growth factor 1(IGF-1) and bone morphogenetic protein 2 (BMP-2), which are potent stimulators of bone formation (Fuller, Lawrence et al. 2008). Therefore, bone loss as a result of long-term usage of glucocorticoids may arise by osteocyte secreted cathepsin K degrading bone extracellular matrix, as well as initiating matrix-derived growth factor degradation, effects which are likely to promote bone loss as a result of GC over-usage.

7.2.1.2 *Cathepsin K production in osteocytes is modulated by PINK1-mediated mitophagy*

After confirming the increased production of cathepsin K in osteocytes following glucocorticoid exposure, possible mechanisms modulating this process were explored. In this regard, it was demonstrated that exposure of osteocyte-like MLO-Y4 cells to glucocorticoid stimulated PINK1-mediated mitophagy, which substantially modulates the production of cathepsin K in osteocytes, thereby contributing to GC-induced bone loss.

The concept of osteocytic osteolysis was firstly proposed in the 1960s based on the observation of enlarged lacunae with irregular borders, rough walls and varying degrees of perilacunar demineralization surrounding mature osteocytes (Baud 1962). Nevertheless, the concept of osteocytes reabsorbing bone was subsequently disregarded as osteoclasts were considered the principal cell responsible for bone resorption. However, studies have now confirmed the earlier observations demonstrating that osteocytes are able to remodel their perilacunar and canalicular matrix and participate in a bone-resorption using the same acid-protease mediators as those used by osteoclasts (Qing, Ardeshirpour et al. 2012, Lotinun, Ishihara et al. 2019). These

findings provide strong justification to reconsider the possibility of 'osteocytic osteolysis' and to consider its role in the regulation of bone and mineral metabolism (Wysolmerski 2012). Our findings in Chapter 4 provide new evidence supporting the concept of osteocytic osteolysis, which help to explain the role of osteocytes in GC-induced bone loss.

7.2.2 Osteocyte-derived sclerostin and AD

7.2.2.1 *Elevated plasma sclerostin levels in cognitively normal adults with high risk of developing AD*

In Chapter 5, we investigated the association between plasma sclerostin, which is a protein primarily produced by osteocytes and brain Aβ load, a pathological hallmark of AD. This study revealed that plasma sclerostin concentrations were significantly elevated in cognitively healthy older individuals classified as having abnormal Aβ deposits in their brain, before and after adjusting for covariates of age, gender and APOE ε4 status. Furthermore, it was demonstrated that sclerostin increased the diagnostic accuracy for AD when combining a base model built with AD risk factors of age, gender and APOE ε4 status compared to using the base model alone. Together these results identified a potential pathogenic association between bone and brain in older individuals, and highlights the possibility that plasma sclerostin levels may represent an early diagnostic biomarker for AD.

Sclerostin, a bone-derived protein has the potential to influence brain function by inhibiting the Wnt signalling pathway, which is important for neurogenesis and maintaining neuronal synaptic plasticity (Chapter 2). For this reason, sclerostin may contribute to the pathogenesis of AD by inhibiting Wnt signalling activity. In support of this, Dickkopf-related protein 1 (Dkk1), another canonical Wnt antagonist, is up-regulated in the brains of AD patients and AD mouse models (Caricasole, Copani et al. 2004, Rosi, Luccarini et al. 2010). Moreover, induced overexpression of Dkk1 in AD models, results in reduced synaptic transmission in the

hippocampus along with long-term memory deficits (Galli, Lopes et al. 2014, Marzo, Galli et al. 2016). In addition, loss of Wnt signalling may contribute to the aggregation of Aβ42 peptide, which is an important molecule found in amyloid plaques and believed to contribute the pathobiology of AD (Tapia-Rojas, Burgos et al. 2016). Therefore, given the above information regarding Wnt signalling antagonist Dkk1 in AD, it is possible that sclerostin activates similar pathogenic mechanisms contributing to AD onset.

