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Establishment of a co-culture model of osteoblasts and osteoclasts to study osteoporotic alterations caused by cigarette smoking: Role of Bisphosphonates

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2D	Two-dimensional
3D	Three-dimensional
AP	Alkaline phosphatase
BMD	Bone mineral density
BMP	Bone morphogenic proteins
BPs	Bisphosphonates
CA	Contrast agent
cAMP	Adenosine monophosphate
CO	Carbon monoxide
CS	Cigarette smoking
CSE	Cigarette smoke extract
CT	Calcitonin
EGF	Epidermal growth factor
FGFs	Fibroblast growth factors
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
HGF	Hepatic growth factor
IGFs	Insulin-like growth factors
MCS-F	Macrophage colony–stimulating factor
OPG	Osteoprotegerin
PDGF	Platelet derived growth factor
PMA	Phorbol-12-myristate 13-acetate
PTH	Parathyroid hormone
RANKL	the Receptor activator of NF- ligand
RNS	Reactive nitrogen species
ROS	Reactive oxygen
S1P	Sphingosine-1-phosphate
SOST	Sclerostin cyclic
SPECT/CT	Single-photon emission computed tomography
SRB	Sulforhodamine B
SRY	Sex-determining region Y
TGF	Transforming Growth Factor
TRAP	Tartrate-resistant acid phosphatase

Chapter 1

Introduction

1.1. Bone is a dynamic and synthetic organ that is regulated by different types of cells and numerous interrelated factors

1.1.1. Bone cells

Bone cells originate from two cell lines: osteoprogenitor cells from the mesenchymal stem cell lineage, which differentiate into osteoblasts and osteocytes; and hematopoietic stem cells, which are able to differentiate into osteoclasts (Florencio-Silva *et al.* 2015). Osteoprogenitor cells are "stem cells" that can undergo migration, proliferation, and differentiation into osteoblasts upon receiving the relevant signal (Clines 2010). Osteoblasts are considered bone forming cells as they synthesize and secrete organic bone matrix (Lee *et al.* 2017). Osteoblast differentiation and activation is modulated by several mediators, including proteins of the transforming growth factor (TGF) and bone morphogenic protein (BMP) families (Wu *et al.* 2016). Osteoprogenitor cells can either remain quiescent osteoblasts, continue to differentiate into osteocytes, or return to bone stem cells. Osteoclasts are multinucleated cells that reside in Howship lacunae (shallow depressions on the bone surface) (Matic *et al.* 2016). During the bone healing and remodeling processes, osteoclasts can also reside in cutting cones (deep resorption cavities) (Buenzli *et al.* 2012). As bone-resorptive cells, osteoclasts solubilize bone matrix via acidification after attaching to it through the brush border, allowing matrix phagocytosis (Yuan *et al.* 2016). A multitude of known factors and cytokines that regulate osteoclast differentiation and function are secreted by osteoblasts, including osteoprotegerin (OPG), the receptor activator of NF-κB ligand (RANKL), and macrophage colony–stimulating factor (MCS-F) (Kim and Koh 2019).

1.1.2. Bone modeling and remodeling

Endochondral ossification and appositional bone growth are two basic modes of osteogenesis, with the former occurring in long bones and the latter in radial bones. Modeling of facial bones occurs primarily though, but not limited to, the process of appositional bone growth (Xie *et al.* 2017). In addition, both endochondral ossification

and intramembranous ossification occur in the craniofacial skeleton during development. Cranium modeling is associated with mechanotransduction signals from potential brain growth (Catala *et al.* 2019). There is a clear relationship between changes in the skeleton and the stresses placed upon it. According to Wolff's law, bones undergo resorption during periods when the bone does not experience stress, while it is reinforced in the presence of stress (Elliott *et al.* 2016).

Bone modeling plays a crucial role in acquiring peak bone mass of human, whereas bone remodeling occurs to modulate the formation, resorption and replacement of bone. Bone remodeling is essential to maintain bone health, regulate calcium homeostasis and repair micro-damaged bones, and this process relies on the balance between bone formation and resorption (Zaidi 2007). Thus, the coupling of osteoclastic and osteoblastic function plays an indispensable role in bone remodeling. The bone turnover rate of an adult is approximately 2% to 3% per year through adulthood until the time of death (Ambroszkiewicz *et al.* 2018).

Six main sequential phases are included in the remodeling cycle: quiescence, activation, resorption, reversal, formation, and termination. Bone remodeling starts upon receiving an initiating remodeling signal after osteoclastic resorption. During the resorption phase, osteoclast precursors are recruited to the target site by osteoblasts that respond to signals from osteocytes or as a result of direct endocrine activation. Almost all osteoclasts disappear in the subsequent reversal phase, and they are completely replaced by osteoblastic cells in the formation phase. Osteoblasts finish differentiation in the termination phase. The result of each cycle of bone remodeling is the production of a new osteon or trabecular packet (Arias *et al.* 2018; Delaisse 2016).

1.1.3. Regulation of bone remodeling

Bone remodeling is regulated by multiple local and systemic factors. Systemic hormonal regulators like Calcitonin (CT), Parathyroid hormone (PTH), vitamin D3 and estrogen play the most important roles in regulating osteoclastic bone resorption (Yu-Yahiro *et al.* 2001). Additionally, growth factors such as insulin-like growth factors (IGFs), BMPs, TGF-β, fibroblast growth factors (FGFs), WNTs, and epidermal growth

factor (EGF) are also currently considered crucial regulators of physiological bone remodeling (Ohba *et al.* 2012). Based on this, hormonal and growth factor-based regulators could be potential alternatives to classical drugs to treat bone diseases such as osteoporosis.

1.1.4. Osteoblast and osteoclast interaction

Osteoblasts and osteoclast are two of the most important cells in the bone remodeling process, and they do not act independently. Several communication pathways have been previously described. Firstly, osteoblast lineage cells have an internal regulatory mechanism; for example, sclerostin (SOST) secreted by osteocytes become a negative feedback for osteoblasts, when the surrounding mineral is overproduced (Spatz *et al.* 2015; Zhu *et al.* 2018). Secondly, osteoblasts/osteocytes can communicate with osteoclasts through direct contact. Gap junctions formed when osteoblasts contact osteoclasts directly, allowing small water-soluble molecules to pass between them. The main molecules include cyclic adenosine monophosphate (cAMP), calcium ions (Weivoda *et al.* 2016), nucleotides (Singh *et al.* 2018), inositol trisphosphate receptor (InsP3R) (Kirkwood *et al.* 1996), amino acids, vitamins, and saccharides (Myneni and Mezey 2017) which are responsible for the proliferation and differentiation of osteoblasts and osteoclasts. Moreover, the paracrine pathway is considered another fundamental communication method for cell-cell communication, where paracrine factors secreted by neighboring cells via diffusion (Wong *et al.* 2019). For instance, osteoblasts secrete OPG and RANKL to regulate osteoclastic differentiation and formation via a paracrine pathway, and osteoclasts secrete sphingosine-1-phosphate (S1P), platelet derived growth factor (PDGF) or hepatic growth factor (HGF) to activate osteoblasts in return (Fouque-Aubert and Chapurlat 2008; Graves *et al.* 1999; Ryu *et al.* 2006). In summary, both the differentiation and function of osteoblasts and osteoclasts are influenced by the communication and interaction between them, thereby regulating bone remodeling (Figure1.1).

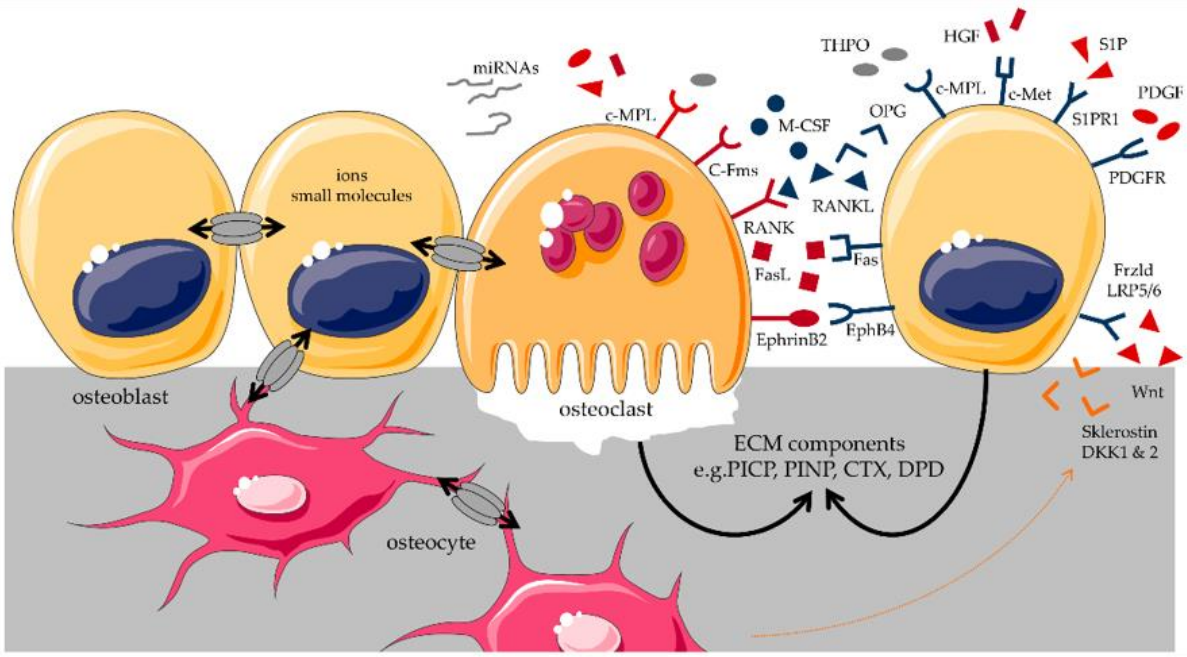


Figure1.1. Communication between osteoblast and osteoclast through cell-cell contact, paracrine factors and their interaction via the bone matrix (Zhu *et al.* 2018) (The figure is published under an open access Creative Common CC BY license for reuse).

1.2. Osteoporosis: the most common bone metabolic disease due to the aging population

1.2.1. Etiology of osteoporosis

The terminal result of an imbalance between osteoblasts and osteoclasts can lead to osteoporosis, which is typically defined by a decrease in bone density, ultimately leading to an increased risk of fracture among older people (Aspray and Hill 2019; Lane 2006). The pathogenesis of osteoporosis is complex and is the result of a comprehensive mixture of genetic, hormonal, dietary, lifestyle and physical factors. Bone mass is acquired early in life, and bone density and structure are maintained during adulthood (Raisz 2005). Damage to bone during skeletal growth and/or an imbalance between factors in the adult skeleton that favor bone resorption and bone formation may be the cause of bone fragility (Licata 2009). Genetic factors mainly affect peak bone mass, whereas systemic hormones or local cytokines are the primary causes of imbalances in bone remodeling (Ralston and Uitterlinden 2010). Briefly, there are 3 main contributors for the development of osteoporosis during remodeling: a failure to reach peak bone mass, compromised bone formation, and excessive bone resorption (Prideaux *et al.*

2016; Sandhu and Hampson 2011).

1.2.2. Epidemiology of osteoporosis

The proportion of older people is increasing rapidly, and it is predicted that by 2050, approximately a quarter of their population of all major regions of the world except Africa will be aged over 60 years (Cooper *et al.* 2011). This aging population will likely have a significant impact on the number of people with osteoporosis (Clynes *et al.* 2020). Due to its prevalence worldwide, osteoporosis is considered one of the most serious public health concerns. It is estimated that over 200 million people suffer from osteoporosis nowadays, and it affects one-third of all postmenopausal women in the Western countries. Osteoporosis patients aged over 50 years are at greater risk of suffering from an osteoporotic fracture, which imposes a devastating burden on patients and their families, and often leads to death. Approximately 15 - 40% of osteoporosis patients sustain osteoporotic fractures at least once in their lifetime (Sozen *et al.* 2017; van Oostwaard 2018). The cost to the health care budget of tackling osteoporosis is also staggering: the European Union spent a total of 37 billion euros in 2010, and the United States spent 20 billion dollars to treat osteoporosis in 2015 (Franic and Verdenik 2018; Pandey *et al.* 2018; Verdonck *et al.* 2019).

1.2.3. Pathogenesis of osteoporosis

Osteoporosis is considered to be a multifactorial disorder that is influenced by numerous factors, and its occurrence in different individuals may be the result of different pathogenic mechanisms (Ahmadi *et al.* 2020). However, there are a set of fundamental and common pathogenic mechanisms that underlie the development of osteoporosis. Firstly, genetic or other factors (anorexia nervosa, corticosteroid use, etc.) contribute to a failure to attain peak bone mass over a patient's lifetime and to impaired bone quality during growth due to trauma or bone diseases (Kabicek *et al.* 2019). Secondly, women face a lost bone mass during menopause, while men experience a gradual onset of sex steroid deficiency which could affect bone cells (Ucer *et al.* 2017). Additionally, age-related metabolic changes have also been shown to be strongly associated with the occurrence of bone loss (Sarbacher and Halper 2019). Finally,

menopausal or age-related bone loss can be accentuated when individuals are exposed to potential causes of bone loss like alcohol consumption, cigarette smoking, glucocorticoid use, and thyrotoxicosis (Medrela-Kuder and Szymura 2018; Payer *et al.* 2010; Siderova *et al.* 2018) (Figure 1.2).

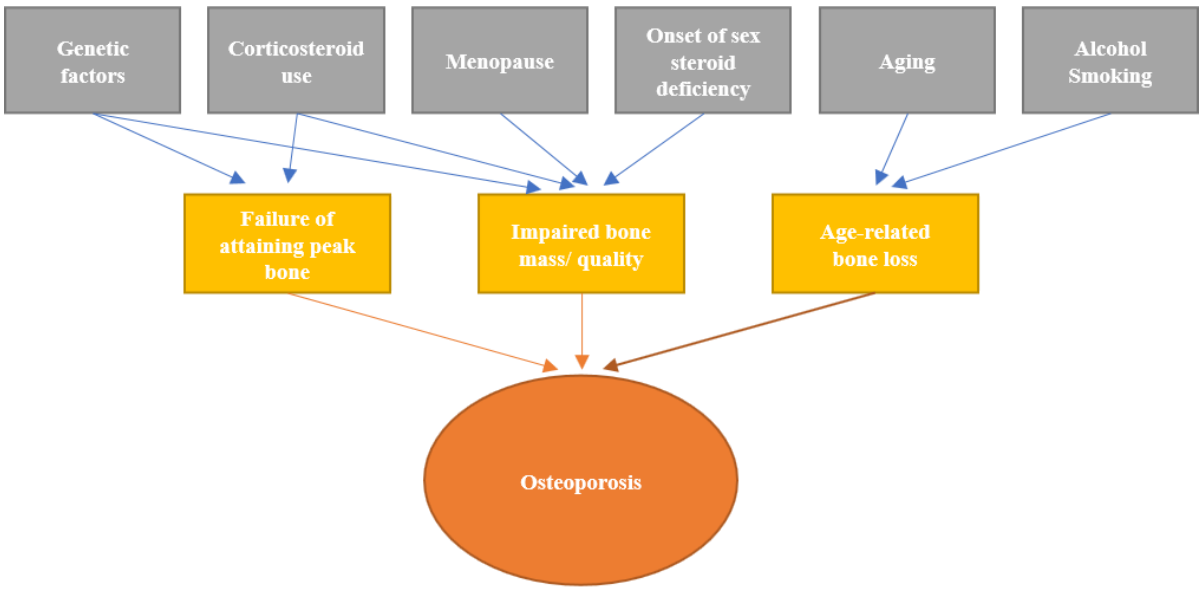


Figure 1.2. Pathogenesis of osteoporosis.

