EFFECTS OF TELOMERASE ACTIVATORS ON MONOCLONAL ANTIBODY-PRODUCING CELL LINES AND STEM CELLS, AND THEIR UTILIZATION IN INDUSTRIAL PRODUCTIONS

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ABSTRACT

EFFECTS OF TELOMERASE ACTIVATORS ON MONOCLONAL ANTIBODY-PRODUCING CELL LINES AND STEM CELLS, AND THEIR UTILIZATION IN INDUSTRIAL PRODUCTIONS

Aging is a physiological and multifactorial biological process of functional decline in any living organism. Telomere shortening, high levels of reactive oxygen species (ROS), and cellular senescence are the primary physiological changes that accompany aging. While there has been an increase in human life expectancy in recent decades, there has not been a concomitant increase in healthy aging. Degenerative diseases, including musculoskeletal disorders such as osteoporosis and osteoarthritis, have been found to be directly linked to aging. Age-related degenerative diseases are devastating diseases that cause millions of deaths worldwide each year and place an economic and psychological burden on society. Due to the ever-increasing number of patients, there is a huge demand for novel therapeutic approaches to treat degenerative diseases. Two main approaches are at the forefront of technology for the treatment of degenerative diseases: stem cell transplantation (regenerative medicine) and monoclonal antibody-based therapy. Indeed, it is well known that there is a strong correlation between disease pathology and telomeres. In fact, the possible therapeutic effects of telomerase activation have been evaluated in diverse backgrounds to cure and prevent various diseases. Within the scope of this thesis, we aim to investigate the effects of telomerase activator novel molecules from *Astragalus* sp., obtained in our previous studies by biotransformation of cycloastragenol (CG) via the plant's endophytic fungi on the health span/lifespan of mesenchymal stem cells (MSCs) during *in vitro* expansion and their osteogenic differentiation. Additionally, the efficacy of these compounds was investigated in the monoclonal antibody (mAb) production process in terms of mAb productivity. Based on the outcomes of the study, novel telomerase activators deriving from natural resources of our country have significant potential in stem cell research, thus regenerative medicine, since promising results were obtained for the clinical use of these novel molecules. Our data also suggest that molecules simultaneously promote osteogenic differentiation and telomerase activation.

ÖZET

TELOMERAZ AKTİVATÖRLERİNİN ENDÜSTRİYEL ÜRETİMLERDE KULLANIMLARI, MONOKLONAL ANTİKOR ÜRETEN HÜCRE HATLARI VE KÖK HÜCRELER ÜZERİNE ETKİLERİ

Yaşlanma, fonksiyonel gerilemenin yaşandığı fizyolojik ve çok faktörlü biyolojik bir süreç olup her canlı organizmayı etkilemektedir. Telomer kısalması, yüksek düzeyde reaktif oksijen türleri (ROS) ve hücresel yaşlanma, yaşlanmaya eşlik eden başlıca fizyolojik değişikliklerdir. Son yıllarda insan ömrü beklentisinde bir artış olmasına rağmen, sağlıklı yaşlanmada eş zamanlı bir artış olmadığı görülmektedir. Osteoporoz ve osteoartrit gibi kas-iskelet sistemi rahatsızlıkları da dahil olmak üzere dejeneratif hastalıkların yaşlanmayla doğrudan bağlantılı olduğu tespit edilmiştir. Yaşa bağlı dejeneratif hastalıklar, her yıl dünya çapında milyonlarca kişinin ölümüne neden olan ve toplum üzerinde ekonomik ve psikolojik bir yük oluşturan yıkıcı hastalıklardır. Giderek artan hasta sayısı, dejeneratif hastalıkları tedavi etmek için yeni terapötik yaklaşımlara büyük bir talep oluşturmaktadır. Dejeneratif hastalıkların tedavisi için uygulanan güncel teknolojide iki ana yaklaşım ön plana çıkmaktadır: kök hücre nakli (rejeneratif tıp) ve monoklonal antikor bazlı tedavi. Diğer yandan, hastalık patolojisi ile telomerler arasında güçlü bir korelasyon olduğu bilinmektedir. Bu nedenle, telomeraz aktivasyonunun olası terapötik etkileri, çeşitli hastalıkları iyileştirmek ve önlemek için birçok hastalık için değerlendirilmiştir. Bu tez kapsamında, daha önceki çalışmalarımızda sikloastragenolün (CG) bitkinin kendi endofitik fungusları ile biyotransformasyonu ile elde edilen *Astragalus* sp. kaynaklı telomeraz aktivatörü yeni moleküllerin mezenkimal kök hücrelerin (MKH) *in vitro* çoğalma ve osteojenik farklılaşmaları sırasında sağlık süreleri/yaşam süreleri üzerindeki etkilerinin araştırılması amaçlanmıştır. Ayrıca, bu bileşiklerin monoklonal antikor (mAb) üretim sürecindeki etkinliği mAb verimliliği açısından araştırılmıştır. Çalışmalar kapsamında elde edilen sonuçlar, ülkemizin doğal kaynaklarından elde edilen yeni telomeraz aktivatörlerinin, osteojenik farklılaşmayı ve telomeraz aktivasyonunu eş zamanlı olarak desteklediğini göstermekte, kök hücre araştırmalarında ve dolayısıyla rejeneratif tıpta önemli bir potansiyele sahip olduğu gösterilmiştir.

Dedicated to my family, with love.

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ABBREVIATIONS

AG	Astragenol
ALP	Alkaline Phosphatase
BCA	Bicinchoninic Acid
BMPs	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
Ca	Calcium
CED	Camurati–Engelmann Disease
c-Fms	Colony Stimulating Factor-1 Receptor (CSF-1R)
CG	Cycloastragenol
CHOs	Chinese Hamster Ovary Cells
CMPs	Common myeloid progenitors
COL-1	Collagen Type-I
CPC	Cetylpyridinium Chloride
DAPI	4',6-Diamidino-2-Phenylindole
ddH_2O	Double Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMP1	Dentin Matrix Protein-1
DMSO	Dimethyl Sulfoxide
DSPP	Dentin Sialophosphoprotein
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
Erk	Extracellular Signal Regulated Kinase
ESC	Embryonic Stem Cell
EtOH	Ethanol
FBS	Fetal Bovine Serum
FBS	Fetal Bovine Serum

FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric Acid
HA	Hydroxyapatite
hAD-MSC	Human Adipose Tissue Derived Mesenchymal Stem Cell
HER2	human epidermal growth factor receptor 2
HSCs	Hematopoietic stem cells
hTERC	Human Telomerase RNA Component
hTERT	Human Telomerase Reverse Transcriptase
IGFs	Insulin-like Growth Factors
IL	Interleukin
iPSC	Induced Pluripotent Stem Cell
IVD	Intravertebral Disc Degeneration
JAKs	Janus Kinases
KPB	Potassium Phosphate Buffer
LDS	Loeys–Dietz Syndrome
L-Gln	L-Glutamine
mAb	Monoclonal Antibody
MAPK	Mitogen-activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
MeOH	Methanol
MEPE	Matrix Extracellular Phosphoglycoprotein
MFS	Marfan Syndrome
MMPs	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cell
mTOR	Mammalian Target of Rapamycin
mtRNA	Mitochondrial RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCPs	Non-collagenous Proteins
NF-κB	Nuclear factor kappa B

OA	Osteoarthritis
OCN	Osteocalcin
OI	Osteogenesis Imperfecta
ON	Osteonectin
OP	Osteoporosis
OPG	Osteoprogerin
OPN	Osteopontin
OPPG	Osteoporosis Pseudoglioma Syndrome
PBS	Phosphate Buffered Saline
*p*NPP	4-nitrophenyl phosphate
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone-related Peptide
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of NF-κB Ligand
ROS	Reactive Oxygen Species
Runx2	Runt-related Transcription Factor 2
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SIBLINGs	Small Integrin-binding Ligand N-linked Glycoproteins
Smad	Mothers Against Decapentaplegic Homolog
STAT	Signal Transducer and Activator of Transcription
TGF-β	Transforming Growth Factor Beta
TL	Telomere Length
TNF-α	Tumor Necrosis Factor Alpha
TSP	Thrombospondin
UPW	Ultrapure Water
Wnt	Wingless-type Mouse Mammary Tumor Virus Integration Site Family
WST-1	4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] 1,3-benzene disulfonate
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
β-gal	Beta-galactosidase

CHAPTER 1

INTRODUCTION

1.1. Aging and Degenerative Diseases

Aging is a physiological and multifactorial biological process of declining functions of every living organism. While there has been an increase in human life expectancy over the past few decades, there has not been an increase in healthy aging. Degenerative diseases such as musculoskeletal disorders, including osteoporosis and osteoarthritis, cardiovascular diseases and neurodegenerative diseases, as well as metabolic disease, have been directly linked to age [1-2]. It is possible to increase the number of examples of degenerative diseases, which are devastating diseases causing millions of deaths worldwide every year. Age-related degenerative diseases have become an economic and psychological burden for the community.

In order to identify the potential therapeutic targets for the attenuation of the pathogenesis aging process, numerous research has been carried out. Multiple biochemical and genetic pathways, including epigenetic deregulations, were found to involve aging pathogenesis, and the hallmarks of aging primarily include telomere shortening, genomic instability, mitochondrial dysfunction, loss of proteases, increased senescence, and stem cell exhaustion [3-6].

Many drug candidates are registered for clinical trials every year for the treatment of degenerative diseases. Still, most of them are discontinued after phase II trials because of a lack of proof between drug and placebo controls [7]. Although there are several outcomes for the treatment of degenerative diseases, even FDA-approved drugs, and other candidates only promise to slow down the disease, but they are still not curative. Obviously, there is still a need for novel methodologies for the treatment of these diseases. Therefore, many studies have focused on understanding the aging process to prevent or

treat age-related diseases rather than treatment since the aging process is irreversible. Since it is well known that telomere length (TL) decreases with age, telomere/telomerase has drawn attention as a potential player in these types of disorders. As a result, extensive studies have indeed shown that there is a link between telomere shortening and several age-related diseases.

On the other hand, the human body can regenerate damaged tissues and organs. But endogenous renewal process is limited and almost all degenerative diseases progressively spread to adjacent tissue [8]. Therefore, stem cell therapy for regenerative medicine is a practical approach for contributing to the complete repair of damaged or lost tissues. From this point of view, within the scope of this thesis, we focused on the efficacy of telomerase activator small molecules on the biological characteristics of stem cells and their osteoblastic differentiation to address their potential usage in regenerative medicine along with stem cells for the treatment of osteodegenerative diseases since bone-associated disorders a significant influence on the population worldwide. Indeed, it has been found that there is a strong correlation observed between osteodegeneration and short telomeres/telomerase deficiency [9].

1.2. Bone Tissue: Structure and Function

Bone tissue, one of the connective tissues, is a multifunctional tissue which is functioning by coordination with other tissues. The bone is one of the strongest biological materials particularly in bearing weight. The bone is the main supporting tissue in the body that builds the majority of the skeletal system, provides movement of the body, protects soft tissues, and contains blood-producing cells. There are two subgroups of the skeleton: axial skeleton (skull, vertebral column, ribs and sternum) and appendicular skeleton (upper and lower limbs). The skeleton of human infants has 270 bones. However, the number decreases to 206 to 213 bones by adulthood as a result of the fusion of some bones (Figure 1.1) [10]. Bones are usually classified according to their shape and size: flat bone (e.g., clavicle-collarbone), long bone (e.g., femur), sesamoid bone (e.g., patella-kneecap), and short bone (e.g., talus) [11-12].

The skeletal system is a multifunctional system; beyond its mechanical functions, together with other connective tissues, it orchestrates physiologically critical functions as an endocrine organ; it protects the mineral (mainly calcium and phosphorus) balance, provides nutrient homeostasis and protects from acidosis [13-15]. The adult bones can be divided into two groups as: spongy bones and compact, or cortical, bones according to their porosities and densities [16]. Bone marrow is found primarily in the long bones. It fills spongy bones and is mainly responsible for hemopoiesis (production of blood cells and cell components) by providing the necessary nutrients and growth factors [17-18]. Bones are composed of special cell types embedded in a unique extracellular matrix (ECM), which consists of numerous inorganic and organic components [19].

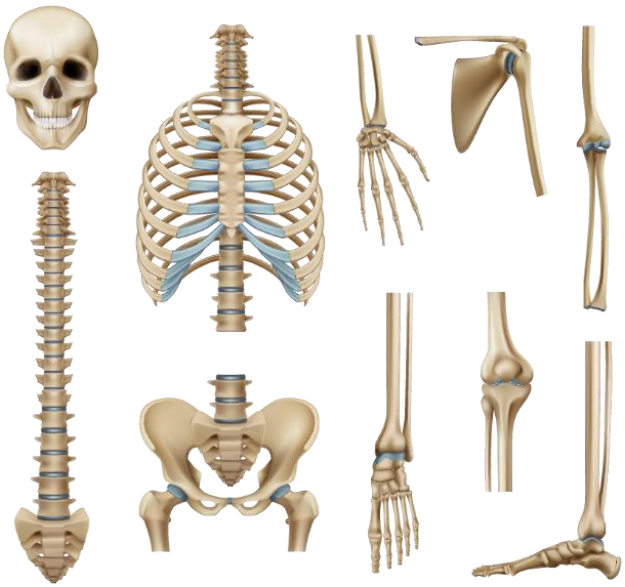

Figure 1.1. The schematic illustration of human skeleton parts

1.2.1. Bone Cells

In order to maintain bone homeostasis, the cells of the bone communicate intensely with each other and act in response to the chemical and mechanical changes in their microenvironment. Due to the fact that dysregulation of the functions of these cells

results in various bone diseases, a better understanding of defining the mechanisms of bone formation, remodeling, and regeneration is crucial for developing novel therapeutic approaches [20]. The primary cells of bone tissue are osteoprogenitor (or osteogenic) cells, osteocytes, osteoblasts, and osteoclasts (Figure 1.2), which are regulated by numerous signaling molecules and work together to form both the organic and inorganic composition of the bone tissue. Bone development and remodeling is a complex process that begins early in embryogenesis and continues throughout lifespan, with compositional changes with age [21-22].

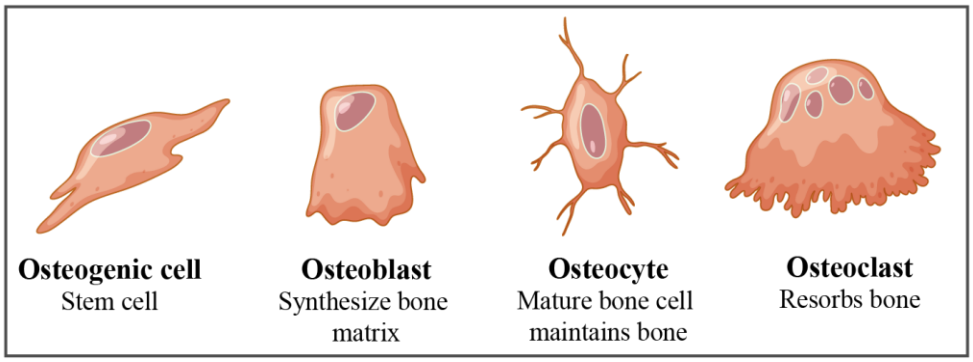

Figure 1.2. The simplified schematic representation of bone cell types

Osteogenic cells are the stem cells that originate from the local periosteum and are capable of self-renewal and differentiate into chondrocytes, osteoblasts, and marrow stromal cells or adipocytes *in vitro* [23-24]. Osteogenic cells form pre-osteoblasts and, eventually, osteoblasts, the primary cell type of bone responsible for bone growth by synthesizing the unique bone ECM. In the human body, osteoblasts have a short lifespan (three months in human bones); therefore, pre-osteoblasts are constantly ready to differentiate into osteoblasts in order to maintain bone tissue [25].

In addition to osteoblasts, the osteoblastic lineage includes osteocytes and bone-lining cells. Osteoblasts secrete an organic matrix of bone (osteoid). Once they complete this function, 50-70% of these cells either become osteocytes by entrapped in the bone matrix or converted into bone-lining cells and remain on the surface of the bone. The remaining osteoblasts undergo apoptosis [26]. Osteocytes -mature bone cells- are

osteoblasts that are trapped in the mineralized bone matrix and lose most of the cytoplasmic organelles [27]. The most abundant cell component of mammalian bones (95% of all bone cells) are osteocytes, which are located in special holes called lacuna and communicate with each other and reach blood supply from the haversian canal through the canaliculus (Figure 1.3) [28]. Bone-lining cells are inactive quiescent cells located on the bone surface as a source for active osteoblasts as well as stem cells [29-30]. The primary function of bone-lining cells is to remove demineralized matrix before bone formation by enwrapping and subsequently digesting collagen fibrils on the bone surface [31-32].

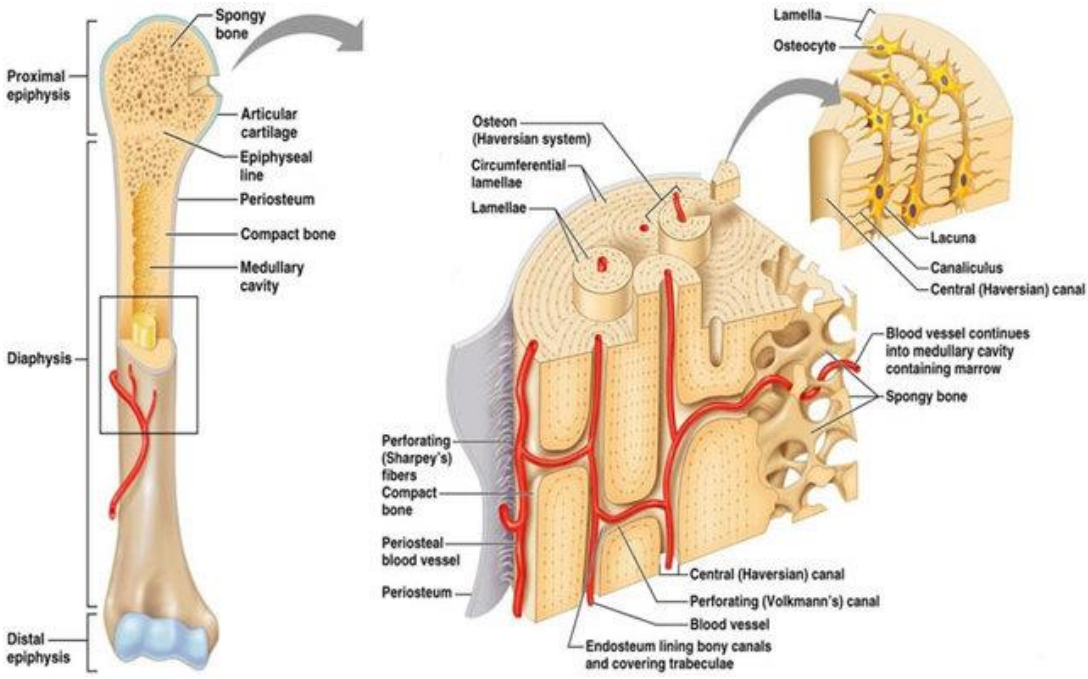

Figure 1.3. The schematic illustration of human long bone cross section [33]

Osteoclasts are multinucleated cells that are responsible for the breakdown of mineralized matrixes for the initiation of bone remodeling. Osteoclasts differ from previously defined bone cells in terms of source lineage since they originate from the precursors belonging to the hematopoietic lineage [34]. Hematopoietic stem cells (HSCs) transform into multipotent progenitor cells, which further differentiate into oligopotent progenitor cells. Common myeloid progenitors (CMPs) are one of the several types of

these oligopotent progenitor cells and give rise to osteoclast progenitor cells [35]. After the formation of osteoclast precursors in the bone marrow, they circulate in the bloodstream and are activated by primarily RANK (receptor activator of NF-κB) / RANKL (RANK ligand) / OPG (osteoprotegerin) system [36-37]. RANKL is an essential stimulator of osteoclastogenesis and is produced by both osteocytes and osteoblasts. Osteoblasts also synthesize OPG, which inhibits osteoclastogenesis by acting as a decoy receptor for RANKL [38]. Once osteoclast precursors are activated, they attach to the bone surface, where the places tagged by osteocytes for remodeling, and fuse to form multinuclear osteoclast cells. Bone degradation via osteoclastic activity occurs lifelong for resorption of aged or damaged bone [39-40]. Osteoclasts are not only responsible for the breakdown of bone, as numerous studies have shown; they also influence osteoblastic activity and regulate the differentiation of osteoblast precursors [41]. Furthermore, osteoclasts regulate HCS movement between bone marrow and bloodstream [42].

Each cell type of bone tissue has a specific role during the different stages of bone formation and remodeling process and responds according to the changes in the bone microenvironment. A variety of growth hormones, cytokines, chemokines, and external biomechanical stimuli orchestrate complex activities of bone cells, such as cellular activation, bone resorption, and matrix secretion, in order to maintain skeletal health. Bone homeostasis mainly depends on the balance between bone formation by osteoblasts and bone resorption by osteoclasts in a healthy bone under normal physiological conditions in the case. Disruption of this balance results in abnormal bone architecture and function and eventually leads to bone metabolic diseases such as osteoporosis.

1.2.2. Bone Extracellular Matrix

The bone matrix is a composite material composed of organic (or protein) and inorganic (or mineral) components. The composition of the bone matrix, including the bone cells, all together determine the mechanical properties of bone. The inorganic matrix provides bone strength and stiffness (the ability to resist deformation), while the organic matrix is responsible for toughness (energy absorption). The cellular part of bone

constantly interacts with each other through the surrounding ECM and these interactions regulate cellular function via a variety of signal transduction pathways [43-44]. The structural components of bone matrix micro- to nanoscale are given in Figure 1.4.

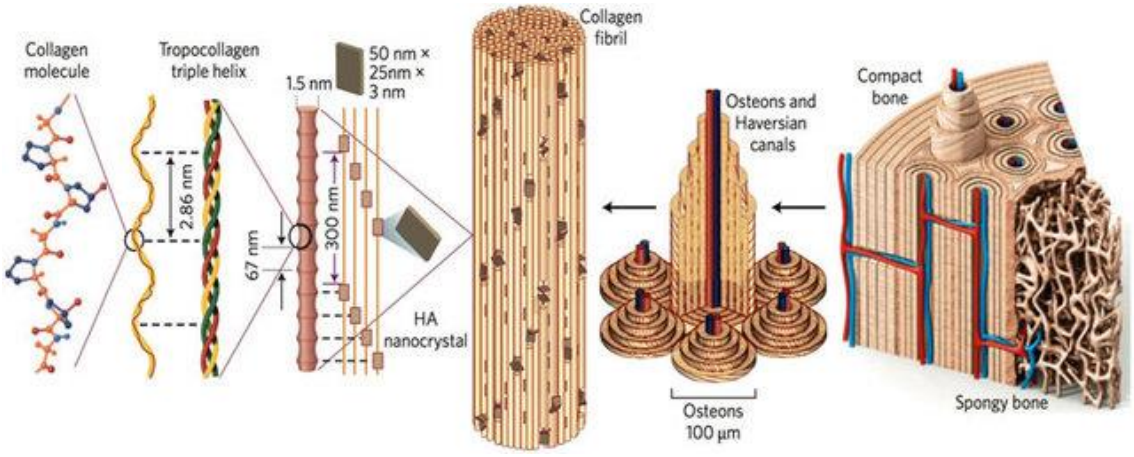

Figure 1.4. The structural components of bone micro- to nano-scale [33]

Apart from the cells, the bone matrix is composed principally of collagen in the organic phase and calcium and phosphorus salts in the inorganic phase [45-46]. The hierarchical organization of bone ECM is the primary determinant of bone mechanical and physiological properties, and alterations of different ECM components could lead to impaired bone function and, thereby, various diseases. The overall bone mass, including both organic and inorganic matrix, accounts for 50 to 70% of bone strength. Additionally, mineral density and porosity, trabecular thickness and heterogeneity, type and degree of collagen cross-linking, crystal size, and crystallinity are important parameters determining the mechanical function of bone in terms of elasticity, plasticity, and bone strength (resistance to fracture) [47-48]. The development, maintenance, and function of bone tissue depend on the proper composition and organization of the bone matrix and are enabled by bone remodeling regulated by complex biochemical and physiological signals provided by the ECM. Thus, it is of great importance to consider the complex effects of ECM signaling for designing therapeutic strategies.

1.2.2.1. Inorganic Matrix

As mentioned previously, the inorganic (or mineral) phase of the bone matrix is composed of calcium and phosphorus salts, mainly hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) form. Due to its biocompatibility and efficient osteointegration property, HA is widely used as bone filling material and to cover bone and dental implant surfaces [49]. Mineralized matrix has different crystal density and composition according to the mechanical demands of bone. The mineralized matrix formation is regulated by ECM proteins, primarily type I collagen (COL-I) and alkaline phosphatase (ALP). Mineralization occurs as a result of the accumulation of calcium phosphate crystals produced by osteoblasts and deposited in COL-1 fibrils of the matrix. Hydroxyapatite crystals grow and aggregate during bone maturation. Among other matrix proteins, proteoglycans and matrix Gla-protein are also essential for bone mineralization. Furthermore, systemic hormones (e.g., parathyroid hormone (PTH)) and vitamin D are known to be responsible for mineral homeostasis via regulating the release and absorption of calcium and phosphorus from bone [43, 50]. Alterations in ECM components in different physiologic conditions are responsible for those changes for sufficient mechanical strength and stiffness. Inorganic matrix mainly determines the strength of the bone tissue, and systemically impaired mineralization results in bone disorders and increases the risk of fracture.

1.2.2.2. Organic Matrix

Most of the organic phase of bone ECM is composed of COL-I, corresponding to 85-90% of the total protein content. However, apart from being different from collagen, more than 100 proteins have been identified in bone ECM, including linking proteins such as fibronectin, laminin and nidogen. The amount of these matrix proteins differs and determines the final functional structure of the bone tissue with appropriate tensile strength and viscoelasticity.[51-52]. Furthermore, several growth factors, cytokines, and

proteinases are embedded into the ECM; the controlled release of these components regulates gene expression levels, which modulates cell signaling cascades [53]. Moreover, the intracellular cytoskeleton between cell surface receptors and ECM plays a crucial role in signal transduction to respond to external biomechanical stimuli. Altogether, the organic matrix regulates proliferation, differentiation, migration, survival and many other cellular processes [54].