7.2.2.2 Sclerostin inhibits neurogenesis and dendritic spine formation in an in vitro neuronal differentiation model

Decreased neurogenesis is believed to be early pathological event contributing to AD (Moreno-Jimenez, Flor-Garcia et al. 2019, Scopa, Marrocco et al. 2020). As mentioned above, Wnt signalling activity is involved in neurogenesis (Garbe and Ring 2012, Schafer, Han et al. 2015, Arredondo, Guerrero et al. 2020, Arredondo, Valenzuela-Bezanilla et al. 2020), and for this reason, we hypothesized that sclerostin inhibits neurogenesis (Chapter 6). In support of this hypothesis, this thesis demonstrated that sclerostin reduced the maturation of *in vitro* NE-4C derived neurospheres, as well as reducing the development of dendritic spines on neurons derived from neurospheres. These findings support a mechanism whereby bone-derived sclerostin inhibits neurogenesis and synaptic activity, thereby contributing to the pathogenesis of AD. However, it still remains to be determined if sclerostin contributes to the amyloidogenic Aβ changes in the brain.

7.2.2.3 Sclerostin and the bone-brain axis

The central nervous system regulates the functions of many organs in the body, however, it is becoming increasingly apparent that some organs also signal back to brain. For example, the gut-brain axis is now well established, in particular the impact of gut microorganism-derived products directly or indirectly impacting the central nervous system. In a similar way to the

gut-brain axis, the bone-brain axis is now recognized to be a novel biological target warranting attention when investigating the pathogenesis of diseases relating to brain. Importantly, sclerostin and osteocalcin are two bone-derived hormones that may be involved in the bone-brain crosstalk. In support of this, a recent study has demonstrated that higher bone mineral density was associated with a lower brain white matter hyperintensity burden and less domain-specific, cognitive impairment, which represent two important indicators of neurodegeneration (Stefanidou, O'Donnell et al. 2021). Another significant finding was that the skeleton is able to influence cognition and contribute to the maternal influence on fetal brain development via a hormone secreted by osteoblasts, namely osteocalcin (Oury, Khrimian et al. 2013). Consistent with these findings, several clinical studies have shown an association between decreased serum osteocalcin levels and cognitive decline (Bradburn, McPhee et al. 2016, Nakamura, Imaoka et al. 2021). Data generated in this thesis now provides further evidence for a bone-brain axis, by demonstrating that sclerostin, a protein predominantly secreted by osteocytes, can negatively impact neurogenesis and dendritic spine formation. Importantly, these findings combined with the observation of increased levels of sclerostin in the plasma of individuals with increased Aβ in their brain, provides evidence that the protein may also contribute to AD onset.

7.3 Limitations

This thesis aimed to explore the local and remote endocrine-like functions of osteocytes, and in particular the protein sclerostin, through *in vitro*, *ex vivo*, and human cohort studies. In doing so, the thesis generated data supporting a role of osteocytes in orchestrating two age-related disease, osteoporosis and AD. However, there are several limitations of this thesis that need to be acknowledged, and these are discussed below.

In Chapter 4, to investigate the role of osteocytes in osteoporosis, glucocorticoid-induced bone loss was employed to mimic the diminished bone density that occurs during ageing. Although endogenous glucocorticoids increase in the body with age and play an important role in ageing physiology, their contribution to osteoporosis may differ to bone loss associated with exogenously administered glucocorticoids. Therefore, the role of cathepsin K studied in glucocorticoid-treated osteocytes may differ in osteocytes in bone during the ageing process.