1.2.4. Prevention and treatment

For the prevention of osteoporosis, weight-bearing exercise with moderate resistance to increase muscle mass is recommended as it can transiently increase bone mineral density (BMD) (Black and Rosen 2016), and shows beneficial effects on skeletal microarchitecture in longitudinal studies (Evans *et al.* 2012). Given that falls frequently lead to osteoporotic fractures and the number of falls increases with advancing age, exercise and balance programs like tai chi and yoga may help to improve balance, increase muscle tone and reduce the risk of falls among elderly people (Zou *et al.* 2017). In addition to exercise, withdrawal of psychotropic medication if possible, awareness of fracture risk assessment, smoking cessation, and limiting alcohol intake play important roles in preventing osteoporosis and osteoporotic fracture (Pfeifer and Sinaki 2019; Soto *et al.* 2019).

Classical treatments that aim to prevent bone resorption include bisphosphonates, peptide hormones, estrogen, and selective estrogen receptor modulators (SERMs)

(Hauk 2018). Additionally, denosumab (a monoclonal antibody), calcium and vitamin D supplementation are also commonly recommended for osteoporosis patients (Qaseem *et al.* 2017). Once recognized, the potential consequences of osteoporosis can be mitigated by the appropriate selection of therapies.

1.3. Cigarette smoking (CS): a major risk factor for osteoporosis

1.3.1. Composition of cigarette smoke

Conventional cigarette smoke consists of more than 6,000 compounds, more than 150 of which are known to be toxic and contribute to the development of various diseases (Aspera-Werz *et al.* 2020; Sung *et al.* 2015). Cigarette smoke inhaled through the filter end of a cigarette, is rich in reactive nitrogen species (RNS) and reactive oxygen species (ROS), both of which can cause cell damage and death through oxidative stress (Comhair and Erzurum 2002; Pryor 1997). Typically, about 20-30 ml of carbon monoxide and 2-3 mg of nicotine can be inhaled via a single cigarette, and soon after smoking, nicotine concentrations in arterial blood rise to 100 ng/ml, which is then distributed to various tissues, including the skeletal system (Sundar 2020).

Nicotine is considered an addictive component of cigarettes, which increases platelet aggregation, decreases microvascular prostacyclin levels and inhibits the biological functions of fibroblasts, erythrocytes and macrophages (Benowitz *et al.* 2016; Hukkanen *et al.* 2005). Cigarette inhalation increases the content of catecholamines in the blood, contributing to the formation of lysergic acid, stimulating an increase in cardiac output and causing adrenergic vasoconstriction, as well as inhibiting epithelialization and disrupting the healing process (Alvarez-Jimenez *et al.* 2018; Glatard *et al.* 2017). As a result of peripheral vasoconstriction, small vessel stasis and microvascular perfusion are widely reduced, blood viscosity and fibrinogen levels are increased, inducing a hypercoagulable state (Hamouda *et al.* 2018). It has been found that smoking only two cigarettes can reduce the blood flow from the heart to the hand by 29% (Vanadrichem *et al.* 1992). Hormone levels are significantly affected by nicotine. Vasopressin, beta-endorphin, adrenocorticotropic hormone and cortisol are found to increase in the circulation in smokers (Windham *et al.* 2005). Furthermore,

nicotine has a detrimental effect on osteoblasts, fibroblasts, and macrophages at the cellular level (Aspera-Werz *et al.* 2018; El-Zayadi *et al.* 2002).

Carbon monoxide (CO) has a 200-fold higher affinity for hemoglobin binding than oxygen, which decreases the oxygen tension in tissues (Takajo *et al.* 2001). CO is produced as a result of incomplete combustion of cigarettes. Cigarettes are known to contain 2-6% CO, which converts up to 15% of hemoglobin into carboxyhemoglobin (Priest *et al.* 2014). The formation of carboxyhemoglobin impairs the transport and function of oxygen, ultimately leading to hypoxia (Barnoya and Glantz 2006). It has been demonstrated that 10 minutes of smoking reduces the oxygen tension in tissues, and smokers who smoke 20 cigarettes per day are in a state of tissue hypoxia for 20 hours per day (Chen *et al.* 2020; Sorensen *et al.* 2009) (Figure 1.3).

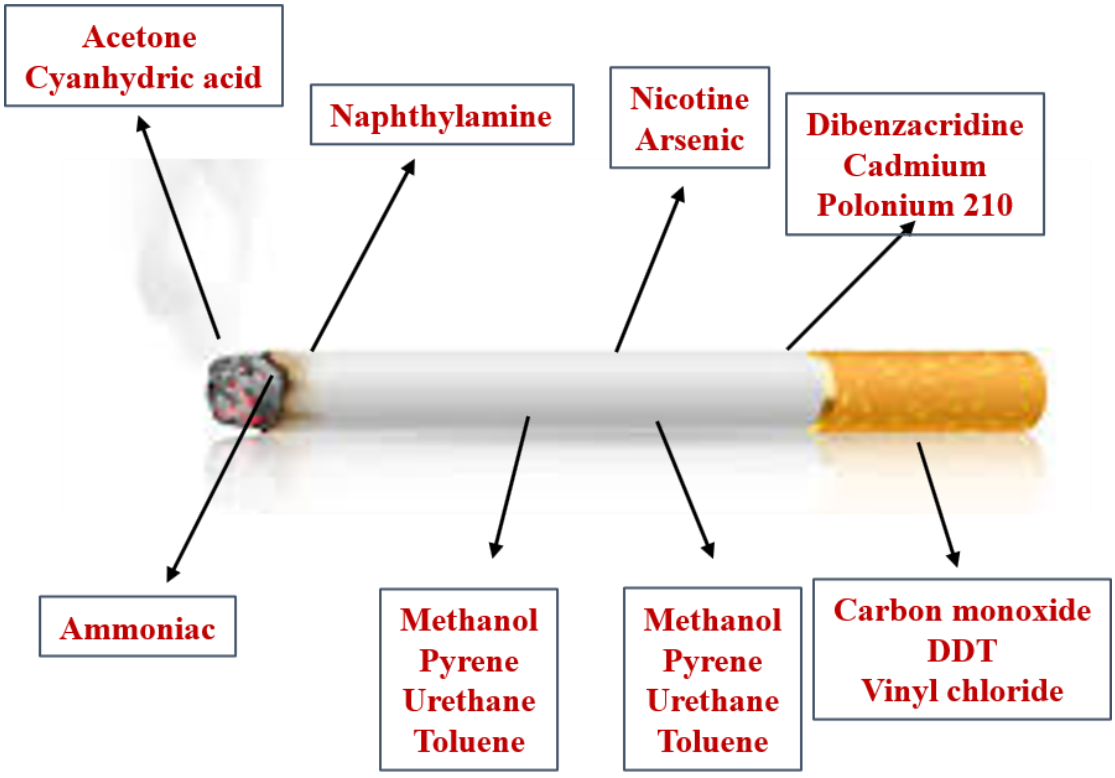


Figure 1.3. Chemical components of CS.

1.3.2. Effects of CS on human health

Active cigarette smokers are exposed to thousands of tobacco toxins, a combination of tobacco constituents and pyrolysis products, by inhaling cigarette

smoke (Onor *et al.* 2017). CS contributes to a major proportion of the population burden of numerous leading diseases, such as cancer, vascular disease, hip fractures, chronic obstructive pulmonary disease and a diminished health status (Kaplan *et al.* 2017). CS during pregnancy is strongly related to low birth weight, fetal growth restriction, and pregnancy complications (Abraham *et al.* 2017). Moreover, passive smoking is an established cause of disease and death (Yang *et al.* 2016).

Because of the non-negligible burden of CS on public health, it is imperative to develop proactive strategies to minimize the prevalence of CS (St Helen and Eaton 2018). At the societal level, many actions can be taken to prevent cigarette use. Evidence supports the effectiveness of several interventions, policies and regulations in reducing access to and consumption of tobacco, such as imposing restrictions on tobacco advertising, and enacting cigarette control legislation (Alberg 2008). At the individual level, the primary aim is to reduce the demand for cigarettes. Strategies can take the form of primary prevention to prevent young people from smoking, or promote cessation among long-term smokers through secondary prevention (Reynales-Shigematsu *et al.* 2019).

1.3.3. CS and osteoporosis

CS has been identified as an indispensable risk factor for osteoporosis, making it an important criterion included in the fracture risk assessment tool (Aspera-Werz *et al.* 2019; Joehanes *et al.* 2016). Multiple pathways are potentially associated with CS-induced osteoporotic changes: CS can lead to changes in hormone household, such as decreases in parathyroid hormone and estrogen levels, and increases in cortisol and adrenaline levels (Kapoor and Jones 2005); CS affects the level of vitamin D (Daniel *et al.* 1992); CS induces oxidative stress that causes osteoblastic damages (Correa *et al.* 2019); CS reduces the blood supply to bone and smokers are at increased risk of peripheral vascular disease (Balaji 2008); and the constituents in cigarettes have direct toxic effects that could affect bone cells (Cyprus *et al.* 2018). However, the underlying mechanisms of CS on osteoporosis remains unclear because few studies have been appropriately designed and are comprehensively enough to investigate the specific

mechanisms (Al-Bashaireh *et al.* 2018). A recent study in an experimental mouse model demonstrated that CS decreased osteogenic differentiation and may compromise bone formation (Cyprus *et al.* 2018). Furthermore, a mouse model with knockout of RANKL and RANK showed an absolute absence of osteoclasts, demonstrating that RANKL is vital for osteoclast differentiation (Leibbrandt and Penninger 2008). Not many studies have investigated the relationship between CS and RANKL/ OPG ratio. A higher RANKL/OPG ratio was found in CS-exposed rats compared to that in untreated rats (Giorgetti *et al.* 2010). A study in humans that explored the relationship between CS and the RANKL/ OPG ratio found that smokers with periodontitis have lower levels of OPG in their blood than non-smokers, which also leads to higher levels of RANKL/OPG ratio (Lappin *et al.* 2007). In addition, a recent human study found significantly lower OPG levels and higher RANKL/ OPG ratio in smokers compared to nonsmokers (Kargin *et al.* 2016). It is of great value to further explore the relationship between CS and the RANKL/ OPG ratio and screen potential treatments that target the RANKL-RANK-OPG pathway in smokers.

1.4. Bisphosphonates (BPs): a classical class of antiosteoporosis drugs

1.4.1. Mechanism of BPs for osteoporosis

Nitrogen-containing BPs are used to treat osteoporosis and other bone disorders associated with excessive bone turnover (Reszka and Rodan 2003). BPs inhibit the synthase of geranylgeranyl pyrophosphate (GGPP) and the formation of farnesyl pyrophosphate (FPP) in the mevalonate pathway (Drake *et al.* 2008; Roelofs *et al.* 2006). Therefore, the main effect of BPs is to promote apoptosis in mature osteoclasts. Additionally, BPs have been suggested to inhibit osteoclast formation possibly by suppressing osteoclast formation and the prenylation of small GTPases *in vitro* (Dunford *et al.* 2006). Recently, BPs were demonstrated to inhibit RANKL-induced osteoclast formation (Tsubaki *et al.* 2014) and enhance RANKL/OPG gene expression (Koch *et al.* 2012) (Figure 1.4).

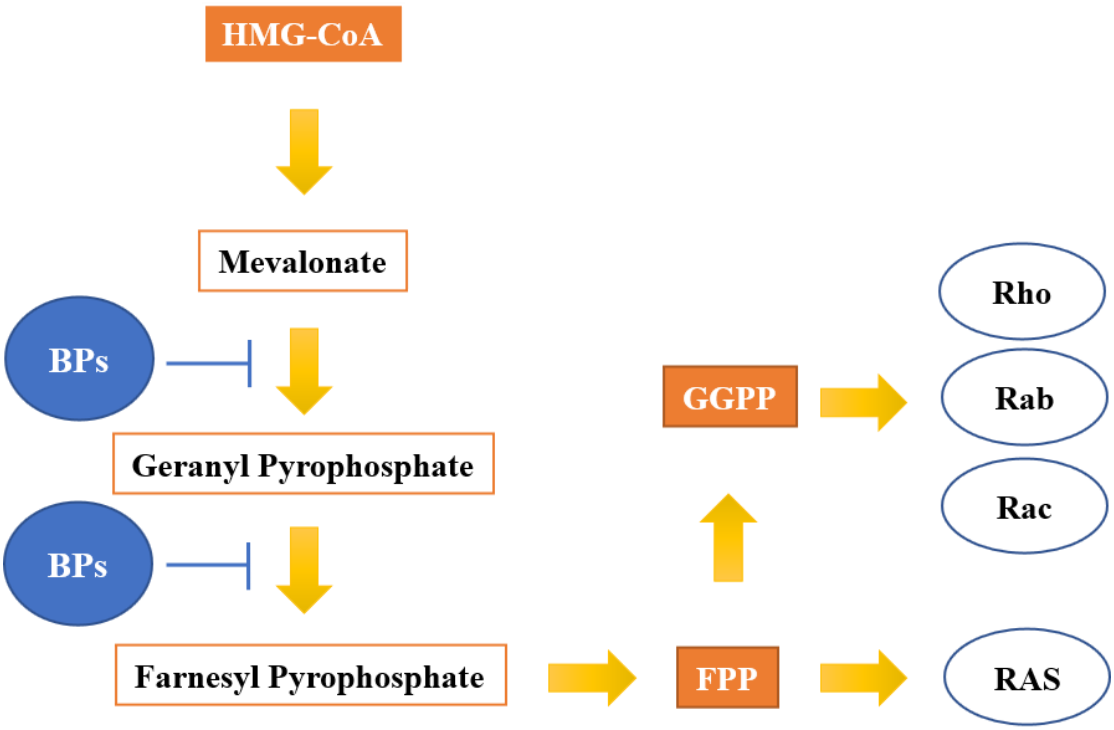


Figure 1.4. Mechanisms of action of BPs at the cellular and molecular level.

1.4.2. Adverse events associated with BPs

BPs are generally well tolerated, but the potential adverse events of BPs could limit their use in some patients (Kennel and Drake 2009). BPs are related to upper gastrointestinal adverse events such as vomiting, nausea, epigastric pain and dyspepsia. Other adverse events like acute phase reactions, musculoskeletal pain, hypocalcemia and secondary hyperparathyroidism, and osteonecrosis of the jaw have been reported to be related to BPs (Papapetrou 2009). Intravenous BPs have also been associated with all of the mentioned adverse events, except for upper gastrointestinal symptoms (Pittman 2014; Wang *et al.* 2016). It is still unclear that the relationship between BPs and atrial fibrillation and atypical fractures of the femoral diaphysis (Lu *et al.* 2020). The onset of oral ulcers, skin reactions, hepatitis, and esophageal cancer have been reported to be associated with BPs (Lu *et al.* 2020). Overall, the appropriate management of BPs confers a clear clinical benefit in osteoporosis patients that outweigh the adverse effects related to their use of BPs.