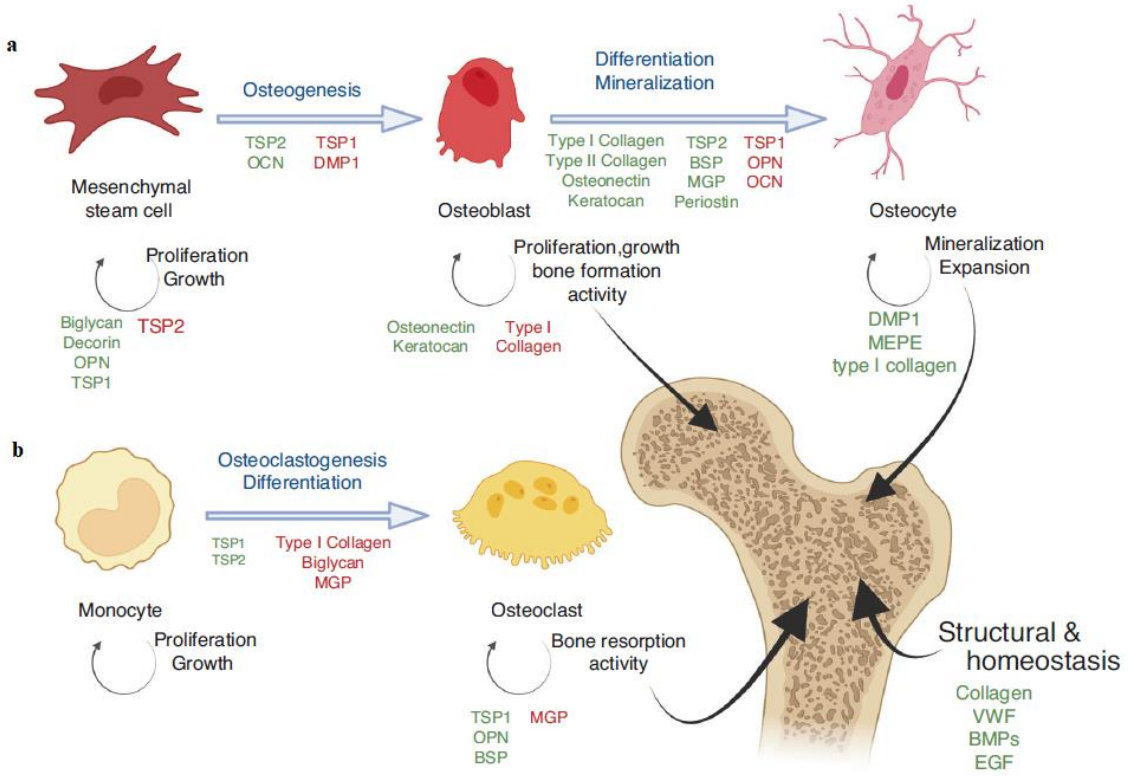

Figure 1.5. The extracellular matrix (ECM) proteins involved in bone formation, remodeling and homeostasis. ECM proteins modulating osteoblast and osteocyte (**a**), and osteoclast (**b**) formation, maturation and transformation through various signaling pathways [55]

Organic matrix proteins can be divided into collagenous proteins and non-collagenous proteins. The collagens primarily construct a scaffold for bone cells, create nucleation sites for mineralization and provide mechanical support. As mentioned before,

approximately 90% of the protein content of organic ECM of bone tissue is COL-I; however, collagen type III and V are also important constituents of bone since they are responsible for the regulation of the fiber diameter and fibrillogenesis. [56]. The tensile strength and viscoelasticity of bone tissue mainly depend on this post-translational modification of collagen, in which enzymatic or nonenzymatic cross-linking in order to assemble the higher-order fibril bundles and fibers [57-58]. Beyond its structural properties, COL-I also promotes BMP-induced osteogenesis and is required for mitogen-activated protein kinase (MAPK) dependent signaling regulation of Runx2, thus maintaining bone formation [59-60].

Noncollagenous proteins (NPCs) consist of proteoglycans (biglycan, decorin, keratocan, asporin), γ-carboxyglutamic acid containing proteins (osteocalcin (OCN), matrix Gla protein (MGP), periostin), glycoproteins (osteonectin (ON), thrombospondins (TSP1, TSP2), R-spondins) and small integrin-binding ligand N-linked glycoproteins (SIBLINGs) (bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE)). NPCs play crucial roles in bone mechanics via reciprocal relationships for the regulation of fundamental cellular processes such as proliferation and differentiation [61-62]. The specific roles of these proteins in bone remodeling and homeostasis were summarized within a schematic illustration in Figure 1.5 [55, 63].

1.2.3. Bone Remodeling and Homeostasis

Bone is a highly vascularized, dynamic and hierarchically organized tissue and bone remodeling is a lifelong process regulated by a complex interplay of various signaling molecules (such as hormones, cytokines, and chemokines) and mechanical stimuli in parallel with the interaction of bone cells, especially the communication between osteoblasts and osteoclasts [64]. The development of novel treatment and prevention strategies, the identification of the key factors involved in bone remodeling and their local and systemic interactions is of great importance.

Bone morphogenesis is mainly started by controlled degradation of the bone matrix via proteases [65]. Metalloproteinases (MMPs) are a group of enzymes that have various roles in maintaining bone homeostasis. For example, MMP2 is responsible for ECM degradation in order to form a lacunar-canalicular network, MMP14 regulates osteoblast to osteocyte transition and MSC osteoblast commitment [66-67]. Another key enzyme for bone development and homeostasis is metalloenzyme alkaline phosphatase (ALP), which catalysis the phosphomonoester hydrolysis, R-O-PO_3 and has a crucial role in bone mineralization [68].

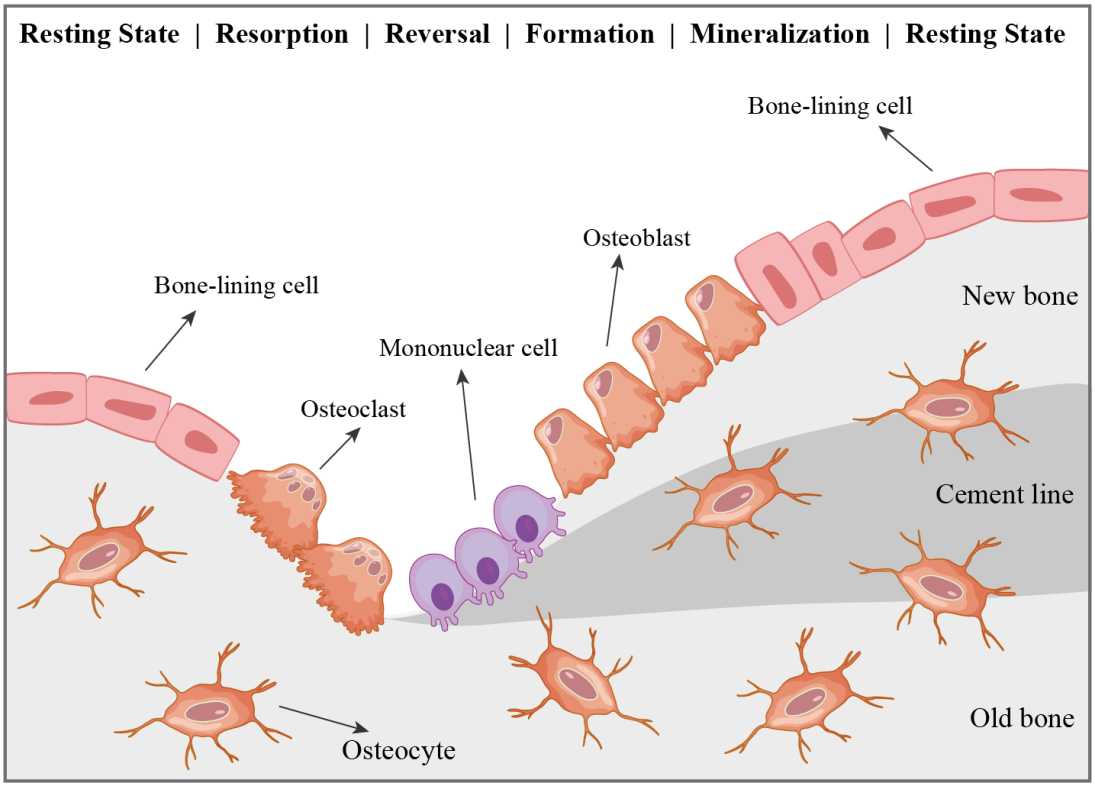

Figure 1.6. The schematic illustration of the bone remodeling

Bone remodeling consists of four main stages: resorption, reversal, formation, and mineralization (Figure 1.6). As mentioned previously, mononuclear cells differentiate into pre-osteoclasts and fuse into multinucleated mature osteoclasts, and bone remodeling begins with the cascade of this event [69]. Once osteoclasts adhere to the bone matrix and

start resorption, quiescent osteoblasts (bone-lining cells) become active, and pre-osteoclasts differentiate into osteoblasts in order to synthesize bone matrix in the gaps formed by osteoclasts, subsequently become mineralized. Osteoclasts die soon after the resorption phase through apoptosis. In contrast, most of the osteoblasts transform into bone-lining cells and remain quiescent until bone resorption occurs again under the control of the immune and endocrine systems [70-71] (see section 1.2.1).

The remodeling cycle, formation by osteoblasts and resorption by osteoclasts, is a balanced lifelong process that periodically renews older bone with new mechanically and physiologically optimum bone. Remodeling allows the bone architecture to adapt to changing mechanical demands and helps repair micro-damage in the bone matrix, preventing the accumulation of old bone. Furthermore, remodeling is also important in maintaining plasma calcium homeostasis. [70, 72]. The balance of the remodeling cycle is crucial for the maintenance of bone homeostasis; several molecular pathways have been implicated in this process, including MAPK, Wnt, Hedgehog, Notch, PI3K/Akt/mTOR, PDGF, IGF, JAK/STAT and Ca^{2+} signaling pathways and many others. The disturbance of this balance between bone formation and bone resorption results in metabolic bone disorders such as osteoporosis, in which bone resorption by osteoclasts is greater than formation by osteoblasts. Hence, chronic bone loss leads to reduce in bone density and increased fracture risks [73].

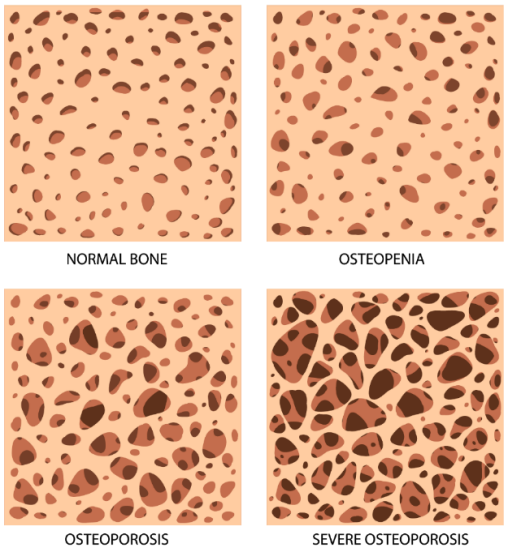

Figure 1.7. The stages of osteoporosis

In healthy individuals, age-related natural bone loss begins after around age 40 (this mass and density loss is called osteopenia), and one in three women and every one in five men aged over 50 are diagnosed with osteoporosis and osteoporosis-related fractures. Osteoporosis is a widespread disorder (worldwide prevalence of 18.3%) that is called "the silent epidemic of the 21st century" since it poses a significant threat to public health, with over 200 million women. Despite the fact that osteoporosis is often seen in postmenopausal women due to estrogen deficiency-driven bone loss, men also suffer from this disease and osteoporosis [38, 74]. The regulation of bone remodeling is both systemic and local; thus, various biological and physical factors are involved in bone remodeling for maintaining homeostasis and skeletal integrity [75-76]. Hormones such as glucocorticoids, growth hormone, parathyroid hormones (PTH), and sex hormones: cytokines, insulin-like growth factors (IGFs), transforming growth factor-beta (TGF-β), prostaglandins, bone morphogenetic proteins (BMPs) are the main factors that involved in systemic regulation. On the other hand, the factors affecting bone cell functions and fate are referred to as local regulation factors of bone remodeling. Similarly, a variety of growth factors and cytokines are involved in local regulation. Most of these factors have an impact on the RANK/RANKL/OPG pathway, which has a major role in bone remodeling [77-79].

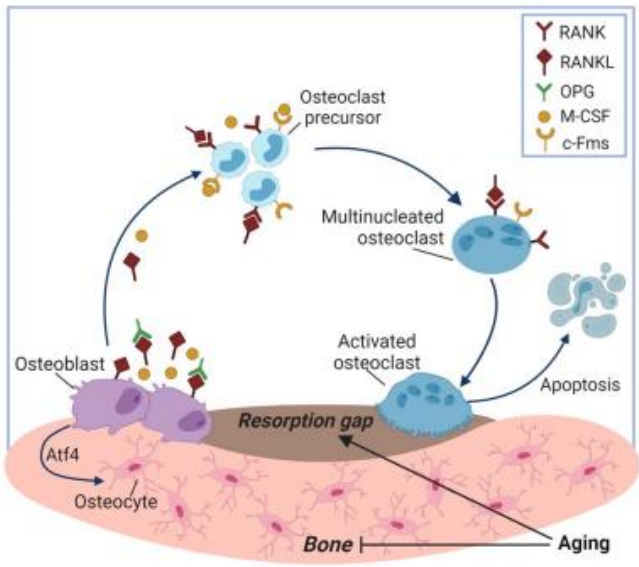

Figure 1.8. The RANK/RANKL/OPG signaling pathway in bone remodeling cycle [80]

Beyond bone matrix formation, osteoblasts secrete several regulatory factors, including RANKL, its antagonist OPG, and the macrophage colony-stimulating factor (M-CSF) (Figure 1.8). M-CSF stimulates the proliferation of osteoclast precursors. Osteoclast precursors express the RANK receptor, and the binding of RANKL/RANK triggers the multinucleated osteoclast formation and osteoclast differentiation, whereas OPG/RANKL binding causes the inhibition of osteoclast differentiation and function, 81-82, thus OPG/RANKL protects bone from destruction due to excessive absorption [81]. Furthermore, it has been known that this complex signaling pathway is in relation to muscle and plays crucial roles in the immune system and cancer [82].

The immune system also regulates bone homeostasis through the RANK/RANKL signaling pathway since T cells and B cells also synthesize RANK, RANKL and OPG, as well as osteogenic cells [83-84]. It can be observed that the immune system modulates the proliferation, differentiation and maturation of both osteoblasts and osteoclasts via not only RANK/RANKL/OPG triad but also cytokines (TNFα, IL-1, IL-11 and IL-17) receptors and transcription factors (Figure 1.9) [80, 85].

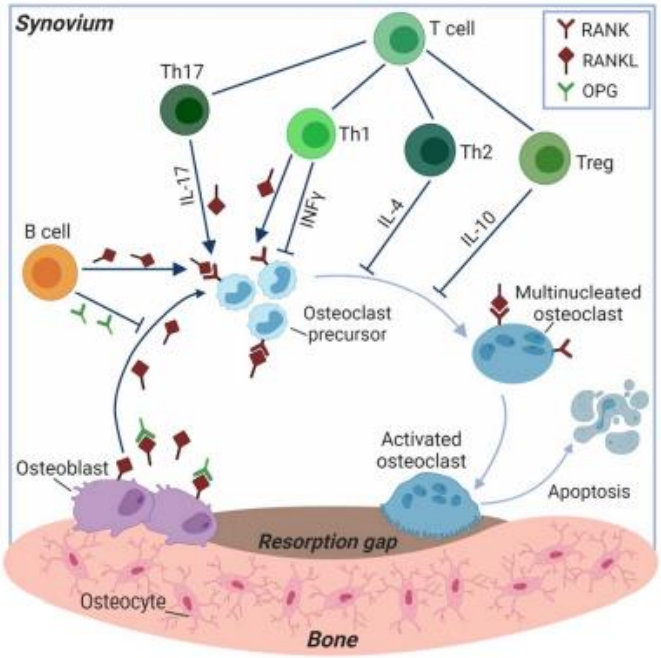

Figure 1.9. The RANK/RANKL/OPG signaling pathway in bone remodeling cycle through osteoimmunology [80]

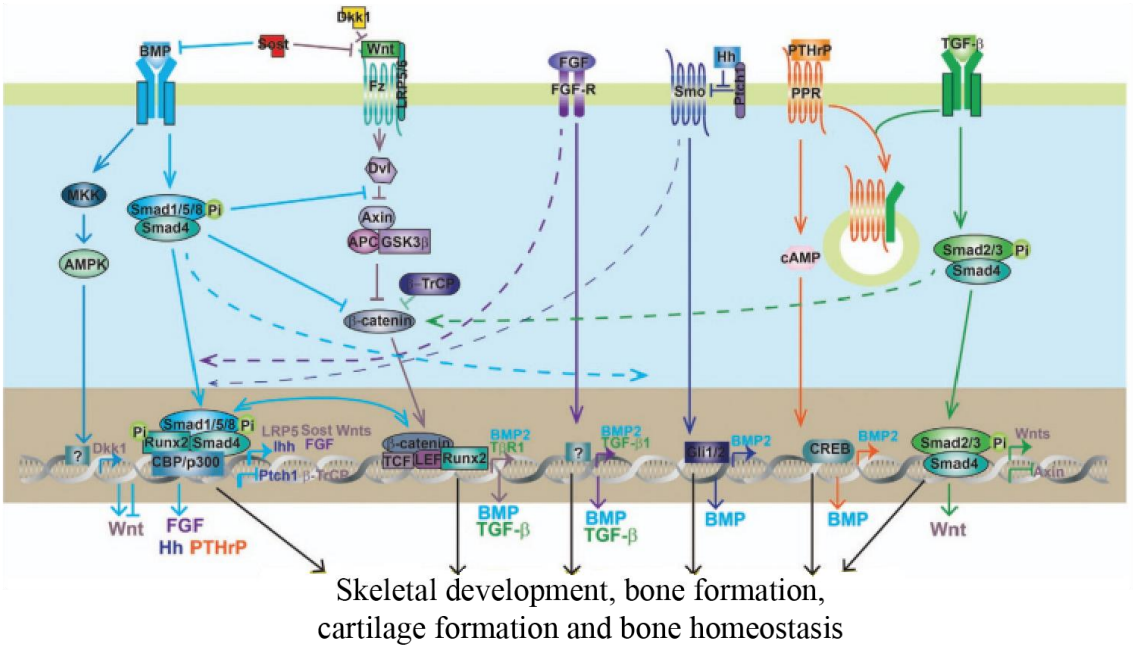

Figure 1.10. The BMP, Wnt, FGF, PTHrP and TGF-β signaling pathways and crosstalk between these during skeletal development and bone remodeling [86]

TGF-βs and BMPs, important cytokines belonging to the TGF-β superfamily, are crucial for skeletal formation during embryonic development, bone remodeling and homeostasis in adulthood. TGF-β is stored in the bone ECM and activated as a result of osteoclastic bone resorption [87]. TGF-β/BMP signaling pathway constantly interacts with various signaling pathways, cytokines, and growth factors such as Wnt, Hedgehog, Notch, PTHrP, MAPK, Erk, and FGF (Figure 1.10) to regulate stem cell differentiation, osteogenesis, chondrogenesis, skeletal development, and bone homeostasis. The TGF-β/BMP signaling pathway also promotes osteogenic gene expression at transcriptional level [88-89].

Previously, it has been shown that dysregulated Smad signaling leads to a number of bone diseases in humans. The regulation of Smad-dependent and non-Smad-dependent TGF-β signaling and BMP signaling pathways in bone tissue remodeling and crosstalk between TGF-β/BMP [90] and other critical signaling pathways have been comprehensibly reviewed by Wu *et al*. [86].

Due to the fact that several signaling pathways contribute to bone homeostasis, it is possible to increase the number of these signaling pathways. The pathways and each component of which have various physiological functions and regulations in bone via inhibitory, stimulatory, or dual effects on the cells [52]. Most importantly, the disturbance in the balance of these factors may result in bone diseases. For example, TGF-β and Wnt signaling pathways primarily regulate bone strength and mutations in TGF-β or Wnt members are known to result in low or high bone disorders such as Marfan (MFS) and Loeys–Dietz (LDS) syndromes and Camurati–Engelmann disease (CED), osteoporosis, osteogenesis imperfecta (OI), and osteoporosis pseudoglioma syndrome (OPPG) [51]. Thus, it could be possible to say that the existing treatment strategies for bone disorders mainly target critical signaling pathways involved in bone homeostasis. However, it should be kept in mind that these signaling pathways are also involved in other tissue and organ systems. For example, it is known that TGF-β regulates bone and cartilage formation in a dose- and stage-dependent manner; hence, insufficient or excessive signaling results in dysregulation of bone homeostasis [91]. On the other hand, the RANK/RANKL pathway has been found to be involved in sarcopenia, atherosclerosis, and cardiovascular diseases [92].

1.3. Therapeutic Treatment Approaches

Age-related bone diseases are thought to be caused by the disruption of the balance between bone formation and resorption as the tissue turnover rate decreases with age. Altered expression of specific signaling molecules, apoptosis of certain cell types, reduced number of osteoprogenitors (or MSCs), or differentiation of MSCs into adipocytes [93-94] could be the reason for the imbalance between bone formation and bone resorption. Furthermore, the age-related decline in sex steroids (estrogen in women and testosterone in men) is known to result in reduced bone strength and increased risk of fractures [95-96]. Basically, the deficiency in sex steroid hormones, cellular senescence and glucocorticoid excess can be considered as the most important causes [72].

The primary goal of potential treatment strategies for bone diseases is to maintain bone homeostasis by modulating the balance between bone resorption and formation for adequate bone strength. For example, targeting the stimulation of osteoblastic bone formation or inhibition of osteoclastogenesis are two main strategies for the treatment of osteoporosis [97]. Based on the current knowledge about the pathophysiology of osteoporosis, there is a wide range of therapeutic approaches to restore bone homeostasis, including biomaterials [98-99], gene therapy [100], monoclonal antibody-based drugs [101], and mesenchymal stem cell transplantation [102].

1.3.1. Stem Cells

Stem cells are undifferentiated cells that are capable of developing new healthy cells instead of damaged or aged cells in multicellular organisms. Stem cells have attracted the attention of scientists due to their two main characteristics: self-renewal and differentiation. Based on their great potential as therapeutic tools for the treatment of many diseases, understanding stem cell biology is a critical aspect of developing new therapies for primarily degenerative diseases, investigating the early-stage development of an organism, and also for drug screening studies. There are three main types of stem cells: embryonic stem cells (ESCs), non-embryonic (adult or tissue) stem cells and induced pluripotent stem cells (iPSCs) [103].

ESCs are pluripotent stem cells, which means that they can differentiate into all cell types of an individual organism. During embryonic development, after the blastocyst phase, they lose this unlimited differentiation feature and begin to differentiate into specific functional cells to form the tissues, organs, and finally, the entire body [104-105]. Despite their great potential, human embryonic stem cells (hESCs) have been limited in their use. However, the groundbreaking invention of induced pluripotent stem cells (iPSCs) in 2005, one year after the application of this procedure into human cells, caused a notable increase in the number of investigations in this area due to ethical concerns [106]. Beyond the ethical issues, studying iPSCs not only prevents immunological responses because of the use of patients' own cells, especially in stem cell-based transplantation

therapies but also provides to develop patient-specific therapy or to investigate illnesses at a patient level. Although iPSCs have unlimited self-renewal and pluripotency like ESCs, there is still a concern about whether iPSCs can totally transform into ESCs [105, 107].

On the other hand, another promising stem cell type, tissue (or adult) stem cells, are multipotent; stem cell pools in each organ or tissue system are responsible for tissue growth, repair, and renewal. This type of stem cell only generates specific cells of the tissue that belongs to [107]. For example, blood originates from hematopoietic stem cells (HSCs), and the nervous system originates from neuronal stem (progenitor) cells throughout the lifespan [108]. Among tissue stem cells, mesenchymal stem cells (MSCs) have an exception by exerting multipotent characteristics, since they can generate bone, cartilage, and muscle. Compared to the other stem cell types, relatively large numbers MSCs can be isolated from bone marrow, adipose tissue, umbilical cord blood, Wharton's jelly, olfactory pulb, etc.). Indeed, they are often preferred for use in stem cell research and regenerative medicine due to their multipotency, sufficient numbers and non-invasive isolation methods [109-110].

Stem cell maintenance *in vitro* is remarkably based on defining the molecular pathways and the control required to proceed with their self-renewal during cultivation [111]. It has been demonstrated that *in vitro* cultivation time needs to be longer along with the protection of their unlimited self-renewal without incurring chromosomal instability and pluripotency, two principal properties of stem cells for their potential use in scientific and medicinal research [112-113]. However, insufficient telomerase activity in stem cells limits their excessive potential. TL shortening and their limited life span during in vitro cultivation diminish the regenerative ability of stem cells. For this purpose, telomerase activation may provide benefits for the production of sufficient quantities and facilitate stem cell differentiation of desired cell types since tissue stem cells, including mesenchymal [107], hematopoietic [114-115], skin [116], hair follicle [117], neuronal [118], pancreatic [119], liver [120], etc. show no or low telomerase activity [121]. Furthermore, TL is critical for successful engraftment and better outcomes in stem cell transplantation [122]. Therefore, telomerase activators are promising agents for stem cell-based therapeutic applications.

Stem/progenitor cell transplantation is an effective approach for contributing to the full repair of damaged lesions in degenerative diseases, during which progressive and irreversible loss of cells occurs [123]. However, insufficient telomerase activity in stem cells

limits their excessive potential. The potential use of stem cells mainly depends on providing chromosomal stability during in vitro expansion without losing two main stemness properties: differentiation capacity and unlimited self-renewal [124]. However, TL shortening and their limited life span during in vitro cultivation diminish the regenerative ability of stem cells. Furthermore, TL is critical for successful engraftment and better outcomes in stem cell transplantation [122]. Interestingly, a dramatic decrease in TL of HSCs was reported after transplantation, which increased the risk of clonal disorders in patients [125]. Therefore, telomerase activators are promising agents for stem cell-based therapeutic applications.

1.3.2. Monoclonal Antibodies

Monoclonal antibodies are defined as identical immunoglobulins that recognize a single epitope on an antigen [126]. Currently, mAbs have a wide range of therapeutic and diagnostic applications, including diagnostic, therapeutic, and analytical/chemical research studies[127].