In addition, to examine the degradation effects of cathepsin K on extracellular type I collagen, an *ex vivo* primary osteocyte culture system was utilized. While the *ex vivo* osteocyte culture system provides a method to mimic the *in vivo* situation, immunohistochemical studies using calvaria sections from glucocorticoid-treated mice are required to confirm the ability of osteocytes to express cathepsin K and degrade type I collagen in bone lacunae. Moreover, it will be critical to confirm that the overproduction of cathepsin K in osteocytes is biologically meaningful, specifically whether glucocorticoid-triggered release of cathepsin K from osteocytes contributes to the pathophysiology of glucocorticoid-induced bone loss. These studies could consist of treating older mice with glucocorticoids followed by immunohistochemical examination of bone sections for osteocytes and cathepsin K expression and bone degradation.

The patient cohort study described in Chapter 5 included an uneven number of male and female individuals, and *AOPE* ε4 allele carrier status was not evenly distributed in both the Aβ- and Aβ+ groups. Thus, the make-up of the patient cohort may explain why a significant difference in plasma sclerostin concentrations was observed in females and *APOE* ε4 non-carriers, but not in male and *APOE* ε4 carriers. Furthermore, the modest sample size and the cross-sectional nature of this cohort study may not have uncovered the full picture regarding sclerostin and its impact in AD. It should also be mentioned that given ageing is a major AD risk factor, the

increased plasma sclerostin levels observed with ageing in this and other studies, may be correlative rather than causative of AD, however, this does not appear to be the case as there was no statistically significant difference between the age of participants in the Aβ- and Aβ+ groups. Nevertheless, the mean age of the individuals of Aβ+ status was slightly higher, than those of Aβ-, and therefore additional studies with larger patient cohorts are required to confirm a causative link between plasma sclerostin levels and brain Aβ amyloidogenesis. Lastly, while this thesis demonstrated an association between elevated plasma sclerostin levels and Aβ status in the brain, a causative relationship between these two biological entities has not been established.

Similarly, the findings obtained in Chapter 6 should be interpreted with caution, given the studies were performed using *in vitro* cell models. Therefore, the inhibitory effects of sclerostin on neurogenesis and dendritic spine formation need to be confirmed in animal models. Furthermore, it needs to be established whether the adverse effects of sclerostin on neurogenesis and synaptic function are directly linked to cognitive dysfunction and other relevant AD symptoms.

7.4 Future directions

Findings from this thesis raise a number of questions regarding the role of osteocytes in osteoporosis and AD. To address these questions, further studies required are discussed below.

Firstly, as mentioned in Chapter 4, the degradative process mediated by cathepsin K on extracellular type I collagen relies on an acidified microenvironment. To degrade bone matrix including type I collagen, osteoclasts release protons (H^+) and chloride ions (Cl^-) into the lacunae to obtain an acidic environment with a pH of 4.5 for optimal cathepsin K activity (Yoneda, Hiasa et al. 2015). Hence, it will be important to demonstrate that osteocytes also have the capacity to release H^+ and Cl- to provide an acidic condition for cathepsin K to degrade

type I collagen. For example, it would be useful to establish an animal model, in which the acidifying function of the osteoclast is modulated and the pH of the lacunae where osteocytes reside could be examined. In addition, to fully simulate the effects of an ageing cellular environment, the impact of osteocytes in osteoporosis pathological mechanisms including glucocorticoid treatment need to be investigated using older animals, for example aged mice.

Secondly, according to the results obtained using a BBB penetrating prediction program (B3Pred), sclerostin possesses an appropriate amino acid content indicating that it is likely to traverse the BBB. However, future studies are required to track the path of bone-derived sclerostin *in vivo* to confirm its capacity to enter the brain. At present, some methodologies have been developed to determine the ability of molecules and proteins in the peripheral serum to enter into the brain and affect neurons. For example, immunocytochemistry has been used to confirm biotin labelled osteocalcin can enter the brain and bind to neurons (Oury, Khrimian et al. 2013). In addition, mesenchymal stem cell-derived exosomes labelled with gold nanoparticles have been observed entering the brain using computed tomography (CT) imaging (Betzer, Perets et al. 2017). Therefore, future studies could use these and similar techniques to verify the capability of sclerostin to cross the BBB *in vivo* and to bind to neurons.