1.4.3. Novel applications of BPs

BPs are initially used in the treatment of bone-related diseases, but have also shown other potential therapeutic options. Studies *in vitro* and *in vivo* have shown that BPs exhibit anticancer activity which can hinder tumor growth and metastasis (Stresing *et al.* 2007). Thus, BPs are commonly used for the prevention and/or management of skeletal-related complications related to advanced cancers or as a result of anticancer treatments (Beuzeboc and Scholl 2014). For example, BPs can be used for multiple myeloma (MM) because skeletal complications occur in almost all MM patients.(Sousa and Clezardin 2018). BPs are also applied in palliative care for patients suffering from bone metastases in order to reduce or offset hypercalcemia (Porta-Sales *et al.* 2017). BPs have been shown to effectively inhibit the proliferation of cancer cells and induce apoptosis. However, studies have found that higher than recommended doses BPs are often required to achieve the anticancer effect, leading to BP-related adverse events (Ouyang *et al.* 2018). Therefore, BPs are often used in combination with other drugs when used as anticancer agents (Modi and Lentzsch 2012). Currently, BPs are frequently used in the drug treatment of breast cancer, lung cancer, renal cancer, and myeloma (Dlamini *et al.* 2019; Hodgins *et al.* 2017). In addition, BPs are used as a contrast agent (CA) in magnetic resonance imaging (MRI) for specific bone-related diagnoses and for visualization of the bone-soft tissue barrier. BPs have also been used as radionuclides to achieve specific bone-sensing features in single-photon emission computed tomography (SPECT/CT) (Kuznik *et al.* 2020).

1.5. Co-culture of osteoblast and osteoclast: an elite *in vitro* model for studying osteoporosis

1.5.1. *In vivo* models vs. *in vitro* models

Most bone-related *in vivo* models are performed in rodent. Compared to mammals, the widespread use of mice is due to relatively minimal public opposition to their use, their low cost and ease of habitation, and their size, which is appropriate for bone-related measurements, such as micro-CT and bone mechanical tests (Bonucci and Ballanti 2014). However, due to species differences, rodent models are not able to

mimic real physiological conditions in humans (Dall'Ara *et al.* 2016). This is demonstrated by the failed translation of many achievements from animal studies to clinical trials (Malfait and Little 2015). Therefore, if preliminary results can be obtained in effective *in vitro* models of human cell lines prior to *in vivo* tests, this could potentially reduce the use of animals and contribute to the eventual successful clinical application (Yildirimer *et al.* 2019).

1.5.2. Types of co-culture models *in vitro*

Mono-culture models of bone cells has been extensively performed over the past two decades (Roeder *et al.* 2011). However, as described above, the physiological metabolism of the skeletal system relies on the interaction of multiple types of bone cells, particularly the communication between osteoblasts and osteoclasts (Pirraco *et al.* 2012). Therefore, even the basic models that try to reflect the real *in vivo* bone environment require co-culture of osteoblasts sand osteoclasts. Direct or indirect co-culture models can be performed. Direct co-cultures involve the cultivation of two types of cells on the same surface, allowing a direct cell-cell contact. This approach includes the possible effects of membrane-bound signaling and soluble factors between cells in co-cultures (Ehnert *et al.* 2018); Indirect co-cultures mainly use transwell devices (Zhang *et al.* 2016), permeable dividers (Bogdanowicz and Lu 2014), and conditioned medium methods (Malekshah *et al.* 2006). These models allow the communication of soluble factors between each cell type and the ability to distinguish different cell types (Figure 1.5). The described co-culture methods are mainly two-dimensional (2D) models, which still have limitations in mimicking the environment in the living organism (Hess *et al.* 2010; Zhu *et al.* 2018). Three-dimensional (3D) co-culture models, which have been recently established and tested in order to better replicate the conditions *in vivo*. However, 3D models need to be optimized and evaluated before they can be widely used to mimic the bone system (Zhu *et al.* 2018).

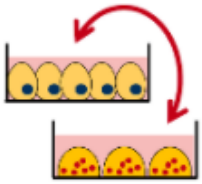
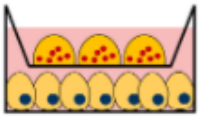
	Figure	Advantages	Disadvantages
conditioned medium		1. simple setup 2. established methods may be used 3. easy to quantify metabolic and functional changes of the different cells	1. no direct cell-cell contact 2. medium has to be carefully selected
transwell co-culture		1. used to investigate paracrine signaling and response to soluble signaling factors 2. cells can be tested separately 3. can be used to analyze cell migration	1. no direct cell-cell contact 2. medium has to be carefully optimized 3. large volumes needed might limit the use of sensitivity-based methods 4. large volumes needed might limit the oxygen supply in the bottom wells
removable permeable divider		1. used to investigate paracrine signaling and response to soluble signaling factors 2. cells can be tested separately 3. requires smaller volumes than in the transwell co-culture (assay sensitivity) 4. same medium height (oxygen supply) for both cell types	1. no direct cell-cell contact 2. medium has to be carefully selected 3. only immature dividers available 4. unknown origin of secreted factors
direct co-culture		1. simple setup 2. allows cell-cell contact 3. partly mimics the in vivo situation 4. requires smaller volumes than in the transwell co-culture (assay sensitivity) 5. same medium height (oxygen supply) for both cell types	1. cell ratios have to be optimized 2. medium has to be carefully selected 3. limited amount of methods available to analyze cells separately 4. unknown origin of secreted factors

Figure 1.5. The overview of co-culture models (Zhu *et al.* 2018) (The figure is published under an open access Creative Common CC BY license for reuse).

1.5.3. Co-culture models of osteoblasts and osteoclasts.

Osteoporosis is becoming the most common metabolic bone disease due to the global aging population currently. It is of great demand to address osteoporosis-related paradoxes and screen for novel anti-osteoporosis drugs (Zhu *et al.* 2018). As biotechnology continues to evolve, co-culture techniques are constantly being developed and optimized for evaluate new treatments that interact with osteoblast and osteoclasts. Menaquinone-4, melatonin, strontium, vitamin D3 and vitamin K2 were recently recognized as potential substances to reduce osteoporotic alterations by co-culture models of osteoblasts and osteoclasts (Maria *et al.* 2018; Maria *et al.* 2017). Moreover, through different osteoblasts and osteoclasts co-culture models, Janus kinases were shown to suppress RANKL expression to hinder osteoclastic

differentiation (Murakami *et al.* 2017), the anti-osteoporotic value of IL-18BP was discovered (Mansoori *et al.* 2016), and traditional Chinese herbs such as Epimedium were found to promote osteogenesis (Ma *et al.* 2011). The future challenges for co-culture models are mainly to complete the translation from 2D to 3D models, such as the optimization of 3D scaffolds, the application of 3D printing technology, and the selection of appropriate measurements.

1.6. Aim of the study

The objective of this thesis is to establish a direct co-culture model of osteoblasts and osteoclasts using human cell lines, mimicking the *in vivo* bone environment and allowing testing of possible mechanism/s of osteoporosis as well as the screening of potential anti-osteoporotic drugs. The following points were addressed:

1. The establishment of co-culture model of osteoblasts and osteoclasts.
2. The optimization of co-culture model of osteoblasts and osteoclasts.
3. The comparison between mono-cultures and co-culture of osteoblasts and osteoclasts.
4. Test the effects of various CSE concentrations on co-cultures of osteoblasts and osteoclasts.
5. Test the effect of BPs (zoledronate and alendronate) on CSE-affected cells in co-cultures of osteoblasts and osteoclasts.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents (Table 2.1)

Table 2.1: List of used chemicals and reagents.

Substance	Company	Article No.
2′, 7′-Dichlorfluorescein-Diacetate (DCFH-DA)	Sigma	21884
4-Nitrophenol solution	Sigma	N7660
4-Nitrophenol solution 10 mM (pNP)	Sigma	N7660
4-Nitrophenyl phosphate disodium salt hexahydrate	Carl Roth	4165.1
Acetic acid	VWR	20104.298
Agarose	Roth	2267.4
Alizarin Red S	Roth	0348.2
Bovine Serum Albumine	Roth	8076.4
Calcein Acetoxymethyl Easter (Calcein AM)	ATT Bioquest	ABD-22002
Calcium chloride	Carl Roth	CN93.1
Cetylpyridiumchlorid monohydrate	Roth	CN27.1
Chloroform	Carl Roth	Y015.1
Cholecalciferol	Sigma	95230
Deoxycholic acid (DOC)	Roth	3484.1
Dexamethason Water Soluble	Sigma	D2915
Diethylpyrocarbonate (DEPC)	Carl Roth	K028.3
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma	D8537
EDTA (Ethylenediaminetetraacetic acid)	Roth	8043.2
Ethanol 99.9% p.a. (EtOH)	VWR	20821.33
Ethidium Bromide 1%	Carl Roth	2218.1
Fetal Calf Serum (FCS)	Invitrogen	41G7141K
First Strand cDNA Synthesis Kit	ThermoFisher	K1621
Formaldehyde	AppliChem	A0823.1000
Glycine	Carl Roth	3908.2
H_2O_2 Solution	Roth	CP26.5
Hepes	Carl Roth	HN78.2
Hoechst 33342	Sigma	14533-100MG
Isopropanol 100%	VWR	20842.33
Isopropanol 100%	Honeywell	33539
L-Ascorbate-2-Phosphate	Sigma	A8960-5G
L-Ascorbic acid-2-phosphate sesqui-magnesium salt hydrate	Sigma	A8960-5G

Substance	Company	Article No.
Luminol	Roth	4203.1
MgCl2	Carl Roth	KK36.2
Milk powder	Roth	T145.2
Na2- Tartrate*2 H2O	Roth	0254.1
p-Cumaric acid	Roth	9908.1
Penicillin/Streptomycin (P/S)	Sigma	P0781
Phalloidin–Tetramethylrhodamine B isothiocyanate	Sigma	P1951-.1MG
pNPP (para- Nitropheyl-Phosphate)	Roth	4165.1
Poncau S	Roth	5938.2
Resazurin Sodium Salt	Sigma	199303-1G
RPMI 1640	Sigma	R8758
Sodium Acetate	Carl Roth	X891.2
Sodium Chloride	Sigma	S7653-1KG
Sodium Chloride	Sigma	S7653
Sodium Hydroxide	Carl Roth	T135.1
Sodiumacetate	Roth	X891.2
Sodiumchloride	Roth	HN00.2
Sulforhodamine B Sodium Salt	Sigma	S1402-1G
Tergitol Solution	Sigma	NP40S-100 ml
TRIS Base	Sigma	T1503-1KG
Trisamine (Tris) Base, >99%, p.a.	Sigma	T1503-1KG
Tris-Base	Sigma	T1503
Trypan Blue	Roth	CN76.1
Trypsin/EDTA	Sigma	T3924
Tween- 20	Roth	9127.1
β-Glycerophosphate disodium salt hydrate	Sigma	G9422-10

2.1.2. Buffers, medium and solutions (Table 2.2)

Table 2.2: List of buffers, medium and solutions.

Buffers/Mediums/Solutions	Compounds and handling
10 % Tween-20 Solution	100 ml Tween-20 900 ml ddH2O
10 X TBE buffer	108 g TRIS 55 g Boric acid 40 ml EDTA (0.5 M, PH 8) 1 L ddH_2O
250 mM Luminol Stock Solution	0.88 g Luminol 20 ml DMSO
Acetic Acid Solution (1%)	100% acetic acid in ddH_2O
Acetic Acid Solution (3%)	100% acetic acid in ddH_2O
Alcian blue solution (1%, PH 2.5)	500 mg Alcian blue (8 GX) 50 ml Acetic Acid (3%)
Alizarin Red Staining Solution	200 mg Alizarin Red S (PH 4.0)

AP Activity Assay buffer	3.75 g Glycine 12.11 g Tris-Base 95.21 mg $MgCl_2$ 1 L ddH_2O (PH 10.5)
AP substrate solution	1.3 mg pNPP 1 ml AP Activity Assay Buffer (pH 10.5)
BSA Blocking Buffer	2.5 g BSA 50 ml TBS-T Washing Buffer
Calcein AM stock solution	502 µl DMSO 1 mg Calcein AM
Calf Thymus DNA stock solution (1 mg/ml)	1 mg Calf Thymus DNA in 1 ml TE buffer
Cetylpyridiumchloride Solution	10 g Cetylpyridiumchlorid monohydrate
Chondrocyte Cells Culture Medium	500 ml DMEM 500 ml Ham's F12 50 ml FCS 10 ml Penicillin/Streptomycin 50 µl L-Ascorbic-2-Phosphate
Chondroitin Sulfate Standards stock solution (1 mg/ml)	10 mg Chondroitin Sulfate Standards in 10 mlPBE buffer
Chondroitin Sulfate Standards working solution (100 µg/ml)	1 mg/ml stock solution in PBE buffer
DMMB solution buffer (PH 3)	304 mg Glycine 160 mg sodium chloride 9.5 ml 0.1 M Acetic acid 90.5 ml ddH2O
DMMB stock solution (8 mg/ml)	8 mg DMMB in 1 ml buffer
DMMB working solution (16 µg/ml)	8 mg/ml stock solution in DMMB buffer
ECL Solution	5 ml 100 mM TRIS 250 mM Luminol Stock Solution 11 µl 90 mM p- Coumaric Acid Stock Solution 5 µl 30 % H2O2 solution (Freshly prepared)
Ethanol solution (70%)	99% Ethanol in ddH2O
Formaldehyde (4%)	Formaldehyde (37%) in ddH_2O
Guanidine Hydrochloride (6 M)	28.65 mg Guanidine Hydrochloride 50 ml ddH2O
Papain stock solution (5 mg/ml)	5 mg papain from papaya latex 1 ml PBE buffer
Papain working solution (25 µg/ml)	5 mg/ml stock solution in PBE buffer
PBE Buffer (PH 6.5)	6.5 mg N-Acetyl-L-Cyteine 138 mg Disodium hydrogen phosphate 14.9 mg EDTA Adjust PH to 6.5 with NaOH Adjust volume to 20 ml with ddH_2O

PCR loading buffer	25 mg Bromophenol blue 5 ml 10X TBE 5 ml Glycerol (20%)
Ponceau S Solution	0.2 g Ponceau S
Resazurin stock solution	0.025% in DPBS
Resazurin working solution	10% Resazurin stock solution in DPBS
RIPA Stock Solution	0.121 g TRIS Base 0.58 g NaCl 500 µl Tergitol Solution 0.3 g DOC 0.372 g EDTA (PH 7.6)
Safranin-O solution (0.1%)	50 mg Safranin-O 50 ml ddH2O
SaOS-2 Cells Osteogenic medium	500 ml RPMI 1640 25 ml FCS 29 mg L-Ascorbic acid 2-phosphate 0.54 g β-Glycerophosphate 2.98 g Hepes 83 mg Calcium chloride 50 µl Cholecalciferol Stock Solution (20 ng/ml)
SaOS-2/ THP-1 Cells Culture medium	500 ml RPMI 1640 25 ml FCS
Sodium Acetate solution (3M, PH 5)	12.3 g Sodium Acetate in 50 ml ddH2O
SRB Solution	0.4% SRB in 1% acetic acid
TBS-T Washing Buffer	100 ml TBS 10X 10 ml 10 % Tween-20 Solution 900 ml ddH2O
TE buffer (PH 7.5-8.0)	10 mM Tris base 1 mM EDTA
TNE buffer (PH 7.4)	121.1 mg Tris Base 37.2 mg EDTA 1.17 g sodium chloride Adjust PH to 7.4 with HCl Adjust volume to 100 ml with ddH_2O
TRAP- Assay buffe	8.2 g Na- Acetate 11.5 g Na2- Tartrate *2 H2O 50 mM 900 ml ddH2O (pH 5.5)
TRIS Buffered Saline (TBS-10X)	12.1 g TRIS 87.66 g NaCl PH 7.6
TRIS Solution (10 mM)	1.2g TRIS in 1L ddH_2O
Trypan blue solution	62.5 mg Trypan blue 50 ml Dulbecco's PBS

2.1.3. Consumables

Table 2.3: List of consumables

Consumable	Type	Serial number	Manufacturer
Cell culture plate	96-well,flat bottom	655180	Greiner bio-one GmbH
Cell culture plate	96-well, V bottom	651101	Greiner bio-one GmbH
Cell culture plate	48-well,flat bottom	3548	Corning Inc.
Cell culture plate	6-well, flat bottom	353046	Corning Inc.
Cell Star Tubes	50 ml	227261	Greiner bio-one GmbH
Cell Star Tubes	15 ml	188271	Greiner bio-one GmbH
Eppendorf tube	0.5 ml, white	72.699	SARSTEDT AG
Eppendorf tube	1.5 ml, white	4182.1	Carl Roth GmbH + Co.KG
Eppendorf tube	1.5 ml, blue	4190.1	Carl Roth GmbH + Co.KG
Eppendorf tube	1.5 ml, green	4209.1	Carl Roth GmbH + Co.KG
Eppendorf tube	1.5 ml, red	4189.1	Carl Roth GmbH + Co.KG
Eppendorf tube	1.5 ml, yellow	4204.1	Carl Roth GmbH + Co.KG
Eppendorf tube	2.0 ml, white	2549	Eppendorf AG
Pipette Tips	0.1 - 10 µl	Colorless	Sorenson BioScience, Inc.
Pipette Tips	2 - 200 µl	Yellow	Sarstedt AG & Co.
Pipette Tips	100 - 1000 µl	Blue	Ratiolab GmbH
Single-channel Pipette	10-100 µl	158240031	Corning Inc.
Single-channel Pipette	20-200 µl	158250088	Corning Inc.
Single-channel Pipette	100-1000 µl	058261237	Corning Inc.
Single-channel Pipette	0.1-2.5 µl	P35434B	Eppendorf AG
Spectrophotometer	Fluostar Omega	415-1264	BMG Labtech GmbH

2.1.4. Equipment

Table 2.4: List of equipment.