The first monoclonal antibody approved for the treatment of cancer: Rituximab (Rituxan®, MabThera®, target: CD20) approved by the U.S. FDA in 1997, is a genetically engineered chimeric murine/human monoclonal antibody used to treat non-Hodgkin's lymphoma [128], and trastuzumab (Herceptin®, target: HER2, human epidermal growth factor receptor 2) approved by the U.S. FDA in 2011, is a humanized monoclonal antibody used to treat metastatic breast cancer [129]. Soon after this success, the FDA approved many other biosimilars that have entered the market for the treatment of a wide range of diseases of chronic lymphocytic leukemia, Wegener's granulomatosis, pemphigus vulgaris, and rheumatoid arthritis for rituximab; gastroesophageal, and gastric cancers for trastuzumab biosimilars [130]. The steady increase in approved mAb-based therapeutics suggests that mAb-based formulations will continue to be the focus of the biotherapeutics industry.

As mentioned in previous sections, bone cells and immune cell precursors that reside in the bone marrow share the same environment; the study of the interactions

between the immune and skeletal systems has revealed the close relationship between these two systems in health and disease. Due to these strong and complex interactions, in 2000, the term 'osteoimmunology' was used by Arron *et al*. for the first time [131], and Srivastava *et al*. introduced the term 'immunoporosis' in 2018 [132]. The contribution of the immune system for treatment purposes is a distinctive approach for bone diseases such as OA and OP, and this strategy may benefit from the regulation of key immune system factors such as interleukins (e.g., IL-1, IL-6), TNFα, and prostaglandins. Monoclonal antibody (mAb)-based therapy has been incorporated into the treatment of bone disorders such as osteoporosis (OP) and rheumatoid arthritis (OA). Some of the examples of successful mAb-based therapeutics approved: tocilizumab (Actemra®, target: IL-6R) [133-134], adalimumab (Humira®, target: TNFα) [135], sarilumab (Kevzara®, target: IL-6) and golimumab (Simponi®, target: TNFα) [136-137] for the treatment of OA, while denosumab (Prolia®, target: RANKL) [138-139], romosozumab (Evenity®, target: sclerostin, an antagonist of the Wnt pathway) [140-141].

1.3.2.1. Monoclonal Antibody Production

Mammalian cells are used for the production of biologics due to their ability to generate recombinant proteins biocompatible with humans owing to their posttranslational modification capability. The hybridoma cell lines were generated by Kohler and Milstein in 1975 for the production of mAbs, the technology used to fuse splenocytes of immunized mice with immortal myeloma cells in order to generate continuously dividing cell lines [142]. The first approved therapeutic recombinant antibody, Muronomab (Muromonab®, target:CD3) produced with hybridoma technology in 1986, which is used as an immunosuppressive therapy in kidney, heart, and liver transplant patients [143-144]. However, hybridoma technology has some limits and drawbacks since this technology is multi-stage and time-consuming, and hybridomas are genetically unstable [145]. Chinese hamster ovary cells (CHOs) were first isolated in 1958; after the parental isolation of CHO, many subtypes were generated. Three common lineages of CHO were CHO-K1 by Kao and Puck in 1968 [146], CHO Pro-3 by Flintoff *et al*. in 1976 [147] and CHO-

S by Tobey *et al*. in 1991 [148], and their subclones were generated in upcoming years (Figure 1.11).

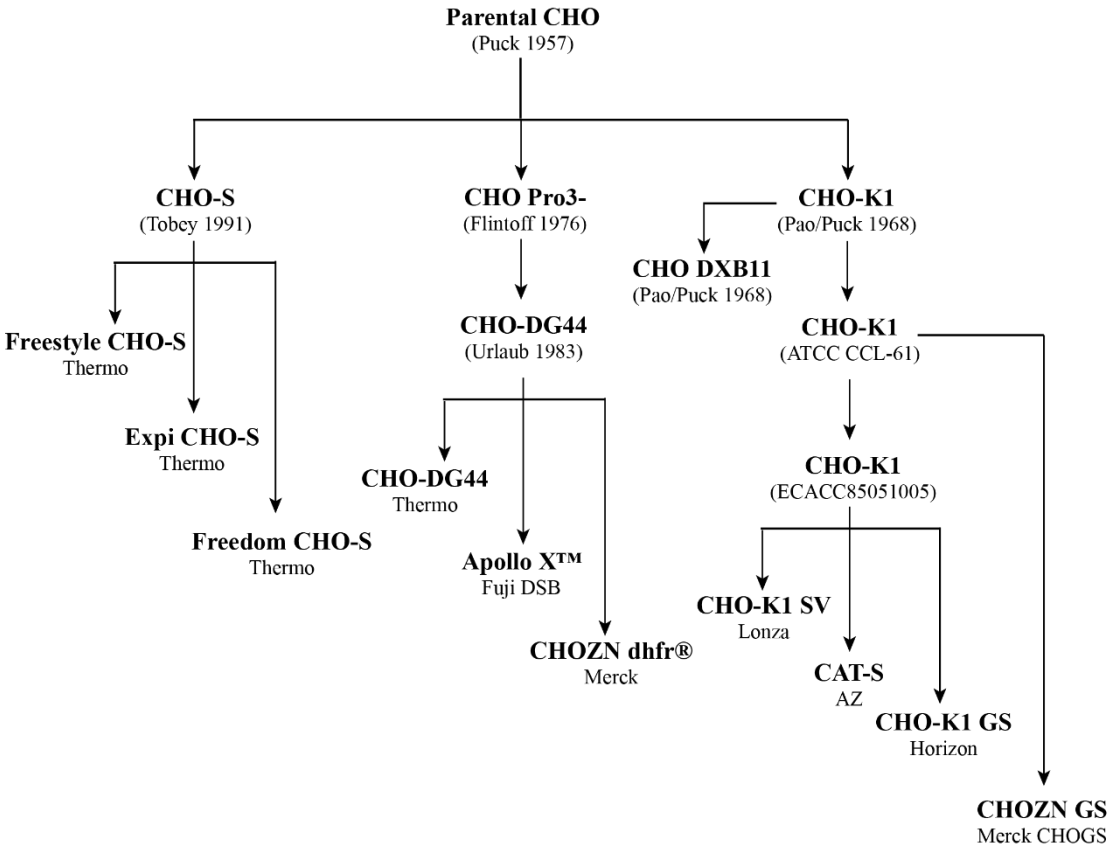

Figure 1.11. The chart of common CHO lineages

The first therapeutic protein, Activase, was produced with the subtype CHO-DG44 isolated in 1983 [149] which is still primary chose for the production of therapeutic proteins including mAbs. The global market of biopharmaceuticals was valued at $237.2 billion in 2018 and is expected to reach $389 billion by 2024. Among these high-value therapeutics, adalimumab was the best-selling biotherapeutic, with $20 billion in sales in 2018. Indeed, it was the first drug that reached $20 billion and still has commercial dominance in the drug market globally [150-151].

Although significant improvements have been made in the production, characterization, and stabilization of mAbs, there are still obstacles to overcome to achieve cost-effective production strategies, including genetic engineering approaches to generating new cell lines and expression systems to create more effective and less immunogenic monoclonal antibodies, however on the other hand process optimization for the production of these high-value biotherapeutics still needs to be under consideration in order to decrease overall production costs by increasing the productivity [152-154]. CHOs are the cells that adopted serum-free and suspension cultures. It is known that they undergo genetic changes in culture due to chromosomal instability. This situation leads to disturbance in cell growth and physiology, resulting in the loss of the expression ability, which, over time, in culture [155]. It is known that Chinese hamster cells have telomere repeats (TTAGGG) at the end of chromosomes and pericentromeric regions. The study by Slijepcevic and Bryant exerted that the telomeric sequences are shorter than 1 kb in chromosomes of immortal CHO lines, including CHO-K1 subtypes CHO-K1a, CHO-K1b, CHO-K1c, and also human-hamster hybrid cell line R342A4 carrying human chromosome 10 [156]. Therefore, telomerase activation may have some benefits in mAb production in terms of productivity by providing chromosomal stability and improving cell growth and physiology.

1.4. Telomere and Telomerase

Telomeres are highly regulated, and dynamic nucleoprotein complexes found at each chromosome ends (Figure 1.12), composed of tandemly repeated short nucleotide sequences (e.g., TTAGGG repeats in all vertebrates) and associated protective proteins, capping and protecting the end of the chromosome from deterioration, fusion with neighboring chromosomes, or preventing being recognized as break points due to DNA end-replication problem in which RNA primer long base lose occurs in each DNA replication. The telomere repeat sequence is different in every living species. Its length may differ from cell to cell between different tissues of the same individual and even within the same tissue [157].

Telomerase is a ribonucleoprotein reverse transcriptase (hTERT), first discovered by Greider *et al.*, responsible for the synthesis of telomere repeat sequences (Figure 1.13). With the presence of telomerase, cells are prevented from losing genetic information and DNA damage responses, and replicative senescence [157-158]. Telomerase maintains telomere homeostasis against aging and environmental stress factors and prevents cells from replicative senescence [159]. It has been known that germ cells and cells in the early development phases of the embryo show high levels of telomerase activity; however, after differentiation into specific tissues and organs, they lose this ability. In other words, somatic cells do not have significant telomerase activity because they lack expression of hTERT, even if they have related sequences in their genome [160].

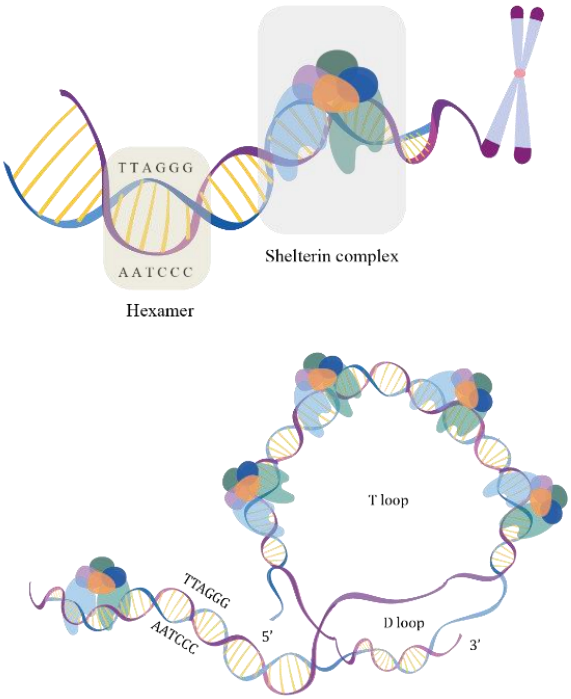

Figure 1.12. The illustration of the telomere and telomere loop

In 2009, Elizabeth Blackburn and her co-workers were awarded a Nobel Prize for their groundbreaking discovery of how telomerase's enzyme impacts telomere length. As

a result of this discovery, many scientists focused on searching for novel telomerase activators for therapeutic purposes. The possible therapeutic effects of telomerase activation have been evaluated in different cellular backgrounds to cure diseases linked to short telomeres or telomerase deficiencies [161-162].

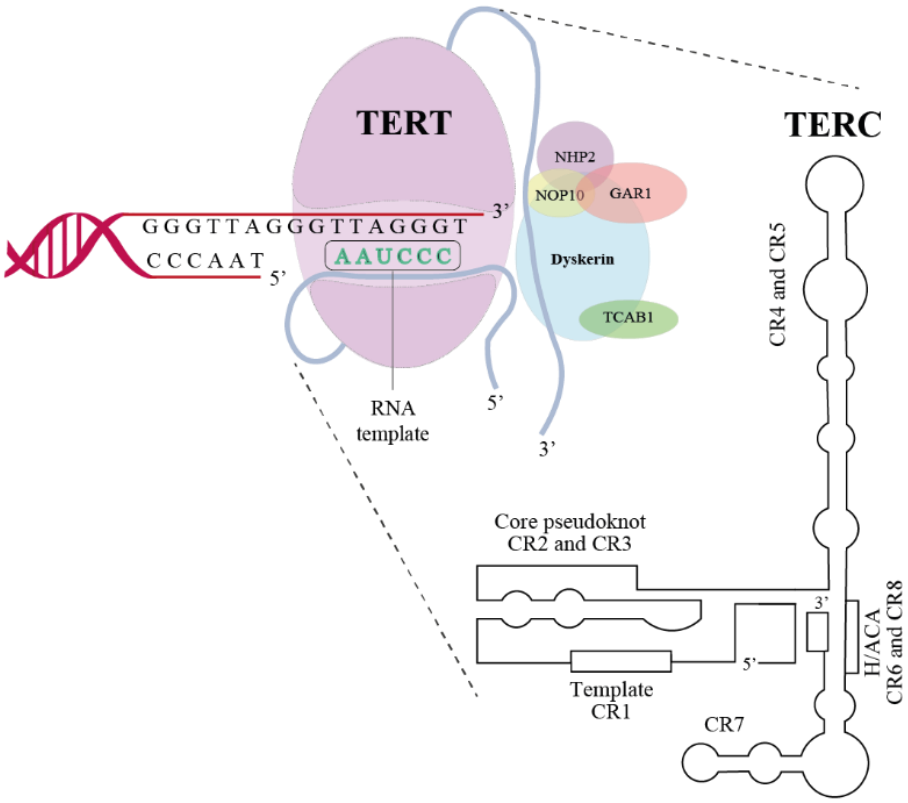

Figure 1.13. The main components of the human telomerase (hTERT, hTERC, Dyskerin, NOP10, NHP2, GAR1, TCAB1); and functional regions of hTERC (template, pseudoknot, CR4-CR5, Box H/ACA, and CR7)

The investigations on different cancer types demonstrate that over 90 percent of cancer cells from different origins show high levels of telomerase activity [163]. Although it has been considered that telomerase activation may cause tissue to become cancerous, controversially, low telomerase activities are one of the reasons for cancer because of

chromosomal instability [164]. Besides, oncogenic transformation was not found in both *in vitro* and in vivo studies on whether telomerase activity causes tumor formation [165-166]. It has been proven that the telomerase genes are not oncogene [167]; after these findings, researchers focused on anti-aging approaches to treat age-related diseases.

1.4.1. Telomere-Independent (non-canonical) Functions of Telomerase and Telomere-Related Proteins

For a long time, telomerase has been thought to be responsible for only telomere maintenance at chromosomes in the nucleus and protecting dividing cells from replicative crisis and senescence. However, scientific studies continue to enlighten mechanisms underlying functions of telomere and telomerase complex proteins one by one, which represents additional protective functions more than a catalytic function of telomerase. The investigations on catalytic protein and RNA components (TERT and TERC) of telomerase have demonstrated that their occurrence differs from tissue to tissue, and environmental stress determines their localization and expression in the cell. Based on this, it has been suggested that subunits of telomerase and related proteins might independently operate protective functions even if significant telomerase activity is not observed [168-169].

The gene sequence of TERT (*tert*) consists of 16 exons and 15 introns [170]. Also, *tert* has a N-terminal leader sequence which is responsible for the transfer of TERT protein by exporting from nucleus and importing it into mitochondria [171-172]. It has been shown that TERT localizes in the nucleus, mitochondria, and cytosol. TERT interacts with messenger RNAs in the cytosol to regulate signaling pathways at the translational level [173]. Moreover, mitochondrial RNAs (mtRNA) represent reverse transcriptase functions in mitochondria [174]. Under stress conditions, for example, dietary restriction causes reduce in mTOR signaling [169, 175] and TERT accumulation in the mitochondrial matrix, which protects cells from apoptosis due to oxidative mitochondrial DNA damage by decreasing the ROS levels and advancing respiration [176-177].

Furthermore, TERT has also been found to be related also signaling pathways that play essential roles for cellular survival by regulating the expression levels of responsible genes such as proinflammatory cytokines, apoptotic signals (e.g., TNFα, TRIAL) or growth factors (e.g. EGFR, FGF). Telomeric repeat binding factors TRF1 and TRF2, TIN2, POT1, TPP1 and RAP1 are regulatory and protective six core proteins belonging telomerase sheltering complex (Figure 1.14). Telomeric and extratelomeric functions of these proteins were well established by Martinez *et al.* besides telomere protection [178]. Additionally, TNF2 was found to bind in extratelomeric regions of chromosomes and has important functions in the maintenance and differentiation of NPCs into neural lineage [179]. RAP1 is another protein that has been shown to function in inflammatory signaling pathways such as Wnt/β-catenin and NF-κB, as well as TERT protein [180].

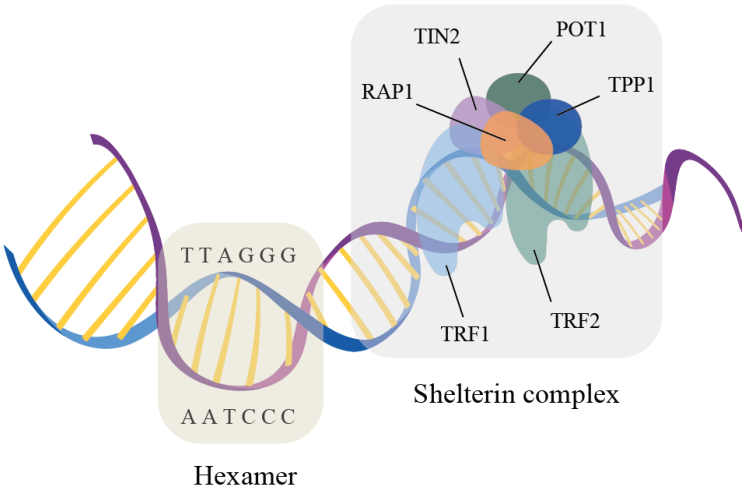

Figure 1.14. The simplified illustration of the human telomere, the shelterin complex proteins (RAP1, TIN2, POT1, TPP1, TRF1, TRF2) and hexamer

As anticipated from the situation, telomere shortening has been found to be related to not only aging and oxidative stress but also inflammation, which is known to take place in several chronic diseases [181]. TERT splice variants are catalytically inactive TERT proteins, which are exon-delated forms of TERT. The most studied splice variants are α

and β forms, which have been shown to increase during brain development and to be responsible for the decrease in telomerase activity as the α-splice variant inhibits activity 184, and they might trigger additional non-canonical functions [182].

Nassour *et al.* has provided strong evidence that telomeric dysfunction causes autophagic cell death through cGAS–STING pathway which is capable of sensing cytosolic chromosomal-DNA fragments; thus, the cells in replicative crisis are prevented from becoming cancerous [183]. Recently, a study conducted by Yilmaz *et al.* showed that proteasome activation by cycloastragenol is mediated through telomerase activation induced by NRF2. Until today, several studies have given evidence for different extratelomeric functions of the proteins of telomerase complex independently. However, the functions of many proteins belonging to the telomerase complex remain unknown [184].

1.4.2. Relation Between Telomere/Telomerase and Degenerative Diseases

The studies targeting telomerase for drug development studies have been increasing due to the significant correlation between telomere dysfunctions. It has been suggesting that dysfunctional telomeres due to either TERT or TERC mutations in degenerative diseases are the cause more than a symptom of the disease. Some of the telomerase associated disease examples are given in Figure 1.15.

Osteodegenerative diseases occur because of disruption of the balance between catabolic and anabolic processes in bone and articular cartilage. The cause of degeneration may occur due to another disease, injury, trauma, or aging. Also, genetic defects and epigenetic alterations are important for disease pathophysiology. Osteoporosis, osteoarthritis, rheumatoid arthritis, and intervertebral disc degeneration are the most common age-related degenerative disorders in skeletal system [185].

Osteodegenerative diseases linked to short telomeres/telomere abnormalities [9] have been investigated: Osteoporosis (OP) is a metabolic disorder characterized by a decrease in bone density, leading to enhanced bone fragility. OP has become more prevalent in both men and women. Interestingly, shorter TL was observed in people with

OP [9]. Osteoarthritis (OA) and rheumatoid arthritis (RA), two of the most common arthritis types, are chronic degenerative diseases leading to cartilage degradation in all joints [186-187]. Previously, it was demonstrated that patients with arthritis had shorter TL, which might be involved in disease development and progress [188-189]. Intervertebral disc degeneration (IVD) is a complex disease, and oxidative stress-induced mitochondrial dysfunction plays a key role in IVD degeneration. Consequently, recent interventions mainly aim to decrease oxidative stress in order to protect IVD homeostasis [190]. For this purpose, treatment strategies focus on antioxidative and antiinflammatory approaches to protect NP cells, which are responsible for IVD hemostasis [191-192]. It is known that IVD degeneration associated with telomere deficiency and short telomeres are one of the reasons for disruption of IVD homeostasis that leads to disease pathogenesis [193].

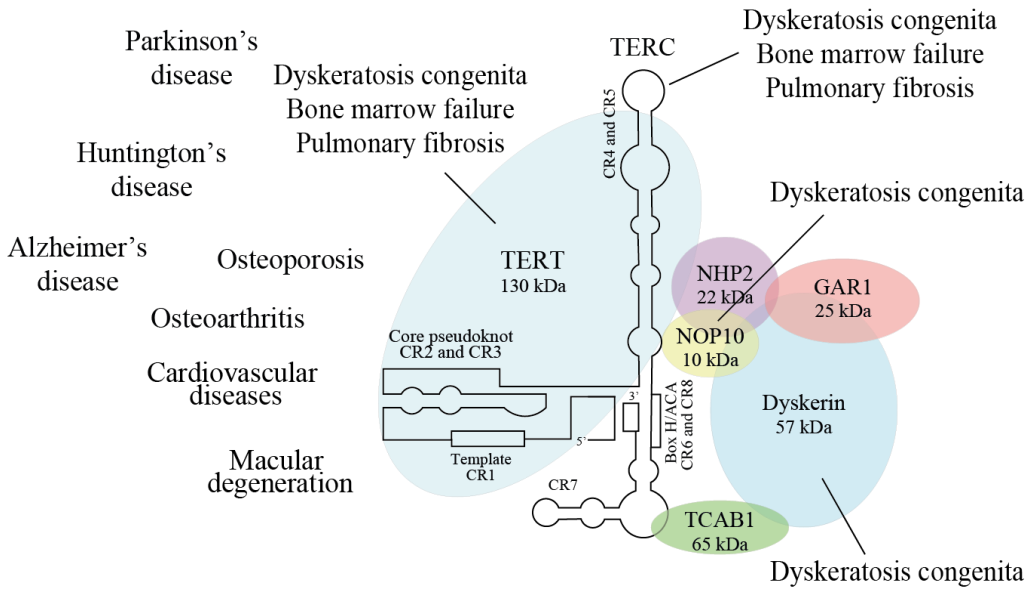

Figure 1.15. Dysfunctional telomerase causes telomere-induced senescence and contributes to cancer and/or degenerative diseases due to genomic instability

Furthermore, studies demonstrated that bone mineral density correlates with TL and short telomeres increase the risk of osteoporosis, especially in women [194]. Similarly, shorter TL in peripheral blood cells was observed in patients with rheumatoid arthritis [195]. Studies with G3 Terc$^{-/-}$ mutant mice demonstrated that the telomere plays a key role in the proliferation and differentiation capacity of osteoprogenitor cells, which are responsible for bone homeostasis [196]. Restoring telomerase activity via gene therapy for the treatment of intervertebral disc degeneration resulted in higher proliferation and matrix production in human nucleus pulposus cells [197-198]. Because of the relation between telomere dysfunction and bone/joint diseases, telomere/telomerase might be an alternative target for the treatment.

1.4.3. Telomerase Activator: Small Molecules from Natural Sources

Telomerase activation is mostly provided by genetic interventions. Gene therapy, used in therapeutic applications, especially in regenerative medicine, has problems such as disruption of genetic integrity, inability to transfer the gene to the desired region, the unpredictability of negative interactions between genes, etc., carrying such risks. Although genetic methods can apply telomerase regulation, obtaining the same effect with a bioactive molecule will reduce the risk ratio as it will eliminate many processing steps in genetic studies. For this reason, the necessity of evaluating the potential of using synthetic, semi-synthetic, or natural origin telomerase activator molecules, which is one of the other ways to increase telomerase activation apart from gene therapy, has come to the fore. In recent years, telomerase activator small molecules have been used to treat or prevent from diseases associated with telomere shortening or telomere dysfunction (Figure 1.16) [199-200].

By the last three decades, telomerase activator small molecules from natural sources have attracted the attention of scientists owing to their great potential as therapeutic tools for healthy aging and preventing age-related degenerative diseases as an alternative methodology to genetic manipulations to control cell fate and also their relatively less toxicity compared to synthetic small molecules. Thus, several research

groups keep searching natural sources to discover new small molecules while semi-synthesis studies are continuing for the production of their improved derivatives. With this regard, many phytoalexins have been isolated and identified, including phenolics, flavonoids, terpenes, iridoids, alkaloids, and many others [168].