Thirdly, this thesis used an *in vitro* culture system to examine the adverse effects of sclerostin on neurogenesis, however, it is still unclear whether sclerostin is involved in Aβ production or accumulation, which is the major pathological alteration of AD. Studies designed to investigate the direct influence of sclerostin on the production of Aβ in neurons using *in vitro* and/or *in vivo* models are thus required to establish a causative relationship between sclerostin and Aβ formation. These studies could consist of exposing *in vitro* cultured primary neurons to sclerostin or treating aged mice or AD mice with the protein. Once a causative link is established between sclerostin and Aβ generation, novel therapies could be designed to

suppress the effects of sclerostin in the brain or inhibiting its ability to traverse the BBB, in addition to its application as a diagnostic marker.

Finally, this thesis demonstrated a significant association between plasma sclerostin levels and brain Aβ load in a cohort of cognitively healthy participants. In addition, further stratified analysis, revealed a positive correlation between sclerostin and Aβ standard uptake value ratio (SUVR) independent of sex and *APOE* ε4 carriage, indicating that plasma sclerostin is a critical factor for abnormal Aβ deposition. These findings now provide the justification for further longitudinal studies employing larger cohorts to extend the link between plasma sclerostin and AD, for example, comparing the levels of sclerostin in healthy participants, individuals with mild cognitive impairment, and in patients with AD.

7.5 Concluding remarks

In this thesis, evidence is provided supporting the novel concept that osteocytes possess an endocrine-like function and have the ability to influence not only bone, but also more remote organs such as the brain. Due to this capability, osteocytes therefore have the potential to participate in the pathogenesis of two common age-related degenerative disorders, osteoporosis and AD. Thus, further studies are now required to investigating the biochemical, molecular and genetic associations between sclerostin secreted by osteocytes and the onset of AD. Specifically, the results provide justification for additionally studies to understand more fully the spectrum of functions of sclerostin in the pathogenesis of osteoporosis and AD. Overall, the findings obtained in this thesis broaden the spectrum of the biological functions of the osteocyte, namely, that it can participate in the pathogenesis of ageing-related diseases affecting both bone and distant body systems, through the secretion of hormone-like proteins.

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APPENDIX

Journal of Alzheimer's Disease 69 (2019) 59–70
DOI 10.3233/JAD-181249
IOS Press

Review

The Potential Influence of Bone-Derived Modulators on the Progression of Alzheimer's Disease

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Accepted 19 February 2019

Abstract. Bone, the major structural scaffold of the human body, has recently been demonstrated to interact with several other organ systems through the actions of bone-derived cells and bone-derived cell secretory proteins. Interestingly, the brain is one organ that appears to fall into this interconnected network. Furthermore, the fact that osteoporosis and Alzheimer's disease are two common age-related disorders raises the possibility that these two organ systems are interconnected in terms of disease pathogenesis. This review focuses on the latest evidence demonstrating the impact of bone-derived cells and bone-derived proteins on the central nervous system, and on how this may be relevant in the progression of Alzheimer's disease and for the identification of novel therapeutic approaches to treat this neurodegenerative disorder.

Keywords: Alzheimer's disease, bone, mesenchymal stem cells, microglia, osteocalcin

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, constituting approximately half of all dementia cases, and predominantly affects individuals older than 65 years of age [1, 2]. Osteoporosis (OP) is a bone degenerative disorder that also occurs mainly in the aging population [3]. Due to an aging human population and increased life expectancy, AD and OP pose a significant social burden worldwide [4, 5]. It is reported that AD ranks as the sixth most common cause of death [4], while the 12-month mortality rate from hip and spine fractures caused by OP is in excess of 20% [6].

Although AD and OP appear as seemingly two independent diseases, they share several epidemiological features, especially among women over

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ISSN 1387-2877/19/$35.00