Equipment	Type	Serial number	Manufacturer
Agitator, magnetic stirrer	RH B2	06.050357	IKA-Werke GmbH
Agitator, magnetic stirrer	MR Hei-Mix L	040700340	Heidolph Instruments GmbH
Centrifuge	Stat Spin	620E50000693	Dako Deutschland GmbH
Centrifuge	Megafuge 40 R	41307652	Thermo Fisher Scientific Inc.
Centrifuge	SI DD 58	DD58-1001	Scientific Industries Inc.
Centrifuge (Mirco)	BN 08060235	C1301B	Labnet International Inc
Centrifuge (Mirco)	Fresco 17	41250019	HERAEUS Med GmbH
Dot Blotter	CSL_D96	190822001	Carl Roth GmbH & Co.KG
Electrophoresispower supplies	Power Pac 200	285BR05538	Bio-Rad Laboratories GmbH
Freezer -20°C	IQ500	GS51NYW41 (01)	BSH Hausgeräte GmbH
Freezer -20°C	Med Line	LGex3410-21K 001	Liebherr AG
Freezer -80 °C	905	827860-2521	Thermo Fisher Scientific Inc.
Freezer -86 °C	ULT1386-9-V17	R10G-333095-RG	Revco Inc
Fridge +4 °C	Comfort	3523-21L	Liebherr AG
Fridge +4 °C	HKMT 040-01	CC00412514	Cool Compact Kühlgeräte GmbH
Ice maker	AF 80	DD 8837 11 X	Scotsmen Inc.
Incubator	Heratherm OMS 60	41296334	Thermo Fisher Scientific Inc.
Incubator	9040-0078	11-22649	Binder GmbH
Incubator	9040-0081	11-22190	Binder GmbH
Laboratory pump (Bench)	Cyclo 2	1109-065	Carl Roth GmbH & Co.KG

Microscope	EVOS-fl	91-AF-4301	PEQLAB Biotechnologie GmbH
Mixer	Vortex Mixer	804995	Corning Inc.
Mixer	LD-76	76000	Labinco BV
Multichannel Pipette	5-50 µl	151620022	Corning Inc.
Multichannel Pipette	20-200 µl	551630277	Corning Inc.
Multichannel Pipette	0.5-10 µl	CH98998 4510	Thermo Electron Co.
Multichannel Pipette	50-300 µl	151640033	Corning Inc.
PCR thermal cyclers	Arktik	10040953	Thermo Fisher Scientific Inc.
PCR thermal cyclers	Forschungslabor	50132	Applied Biosystems GmbH
pH meter	Five Easy FE 20	1232315296	Mettler-Toledo GmbH
Pipette controller	Pipetboyacu	629619	Integra GmbH
Pipette controller	Rota-Filler 3000	HSA05119	Heathrow Scientific LLC
Refrigerator	HKMT 040-01	CC 00412516	Cool Compact Kühlgeräte GmbH
Refrigerator	HKMN 062-01	CC 00412513	Cool Compact Kühlgeräte GmbH
Safety workbench	Maxisave S20201.8	41293949	Thermo Fisher Scientific Inc.
Safety workbench	Maxisave S20201.8	41293948	Thermo Fisher Scientific Inc.
Scale	ABJ 120-4M	WB 1140084	Kern & Sohn GmbH
Shaker, laboratory	DRS 12	11DE243	LTF Labortechnik GmbH & Co KG
Shaker, laboratory	ES-20	010111-1107-0119	PeqlabBiotechnologie GmbH
Shaker, laboratory	DRS 12	11DE090	LTF Labortechnik GmbH & Co KG
Shaker, Laboratory	LSE Vortex Mixer	1101260	Corning Inc.
Single-channel Pipette	0.5-10 µl	158220060	Corning Inc.
Single-channel Pipette	2-20 µl	158230441	Corning Inc.

Single-channel Pipette 10-100 µl		**158240031**	**Corning Inc.**
Single-channel Pipette 20-200 µl		158250088	Corning Inc.
Single-channel Pipette 100-1000 µl		058261237	Corning Inc.
Single-channel Pipette 0.1-2.5 µl		P35434B	Eppendorf AG
Spectrophotometer	Fluostar Omega	415-1264	BMG Labtech GmbH
Water-bath	ECO ET 20	LY 06.1	Lauder Dr. R. Wobser GmbH

2.2. Methods

2.2.1. Cell culture

2.2.1.1 Cell lines

To achieve a replicable co-culture model of osteoblast-like and osteoclast-like cells and the best mimicry of the *in vivo* environment, selected cell lines of human tumor origin were selected. SaOS-2 is a commonly used pre-osteoblastic cell line with a potent differentiation capacity. THP-1 cells derive from a female leukemia patient, which can differentiate from neutrophils to osteoclast-like cells with the supplement of RANKL and MCS-F (Villagran *et al.* 2015).

2.2.1.2. Cultivation of SaOS-2 and THP-1 cells

RPMI 1640 medium (supplements are shown above in Materials section) was used for SaOS-2 and THP-1 cells cultivation, medium change was performed every 3-4 days and cell subculture of cells was carried out when cells reached on the confluence of 80%. For cell seeding, SaOS-2 cells were detached with Trypsin/ EDTA while THP-1 is a suspended cell line. Trypan blue solution was used to stain cells for cell counting. Cells were then centrifuged and re-suspended with SaOS-2/ THP-1 culture medium. Re-suspended SaOS-2 and THP-1 cells were seeded in the ratio of 1: 2 (for 96-well plate, 1×10^4 SaOS-2 and 2×10^4 THP-1 cells per well). In particular, supplement of phorbol-12-myristate 13-acetate (PMA) is required when seeding THP-1 cells due to their suspension characteristic.

2.2.1.3. Cell differentiation

For SaOS-2 mono-culture, SaOS-2 osteogenetic medium (components are shown

in metiral section) was used to replace culture medium on the secound day of cell seeding. Differentiated SaOS-2 cells can secrete RANKL and MCS-F in the supernatant, so cell culture medium was repleced by SaOS-2-comditioned supernatant of differentiaed SaOS-2 cells in 6-well-plates (30×10^4 cells per well) for THP-1 mono-culture. For SaO-2 and THP-1 co-cultures, as highly differentiated pre-osteoblasts, SaOS-2 started to secrete factors in the shared medium for THP-1 cells differentiation after culture medium was changed by SaOS-2 osteogenetic medium (Weng *et al.* 2020).

2.2.2. CSE preparation

Cigarettes used in the thesis are commercial Marlboro products from Philip Morris company. Plain SaOS-2/ THP-1 cell culture medium (RPMI 1640 medium without any supplement) was used to dissove cigarette smoke. One cigarette was bubbled by using a gas wash bottle and a peristaltic pump (100 bubbles per minute) (Figure 2.1). The optical density of CSE-dissoved medium was determined by the plate reader at 320nm, and an optical desity of 0.7 is considered a CSE concentration of 100% (10% of CSE is assumed to be equivalent to smoke 20 cigarettes a day for human). CSE was sterilized by 0.22 μm filter and diluted by SaOS-2 osteogenic medium to reach different concentrations.

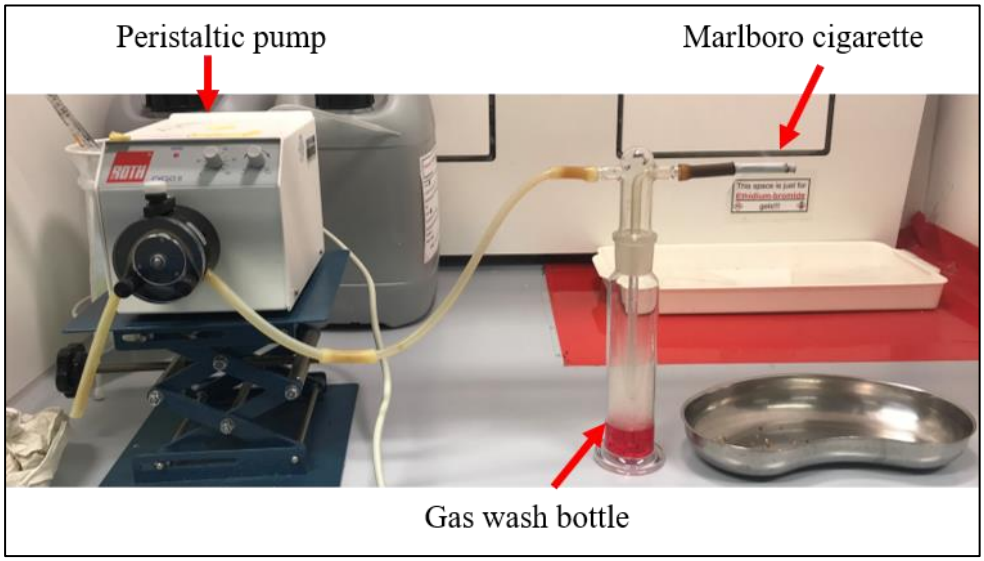


Figure 2.1. CSE preparation.

2.2.3. Sulforhodamine B (SRB) staining

Total protein of cells can be determined by SRB staining (Aydinlik *et al.* 2020). Cells need to be fixed in ethanol (99%) at -20 ℃ for at least 24 h. After fixation, cells were washed three times by PBS and incubated with SRB solution (0.4 %, can be recycled and resued) for 30 minutes under light-avoiding conditions at room temprature (RT). Acetic acid (1%) was used to remove unbound SRB solution and TRIS solution (10mM, PH = 10.5) was used to resolve bound SRB. Resolved SRB quantification was measured photometrically (λ = 565 nm).

2.2.4. Alkaline phosphatase (AP) activity

AP activity is a marker of early osteoblast differentiation and can be an indicator for osteoblastic function (Kyyak *et al.* 2020). After washing celss with PBS, AP reaction solution was added and then measured with absorbance by a plate reader (λ = 405 nm) for 30 min. AP activity results were eventually normalized to SaOS-2 cell number.

2.2.5. Tartrate-resistant acid phosphatase (TRAP) 5b activity

Macrophages secrete TRAP 5a and TRAP 5b, while mature osteoblasts secrete only TRAP5b. Therefore, TRAP 5b was measured to represent diffrentiated osteoclats and osteoclatic activity for THP-1 cells (Rossler *et al.* 2018). 30 μL of cell supernatant was incubated with 90 μL TRAP 5b reaction solution for 6h, and 90 μL NaOH was used to stop reaction. The supernatant was then directly measured photometrically (λ = 405 nm). TRAP 5b results were eventually normalized to relative THP-1 cell number.

2.2.6. Actin ring/ Nuclei staining

The formation of actin ring and the presence of multinucleated cells are both typical morphological characteristics of osteoclasts (Boyce *et al.* 2012; Wilson *et al.* 2009). After washing with PBS twice, cells were incubated with Triton X-100 (0.2%) for 20 min to penetrate cell membrane and then fixed with formaldehyde (2%). Cells were washed with PBS and incubated with BSA (5%) for 1 h to block non-specific bingdings. After washing with PBS, cells were incubated with staing soluntion (Phalloidin-TRITC and Hoechst 33342, 1: 1000 in PBS) for 30 min. A fluorescence microscope was used to identify osteoblasts (actin filament structures and one nucleus) and osteoclasts (actin ring formation and/ or two nuclei or more in single cell).

2.2.7. Matrix determination

Alizarin-red staining was measured to determine mineralized matrix (Kannan *et al.* 2020). Cells were fixed with ethanol (99%) at -20 ℃ for at least 24 h after washing with PBS twice. After fixation, cells were washed by tap water three times and then incubated with alizarin-red staining solution for 30 min at RT. Stained matrix can be detected by microscope after washing with tap water in this stage. After resolving with cetylpyridinium chloride, alizarin-red quantification can be determined photometrically (λ = 565 nm).

2.2.8. PCR measurement

Self-made Trifast reagent (coponents are shown in Material section) was used to isolate total RNA of cells, and first strand cDNA synthesis kit from thermofisher company were used to achieve complementary DNA synthesis. cDNA samples were normalized with DEPC water to 10 ng per μL. A mixture of 7.5 μL Red HS Taq Master Mix, 20 μL cDNA sample, 20 μL DEPC water, and 7,5 ul former/ revers primer was used for RNA reaction for each sample (Target primers are shown in Table 2.5). Agrose gels (1.5 – 1.8%) with Etbr were used to load samples, and gel electrophoresis (80 - 90 V for 40 - 50 min) was performed for gel seperation. The intesity of detecable bands of PCR was measured by ImageJ software.

Table 2.5. Primer sequences and PCR conditions for the investigated genes.

Gene	Accession Number	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Product Length (bp)	Annealing Temperature (°C)	Cycles
OPG	NM_002546.3	CCGGAAACAGTGAATCAACTC	AGGTTAGCATGTCCAATGTG	313	60	35
RANKL	NM_033012.3	TCCCAAGTTCTCATACCCTGA	CATCCAGGAAATACATAACAC	245	56	35
NFATC1	NM_172390.2	TGCAAGCCGAATTCTCTGGT	CTTTACGGCGACGTCGTTTC	228	64	35
β-Actin	NM_001101.3	CGACAACGGTCCGGCATGT	GCACAGTGTGGGTGACCCCG	461	64	30

2.2.9. Dot blot measurement

Secreted proteins are present in the supernatant of the cells, so extracting the supernatant of cells can be used to determine protein levels of OPG, RANKL and TRAP 5b which secreted by either osteoblasts or osteoclasts. Supernatant of cells was

transferred to a nitrocellulose membrane by a Dot blotter, the total protein of supernatant was confirmed by Poceau staining. After removing poceau by washing with tap water, membranes were blocked by BSA (5%) for 1 h. After washing with TBS-T buffer three times, membranes were incubated with primary antibodies agianst OPG, RANKL and TRAP 5b overnight in a ice box (The informaton of used antibodies are shown in Table 2.6). Membranes were washed with TBS-T buffer and then incubated with secoundary antibodies for 2 h at RT. ECL solution was used to develop chemiluminescent signals which can be detected by a CCD camera. The intensity of Dot blot signals were measured by using imageJ software.

Table 2.6. Antibodies used in Dot blot measurements.