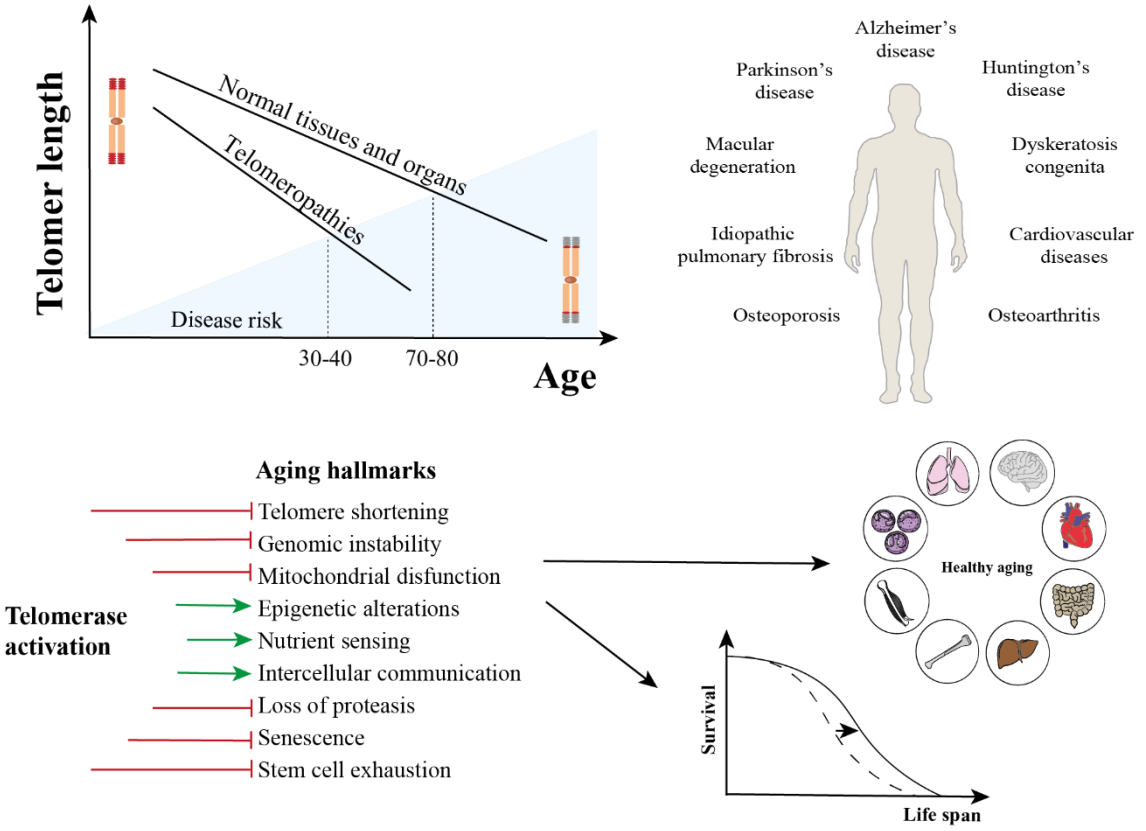

Figure 1.16. The telomere shortening continues lifelong and may accelerate due to genetic or environmental factors (upper left). The supplementation of telomerase activator small molecules can restore/ameliorate telomerase function by targeting aging hallmarks (lower left), protecting tissues and organs and providing healthy aging (upper right) and longer life span (lower right)

Cycloastragenol (CG) is a triterpenoid sapogenin hydrolyzed form of astragalosides such as astragaloside IV (Figure 1.17), the main secondary metabolites of *Astragalus membranaceus* which have been used in Chinese Traditional Medicine for centuries [201]. In 2006, because of long screening efforts by Geron Inc., CG was found to

be a potent telomerase activator. Soon after, in 2007, CG was licensed by TA Sciences and was placed on the market as a dietary supplement with the trade name TA-65, the first telomerase activator on the market [202].

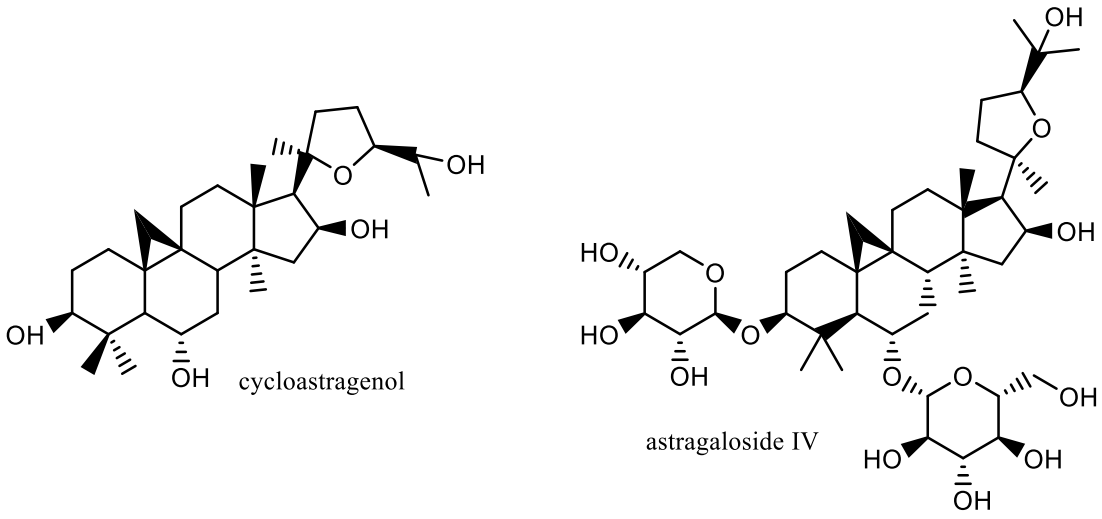

Figure 1.17. The chemical structures of cycloastragenol and astragaloside IV

Both *in vitro* and *in vivo* studies showed that CG is a promising anti-aging agent not only for the treatment of age-associated diseases but also for the prevention of those by providing healthy aging [203-206]. Additionally, ***astragaloside IV (AST IV)*** was also reported as a telomerase activator. Both AST IV and CG were found to activate telomerase and epidermal growth factor receptor (EGFR)/Ras-extracellular signal-regulated kinase (ERK) signaling via phosphorylation of ERK in the cells from different origins [207].

Resveratrol (trans-3,5,4′-trihydroxystilbene) (Figure 1.18) is a phenolic compound, first isolated from the roots of *Veratrum grandiflorum* O. Loes by Takaoka in 1939 [208-209], and it has been shown that many plants including berries, grapes, and peanuts contain a high amount of resveratrol. Resveratrol is one of the most studied natural products exhibiting several bioactivities, viz. antioxidative, anti-inflammatory, chemopreventive, and neuroprotective effects [210-211]. Telomerase activation is reported as one of its molecular mechanisms of action. Resveratrol was shown to enhance proliferation as well as telomerase activity in EPCs through PI3K-Akt signaling [212-213].

Moreover, continuous supplementation of resveratrol was shown to repress telomere shortening by 45%. However, the negative effect of resveratrol on TL is also reported in different cancer cell types [214-216].

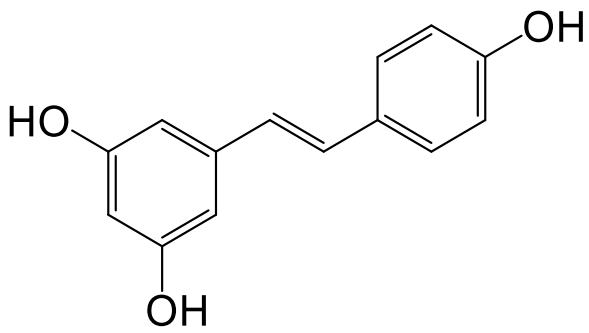

Figure 1.18. The chemical structure of trans-resveratrol

Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione) (Figure 1.19) is a natural phenolic found mainly in *Curcuma longa* (known as turmeric). Turmeric has been consumed as spice or used in traditional medicine for centuries against mainly chronic diseases because of its therapeutic effects such as antioxidative, antiinflammatory, and neuroprotective, attributed to the major bioactive ingredient curcumin (75% of turmeric) [217]. According to Taka *et al.*, curcuminoids activate telomerase in a different manner compared to other activators by directly binding to telomerase itself [218]. Although numerous studies demonstrated that curcumin (50-100 μM) had anti-proliferative properties on human cancer cell lines by decreasing hTERT expression and inhibiting telomerase activity [219-220], relatively lower concentrations of curcumin (0.02 μM and 10 μM) induced telomerase activation [221-222].

In 2019, Tsoukalas and colleagues screened several natural products for telomerase activation in peripheral blood mononuclear cells. Pentacyclic triterpenoids ***oleanolic acid (OAC)*** (Figure 1.20) and maslinic acid are found as strong telomerase activators, while ***Centella asiatica* extract *(CAE)*** has superior activity compared to TA-65. At the same time, the CAE caused an approximately 9-fold increase in telomerase activity, only.

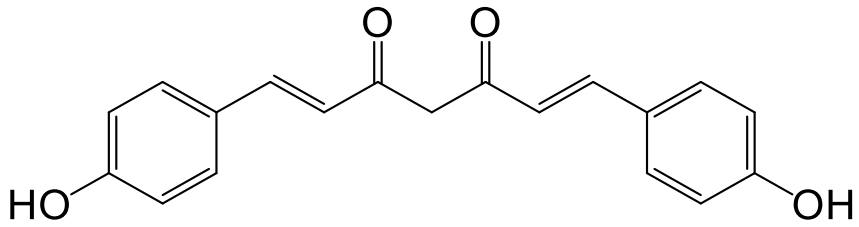

Figure 1.19. The chemical structure of curcumin

A 2-fold increase was detected in the case of TA-65 treatment. However, the study did not directly compare TA-65's active constituent CA and CAE towards telomerase activation [223]. Another study proved that CAE repressed TL decrease during aging of HEK293 cells while attenuated reduction in Sirtuin 1 (SIRT1), Sirtuin 2 (SIRT2), CMYC, and KL expressions that are related to aging process. Moreover, CAE increased the survival of *Drosophila melanogaster*. Importantly, CAE did not lead to tumorigenicity in cells [224]. CAE was placed on the market as a dietary supplement with the trade name Reverse™. Recently, it was revealed that Reverse™ supplementation for 3 months resulted in increased telomerase activity via restored TERT expression in the brains of naturally aged rats [225].

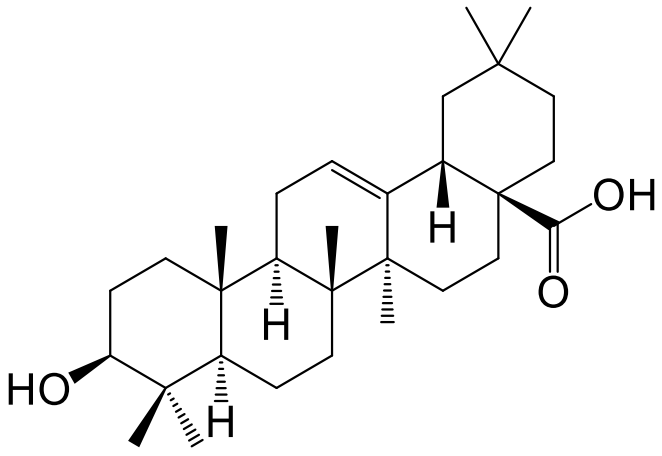

Figure 1.20. The chemical structure of oleanolic acid

Ginkgo biloba, which belongs to the Ginkgoaceae family, is used in traditional medicine for various purposes, such as improving memory and treating neuronal disorders. Nowadays, Ginkgo biloba products are one of the top-selling phytopharmaceuticals worldwide [226-227]. *Ginkgo biloba* extract (GBE) has been recently reported to increase endothelial progenitor cell (EPC) numbers and functional activity through enhancement of telomerase activity based on the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) signaling pathway [228]. Accordingly, ***quercetin*** (Figure 1.21), a flavonoid found in *Ginkgo biloba* leaves, is also reported as a telomerase activator. Quercetin was found to protect telomeres from shortening and TRF2 loss [229]. It was reported that quercetin increased the average TL of hMSCs. On the contrary, a higher concentration of quercetin induces apoptosis involving telomerase inhibition in cancer cells [230-232], suggesting that it has a dose-dependent or cell type-dependent bilateral effect.

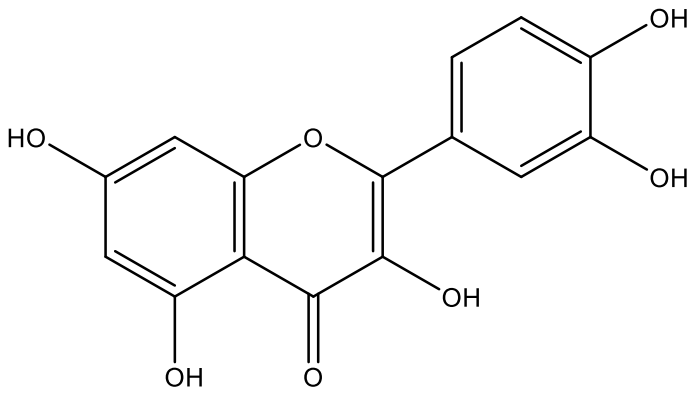

Figure 1.21. The chemical structure of quercetin

It is important to note that while these natural small molecules exert promise, their efficacy can be strongly influenced by factors such as dosage, bioavailability, and individual variability. Moreover, most of the evidence comes from preclinical studies, and more rigorous clinical trials are needed to understand their potential fully. Additionally, these natural compounds should be considered as part of a broader treatment strategy.

1.5. Aim of The Thesis

The main focus of this thesis is to demonstrate the usage of telomerase activators in stem cell research for regenerative medicine by addressing the issue of cellular aging and senescence, which are significant barriers to tissue regeneration and repair. Additionally, we aimed to investigate the usage of telomerase activators as medium supplements in monoclonal antibody production by CHOs. Based on the scope of the study, the main aims were to (i) enhance the viability and survival of human mesenchymal stem cells, (ii) decrease the number of senescent cells in the population (iii) protect differentiation potential of the cells during continuous passaging (iv) accelerate osteoblastic differentiation (v) enhance viability, productivity and stability of CHO cells and (vi) investigate the role of telomerase activators on the productivity of monoclonal antibody production.

The potential use of the telomerase activator small molecules in regenerative medicine protects from cellular aging and, thus, senescence, which are considered the main obstacles in tissue regeneration and repair. It has been known that telomerase activity in tissue stem cells is insufficient or absent as well as in somatic cells, and the progressive shortening of telomeres with each cell division, eventually, telomeres become critically short, resulting in cellular senescence or leading DNA damage responses (e.g., apoptosis) due to the chromosomal instability.

In the context of regenerative medicine, the activation of telomerase in stem cells may improve genetic stability and is expected to:

→ extend the replicative lifespan of stem cells, which is crucial for obtaining the number of cells required for tissue engineering and regenerative therapies.

→ prevent cellular senescence by delaying the onset of cellular senescence, which provides to maintain stemness characteristics for tissue repair and regeneration.

→ enhance differentiation/tissue regeneration potential.

On the other hand, the usage of telomerase activator small molecules as a medium supplement in monoclonal antibody production with CHO cells may provide benefits in terms of enhancing the longevity of the cells by protecting chromosomal stability from

environmental stress factors, thus enhancing productivity and stability instead of preventing cellular aging.

In summary, within the scope of this thesis, we mainly focused on stem cells and monoclonal antibody production in terms of their potential use in regenerative medicine, together with novel telomerase activators for the treatment of osteodegenerative diseases. The usage of telomerase activator small molecules in regenerative medicine offers a promising approach to overcoming the limitations imposed by cellular aging, enhancing the regenerative capacity of stem cells, and improving the outcomes of regenerative therapies. Furthermore, it has potential benefits in the industrial production of valuable biotherapeutics.

CHAPTER 2

SELF-RENEWAL, SENESCENCE CONDITION AND THE OSTEOGENIC POTENTIAL OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS DURING EXTENSIVE SUB-CULTIVATION

2.1. Background

Mesenchymal stem cells (MSCs) are defined as adult stem cells that can be obtained from bone marrow, cord blood, and many other tissues and can potentially differentiate into various cell types, such as osteoblasts, chondrocytes, adipocytes, muscle cells, and nerve cells [233-234]. Stem cell applications, which have a high potential for treating diseases that reduce the quality of life, allow tissue regeneration with tissue engineering studies. Stem cells for this purpose can be isolated from the individual's organs or tissues or obtained from compatible/semi-compatible donors to regenerate the specific cells, tissues, or organs [235]. MSCs are frequently preferred in tissue engineering studies. Stem cell isolation from adipose tissue is a faster and simpler method (liposuction/fat removal) compared to bone marrow; it provides advantages for older patients as it is a non-invasive isolation method, and more cells can be obtained [236].

Based on their great potential as therapeutic tools for the treatment of many diseases, understanding stem cell biology is a critical aspect of developing new therapies for especially degenerative diseases, investigating the early-stage development of an organism, and also for drug screening studies. It is possible to cultivate stem cells *in vitro* for these purposes. Still, it has been demonstrated that the longest cultivation time is approximately six months (over 47 passages) for hESCs with unlimited self-renewal without incurring chromosomal instability and pluripotency [237], which are two essential properties for the potential use of stem cells in scientific research.

The potential use of these stem cell cultures mainly depends on providing chromosomal stability during unlimited self-renewal also conservation of differentiation capacities at the same time. As mentioned previously, unlike immortal cell lines, stem cells undergo changes with age and cannot be cultured for long-term periods [121, 238]. The aging process of stem cells has not been clearly identified yet, and that is the main challenge in stem cell research [104, 239]. Once the mechanisms and regulators are understood, it will be possible to cultivate enough numbers of stem cells. Thus, master stem cell lines can be generated for further connected studies.

It is known that senescence and cell aging are also seen in stem cells, along with the risk of loss of self-renewal and differentiation abilities, which are the two main features of stem cells [240-241]. In other words, stem cells age due to telomere shortening due to unfavorable external factors (reactive oxygen species, DNA damage, disruption of protein homeostasis, systemic factors, etc.) and genetic factors [242-243]. For this reason, it is necessary to increase the resistance of stem cells to *in vitro* conditions and to eliminate the adverse effects of replicative stress for use in clinical applications [3, 244-245]. In this context, when stem cell physiology is closely examined, it has been reported that adult human MSCs grown *in vitro* do not show detectable telomerase activity [246-248]. When the *in vitro* osteoblast differentiation of MSCs from telomerase-negative mice was examined, it was observed that the proliferation of the cells was reduced, the doubling times were prolonged, and sufficient mineral accumulation could not be achieved [249].

2.2. Materials and Methods

2.2.1. Cell Culture

Human adipose tissue-derived mesenchymal stem cells MSCs), obtained from KÖGEM -Stem Cell and Gene Therapies Research and Application Center- (Kocaeli, Turkey), were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco) without

antibiotics, and cultured in a humidified incubator with 5 % CO_2 at 37 °C. The medium was refreshed every 3-4 days until 80-90 % confluence was achieved.

2.2.2. Molecules and Treatment

Cycloastragenol **1** (>98 % purity) was kindly provided by Bionorm Natural Product Co. (İzmir, Turkey). The derivatives of **1,** E-CG-01 (**2**), E-AG-01 (**3**), and E-AG-02 (**4**) were produced and purified as described previously [250]. The chemical structures of the molecules are shown in Figure 2.1. Stock solutions (5 mM) in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) were prepared with lyophilized powders of the molecules and stored at -20 °C until use. Before the experiment, each stock solution was diluted in DMSO as appropriate concentrations and final molecule concentrations were achieved with 0.1% DMSO (v/v) [251].

2.2.3. Cell Viability

Cell viability of MSCs under specific concentrations of telomerase activators was investigated. For this purpose, cells were seeded to the 96-well plates with $5x10^3$ cells/well in DMEM (with 10% FBS) and incubated overnight at 37 °C in a humidified incubator with 5% CO_2. After cells were attached to the surface, media including molecules (0.1 to 10000 nM concentrations) was added to the wells as triplicates and incubated for up to 28 days. At certain time points, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Cat# M2128-1G) procedure was applied according to the manufacturer's instructions. Briefly, the existing medium was replaced with 100 µl medium supplemented with 5 % MTT; after three hours of incubation, the MTT solution was discarded, formed formazan crystals were dissolved with DMSO (100 µl/well), and measured by reading absorbance at 570 nm via a micro-plate reader (Synergy™ HTX- BioTek, Winooski, VT, ABD). The metabolic activity of viable cells

was calculated by normalization of absorbances to the DMSO control group and represented as percentages.

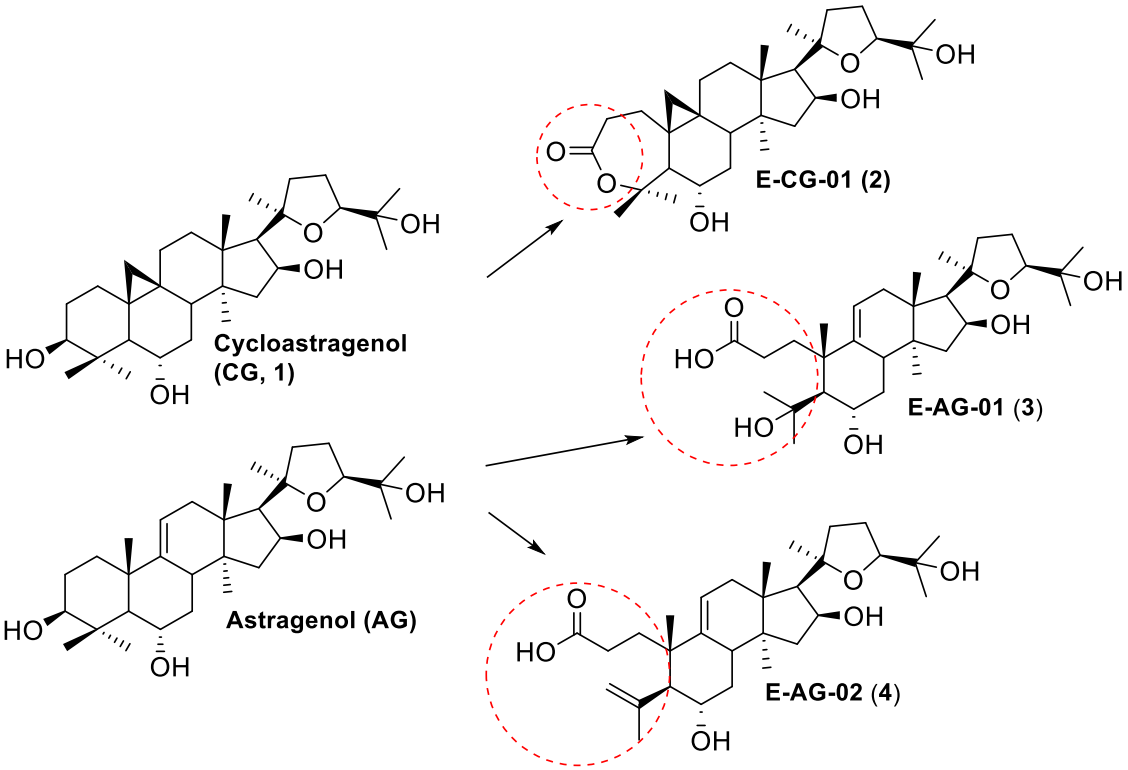

Figure 2.1. Chemical structures of the telomerase activator molecules used in the studies

2.2.4. Wound Repair (*in vitro* Scratch Assay)

The wound-healing effect of telomerase activators was studied using the method adapted from Karaman *et al.* (2017). Briefly, cells were cultivated with standard culture methods (DMEM supplemented with 10% FBS and 1% L-glutamine, without antibiotics). The cells were seeded on 48-well plates ($5x10^4$ cells/ml) and incubated at 37 °C for 24 hours in the 5% CO_2 atmosphere. Afterward, a linear scratch was created in the confluent monolayer by gently scraping with a sterile 200 µl pipette tip, and the cellular debris was removed by washing the cell surface twice with PBS. The serum-free medium (SFM)

containing the compounds at concentrations ranging from 0.5 to 100 nM (0.5, 2, 10, 30, and 100 nM) was applied to the wells, and SFM with 0.1% DMSO was used as a control group for calculations, and EGF (10 ng/mL, ~1.67 nM) was used as positive control. The promotion of wound closure was observed by capturing micrographs with a light microscope (Euromex Oxion Inverso, OX.2003-PL, Holland) at 0h, 24h, 48h, and 72h, respectively. The percentage of healed wound area at each time point was analyzed by using the ImageJ software (NIH, Bethesda, MD, http://www.rsb.info.nih.gov/ij) [252-253] and wound closure at each time point was quantified (Eq. 1-2) and to DMSO control.

In order to evaluate the effects of the molecules on cell migration, the same procedure was applied after Mitomycin C (MMC, 25 µM) treatment for two hours prior to scratching to suppress cell proliferation.

$$\% \text{ of wound area at } t(x) = b/a * 100 \qquad \text{Equation 1}$$

$$\% \text{ of wound closure after } x\text{h} = 100-(b/a * 100) \qquad \text{Equation 2}$$

Where, x: time point, a: cell-free area of the initial, b: cell-free area after x hour.

2.2.5. Growth Kinetics

MSCs cells were seeded on 6-well plates with $3x10^4$ cells/well and cultured for up to 40 days. During incubation, randomly selected 3 wells were subjected to cell counting with a Neubauer hemacytometer after trypsinization every other day. The cell numbers and appropriate days of incubation were used to draw a growth curve for the calculation of population doubling time according to the following equation (Eq. 3).

$$t_d = \ln 2/\mu \qquad \text{Equation 3}$$

Where, t_d: doubling time, µ: specific cell growth rate.

2.2.6. Population Doubling Level

MSCs, at earliest passage number (P4), were cultivated with standard culture methods (DMEM supplemented with 10% FBS and 1% L-glutamine, without antibiotics) incubated at 37 °C for 24 hours in the 5% CO_2 atmosphere until 70-80% confluency. Then, the cells were detached via trypsinization and seeded on 12-well plates ($3x10^4$ cells/ml). After 24 hours of incubation, cells were treated with molecules and cultivated with medium renewal and molecule treatment every other day until cells reached approximately 80% confluency. Before reaching full confluency, cells were passed into new 12-well plates after cell counting for every condition separately and continued until the cells were no longer attached to the plate surface and proliferated. The cell number for every passage, the population doubling length (PDL), was calculated according to the formula $PDL = PDL_0 + 3.322(\log C_f - \log C_i)$ where PDL_0: population doubling length of the passage number at the start, C_i: the initial cell number that seeded into the plates, C_f: the final cell number counted at the end of the passage [254-255]:

2.2.7. β-Galactosidase Staining

β-Galactosidase Staining Kit (BioVision, Cat. No. K802-250) was used for this purpose according to the manufacturer's instructions. Briefly, cells were washed once with 1 mL of 1X PBS and fixed with 500 µl of fixative solution for 15 minutes at room temperature. The staining solution mix was prepared while the cells were in the fixative solution. After fixation, the fixative solution was aspirated, and cells were washed (×2) with PBS. Afterward, stained with 500 µl of staining solution mix was added to each well. The plate was covered and incubated overnight at 37 °C. The development of the blue color was observed under the microscope. The staining solution was removed for long-term storage of the stained plates, and the plate was overlayed with 70% glycerol and stored at 4°C. The development of the blue color was observed under the light microscope.

2.2.8. Osteogenic Differentiation

MSC (HMSC-AD-500, CLS cell lines Service, Lot #102, Eppelheim, Germany) was cultivated based on standard cell culture techniques using basal medium DMEM (supplemented with 10% FBS and 1% L-Gln) in a humidified incubator with 5% CO_2 at 37 °C.