Antibody	Catalog No.	Company	Dilution
OPG	500 - P149	Peprotech	1: 1000
RANKL	500 - M46	Peprotech	1: 1000
TRAP 5b	Sc - 376875	Santa Cruz Biotech	1: 1000
Goat anti-rabbit IgG-HRP	Sc - 2004	Santa Cruz Biotech	1: 10000
Goat anti-mouse IgM	Sc - 2064	Santa Cruz Biotech	1: 10000

2.2.10. Total DNA measurement

Cells were incubated with pre-warmed NaOH (50mM) for 5 min after washing with PBS twice to penetrate cell membranes. Supernatant with cell lysates was incubated in a thermoshaker at 98 ℃ for 30 min, and then mixed with 50 µL ddH_2O and 5 µL TRIS solution (1M, pH = 8). The mixture was centrifuged for 10 min (14,000 x g) at 4 ℃ and supernatant was transffered to a new tube. 2 µL of supernatant samples were measured with a plate reader for DNA quantification.

2.2.11. Cell-type-specific normalization

For co-cultures, the DNA samples contain both SaOS-2 and THP-1 cells, so identification of both cell types is required. As mentioned, THP-1 cell line is of male origin while SaOS-2 cell line dirives from a female patients. Therefore, the sex-determining region Y (SRY), a gender-determined gene for males, was selected to determine the DNA amount of THP-1 cells. The SaOS-2 DNA amount was obtained by substracting THP-1 DNA amount determined by SRY from total DNA. In brief, using

the PCR measurement described above, a standard curve was first measured using a gradient DNA amount of THP-1 cells and their SRY gene expression. The SRY expression in co-cultures was then measured by PCR measurements, the THP-1 DNA amount in co-cultures was calculated by SRY expression and the standard curve (Figure 2.2).

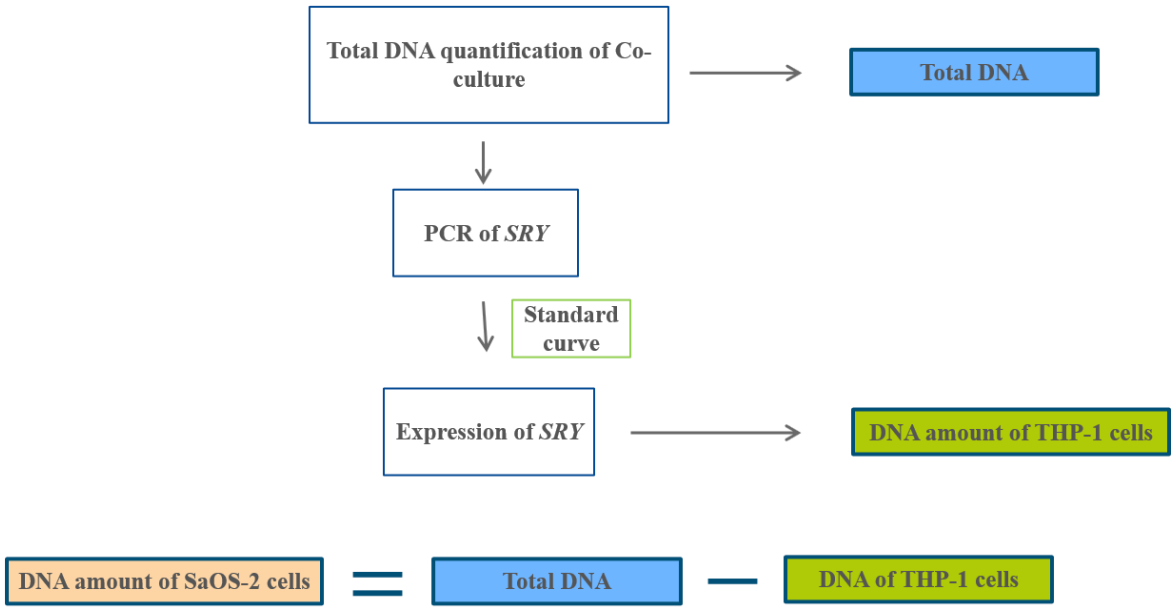


Figure 2.2. The cell-type-specific normalization method in the co-culture of SaOS-2 and THP-1 cells.

2.2.12. Statistics

Results are presented in the form of line/bar chart (mean ± SEM), and each experiment repeated at least three times.Graphpad prism software was used for data analysis, and non-parametric one-way ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparison test was used to compare data sets. The minimum level of significance is $P < 0.05$.

Chapter 3

3. Results

3.1 THP-1 cells can be induced to differentiate into osteoclast-like cells by SaOS-2 conditioned medium and can maintain cell survival for 7 days.

One of the main aims of the thesis is to develop a supplement-free co-culture model of osteoblasts and osteoclasts. SaOS-2 cells are pre-osteoblasts which can be induced to differentiate by SaOS-2 osteogenic medium, so the first challenge in our study was to induce the differentiation of osteoclasts from THP-1 cells and maintain the survival of osteoclast-like cells. Previous studies mainly used additional M-CSF and RANKL for induction of osteoclast differentiation of THP-1 cells (Zhang *et al.* 2020). However, SaOS-2 are well-differentiated pre-osteoblasts that can secrete M-CSF and RANKL after differentiation induction. Therefore, THP-1 cells mono-cultures with diffenretiated SaOS-2 cells conditioned medium was first preformed to verify whether THP-1 cells can be induced to differentiate by secretions from SaOS-2 cells. According to the results of SRB staining indicating the cell viability, cells decreased significantly over time points (Figure 3.1A). Calcein-AM staining representing living cells showed the same tendency as SRB staining that cells can remain viable for at least 7 days, but not after 10 days (Figure 3.1B). TRAP-5b is a specific indicator of osteoclasts reflecting the osteoclastic function (Song 2017), and an actin ring structure under fluorescent staining is characteristic of mature osteoclasts (Matsubara *et al.* 2017). TRAP 5b activity was detected in THP-1 mono-cultures on day 4, and decreased over time points (Figure 3.1C). Clear actin ring structures with multiple nuclei were observed by the fluorescent-actin staining (Figure 3.1 D). These results indicate that THP-1 cells can be successfully induced to differentiate into osteoclast-like cells by SaOS-2 secretions, and the optimal time frame to investigate osteoclasts for THP-1 mono-cultures is from day 4 and day 7.

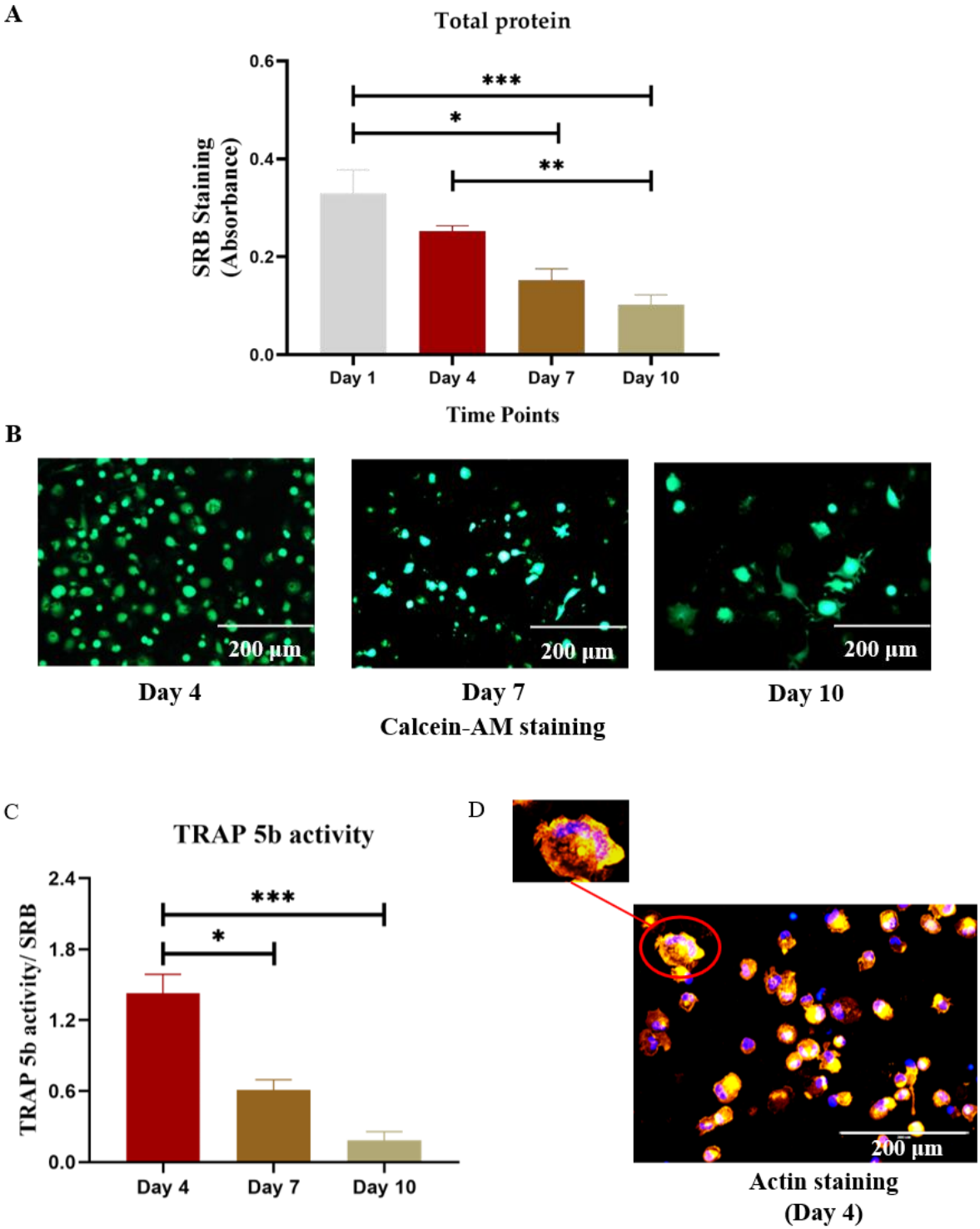


Figure 3.1 Differentiation of THP-1 cells by SaOS-2 conditioned medium in mono-cultures **A.** SRB staining representing total protein results of THP-1 cells on different time points. **B.** Representative Calicein-AM staining of THP-1 cells on day 4, day 7, and day 10. Green structures represent living cells. **C.** TRAP 5b activity of THP-1 cells on different time points. **D.** Representative Actin staining of THP-1 cells on day 4. Osteoclasts were determined by the presence of actin ring formation and the presence of at least two nuclei. Data are represented the mean ± SEM, and the significance was determined as $^{**}p < 0.01$ and $^{***}p < 0.001$ (N = 3, n = 3).

3.2 Comparison of SaOS-2 and THP-1 cells mono-culture and co-culture.

As THP-1 cells can be induced to differentiate by SaOS-2 conditioned medium, THP-1 cells is expected to be induced to differentiate in co-culture because of their direct exposure to SaOS-2 secretions in the shared supernatant. SRB staining results showed cells in the co-culture system had significantly higher cell viability compared to cells in mono-cultures. Co-culure of SaOS-2 and THP-1 cells maintained cell survival up to 14 days, while almost all cells died on day 10 in mono-cultures (Figure 3.2A). TRAP 5b activity in co-cultures was significantly higher than mono-cultures (Figure 3.2B), indicating that THP-1 cells were better differentiated into osteoclast-like cells in co-cultures than cells in mono-culture did. The actin staining on day 10 confirmed SRB results, and clear actin ring structures with multiple nuclei were observed (Figure 3.2C).

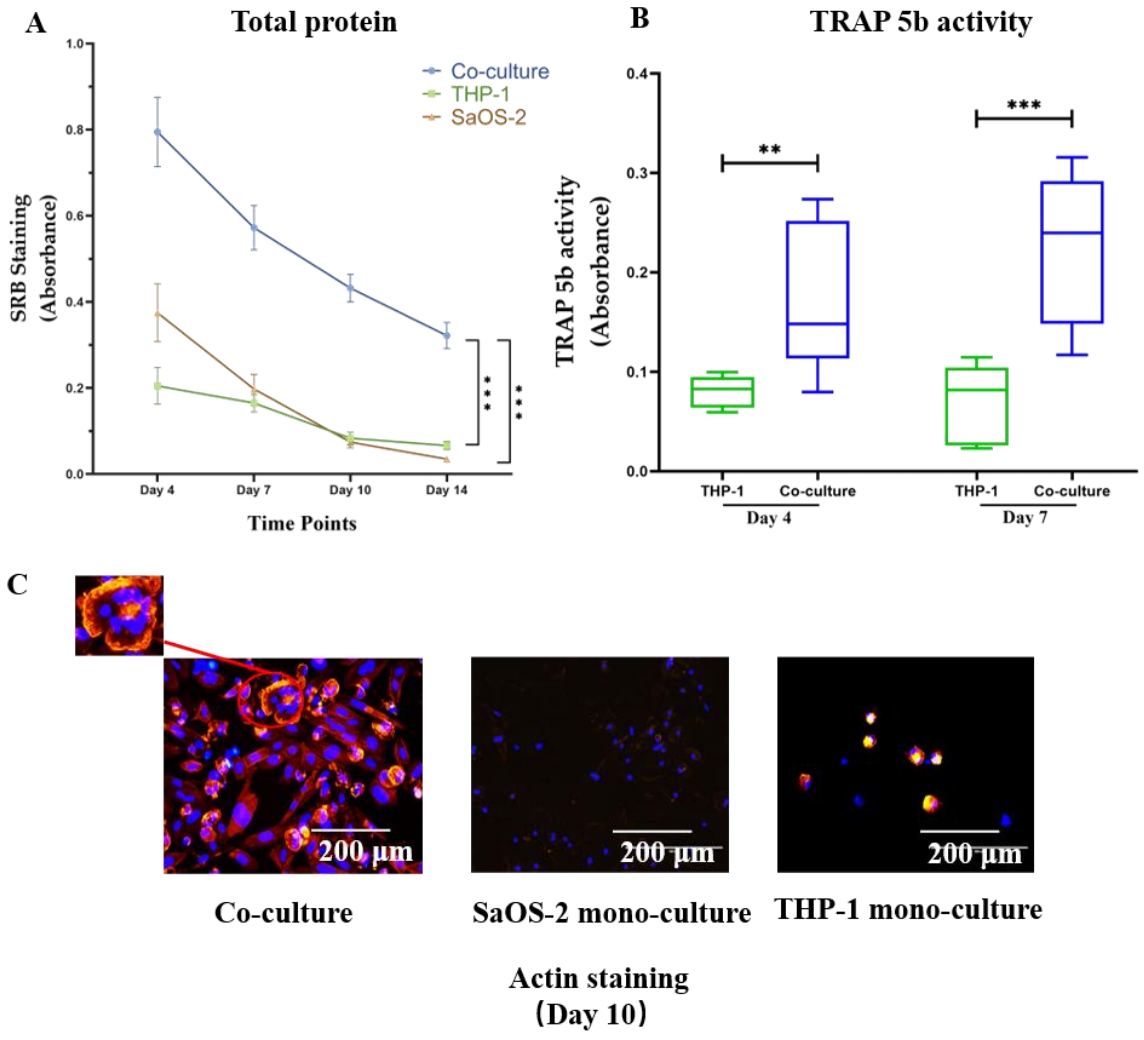


Figure 3.2 Comparison of SaOS-2 and THP-1 mono-culture and co-culture. **A.** SRB staining of co-culture of SaOS-2 and THP-1 cells/ THP-1 mono-culture/ SaOS-2 mono-culture on day 4, 7, 10 and 14.. **B.** TRAP 5b activity of co-cultures of SaOS-2 and THP-1 cells and THP-1 cell mono-cultures. Data are represented the mean ± SEM, and the significance was determined as $^{**}p < 0.01$ and $^{***}p < 0.001$ (N = 3, n = 3). **C.** Representative actin ring/ nuclei staining in co-cultures/ SaOS-2 mono-culture/ THP-1 mono-culture on day 10. Osteoclasts were determined by the presence of actin ring formation and the presence of at least two nuclei, while Osteoblasts were identified by actin filament structures and one nucleus.

3.3. CSE decreased cell viability in a dose dependent manner in the co-culture of SaOS-2 and THP-1 cells

CSE with the concenrations ranged from 0% to 10% was tested in the co-culture system up to 14 days. Cells decreased with increasing CSE concentrations, and 10% of CSE was too toxic for cells to maintain their survival after 7 days (Figure 3.3A). Actin staining showed typical osteoclastic structures in the co-cultures and confirmed SRB results (Figure 3.3B).

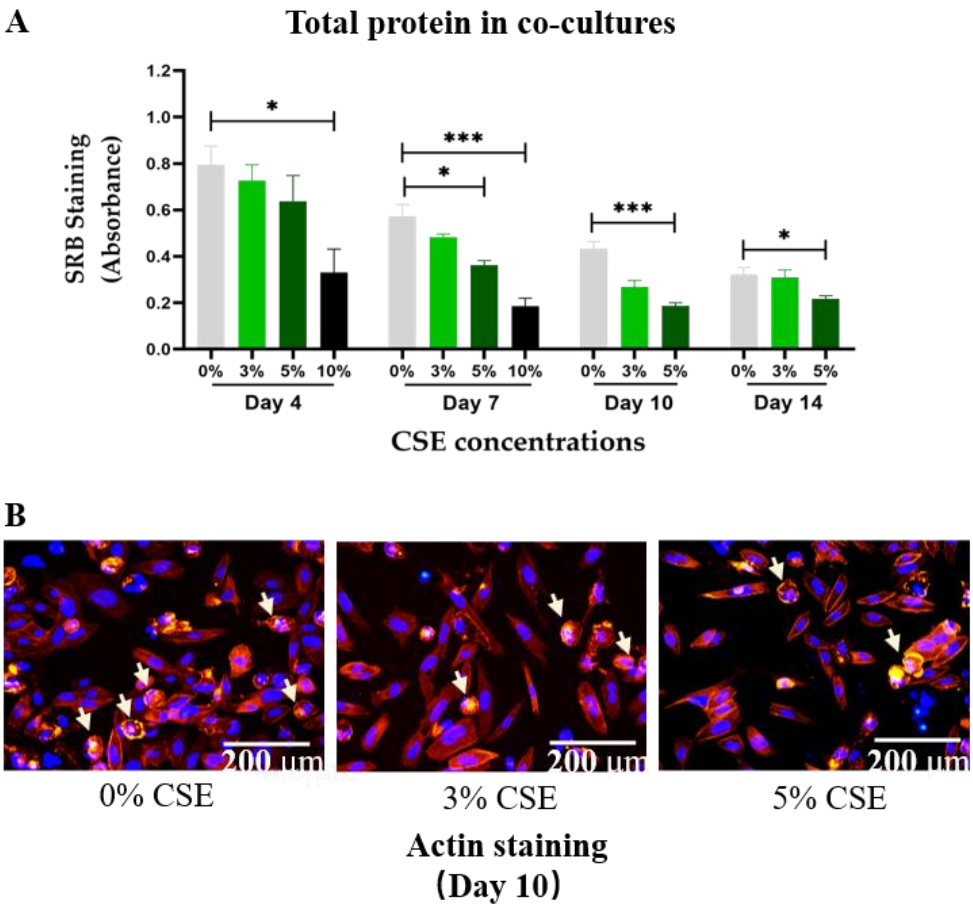


Figure 3.3. The effect of CSE concentrations on cell viability in the co-culture of SaOS-2 and THP-1 cells. **A.** SRB staining of co-cultures of SaOS-2 and THP-1 cells with exposure to CSE concentrations on day 4, 7, 10 and 14 ($N \geq 3$, $n = 3$). Data are represented as the mean ± SEM, and the significance was represented as $*p < 0.05$, and $***p < 0.001$ vs. 0% CSE group. **B.** The representative actin ring/ nuclei staining of co-cultures of SaOS-2 and THP-1 cells exposed to different CSE concentrations on day 10. The white arrows indicate actin rings which are formed by mature osteoclasts.

3.4. CSE enhanced osteoclastic function in the co-culture of SaOS-2 and THP-1 cells in a dose dependent manner.

CA II and TRAP 5b are markers of early and late stages of osteoclast differentiation, respectively (Jeong *et al.* 2019; Lehenkari *et al.* 1998). CA II and TRAP 5b activity in the co-cultures were up-regulated by CSE in a dose dependent manner (Figure 3.4A and 3.4 B).

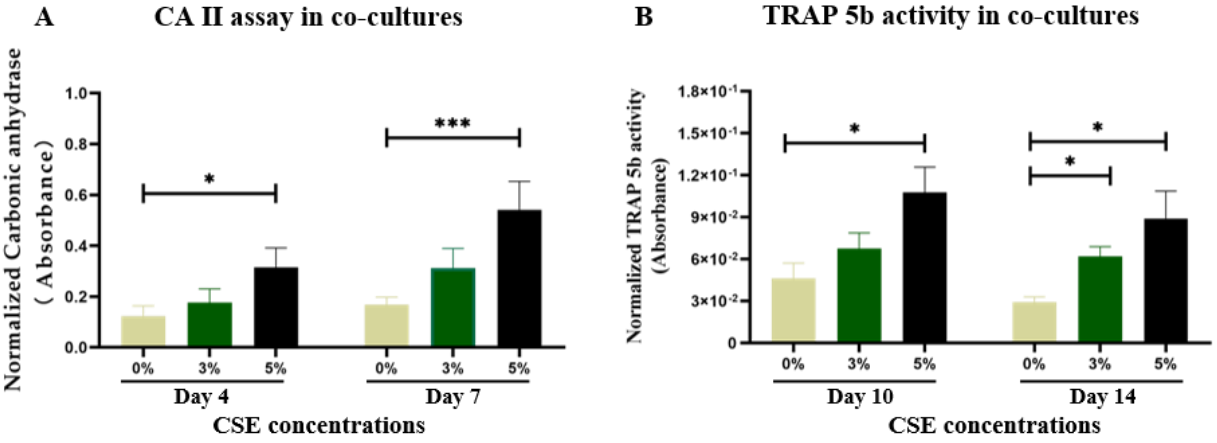


Figure 3.4. The effects of CSE concentrations on cell function in the co-culture of SaOS-2 and THP-1 cells. **A.** CA II assay representing the osteoclastic differentiation of co-cultures. **B.** TRAP 5b activity representing the mature osteoclastic function of co-culture of SaOS-2 and THP-1 cells on day 10 and day 14. Data are representing the mean ± SEM. Significance was represented as $*p < 0.05$, and $**p < 0.01$ ($N = 3$, $n = 3$).

3.5. CSE decreased matrix remodeling in the co-culture of SaOS-2 and THP-1 cells in a dose dependent manner.

Alizarin-red staining representing bone matrix remodeling results from the interaction between osteoblasts and osteoclasts. Alizarin-red staining was decreased by CSE in a dose dependent manner in the co-cultures of SaOS-2 and THP-1 cells, and 5% of CSE significantly decreased matrix remodeling compared to control group (Figure 3.5).

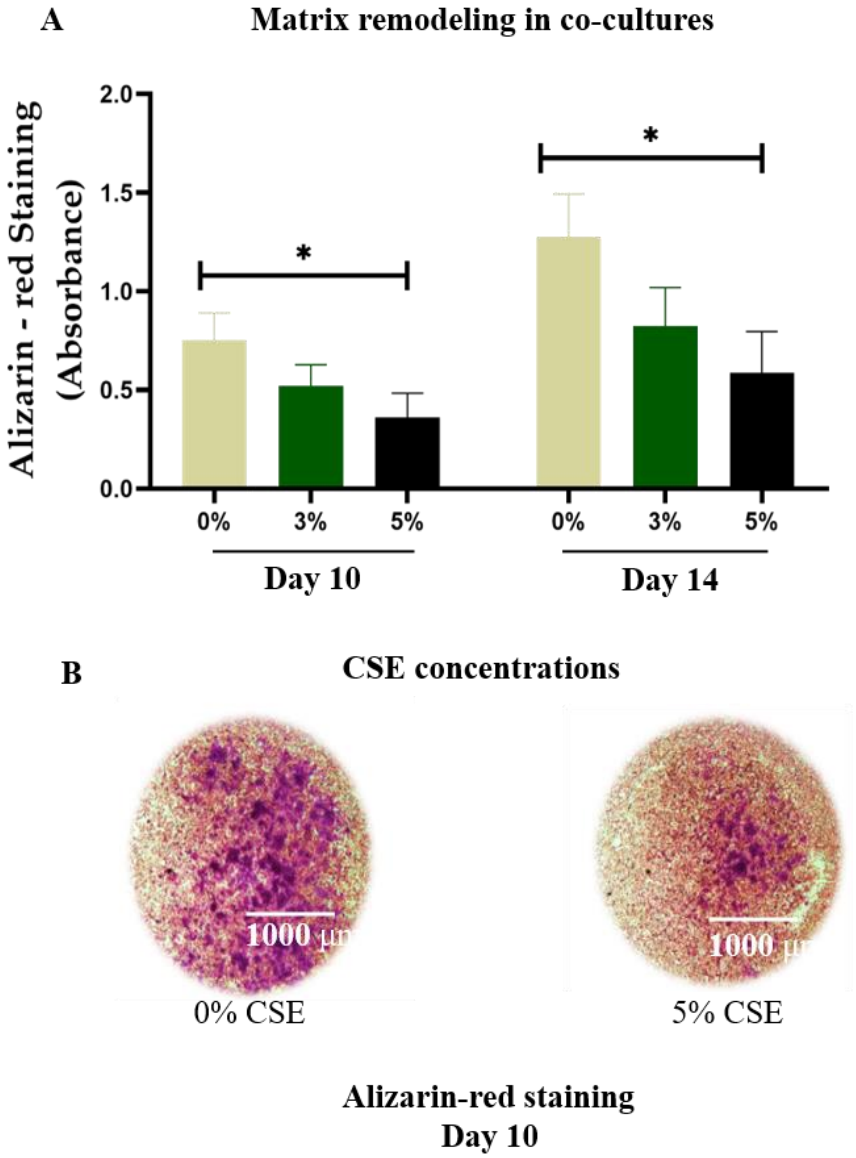


Figure 3.5. The effects of CSE concentrations on matrix remodeling in the co-culture of SaOS-2 and THP-1 cells. **A.** Alizarin-red staining of co-culture of SaOS-2 and THP-1 cells on day 10 and day 14 (N = 3, n = 3). Data are representing the mean ± SEM. Significance was represented as $*p < 0.05$. **B.** The representative microscopic representative images showed Alizarin red staining of 0% CSE (control group) and 5% CSE group on day 10.

3.6. BPs reduced the effects of CSE on the co-culture of SaOS-2 and THP-1 cells.

CSE at the concentration of 5% was shown to improve osteoclast function and reduce bone remodeling in the co-cultures, therefore 5% of CSE was used in the subsequent experiments. BPs (zoledronate and alendronate) as a classical anti-osteoporotic drug were tested in CSE-affected cells in the co-culture model. Zoledronate and alendronate both decrased TRAP 5b activity elevated by CSE, and

alendronate group showed significant difference compared to CSE group (Figure 3.6A). Zoledronate and alendronate showed a positive effect on matrix remodeling decresed by CSE in the co-culture of SaOS-2 and THP-1 cells (Figure 3.6B), and the microscopy images confirmed the alizarin-red results (Figure 3.6C).

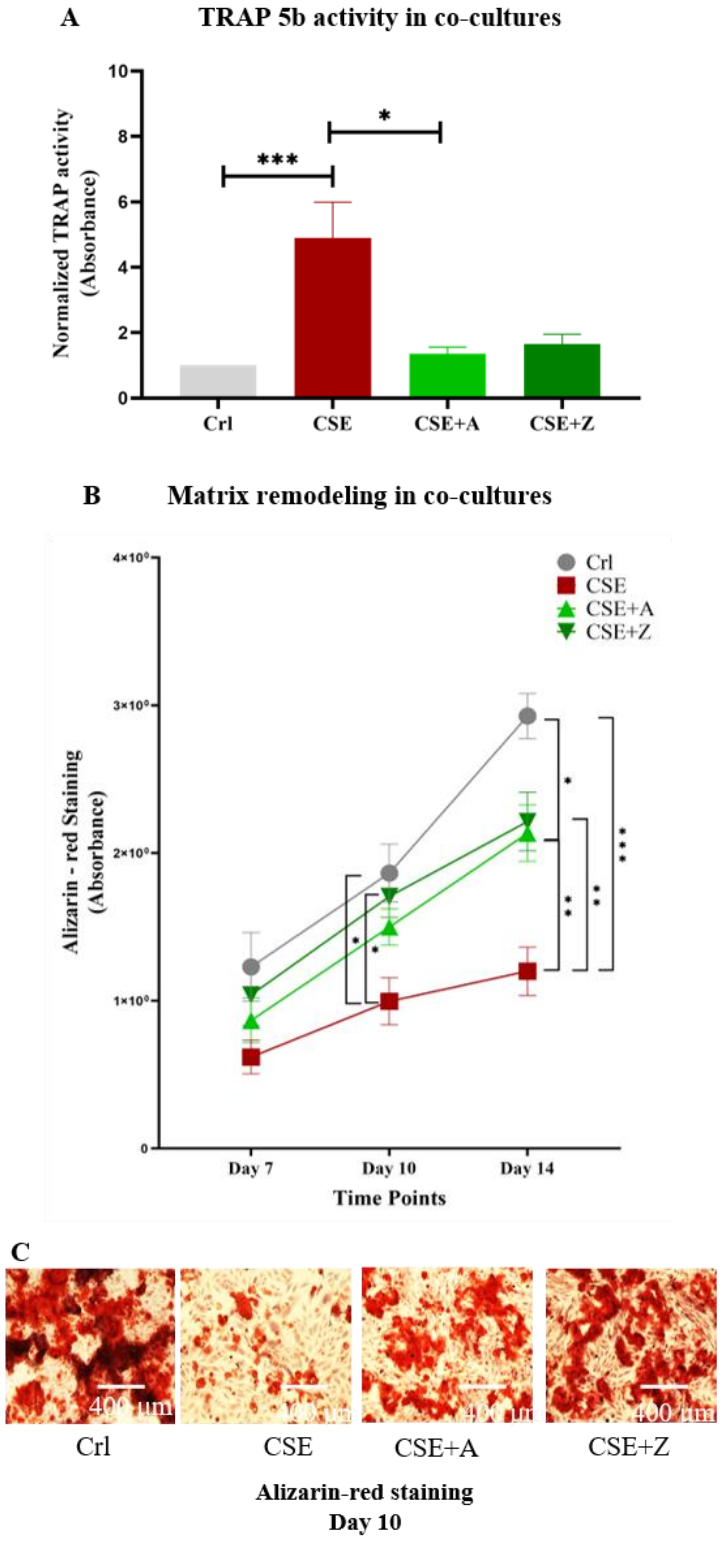
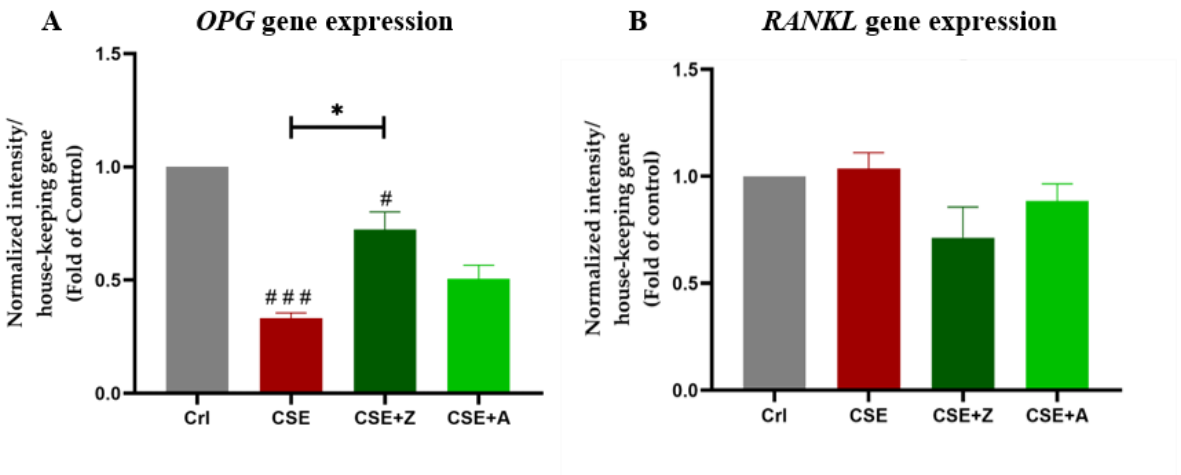


Figure 3.6. The effects of BPs on CSE-affected cells in the co-culture of SaOS-2 and THP-1 cells. **A.** TRAP 5b activity results of the co-cultures exposed to the same experimental set-up as for 7A and /B on day 7.**B.** Alizarin red staining of co-cultures exposed to 5% CSE with or w/o alendronate or zoledronate on day 7, 10, 14. **C.** A representative microscopy image showing Alizarin red staining of 0% CSE (Crl group) and 5% CSE group with or w/o alendronate or zoledronate on day 10. Data are shown as the mean ± SEM, and the significance was set as $*p < 0.05$, $**p < 0.01$ and $**p < 0.01$ (N=3, n=3).