The cells grown with serial sub-cultivation with or without molecule treatment were subjected to osteogenic differentiation. For this purpose, cells from each condition were seeded on well plates. After 24 h incubation for cell adhesion, the medium was replaced with osteogenic medium (DMEM supplemented with 10% FBS, 100 nM dexamethasone, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate) including different concentrations of telomerase activators (1 to 1000 nM) and cultured up to 21 days. To observe osteogenic differentiation, Alizarin red S (ARS) staining was applied. Briefly, cells were rinsed with PBS (×3) and then fixed with ice-cold MeOH for 15 minutes at room temperature. Afterward, MeOH was removed, and cells were washed (×2) with deionized water and stained with Alizarin red S dye solution (ARS, 2% w/v) at 37 °C for 15 min. Afterward, stained cells were rinsed (×2) with PBS and incubated in PBS at room temperature for 15 min with rotation to remove nonspecific dye binding.

2.2.9. Immunoblotting

Preparation of cell extracts and western blot scraped monolayer cells for 48 h were collected at 1,500 g for 5 min, washed twice with ice-cold PBS, and whole protein lysates were extracted using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM ethylene glycol tetra-acetic acid, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethanesulfonyl, 2 mM iodoacetamide, and 1x protease inhibitor cocktail) (Roche, Indianapolis, IN, USA). Cell extracts were centrifuged at 16,000 g for 10 min at 4˚C. Supernatants were used for protein quantification using the Pierce1 BCA Protein Assay Kit, followed by denaturation of the sample with Laemmli buffer.

For the Western Blot analysis, 60 µg proteins were loaded per lane on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Following electrophoresis and transfer to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA), the blots were incubated in 5% nonfat milk in TBS-T (20 mM Tris, 150 mM NaCl, Tween 0.2%, pH 7.6). Membranes were then incubated with rabbit anti-Galectin1 1:5,000 (Thermo Fisher Scientific), rabbit antiGLUT1 1:1,000 (Thermo Fisher Scientific), and rabbit anti-β-actin 1:1,000 (Thermo Fisher Scientific) antibodies. After washing, the membranes were incubated with IRDye1 800CW antiRabbit IgG 1:15,000 (LI-COR Biosciences, Lincoln, NE, USA) and imaged on the Odyssey Infrared Imaging System (LI-COR Biosciences) followed by densitometric analysis.

Table 2.1. Preparation of resolving and stacking gels

Resolving Gel				**Stacking Gel**	
Gel percentage	**10%**	**12%**	**15%**	**Gel percentage**	
Acrylamide (30%)	3.33 ml	4 ml	5 ml	**Acrylamide (30%)**	0.35 ml
4x Resolving buffer	2.5 ml	2.5 ml	2.5 ml	**4x Stacking buffer**	0.75 ml
Distilled water	4.1 ml	3.5 ml	2.5 ml	**Distilled water**	1.9 ml
AP (10%)	75 µl			**AP (10 %)**	25 µl

2.2.10. Statistical Analysis

All experiments were conducted at least in triplicates, and GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) was used for statistical analyses. Data were presented as mean ± standard deviation. Statistical significance was determined by Student's t-test (two-tail) or two-way analysis of variance (ANOVA) with Sidak's post hoc correction through GraphPad Prism version 6.0 (GraphPad Software), and $p < 0.05$ was considered statistically significant. Significance on the graphs was indicated with *, where $p < 0.05$; **, where $p < 0.01$; ***, where $p < 0.001$; ****, where $p < 0.0001$. Non-significant correlations ($p > 0.05$) were not indicated on the graphs.

2.3. Results

2.3.1. The Effects on the Proliferation of MSCs

One of this thesis's primary goals is to define the role of telomerase activators in the differentiation of mesenchymal stem cells to investigate their improved usage in research and clinical applications. Based on this purpose, firstly, MTT assays were constructed in order to determine the effective concentration range for each molecule.

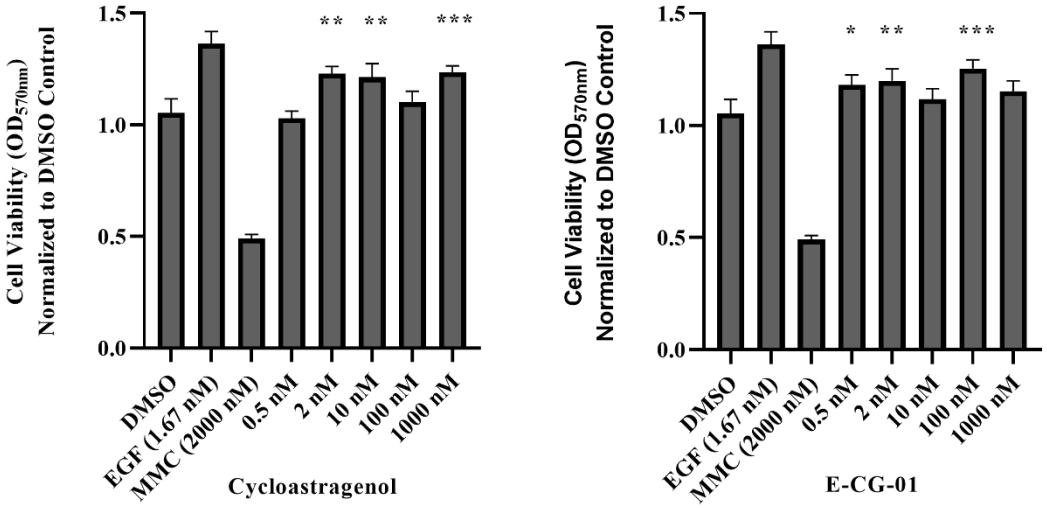

Figure 2.2. Effect of CG and E-CG-01 molecules on cell proliferation (concentration range 0.5 - 1000 nM, 48 h treatment). Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way analysis of variance (one-way ANOVA, Tukey's test) and significance levels are presented as *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 compared to vehicle control, DMSO

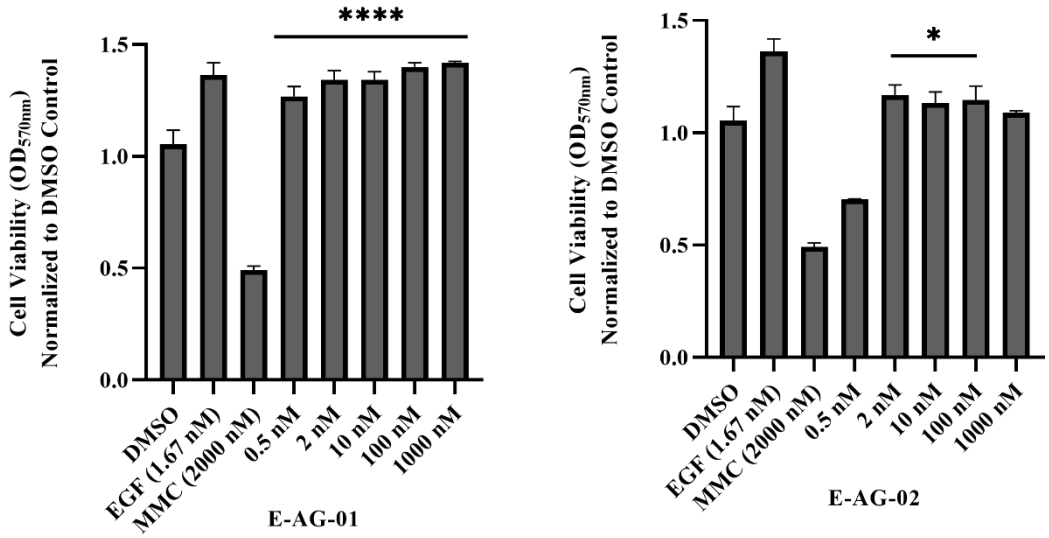

Figure 2.3. Effect of E-AG-01 and E-AG-02 molecules on cell proliferation (concentration range 0.5 - 1000 nM, 48 h treatment). Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way analysis of variance (one-way ANOVA, Tukey's test) and significance levels are presented as *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 compared to vehicle control, DMSO

2.3.2. Wound Healing

In order to evaluate the effect of the molecule on wound healing in cells, an *in vitro* wound model was created, and the behavior of the cells in closing the wound area was examined. For this purpose, the effect of molecule application at different concentrations (0.5 - 1000 nM) on cell proliferation/viability after 48 hours was evaluated, and the results are presented in Figure 2.4. When the results are considered, it is seen that the molecules increase cell proliferation in competition with the growth factor EGF. On the other hand, the MMC molecule, which is planned to be used in migration experiments, has been shown not to kill cells at the applied concentration but only to stop proliferation.

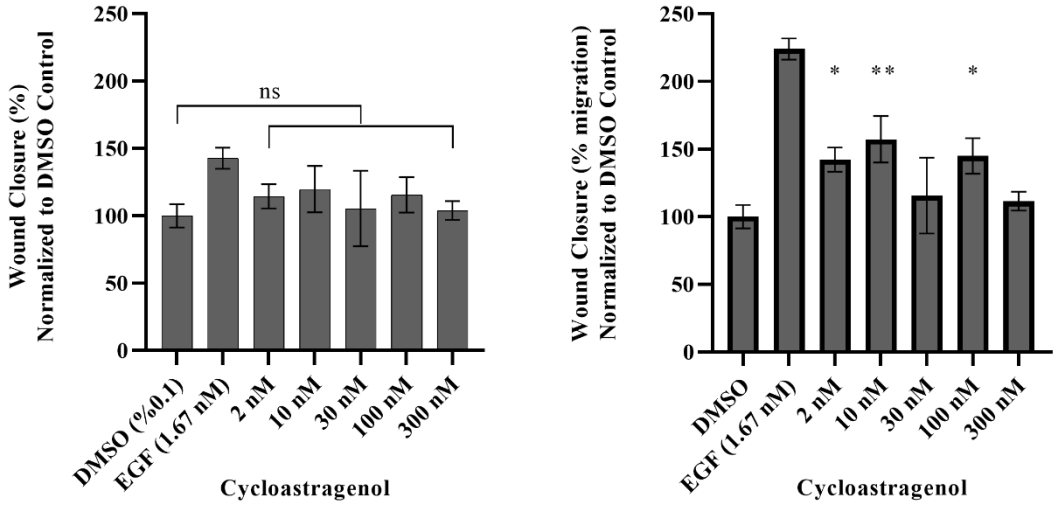

Figure 2.4. Effects of CG on *in vitro* wound healing. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way ANOVA, Tukey's test, *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001

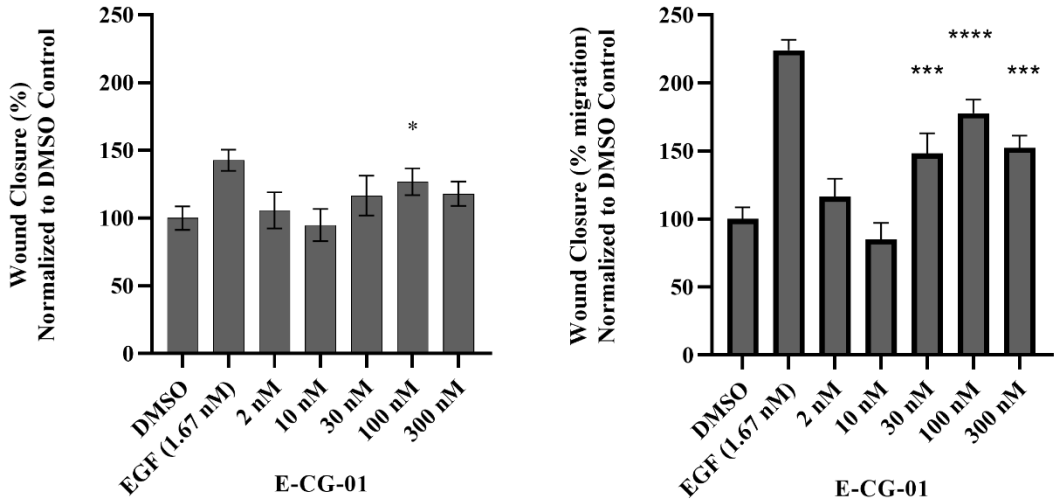

Figure 2.5. Effects of E-CG-01 on *in vitro* wound healing. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way ANOVA, Tukey's test, *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 ns: not statistically significant compared to vehicle control, DMSO

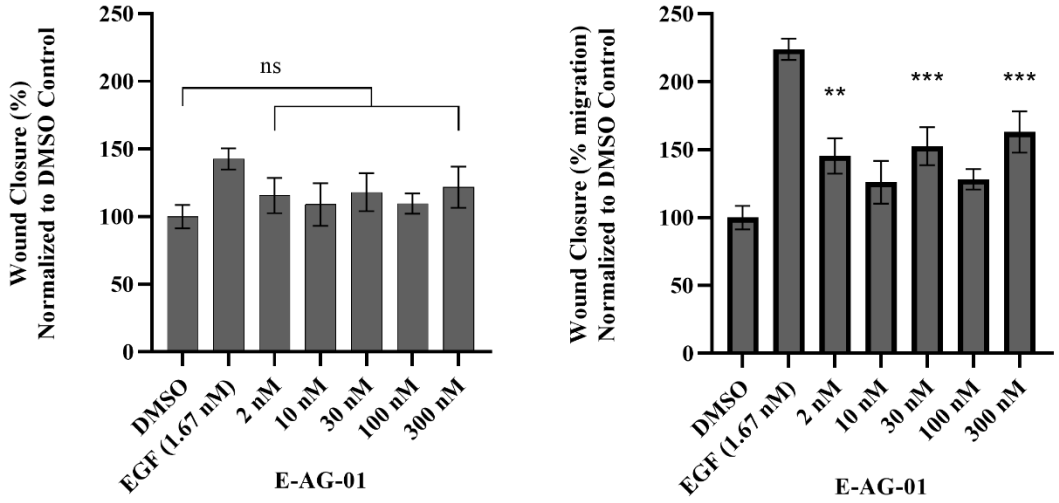

Figure 2.6. Effects of E-AG-01 on *in vitro* wound healing. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way ANOVA, Tukey's test, *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001

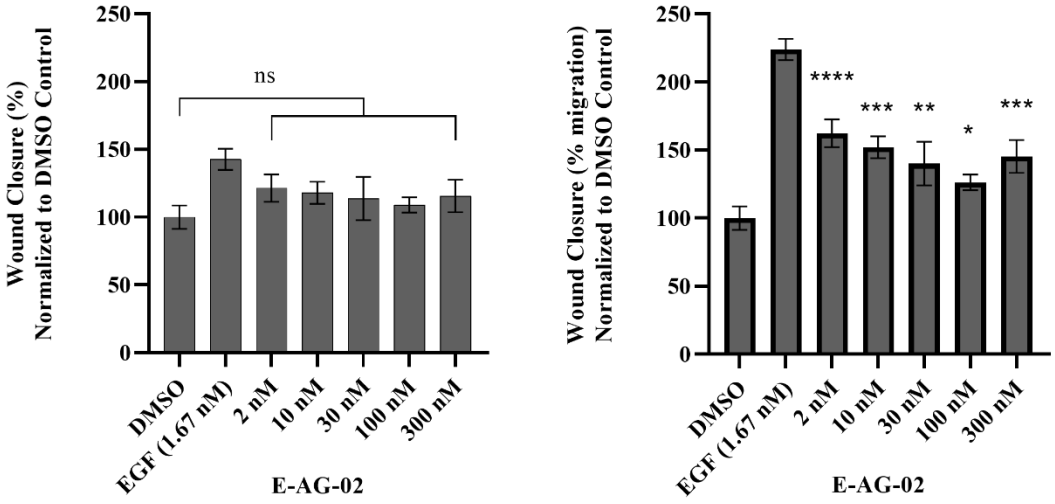

Figure 2.7. Effects of E-AG-02 on *in vitro* wound healing. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way ANOVA, Tukey's test, *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001
ns: not statistically significant compared to vehicle control, DMSO

2.3.3. Growth Kinetics and Senescence Condition of MSCs

In differentiation studies of stem cells, it was thought that differentiation should be initiated by applying differentiation-inducing factors in the period when the growth is in the exponential phase. For this reason, the growth behavior of MSCs used in the project was examined. Looking at the cell number versus time graph (Figure 2.4) obtained by cell counting at certain time intervals, it is seen that the cells enter the exponential phase on the 2nd to 3rd day after the lag phase and remain in this phase until the 15th day. After day 15, the stationary phase, in which the number of cells remained constant, lasted until day 28, and then the death phase started. Therefore, in osteogenic differentiation studies, it was decided to replace the medium with osteogenic differentiation medium on the 2nd or 3rd day of cultivation, considering the confluency of the cells.

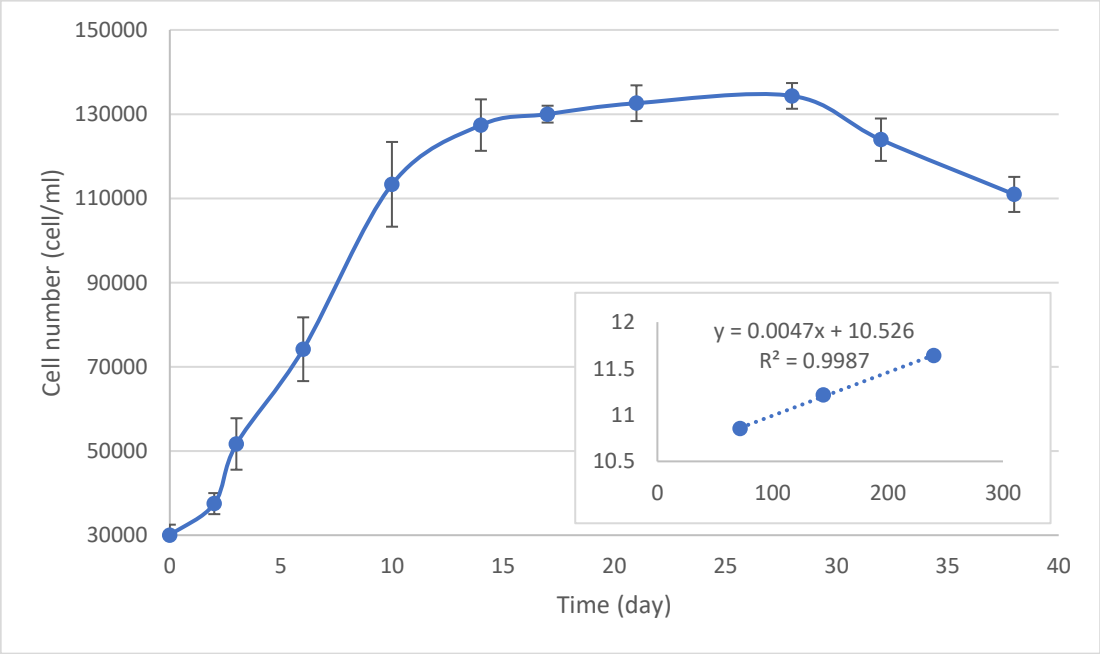

Figure 2.8. The Growth curve of MSCs under standard culture conditions

By preventing cellular senescence through telomerase activation, it is aimed to extend the manipulation time of stem cells *in vitro* and to improve stem cell therapies by transferring their differentiation properties to advanced passages. In this direction, firstly, the senescence status of MSCs was cultured and carried to further passages by sub-cultivation, β-galactosidase activity and the levels of telomerase-associated hTERT protein levels were examined (Figure 2.9).

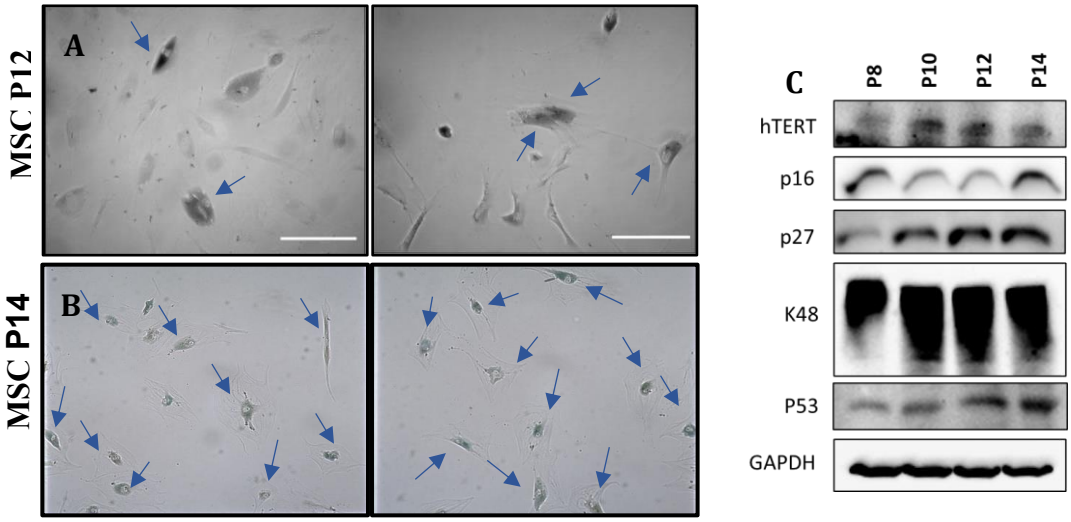

Figure 2.9. Senescence markers of MSCs at different passages. Images of β-galactosidase staining (A and B, senescent cells were indicated with blue arrows) and Western blot analysis of senescence markers and hTERT levels (C) of MSCs for different passages (left). The change in osteogenic marker proteins and p27 levels of vehicle controls during osteogenic differentiation of MSCs (right)

2.3.4. Population Doubling Length

In order to evaluate the effects of the molecules on the population doubling length (PDL) of MSCs during continuous sub-cultivation, molecule administration was started

at the earliest passage (P4) with a concentration range between 1 to 1000 nM. At every passage, cells were counted via a hemocytometer, and PDL was calculated for each condition separately. Since overgrowth may cause senescence, sub-cultivation was performed before cells reached full confluency. The sub-cultivation was continued until P15 and the morphology of the MSCs at P15 was given in Figure 2.10. The results of PDL of MSC at passages between P4 to P15 were represented for each molecule in Figures 2.11 – 2.14.

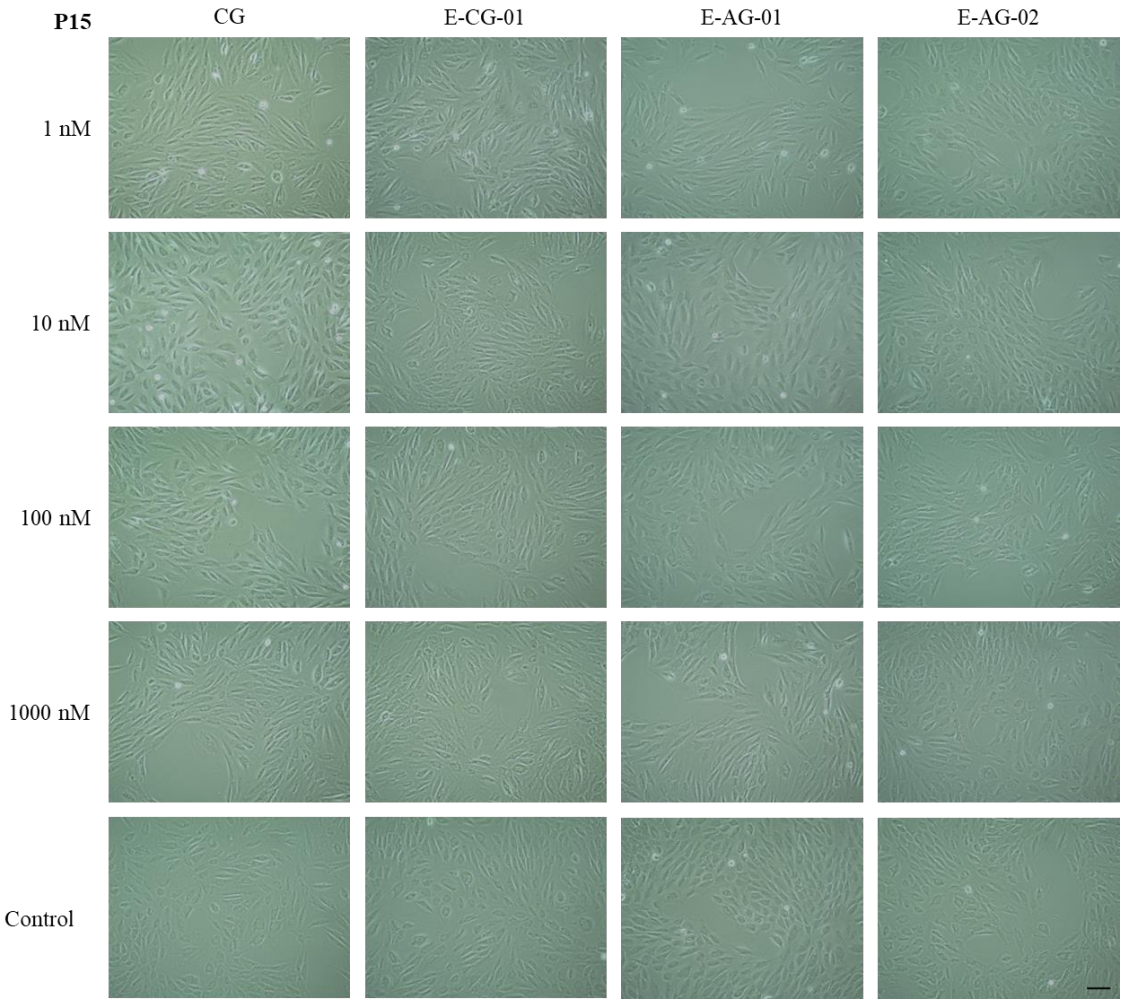

Figure 2.10. Light microscopy images of MSCs at P15 (10x objective, Scale bar 100 µm)