3.7. CSE up-regulated the RANKL/ OPG ratio and osteoclastic markers at the gene level, BPs can reverse the effects of CSE on co-cultures.

OPG and *RANKL* which expressed by osteoblasts, and *NFATC1* which exressed by osteclasts were tested by PCR measrements in the co-cultures. Osteoclast differentiation relies on the RANKL/ OPG ratio and *NFATC1* is considered an crucial regulator for osteoclast differentiaon. The results showed 5% of CSE siginificantly down-regulated the *OPG* expression, but up-regulated the *NFATC1* expression. Although no obvious difference was found in the *RANKL* gene expression, the calculated RANKL/ OPG ratio was significantly enhanced by CSE in the co-cultures. Zoledronate and alendronate were shown to reduce the enhanced effect of CSE on *OPG* gene expression, resulting in a decreased RANKL/ OPG ratio elevated by CSE. The BPs also decreased the *NFATC1* gene expression enhanced by CSE (Figure 3.7).



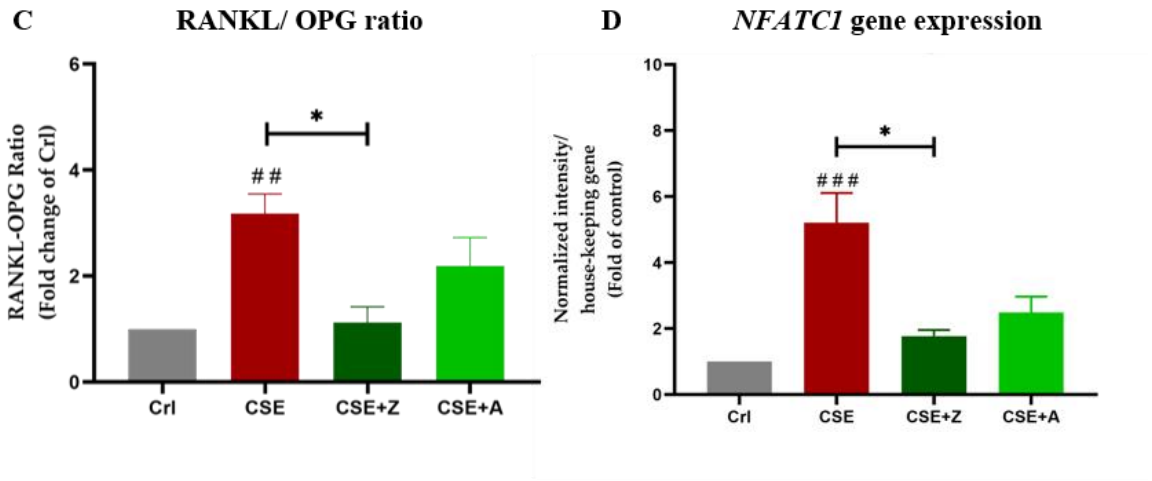
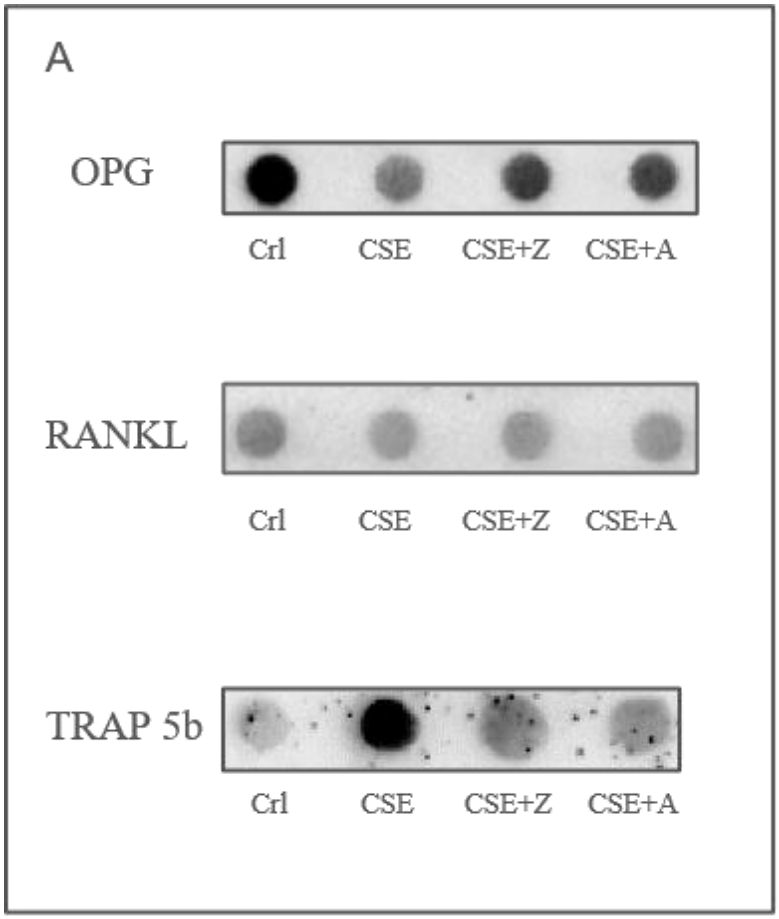


Figure 3.7. The effect of BPs on CSE-affected gene expression of osteoblastic and osteoclastic markers in the co-culture. **A.** PCR of *OPG* gene expression in co-cultures exposed to CSE with or w/o zoledronate and alendronate on day 4. **B.** PCR of *RANKL* gene expression in co-cultures exposed to CSE with or w/o zoledronate and alendronate on day 4. **C.** The RANKL/ OPG ratio of gene expression in co-cultures on day 4. **D.** PCR of *NFATC1* gene expression in co-cultures exposed to CSE with or w/o zoledronate and alendronate on day 4. Data are shown as the mean ± SEM, and the significance was set as $^{*}p < 0.05$, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. Crl group (N = 3, n ≥ 2).

3.8. CSE decreased OPG protein levels but increased TRAP 5b protein levels in the co-cultures. BPs counteracted the effects of CSE on TRAP 5b protein levels and the RANKL/ OPG ratio

OPG and RANKL are osteoblast-secreted proteins and co-regulate osteoclast differentiation. CSE showed a significantly negative effect on OPG levels, but slight effect on RANKL levels in the co-cultures. Therefore a increased RANKL/ OPG ratio was shown in the CSE group, indicating an enhancement of osteoclastic differentiation. Consistantly, TRAP 5b protein levls representing osteoclastic function was increased in the CSE group. BPs was able to reverse the effects of CSE on protein levels in the co-cultures, resulting in a lower RANKL/ OPG ratio and TRAP 5b levels compared to the CSE group (Figure 3.8).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TRAP 5b
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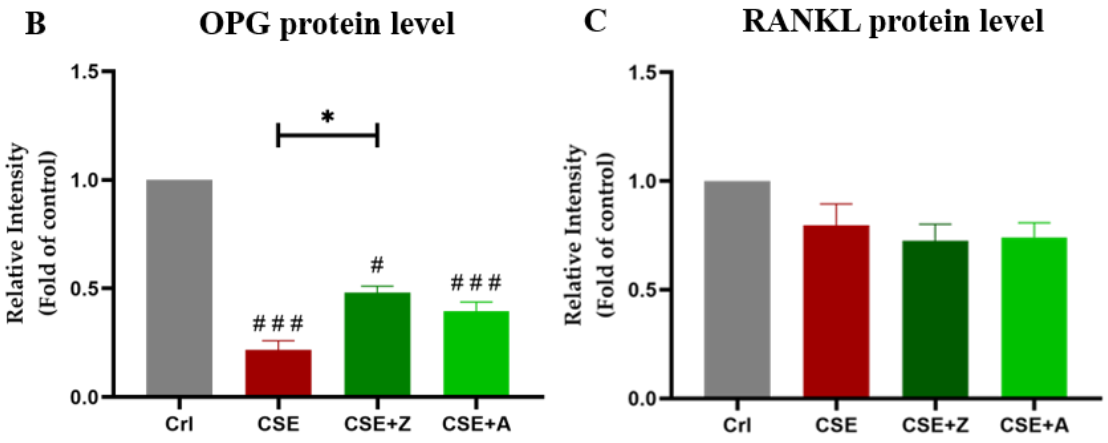
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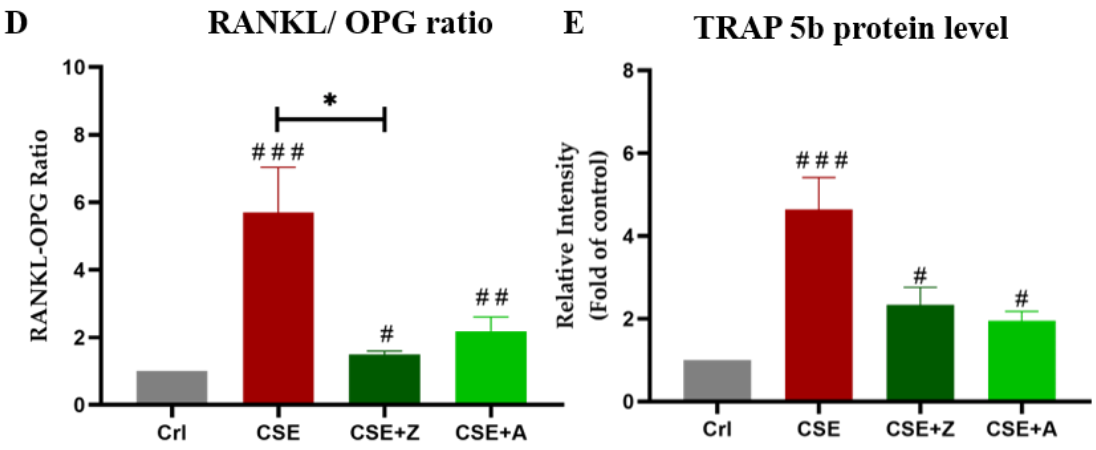

Figure 3.8. The effect of BPs on CSE-affected protein levels of osteoblastic and osteoclastic markers in the co-culture. **A.** Representative Dot blot images of protein levels of OPG, RANKL and TRAP 5b. **B.** Dot blot analysis of the OPG protein level in co-cultures exposed to CSE with or w/o zoledronate and alendronate on day 4. **C.** Dot blot analysis of the RANKL protein level in co-cultures exposed to CSE with or w/o zoledronate and alendronate on day 4. **D.** The RANKL/ OPG ratio of protein levels in co-cultures on day 4. **E.** Dot blot analysis of the TRAP 5b protein level in co-cultures exposed to CSE with or w/o zoledronate and alendronate on day 4. Data are represented as the mean ± SEM, and the significance was represented as $*p < 0.05$, $**p < 0.01$, and $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ vs Crl group (N = 3, n = 3).

Chapter 4

4. Discussion

Bone, as a dynamic tissue, is continually formed and absorbed throughout a human life. (Grabowski 2009). The balance between bone formation and resorption is inextricably linked to bone strength, and any disturbance in this balance can lead to bone metabolic diseases (Myneni and Mezey 2017). Osteoporosis has currently become the most prevalent bone metabolism disease, resulting in significant economic burden, compromised life quality and considerable morbidity of patients (Kanis *et al.* 2019). Therefore, it becomes increasingly demanded to establish a cell culture model that can effectively mimic various osteoporotic alterations *in vivo* to investigate its pathological mechanisms and potential therapeutic agents. The basic pathogenesis of osteoporosis is osteoblast inhibition and osteoclast activation, leading to a loss of bone mass (Liu *et al.* 2019). The communication and interaction between osteoblasts and osteoclasts have been extensively demonstrated to be related to the development of osteoporosis (Kendler 2011; Rachner *et al.* 2011). Cell cultures allowing cell-cell communications are better models to reflect osteoporosis than mono-cell cultures. Thus, to establish a feasible and replicable co-culture model of osteoblasts and osteoclasts is becoming imperative. Although some exiting co-culture models have been used for osteoporosis research, limitations are still evident. For example, Maria *et al.* used an indirect co-culture model of osteoblast and osteoclast based on transwell technics *in vitro*, but direct cell-cell contacts could not be approached in this set-up (Maria *et al.* 2017). Additionally, Schulze *et al.* included a direct co-culture allowing cell-cell contact in their pre-clinical cell culture models, but an effective method for distinguishing the two cell types had still not been solved (Schulze *et al.* 2018). In the present study, a supplement-free co-culture model of SaOS-2 and THP-1 cells mimicking the *in vivo* cell-cell contact and allowing to distinguish two cell types by the gender-specific DNA quantification method was established. Based on our previous study, SaOS-2 cells showed advantages of maturity, protein expression and matrix formation among human osteoblastic cell lines, and THP-1 cells had a better compatibility and stability in co-

cultures compared to other human osteoclastic cell lines (Ehnert *et al.* 2020). Most osteoclast-related studies require additional M-CSF and RANKL to induce differentiation of osteoclast precursors, however SaOS-2 was expected to directly induce THP-1 cells differentiation in this study due to its strong capacity to secrete proteins. In this regard, THP-1 mono-culture was first performed with conditioned medium of SaOS-2 cells, and cells could maintain survival and osteoclastic function till 7 days. Clear actin-ring structures with multi nuclei indication mature osteoclasts were found, and TRAP 5b activity representing osteoclastic function was also detected on day 4 and day 7 proving the successful differentiation of THP-1 cells. These results provided solid bases for the next step in the development of the co-culture model of SaOS-2 and THP-1 cells. In the co-cultures, whether SaOS-2 cells can coexist with THP-1 cells in a shared medium is the primary question to be investigated. According to the SRB staining results, cells in co-cultures not only remained viable until 14 days, but also had significantly higher cell viability than that in mono-cultures. The results demonstrated that co-cultures of SaOS-2 and THP-1 cells were more stable than mono-cultures. Moreover, significantly higher TRAP 5b activity was detected in co-cultures than in mono-cultures, demonstrating that THP-1 cells were better differentiated in co-cultures than in mono-cultures. Overall, the co-cultures of SaOS-2 and THP1 cells emphasized the importance of cell-cell contact and communication for cell survival and cell function.