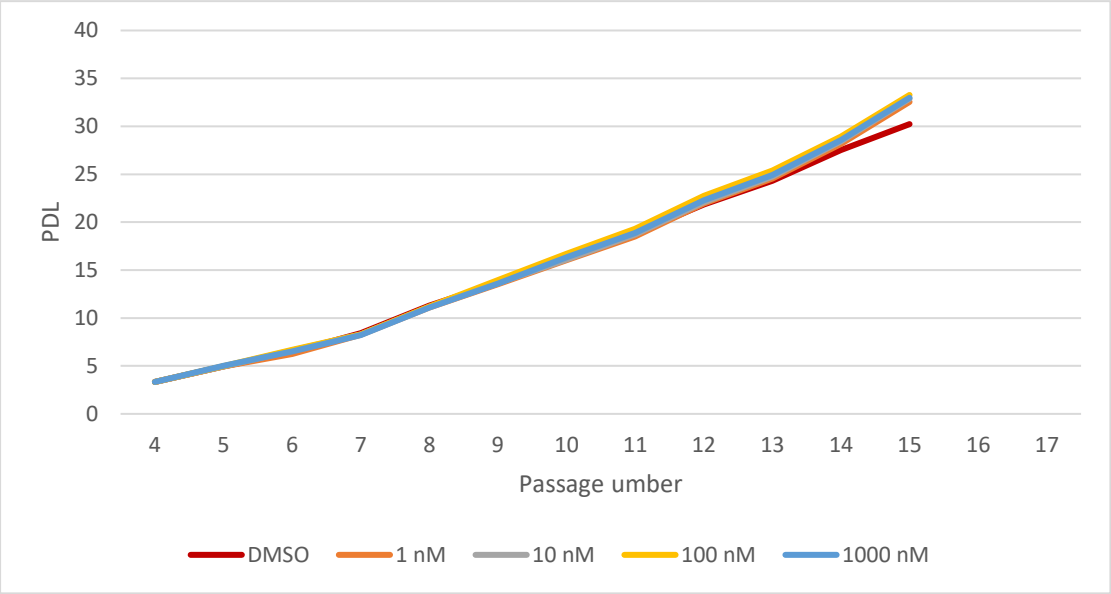

Figure 2.11. The effects of CG on population doubling length of MSC during serial sub-cultivation with continuous treatment

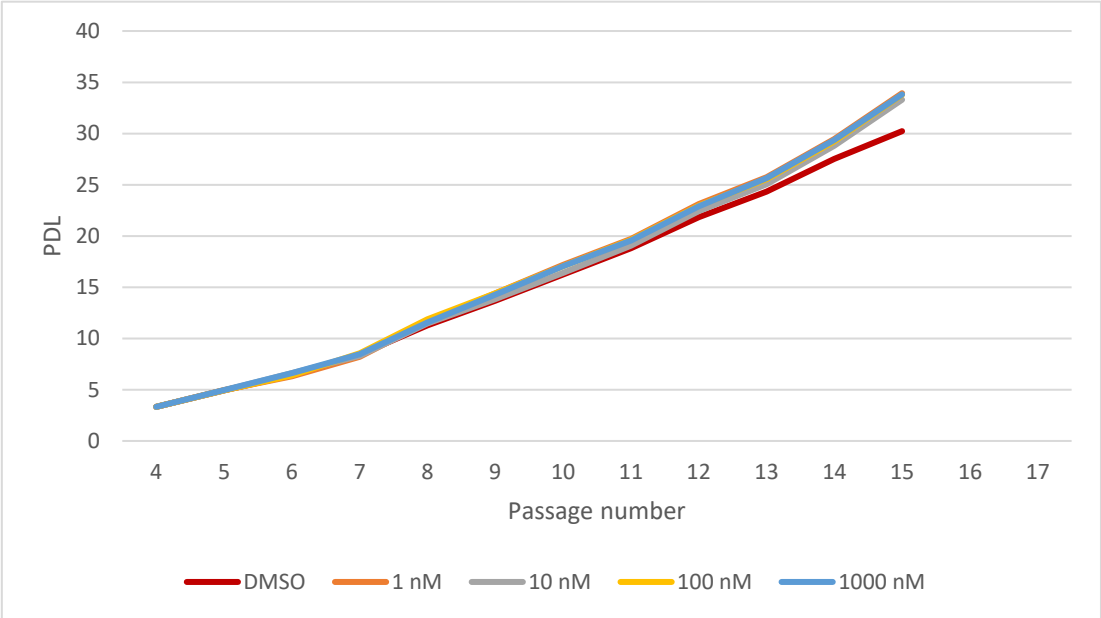

Figure 2.12. The effects of E-CG-01 on population doubling length of MSC during serial sub-cultivation with continuous treatment

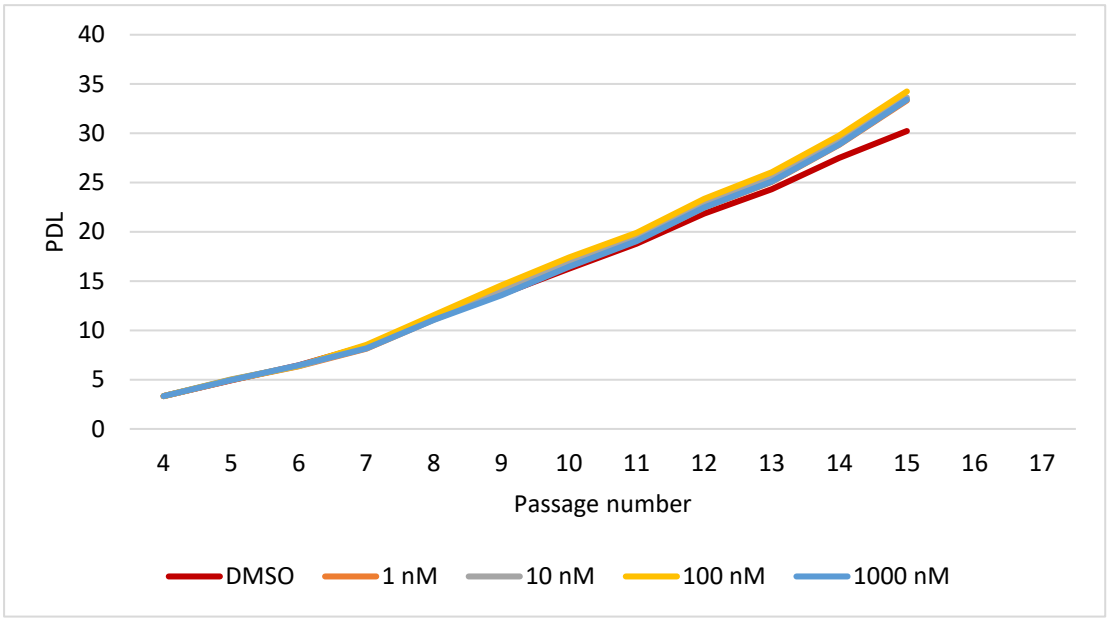

Figure 2.13. The effects of E-AG-01 on population doubling length of MSC during serial sub-cultivation with continuous treatment

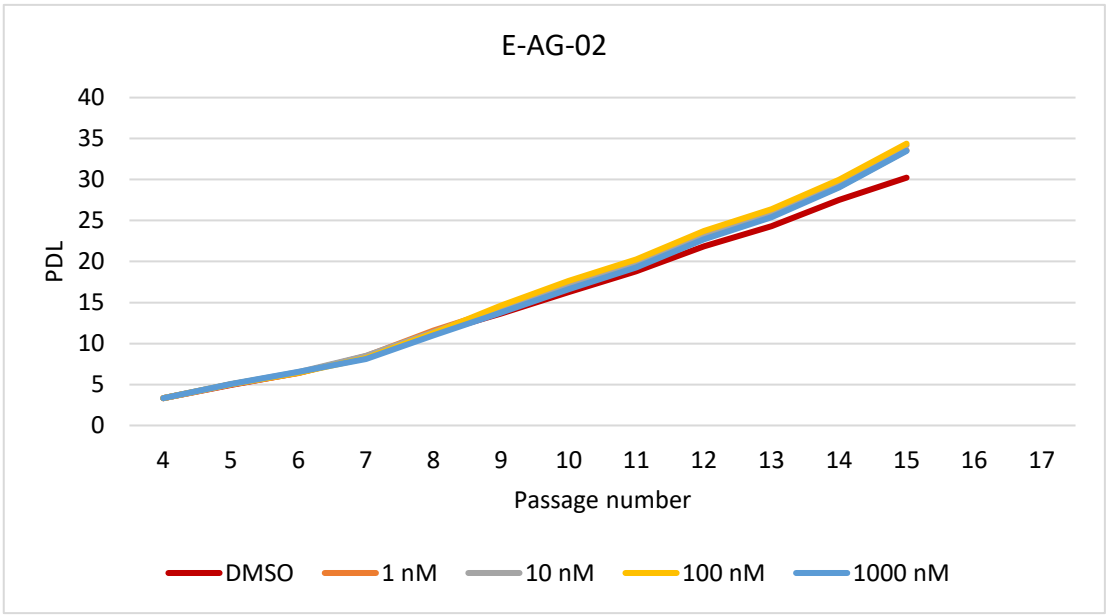

Figure 2.14. The effects of E-AG-02 on population doubling length of MSC during serial sub-cultivation with continuous treatment

2.3.5. β-Galactosidase Staining

The senescence conditions of MSCs were evaluated via -galactosidase staining during continuous sub-cultivation. For this purpose, cells at P15 where the blue-colored senescent cells were observed for the first time. The overgrowth and starved MSCs were used as the positive control for the staining procedure (Figure 2.15). Light microscopy images of β-gal stained MSCs at P15 via sub-cultivation with continuous molecule treatment were given in Figures 2.16 – 2.19.

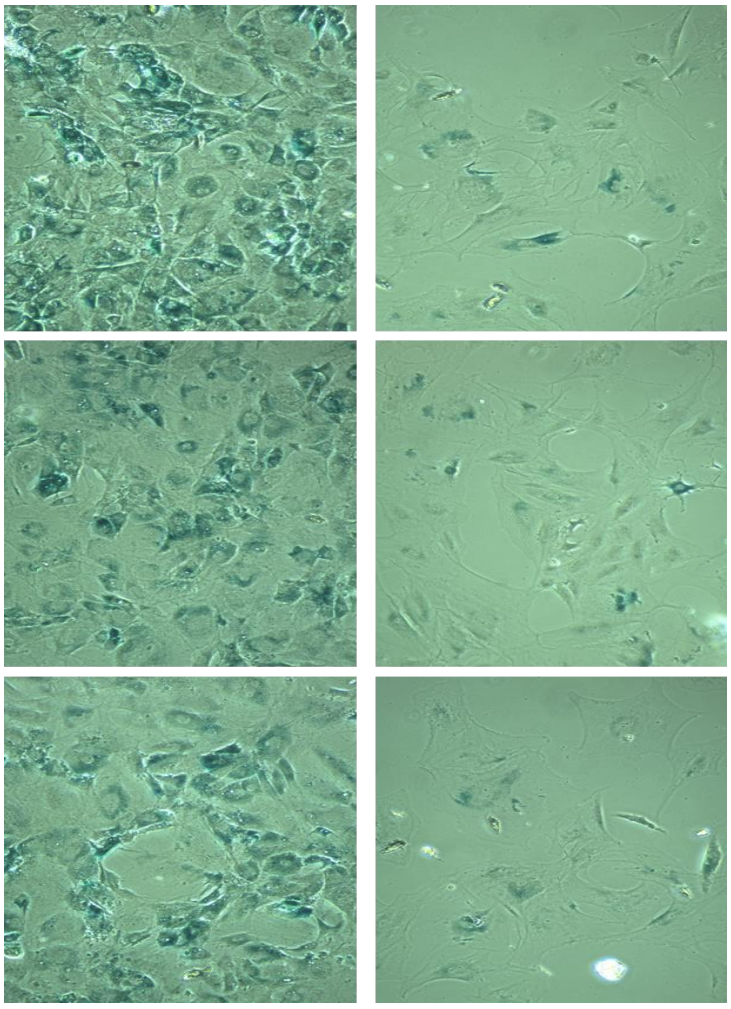

Figure 2.15. Light microscopy images of β-gal positive MSCs at high and low densities

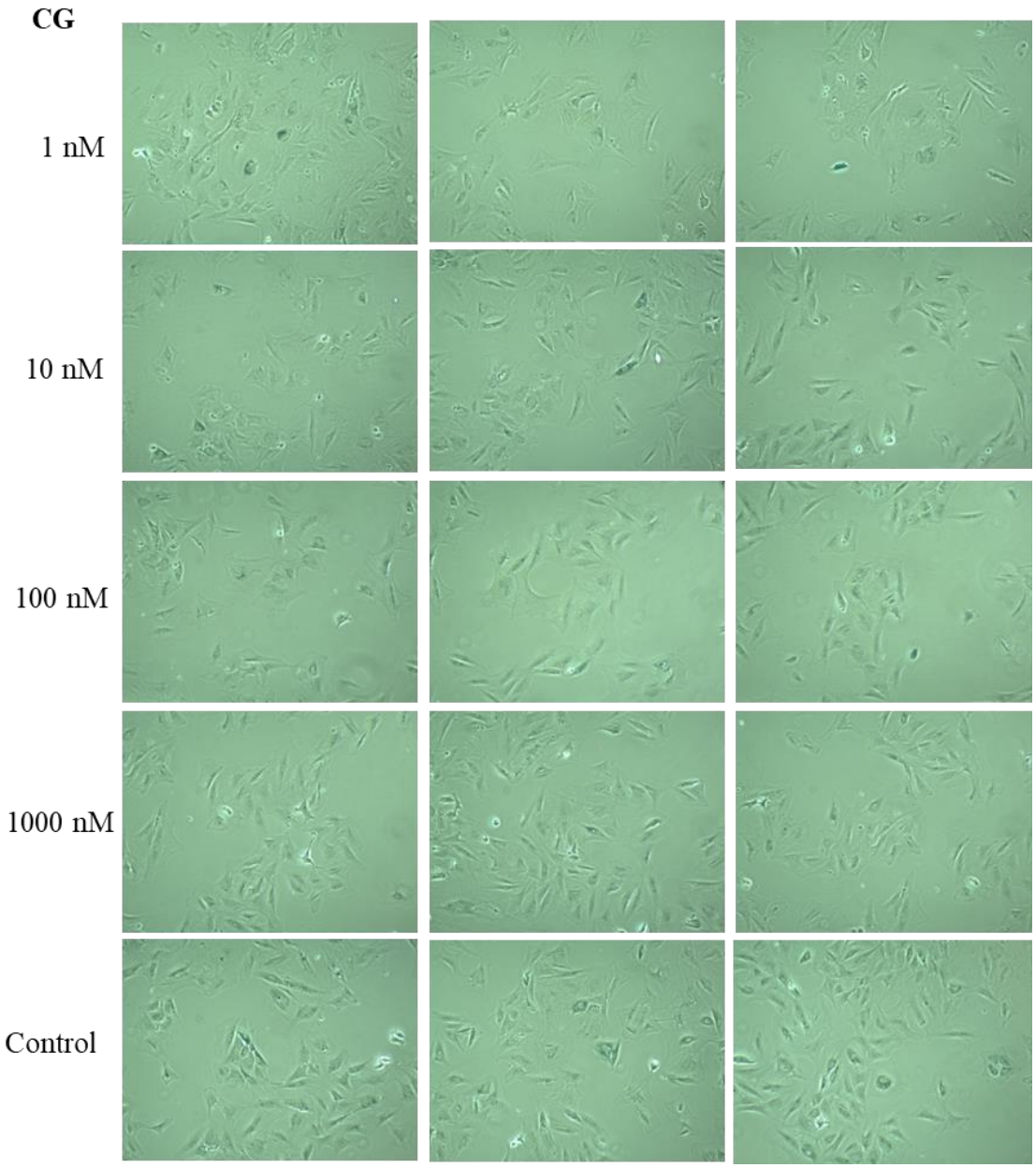

Figure 2.16. Light microscopy images of β-gal stained MSCs at P15 via sub-cultivation with continuous CG treatment. Micrographs represent randomly selected 3 replicates for each condition

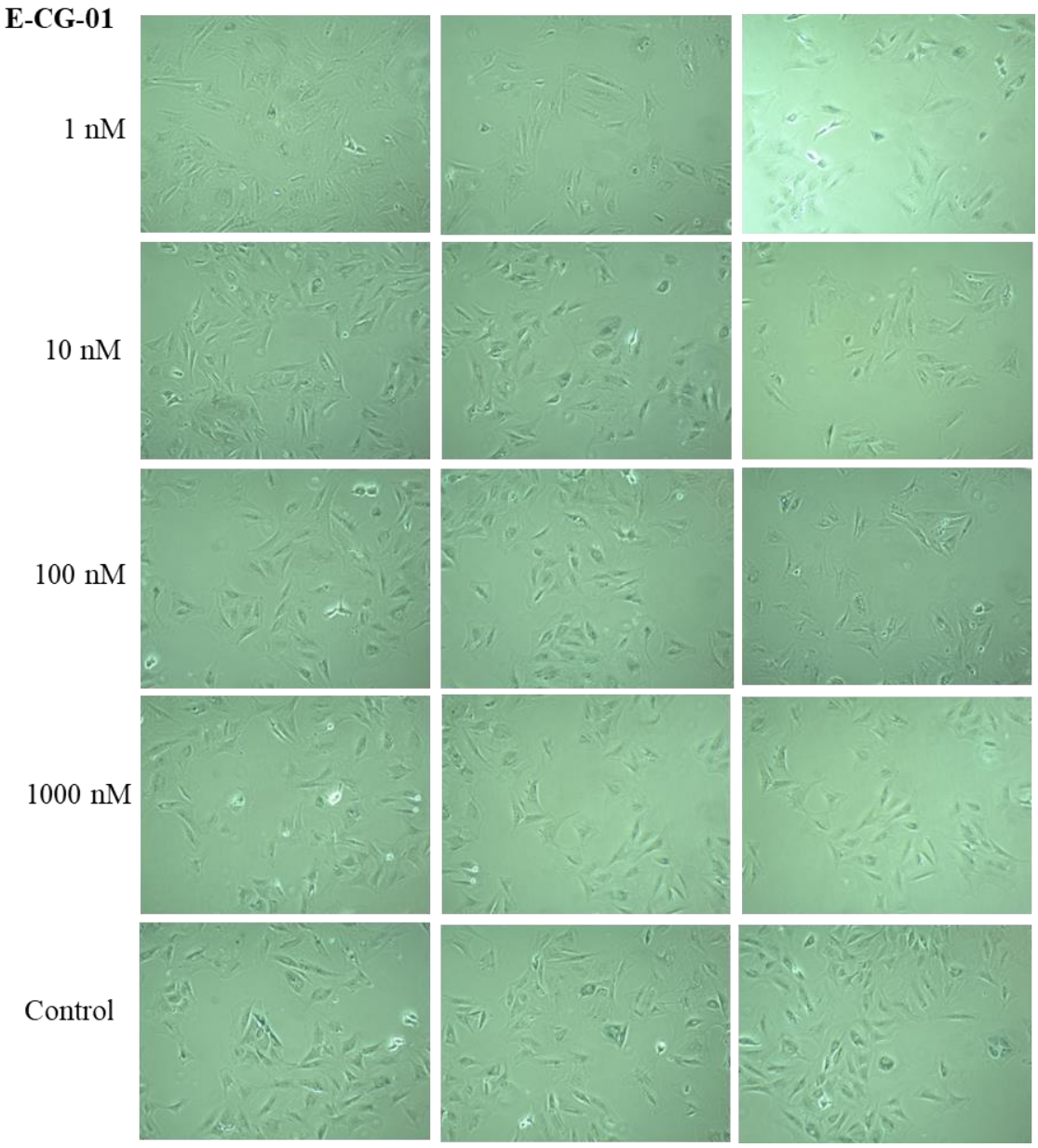

Figure 2.17. Light microscopy images of β-gal stained MSCs at P15 via sub-cultivation with continuous E-CG-01 treatment. Micrographs represent randomly selected 3 replicates for each condition

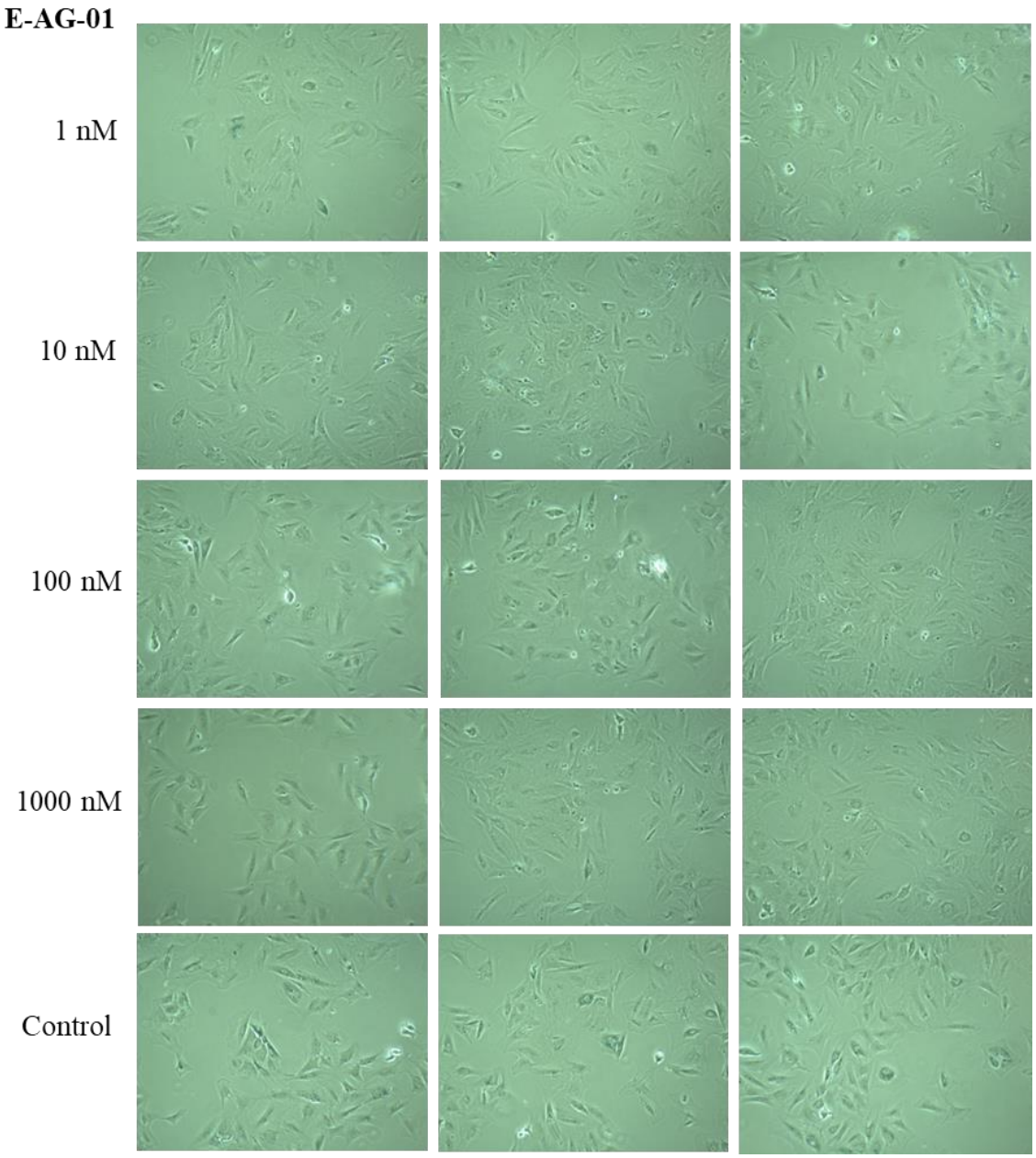

Figure 2.18. Light microscopy images of β-gal stained MSCs at P15 via sub-cultivation with continuous E-AG-01 treatment. Micrographs represent randomly selected 3 replicates for each condition

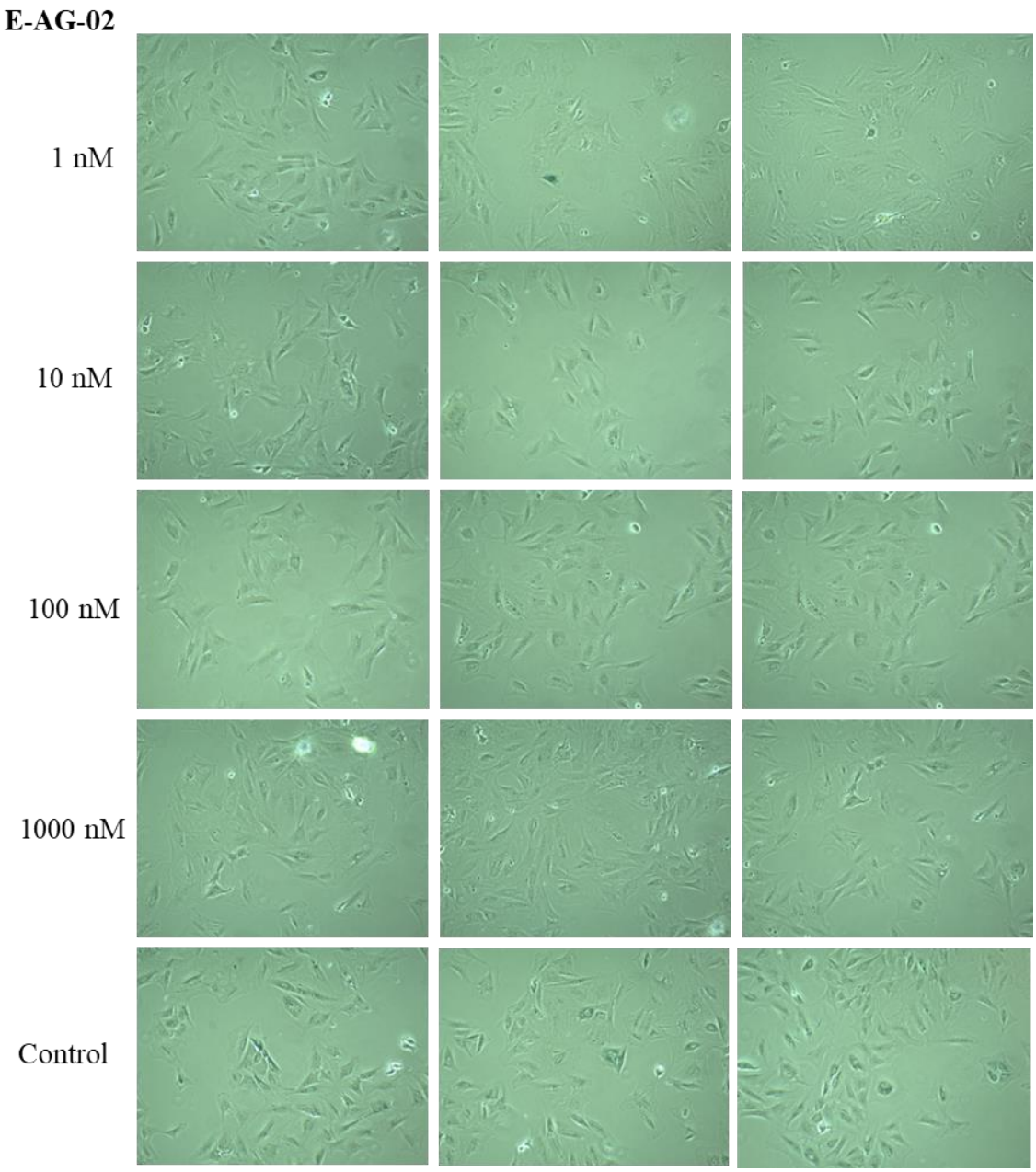

Figure 2.19. Light microscopy images of β-gal stained MSCs at P15 via sub-cultivation with continuous E-AG-02 treatment. Micrographs represent randomly selected areas of 3 replicates for each condition

2.3.6. Osteogenic Differentiation

In order to evaluate the osteogenic differentiation potential of MSC at further passages, cells were serially passaged starting from P4, and after P10, cells were subjected to osteogenic differentiation at every other passage. At the end of 21 days, differentiation was checked with ARS staining. The photographs of stained wells for P10 to P14 were given in Figure 2.20 for each condition.

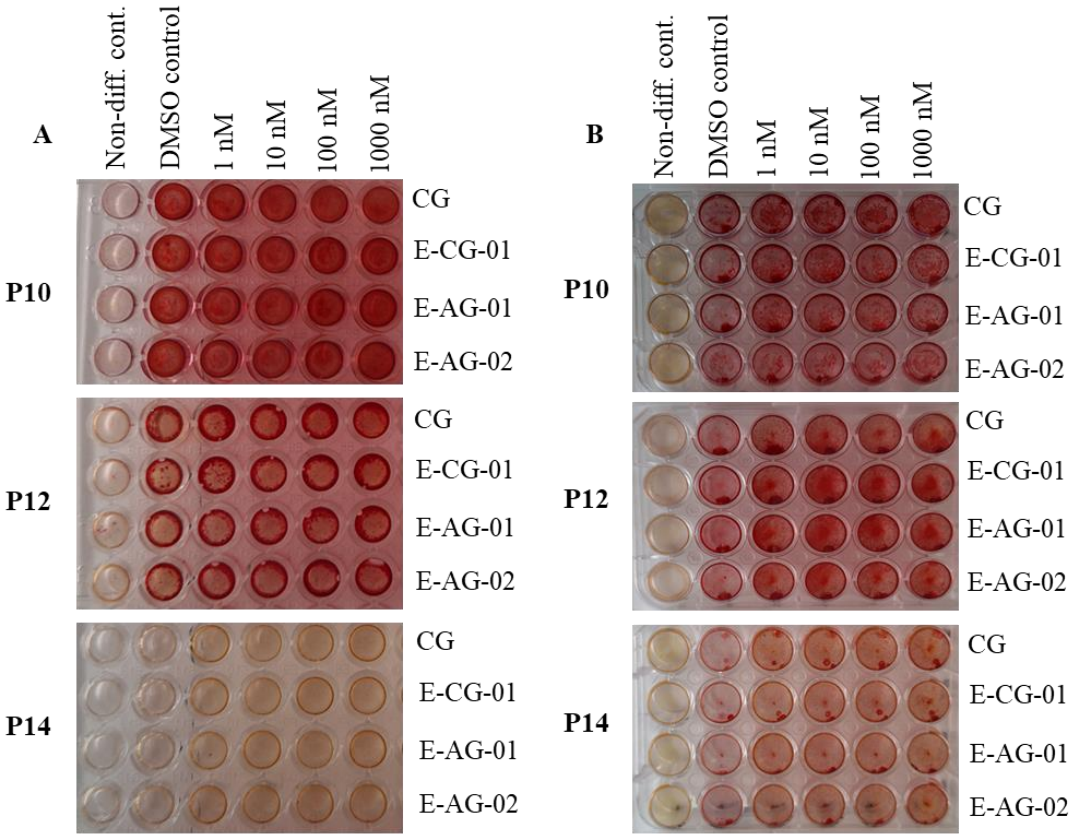

Figure 2.20. The images of AR-S stained MSCs at different passages (P10, P12 and P14) via sub-cultivation with or without continuous compound treatment for 21 days. Serial passaging without treatment (**A**) serial passaging with continuous molecule (1 to 1000 nM) treatment (**B**). Non-diff. and DMSO controls represent undifferentiated control group and vehicle control group, respectively

Continuous sub-cultivation was carried out until P20. However, osteogenic differentiation was not observed after P14. The ARS staining results of P15 are given in Figure 2.21.

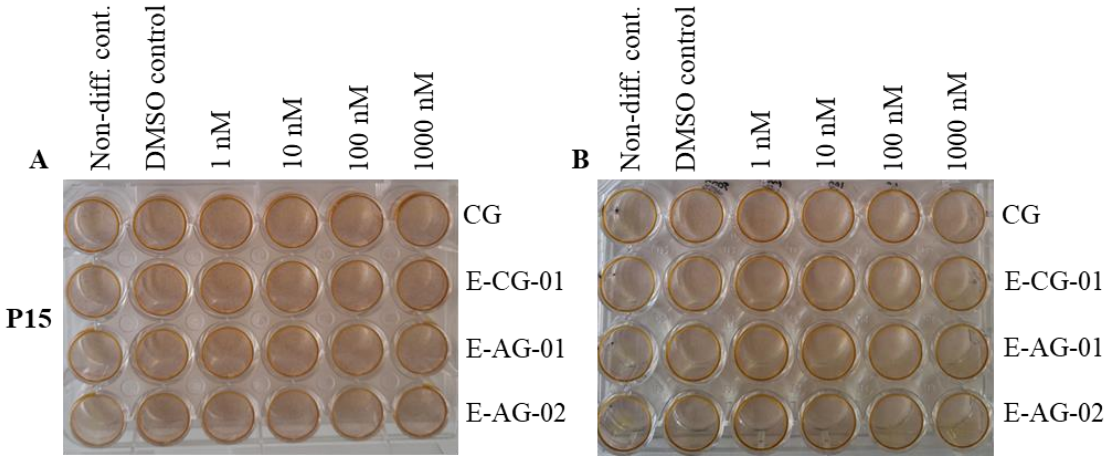

Figure 2.21. The images of AR-S stained MSCs at P15 via sub-cultivation with or without continuous compound treatment for 21 days. Serial passaging without treatment (**A**) serial passaging with continuous molecule (1 to 1000 nM) treatment (**B**). Non-diff. and DMSO controls represent undifferentiated control group and vehicle control group, respectively

2.4. Discussion

In a study by Zhou and colleagues, telomerase activation was shown to increase the lifespan and reproductive capacity of stem cells significantly. Similarly, within the scope of the project, the molecules were found to act in competition with the growth factor EGF, given the data obtained from cell proliferation and wound healing experiments. Another study showed that ectopic expression of telomerase in stem cells isolated from adipose tissue does not negatively affect the normal metabolism of the cells, increases the sustainability of the osteogenic cell pool *in vitro* and helps to maintain the multipotent properties of the cells. In the same study, it was found that control group cells without

telomerase expression lost their self-renewal properties at the end of 12 passages. In comparison, telomerase-expressing cells maintained their differentiation ability for 50 passages, and there was no change in growth kinetics during this period (Zhou *et al.*, 2020). While CG, the starting molecule, increased cell proliferation by 10%, it was observed that E-AG-01, one of the CG derivatives, increased proliferation above 20% at all applied concentrations. Similarly, E-CG-01 and E-AG-01 molecules also increased proliferation by up to 20%. In wound healing experiments, unlike previous studies (Sevimli-Gür *et al.*, 2011), cell proliferation was stopped by mitomycin C application, and the migration effect of the molecules was also examined. When the migration results were examined, it was seen that the molecules provided wound closure with a migration effect rather than a proliferative effect. Thus, while CG (300 nM) provided 50% wound closure in mesenchymal stem cell wound healing, CG derivatives carried this rate above 50% in the 2-300 nM concentration range and especially E-CG-01 (100 nM) molecule was the most effective molecule in wound closure. Cao and colleagues reported that CG (300 nM) increased proliferation and migration in human epidermal stem cells (Cao *et al.*, 2019). Specifically, E-CG-01 has a lactone framework in the ring A of CG, whereas E-AG-01 and E-AG-02 are 3,4-seco forms of the same ring in astragenol skeleton (AG; cyclopropane ring cleaved form of CG obtained upon strong acid treatment). Both groups significantly increased the activities in comparison to starting molecule CG. On the other hand, our results indicated that telomerase activators provide cell-specific responses that need to be evaluated case by case since a similar trend was not evident in all cell types in terms of concentration and molecule type.

As a result of our studies, it has been shown that the doubling times are prolonged with the increase in senescence-related protein (p16, p27 and K48) levels with increasing passage numbers. Histochemical staining for β-galactosidase (β-gal), which is closely related to senescence, was performed on cell samples taken by skipping one passage from P8, and the prepared cell preparations were examined under a light microscope. It was observed that the number of blue cells was almost negligible until P12; this number started to increase from P14, and at P16, the number of cells stained blue due to β-gal activity was at high levels. In a study on stem cell senescence conducted by Gruber and colleagues with adipose tissue-derived mesenchymal cells, it was similarly reported that the doubling time of cells increased with increasing passage number, and this value

exceeded 200 hours at P8. Since stem cell metabolism can vary according to the health status and age of the isolated donor [256], it is thought that the data obtained within the scope of the thesis are consistent with the literature [257-258].

According to MTT assay results, it has been shown that telomerase activators caused an increase in cell proliferation by up to 80% compared to the control group (DMSO control, 0.1%). Additionally, results demonstrated that the effect of telomerase activators is higher for P10 and P12 compared to early and late passages (P8 and P14).

Our results showed that telomerase activators significantly ameliorated *in vitro* wound repair. These findings were compatible with previous works by Sevimli-Gür *et al.* with *Astragalus* cyclobutane-type saponins, in which 2nM of CG was the most effective concentration for *in vitro* (HS2 cells) wound healing. Specifically, E-CG-01 has a lactone framework in the ring A of CG, whereas E-AG-01 and E-AG-02 are 3,4-seco forms of the same ring in astragenol skeleton (AG; cyclopropane ring cleaved form of CG obtained upon strong acid treatment). Both groups significantly increased the activities in comparison to starting molecule CG. On the other hand, our results indicated that telomerase activators provide cell-specific responses that need to be evaluated case by case since a similar trend was not evident in all cell types in terms of concentration and molecule type.

Senescent cells were determined in different passage numbers via the β-galactosidase staining procedure, and senescent cells (blue) were investigated under the light microscope. A few numbers of senescent cells were observed first at P12, and it was seen that blue-colored senescent cells dominated the culture after P14. Furthermore, we performed an immunoblotting assay, Western blot analysis, to investigate senescent-associated proteins and hTERT protein levels, closely related to telomerase activity. The Western blot results showed that during long-term incubation periods, senescence-associated protein levels increase starting from P10. Also, it can be concluded that there is no significant hTERT protein secretion in MSCs. Thus, we expect the telomerase activator molecules to increase the hTERT expression and prevent stem cells from senescence during *in vitro* cultivation.

Based on these results, we continue to incubate cells (P4) with small telomerase activator molecules to identify the effects of the molecules on the senescence and differentiation capacity of MSCs during long-term cultivation. The cell samples from

every other passage were tested separately for their senescence condition and differentiation ability through β-galactosidase and ARS staining, respectively.

It has been reported that adult human MSCs and osteoblasts propagated *in vitro* show no detectable telomerase activity. When telomerase enzyme was expressed in bone marrow stromal stem cells, their lifespan and reproductive capacity were significantly increased, and their bone formation potential was increased *in vitro* [247-248]. When these studies were examined, it was seen that genetic interventions provided telomerase enzyme expression.

The decrease in the ossification and bone healing process with aging and the decrease in the regenerative potential of MSCs as the age of the donor increases in stem cell therapy are the most significant problems to be overcome for cellular clinical treatments [259]. In addition, the amount of MSC in the bone marrow decreases with age; during *in vitro* propagation, it is lost because they enter a phase called senescence (cell cycle arrest), or tissue loss may occur shortly after transplantation due to the lack of tissue integration [260-261].

Telomerase activation in MSC is predicted to improve tissue engineering studies and accelerate the transition to clinical applications. The literature has reported that telomerase activation in stem cells significantly increases the lifespan and reproductive capacity of cells while increasing their bone formation potential *in vitro* [245]. Telomerase activation accelerates osteogenic differentiation both *in vitro* and in vivo by causing an increase in the expression of genes directly related to bone development, such as osterix and osteocalcin, accelerates the process by increasing calcium accumulation, and is 6 times more likely than the control group in the 2-6 weeks post-transplantation period. It has been shown that more bone formation is provided [262]. Another study showed that ectopic telomerase expression in stem cells isolated from adipose tissue does not adversely affect the normal metabolism of cells, increases the *in vitro* sustainability of the osteogenic cell pool, and helps preserve the multipotent properties of the cells. The same study determined that the control group cells without telomerase expression lost their self-renewal properties at the end of 12 passages. In contrast, telomerase-expressing cells retained their differentiation ability for 50 passages, and there was no change in their growth kinetics during this period. The fact that no malignant transformation was

observed with the increase in telomerase activity also proves that the method has a high potential for tissue engineering and regeneration studies [263].

2.5. Conclusion

The fate of both stem cells is hardly regulated by also their niche, the local microenvironment. The determination of the regulatory factors from the niche and their biological targets is an important aspect to understand and guide cell biology [240]. Due to their dependence on unknown regulatory factors, stem cells mostly need feeder cells and serum-containing media to survive *in vitro*. Unfortunately, using feeder cells and serum results in an increasing number of undefined regulatory factors related to stem cell differentiation, self-renewal, and pluripotency [237, 264]. To overcome the complexity owing to secretions of feeder cells or unknown ingredients from serum, chemically defined xeno- and feeder-cell-free media compositions can be used by supporting small molecules instead of growth factors [237]. These chemically defined media and small molecules provide not only defined conditions to discover certain molecular mechanism effects but also result in decreased cost due to the use of growth factors to control and investigate cells and, importantly, decrease rejection of transplanted stem cells [264-265].

CHAPTER 3

EVALUATION OF THE EFFECTS OF TELOMERASE ACTIVATORS ON OSTEOGENIC DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

3.1. Background

Strong evidence has shown that osteodegenerative diseases such as osteoporosis are associated with short telomeres. When the *in vitro* osteoblast differentiation of MSCs from telomerase-negative mice was examined, it was observed that the proliferation of the cells was reduced, the doubling times were prolonged, and the mineral deposition could not be achieved [249].

Obtaining MSC from bone marrow has been possible for many years. However, obtaining it from bone marrow is a challenging method for regenerative medicine as it is an invasive and painful procedure for patients. In addition, the amount of MSC in the bone marrow decreases with age. It is lost during *in vitro* propagation, mainly because they enter senescence or are lost shortly after transplantation [260-261, 266]. It is known that it can also be isolated from other tissues such as adipose tissue, skin, amniotic fluid, amniotic membrane, placenta, umbilical cord, cord blood, etc. [236, 267]. Among these tissues that are the source of MSCs, the fact that adipose tissue contains higher amounts of MSCs and can be easily obtained with a simple surgical procedure has increased the use of adipose MSCs for therapeutic purposes in recent years [268].

Increasing the TERT gene's co-ectopic expression, the telomerase's catalytic subunit and VEGF (vascular endothelial growth factor) have been shown to prolong the *in vitro* cultivation time and accelerate vascularization both *in vitro* and *in vivo* in bone marrow stromal stem cells from aged donors. In the karyotype analysis, no chromosomal

differences were observed, and no tumorigenic risk was detected [166]. In studies on increasing telomerase activity, it is concluded that in addition to increasing telomere length, telomerase also positively affects and accelerates stem cell differentiation. When the multipotent properties of mesenchymal stem cells are added, it is possible to obtain similar results for more than one tissue type.

In the study by Shi *et al.* in 2002, hTERT expression of human bone marrow stem cells was increased by retroviral transduction. As a result, the number of passages could be increased above 80 by prolonging the lifespan of cells that showed senescence after 30 passages and were known to lose their osteogenic differentiation capacity. It has been reported that cells subjected to osteogenic differentiation showed twice as much calcium accumulation as the control group at the end of 4 weeks. In addition, the effect of telomerase expression on bone tissue formation was examined in an *in vivo* study. It was determined that telomerase active stem cells transplanted in the 20th passage formed 5 times more bone tissue than the control group. The effect of telomerase expression on preserving osteogenic differentiation capacity was tested by transplanting cells of different passage numbers. It was observed that cells in passage 80 also had osteogenic differentiation capacity. However, it was stated that the cells with the best bone-forming capacity were telomerase-active cells in the 40th passage. In the experiment on whether telomerase activity causes tumor formation, oncogenic transformation was not found *in vitro* and *in vivo* [247].

In 2003, Gronthos *et al.* conducted a study with bone marrow stromal cells to investigate the functional role of telomerase during osteogenesis. It has been shown to accelerate both *in vitro* and *in vivo*. In the *in vitro* study, it was observed that a significant amount of calcium accumulation took 4 weeks in the control group without telomerase activity. The exact amount of calcium accumulation could be achieved in only 2 weeks in cells with increased telomerase expression. It has been reported that the number of viable cells is higher than the control group in transplantation with telomerase-activated cells, and 6 times more bone formation is achieved in the 2-6 weeks post-transplantation period than in the control group [262]. In the study published by Kang *et al.* in 2004, the effect of telomerase on the *in vitro* lifespan of stromal cells isolated from adipose tissue and its effect on osteogenic differentiation were investigated. *İn vitro* sustainability of the osteogenic cell pool of ectopic expression of telomerase.

3.2. Materials and Methods

3.2.1. Osteogenic Differentiation

MSC (HMSC-AD-500, CLS cell lines Service, Lot #102, Eppelheim, Germany) was cultivated based on standard cell culture techniques using basal medium DMEM (supplemented with 10% FBS and 1% L-Gln) in a humidified incubator with 5% CO_2 at 37 °C.

After 24 h incubation for cell adhesion, the medium was replaced with osteogenic medium (DMEM supplemented with 10% FBS, 100 nM dexamethasone, 50 µg/mL ascorbic acid, 10 mM β-glycerophosphate) including different concentrations of telomerase activators (0.1, 2, 10 30, 100, 300 and 1000 nM) and cultured up to 28 days. To observe osteogenic differentiation of MSCs, total DNA content, alkaline phosphatase (ALPase), and calcium (Ca^{2+}) assays were evaluated every week according to manufacturer's instructions (QuantiChrom ALPase Assay Kit Cat# and QuantiChrom Calcium Assay Kit Cat#). ALPase activity and Ca^{2+} contents were normalized to DNA content for comparison groups.

3.2.1.1. Alizarin Red S Staining and Quantification

Cells were rinsed with PBS (×3) and then fixed with ice-cold MeOH for 15 minutes at room temperature. Afterward, MeOH was removed, and cells were washed (×2) with deionized water and stained with Alizarin red S dye solution (ARS, 2% w/v) at 37 °C for 15 min. Afterward, stained cells were rinsed (×2) with PBS and incubated in PBS at room temperature for 15 min with rotation to remove nonspecific dye binding. After taking micrographs under the light microscope, cells were destained with 10% cetylpyridinium chloride (CPC, in 10mM potassium phosphate buffer pH 7.0) solution with rotation at room temperature for 20 min. The samples were transferred into 96-well

plates, and absorbance was measured at 562 nm via a micro-plate reader (Synergy™, HTX- BioTek, Winooski, VT, ABD). Quantification of calcium deposits was performed by using the equation from the ARS standard graph (absorbance vs ARS amount) given in Figure 3.1.

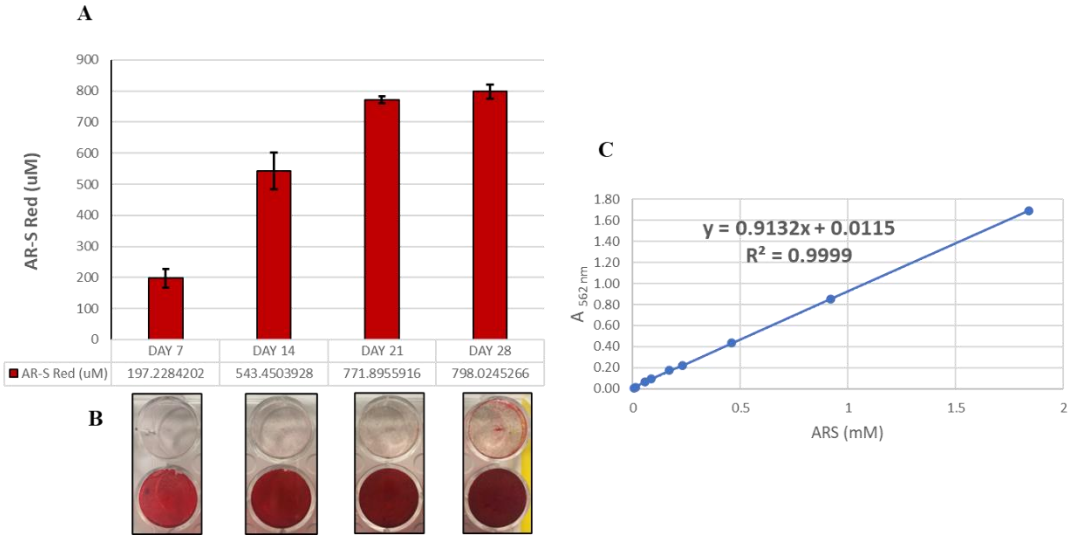

Figure 3.1. Quantification of ARS-stained calcium deposits at 14, 21 and 28 days of differentiation of MSC. Error bars mean ± standard deviation (n = 3). The change in calcium deposits of MSC during four weeks of differentiation (**A**), photographs of the differentiated (lower) and undifferentiated (upper) MSC for each week (**B**), and the standard curve graph and equation for the ARS quantification (**C**)

3.2.1.2. Calcium Assay

The total calcium amount was determined by using the QuantiChrom calcium assay kit (DICA-250, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, the cell pellets were lysed on ice using 10 mM Tris-HCl with 0.1 % Triton X-100 in PBS by shaking at room temperature at 150 rpm for 20

min and centrifuged at 21,000 g (4 °C) for 10 minutes. The supernatants were used for the calcium assay. Total protein concentration and double-stranded DNA amount were determined by bicinchoninic acid (BCA) method by using Pierce protein assay kit (ThermoFisher Scientific, Waltham, MA, USA) and DNA Quantification Kit (Sigma Aldrich, St. Louis, MO, USA) respectively according to the manufacturer's instructions.

3.2.1.3. Alkaline Phosphatase Activity Assay

ALP activity assays were performed using a QuantiChrom ALP assay kit (DALP-250, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, the cell pellets were lysed on ice using 10 mM Tris-HCl with 0.1 % Triton X-100 in PBS by shaking at room temperature at 150 rpm for 20 min and centrifuged at 21,000 g (4 °C) for 10 minutes. The supernatants were used for the ALP activity assay. Total protein concentration and double-stranded DNA amount were determined by bicinchoninic acid (BCA) method by using Pierce protein assay kit (ThermoFisher Scientific, Waltham, MA, USA) and DNA Quantification Kit (Sigma Aldrich, St. Louis, MO, USA) respectively according to the manufacturer's instructions.

3.2.1.4. Immunofluorescence

Following the treatment with molecules for 28 days, cells were rinsed with PBS and fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) for 30 minutes at 4 °C and washed three times with ice-cold PBS. The samples were immersed with 0.1% Triton X-100 in PBS for 1 hour and blocked with 1% BSA in PBS for 1 hour. After removal of BSA solution, the samples were incubated with primary antibodies in 1% BSA overnight at 4 °C. Primary antibodies (Santa Cruz Biotechnology Inc., California, USA) mouse monoclonal antibody against OP antibody (1:100, #21742;), mouse monoclonal antibody against OC (1:100; #365797), mouse monoclonal antibody

against COL I (1:100, #59772;), and mouse monoclonal antibody against ALP (1:100, #365765,) were used for osteogenic markers. After washing primary antibodies, the samples were incubated with secondary antibody m-IgG kappa BP-FITC (1:500, #516140; Santa Cruz Biotechnology Inc., California, USA) in 1% BSA for 90 min at 37 °C. At the end of the incubation, the samples were stained with 4,6-diamidino- 2-phenylindole (DAPI, Sigma Aldrich, St. Louis, MO, USA) for 5 min at room temperature. The images were captured with the same exposure time and light intensity via an inverted fluorescence microscope (Axio observer Z1, Zeiss, Jena, Germany).

3.2.1.5 Telomerase Activity Assay

Telomerase activity was quantified with a TeloTAGGG Telomerase PCR ELISA^{PLUS} kit according to the manufacturer's instructions (Roche Diagnostics, Meylan, France).

3.2.2. Statistical Analysis

All experiments were conducted at least in triplicates, and GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) was used for statistical analyses. Data were presented as mean ± standard deviation. Statistical significance was determined by Student’s t-test (two-tail) or two-way analysis of variance (ANOVA) with Sidak’s post hoc correction through GraphPad Prism version 6.0 (GraphPad Software), and $p < 0.05$ was considered statistically significant. Significance on the graphs was indicated with *, where $p < 0.05$; **, where $p < 0.01$; ***, where $p < 0.001$; ****, where $p < 0.0001$. Non-significant correlations ($p > 0.05$) were not indicated on the graphs.

3.3. Results

3.3.1. Alizarin Red S Staining

In order to follow the formation of extracellular calcium deposits, which is one of the important markers of osteogenic differentiation, i.e., bone tissue-specific mineralization, osteogenic differentiation studies were performed with molecule application to stem cells seeded in 48-well culture dishes. AR-S staining was performed on the 7, 14, 21 and 28 days of differentiation. The images of the differentiation in control groups are presented in Figure 3.2. The quantification of AR-S stained calcium deposits of experimental groups is given in Figure 3.3. Accordingly, it is seen that the molecules used significantly increased mineralization during osteogenic differentiation.