CS is a recognized risk factor for osteoporosis, and smoking cessation is considered an effective strategy for maintaining bone mass (Farrah and Jawad 2020). Unfortunately, it is not always a feasible alternative for long-term smokers to quit smoking (Murphy-Hoefer *et al.* 2020). Substitutes of traditional cigarettes such as E-cigarettes, tobacco heating system have been proposed and studied recently, which have been demonstrated to cause less damage to the skeletal system than traditional cigarettes (Aspera-Werz *et al.* 2020). However, these alternatives have not been widely accepted by smokers, and their health benefits are still controversial (Sutanto *et al.* 2020). Therefore, it remains essential to understand the specific mechanisms by which

traditional cigarettes cause skeletal disorders such as osteoporosis and to screen possible treatments for traditional cigarette users. Our previous studies have demonstrated a significantly detrimental effect of CSE on bone mesenchymal stem cells (Aspera-Werz *et al.* 2019), osteoblasts (Holzer *et al.* 2012), and another study have shown the potential activation of osteoclast-like cells by CSE (Igari *et al.* 2019). In the present study, CSE was novely evaluated in the described co-culture model of SaOS-2 and THP-1 cells. SRB staining results showed that cell viability in co-cultures was decreased by CSE in a dose dependent manner. CA II and TRAP 5b can be used as markers of osteoclast differentiation and maturation, respectively. CA II and TRAP 5b activity were both elevated by CSE in co-cultures, demonstrating the positive effect of CSE on osteoclasts. CSE also showed a significantly negative effect on matrix remodeling according to the alizarin-red results, confirming the detrimental effect of CSE on bone mass. These results showed that CSE could cause osteoporosis-like alterations of cells in the co-culture model.

As CSE has effectively stimulated osteoporosis-like alterations in the co-culture of SaOS-2 and THP-1 cells, it is of great value to screen potential treatments to reverse these alterations by the co-culture model. BPs are the most commonly used anti-osteoporosis drugs, and their anti- resorptive properties have been widely recognized (Martiniakova *et al.* 2020). Zoledronate and alendronate, the most frequently prescribed BPs by clinicians, were tested on CSE-affected cells in the co-cultures. Alizarin-red staining results showed that zoledronate and alendronate both significantly increase matrix remodeling reduced by CSE in co-culturs on day 14. The tested BPs also decreased TRAP 5b activity elevated by CSE confirming the anti-osteoclastic effect of BPs. Overall, CSE induced osteoporotic-like changes, while BPs reversed these changes and displayed their anti-osteoclastic effects in the co-culture system. This demonstrated that the co-culture system was able to mimic the real *in vivo* environment and respond to stimuli sensitively.

Although it is currently recognized that BPs inhibit osteoclasts via the mevalonate pathway (Drake *et al.* 2008), the mechanisms of actions of BPs on bone cells remains

to be clarified (Bellido and Plotkin 2011). For example, BPs was shown to reduce matrix mineralization by affecting osteoblasts (Huang *et al.* 2015), while another study suggested BPs to promote osteoblast proliferation and maturation (Im *et al.* 2004). Moreover, in addition to the mevalonate pathway, recent evidence indicated that BPs could also affect osteoclast differentiation via the RANKL/ OPG pathway. (Dobnig *et al.* 2006; Pan *et al.* 2004). However, the relationship between BPs and the RANKL/ OPG ratio is still being debated. Some studies showed no significant effects from BPs on the RANKL/OPG ratio (Kim *et al.* 2002; Verde *et al.* 2015), while BPs was shown to affect all three stages of bone remodeling, including regulating RANKL and OPG levels and osteoclastogenesis (Mulcahy *et al.* 2015). BPs were shown to enhance the RANKL/OPG expression of osteoblasts *in vitro*, suggesting that BPs may promote osteoclast differentiation under physiological conditions (Koch *et al.* 2012). On the contrary, several clinical studies demonstrated that BPs could reduce RANKL expression and promote OPG expression in patients with osteoporosis (D'Amelio *et al.* 2010; Dobnig *et al.* 2006). Other *in vitro* studies also suggested that BPs enhanced OPG expression but inhibit RANKL expression (Tsubaki *et al.* 2012; Viereck *et al.* 2002). In the present study, RANKL and OPG of cells in co-cultures were evaluated at the gene and protein levels. CSE significantly down-regulated OPG levels, but had no obvious effect on RANKL levels, resulting a significantly increased RANKL/ OPG ratio. The results are consistent with an *in vivo* study showing smokers have a significantly suppressed OPG production and an increased RANKL/ OPG ratio (Tang *et al.* 2009). Zoledronate and alendronate could reduce the effect of CSE on OPG gene expression and protein levels in co-cultures, leading to a lower RANKL/ OPG ratio compared to that in CSE group. The RANKL/ OPG ratio can be used as an important indicator of osteoclast differentiation and survival, and subsequently, bone mass and skeletal integrity (Yang *et al.* 2020). In theory, RANKL is required for osteoclast differentiation, while OPG competes with RANKL for RANK receptors thereby hindering osteoclast differentiation. Thus, the increased RANKL/ OPG ratio is considered an important indicator of activated osteoclastic differentiation and function *in vivo* (Lenneras *et al.*

2015). As osteoclastic markers, such as NFATC1 gene expression and TRAP 5b protein levels are consistent with the RANK/OPG ratio in the present study. Both osteoclastic makers were up-regulated by CSE, and zoledronate and alendronate could counteract the effect of CSE in co-cultures. In summary, BPs could inhibit CSE-induced osteoclastic differentiation by down-regulating the RANKL/ OPG ratio (Figure 4.1).

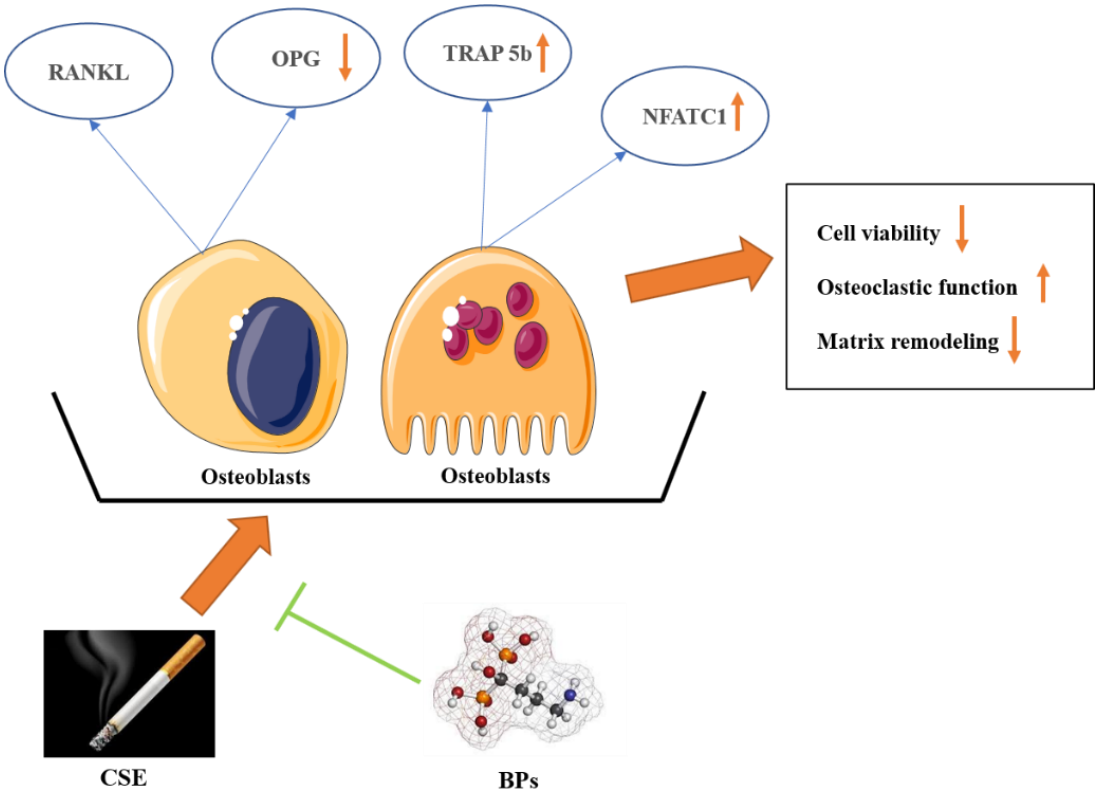

Figure 4.1. Summary of the results in the thesis. OPG levels in the co-culture of osteoblasts and osteoclasts were reduced by CSE, resulting in the increased RANKL/ OPG ratio and osteoclastic function and decreased cell viablity, matrix remodeling. BPs reversed the effect of CSE on the co-cultures by modulating the RANKL/ OPG ratio.

In the clinic, there is no treatment for osteoporosis in smokers specifically. BPs could be potentially used for osteoporotic smokers based on our results. However, it is still needed to validate the effects of BPs on smoking patients by *in vivo* studies and clinical trials, and BPs-related administration (*e.g.* dosage and duration) (Ko *et al.* 2017) and adverse events (musculoskeletal pain, atrial fibrillation, and hypocalcemia) need be carefully monitored (Reyes *et al.* 2016). Osteonecrosis of the jaw is an important

potential side event of bisphosphonate therapy, and smoking patients treated with BPs have a higher risk of osteonecrosis of the jaw than non-smokers (Adler *et al.* 2016). CS has also been indicated to be associated with poor meication adherence among bisphosphonate-treated patients (Yeam *et al.* 2018). These aspects are worthy of attention and further research when applying bisphosphonates to smokers. Furthermore, the co-culture model presented in this thesis could be applied to explore other risk factors or comorbidities of bone metabolic diseases. For example, alcohol use or diabetic conditions could be simulated in this model and specific treatments could be tested. In the future, this 2D model is expected to transform into a 3D model to better mimic the *in vivo* physiological environment. The fabrication of 3D scaffolds, maintenance of cell survival, and selection of appropriate readouts will be the main challenges.

Chapter 5

5.1. Summary

Osteoporosis has become a bone metabolism disease that currently afflicts elderly patients, leading to a huge financial burden and impairment of life quality. CS has been shown to be associated with osteoporosis by *in vivo* studies, however treatments for osteoporosis in smokers have yet to be investigated. To design a cell culture model of osteoporosis, the combined effects of osteoblasts and osteoclasts must be taken into account due to the importance of the communication between these two types of cells. BPs are used for osteoporosis treatment mainly due to their anti-osteoporotic function, but other potential mechanisms of BPs and their effects on osteoporotic smokers are unclear.

SaOS-2 and THP-1 cells were selected to establish a supplement-free direct co-culture in the present thesis. THP-1 cells were successfully induced by SaOS-2 secretions to differentiate into osteoclast-like cells in the co-culture model. CSE had a negative effect on cell viability and matrix remodeling in the co-cultures. A possible possible mechanism was to increase the RANKL/ OPG ratio by significantly inhibiting OPG levels. BPs demonstrated a property to counteract the effects of CSE on cells in the co-culture model, resulting in a decreased RANKL/ OPG ratio (Figure 5.1).

Based on these results, BPs could be potentially used for osteoporotic smokers with the cautious monitoring of adverse reactions. Clinical trials are needed to validate the effects of BPs on smoking patients. Furthermore, the described co-culture model of osteoblasts and osteoclasts could be applied to explore other risk factors or comorbidities of bone metabolic diseases, and transformed into a 3D cell culture system in the future.

5.2. Zusammenfassung

Osteoporose ist zu einer Erkrankung des Knochenstoffwechsels geworden, von der zunehmend ältere Patienten betroffen sind und die zu einer enormen finanziellen Belastung und Beeinträchtigung der Lebensqualität führen. *In vivo* Studien haben gezeigt, dass Zigarettenrauch mit Osteoporose in Verbindung gebracht werden kann, doch die Behandlung von Osteoporose bei Rauchern muss noch untersucht werden.

Da die Kommunikation zwischen Osteoblasten und Osteoklasten eine wichtige Rolle spielt, müssen die Wirkungsweisen beider Zelltypen bei der Entwicklung eines Zellkulturmodells für Osteoporose berücksichtigt werden.

Bisphosphonate werden hauptsächlich wegen ihrer antiosteoporotischen Funktion zur Osteoporosebehandlung eingesetzt, wobei andere potenzielle Mechanismen der Bisphosphonate und ihre Auswirkungen auf osteoporotische Raucher unklar sind.

In der vorliegenden Arbeit wurden SaOS-2- und THP-1-Zellen ausgewählt, um eine direkte Kokultur zu etablieren, ohne weitere Zusätze hinzuzufügen. Aufgrund der Produktion von bestimmten Stoffen durch SaOS-2-Zellen, wurden die THP-1 Zellen dazu gebracht, sich im Kokulturmodell zu osteoklastenähnlichen Zellen zu differenzieren. Zigarettenrauchextrakt hatte einen negativen Effekt auf die Zelllebensfähigkeit und den Matrixumbau in den Kokulturen. Ein möglicher Grund war der Anstieg des RANKL/OPG-Verhältnisses durch eine signifikante Hemmung der OPG-Konzentration. Bisphosphonate wirkten dem Einfluss des Zigarettenrauchextrakts auf die Zellen im Kokulturmodell entgegen, was zu einem verringerten RANKL/OPG-Verhältnis führte.

Auf der Grundlage dieser Ergebnisse könnten die Bisphosphonate bei osteoporotischen Rauchern eingesetzt werden, wobei potenzielle Nebenwirkungen umsichtig überwacht werden müssen. . Klinische Studien sind erforderlich, um die Auswirkungen von Bisphosphonaten bei rauchenden Patienten zu validieren. Darüber hinaus könnte das beschriebene Kokulturmodell von Osteoblasten und Osteoklasten zur Erforschung anderer Risikofaktoren oder Komorbiditäten von Knochenstoffwechselerkrankungen angewandt und in Zukunft in ein 3D-Zellkultursystem umgewandelt werden.

Chapter 6

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Chapter 7

7. Declaration

All work included in the thesis was performed in Siegfried Weller Institute for Trauma Research, Eberhard Karls Universität Tübingen, Tübingen.

The thesis and related work were supervised by Prof. Dr. A.K. Nüssler.

Prof. Dr. A.K. Nüssler, Dr. Sabrina Ehnert and me conceptualized the thesis. Dr. Romina H. Aspera-Werz and me designed the experiments. All experiments were performed and analyzed by myself. Prof. Dr. A.K. Nüssler, Dr. Sabrina Ehnert, Dr. Romina H. Aspera-Werz, Dr. Tao Chen, Victor Häussling, Bianca Braun, Weidong Weng reviewed and discussed results and conclusions of the thesis.

I declare that all the results are from my own research data, except for the quoted references and figures. Also, all the figures from the website or other papers have permission licenses.

I hereby declare that the submitted thesis entitled: “Establishment of a co-culture model of osteoblasts and osteoclasts to study osteoporotic alterations caused by cigarette smoking: Role of Bisphosphonates” has been composed by myself and that the work has not be submitted for any other degree or professional qualification.

_____

Place/date/signature of doctoral candidate

Chapter 8

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Chapter 9

9. Curriculum vitae

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- **Zhu S**, Ehnert S, Rouß M, Chen T, Nüssler AK, *et al.* From the Clinical Problem to the Basic Research- Co-Culture Models of Osteoblasts and Osteoclasts. Int J Mol Sci, 2018, 19 (8). IF 4.556
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