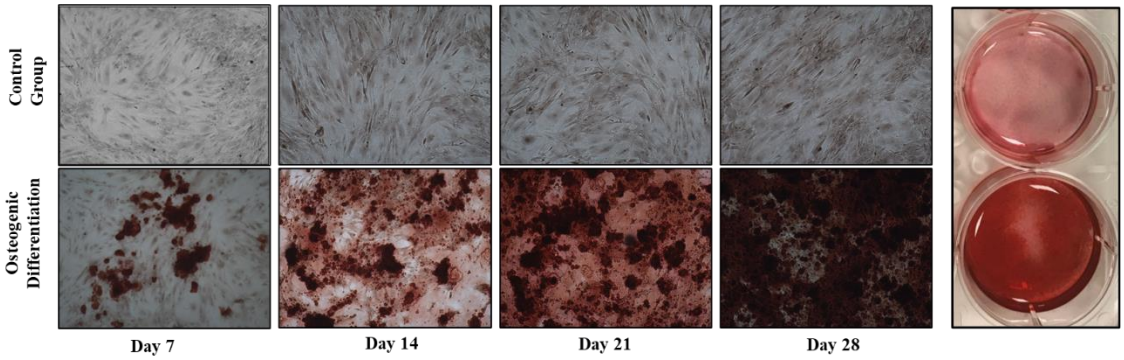

Figure 3.2. The images of ARS-stained calcium deposits at 14, 21 and 28 days of differentiation of MSC

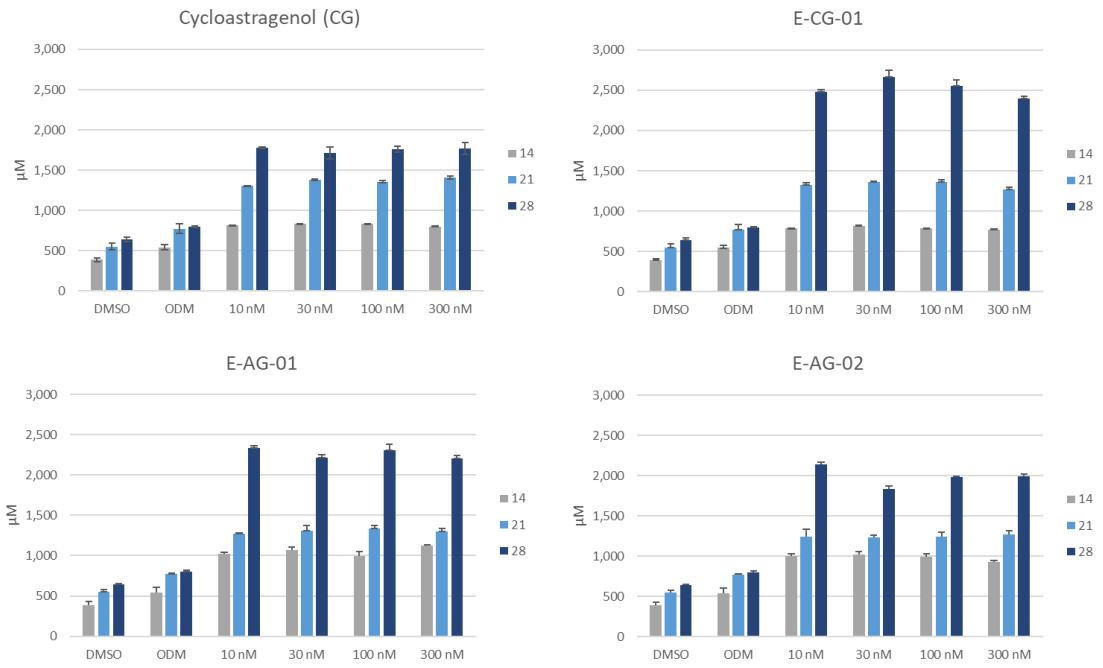

Figure 3.3. Quantification of ARS-stained calcium deposits at 14, 21 and 28 days of differentiation of MSC with molecule treatment (10, 30, 100 and 300 nM). Error bars mean ± standard deviation (n = 3). DMSO, vehicle control; ODM, osteogenic differentiation medium only

3.3.2. DNA Amount, ALP Activity and Total Calcium Content

In order to follow the changes in ALP activity and calcium content, which are important markers of osteogenic differentiation, osteogenic differentiation studies were performed with molecule application to stem cells seeded in 12-well culture dishes. On the 7th, 14th, 21st and 28th days of differentiation, the total DNA amount was determined. ALP activity and calcium content were ratioed to the total DNA amount and the results are presented in Figure 3.4 – 3.7.

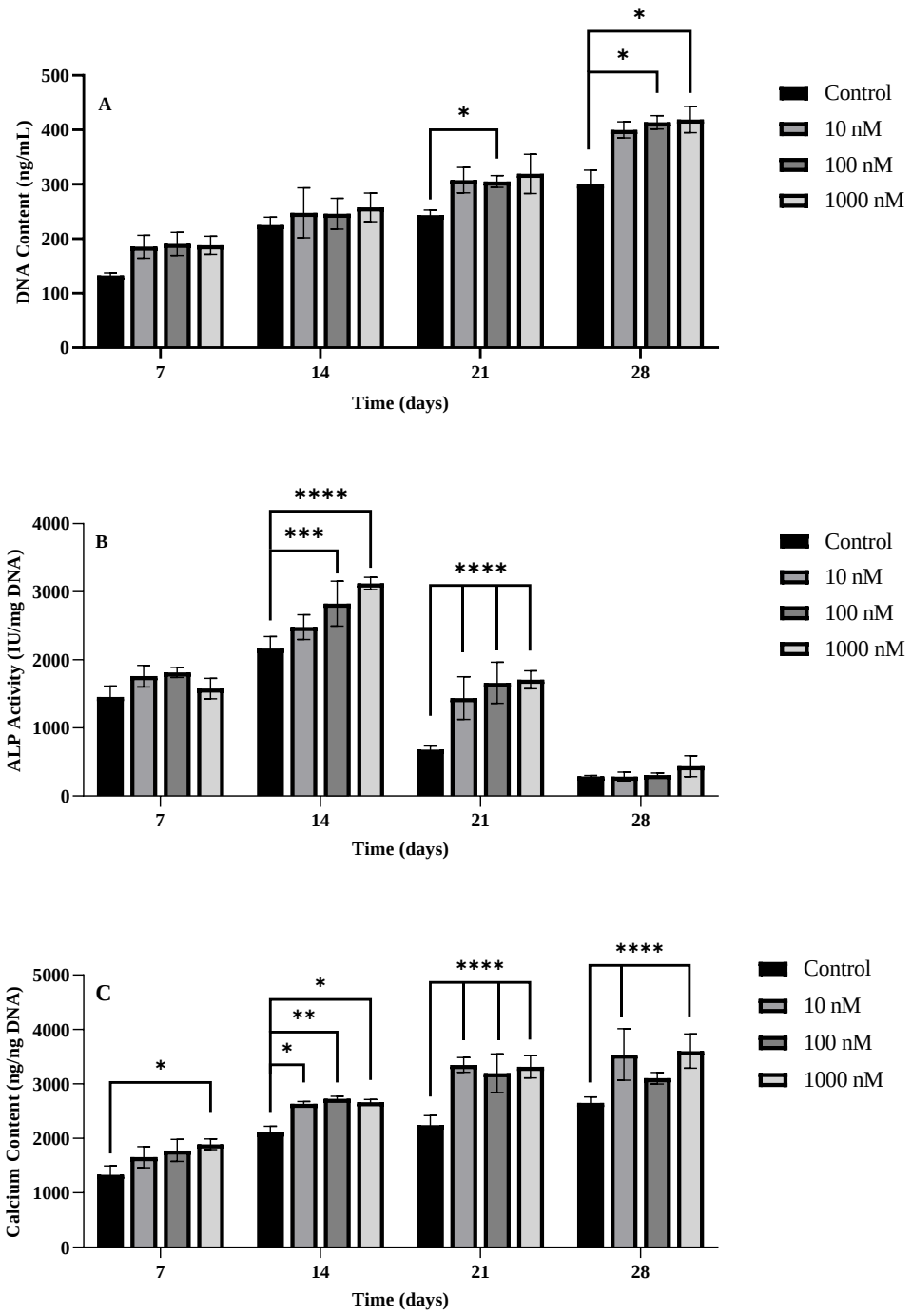

Figure 3.4. Effect of CG on DNA amount (A), ALP activity (B) and calcium content (C) during osteogenic differentiation. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way analysis of variance (one-way ANOVA, Sidak test), *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 compared to vehicle control, DMSO

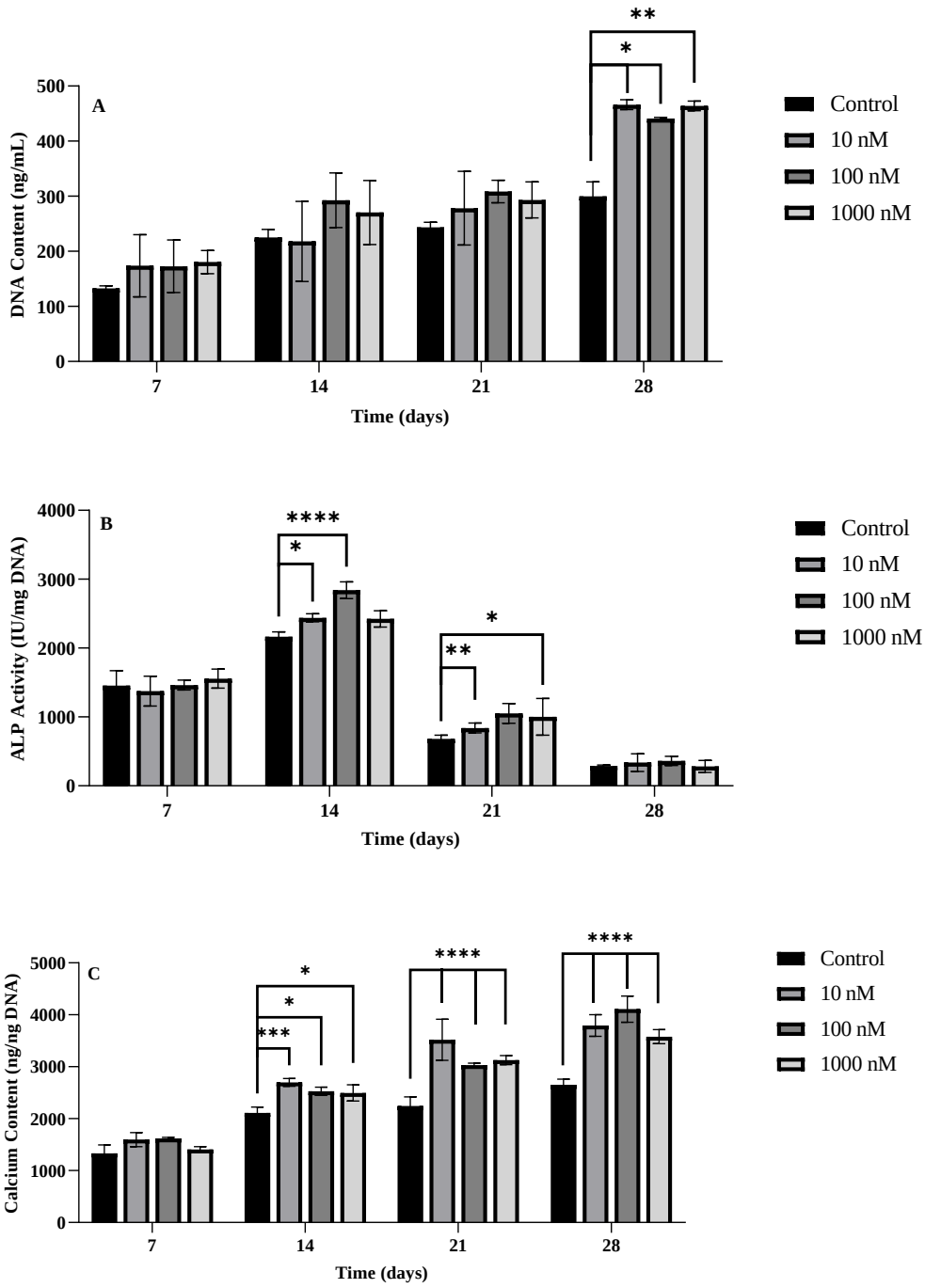

Figure 3.5. Effect of E-CG-01 on DNA amount (A), ALP activity (B) and calcium content (C) during osteogenic differentiation. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way analysis of variance (one-way ANOVA, Sidak test), *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 compared to vehicle control, DMSO

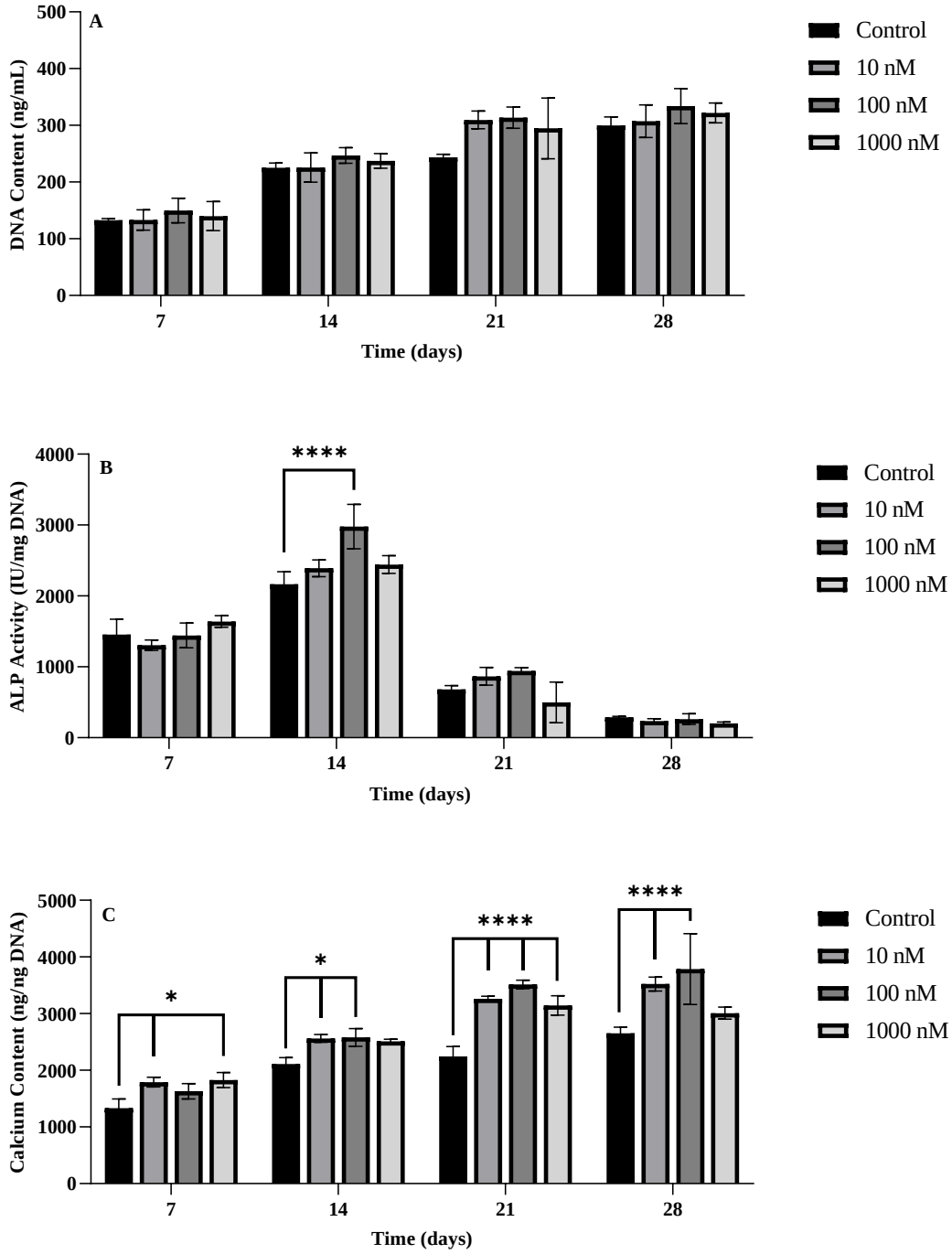

Figure 3.6. Effect of E-AG-01 on DNA amount (A), ALP activity (B) and calcium content (C) during osteogenic differentiation. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way analysis of variance (one-way ANOVA, Sidak test), *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 compared to vehicle control, DMSO

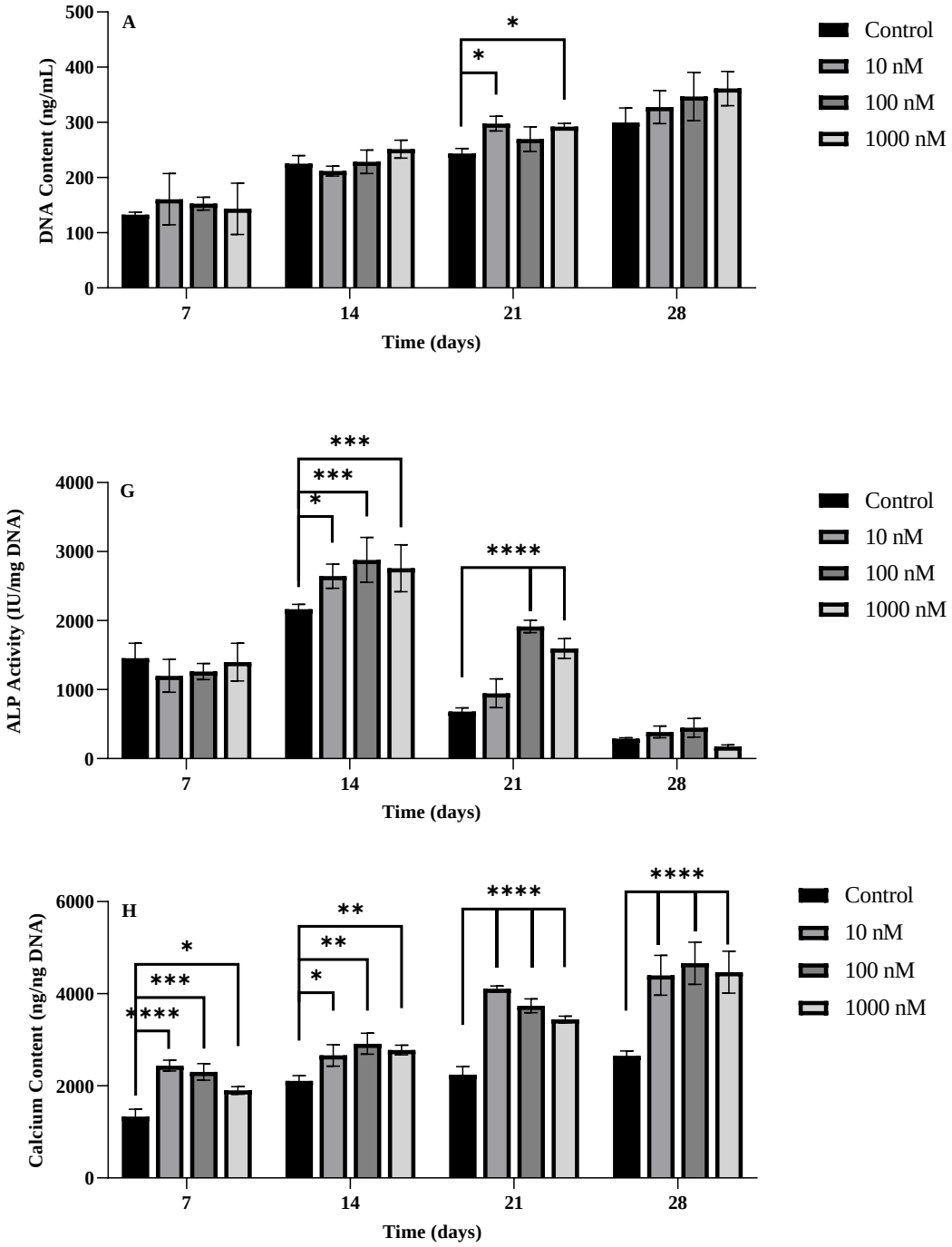

Figure 3.7. Effect of E-AG-02 on DNA amount (A), ALP activity (B) and calcium content (C) during osteogenic differentiation. Error bars mean ± standard deviation (n = 3) (significant differences were determined by one-way analysis of variance (one-way ANOVA, Sidak test), *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 compared to vehicle control, DMSO

3.3.3. Immunofluorescence

Before immunofluorescence staining, a control group of cells subjected to osteogenic differentiation was stained with Alizarin red (AR-S) to confirm that osteogenic differentiation had occurred, and fluorescence staining was started. The mineralized nodules initiated by osteogenic differentiation and formed as a result of alkaline phosphatase (ALP) enzyme activity are shown in Figure 3.8. Immunofluorescence staining was performed after it was shown that osteogenic differentiation was performed smoothly. Cells were subjected to osteogenic differentiation with molecule application, and in the third week of differentiation, cytoskeleton (actin, phalloidin), collagen type 1 (COL-1), osteopontin (OPN) and osteocalcin (OCN) were visualized under fluorescence microscope by immunofluorescence staining method. The obtained actin filament images are presented in Figure 3.9, COL-1 in Figure 3.10, OPN in Figure 3.11 and OCN in Figure 3.12.

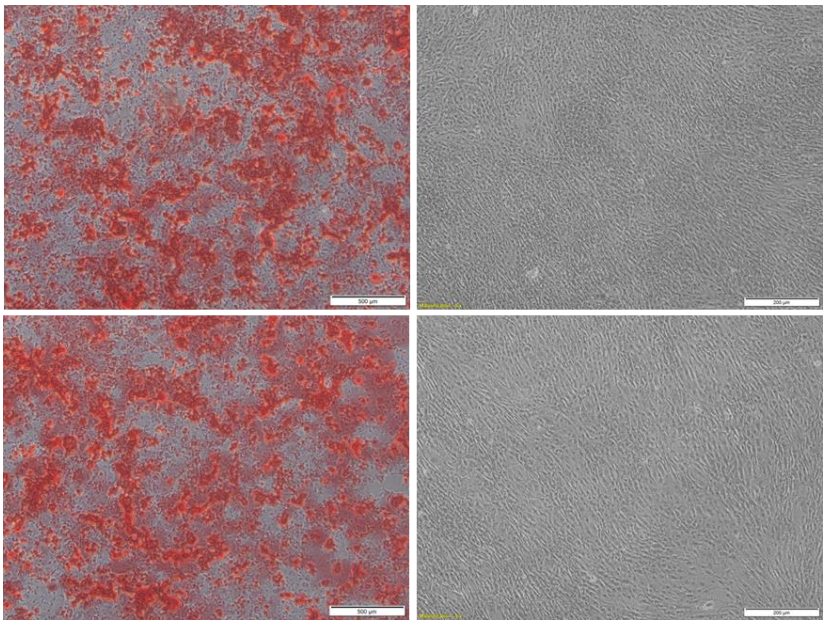

Figure 3.8. The images of ARS staining of differentiated (left) and non-differentiated (right) MSC at 21 days of incubation for immunofluorescence staining (objective 4x, scale bars left 500 µm and right 200 µm)

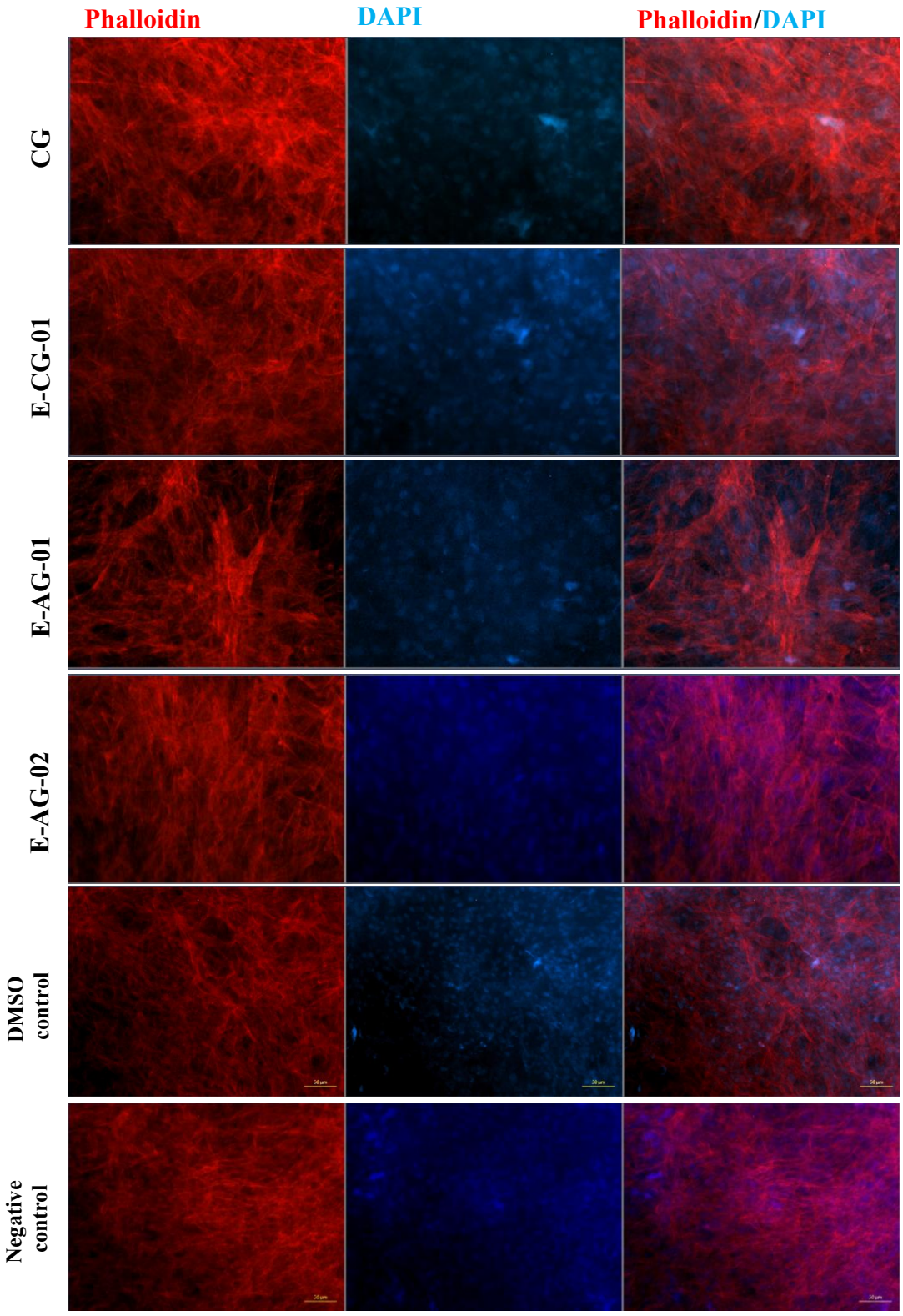

Figure 3.9. Cytoskeleton images from the third week of differentiation (100 µM treatment). Actin filaments were stained with phalloidin (red) and the cell nucleus with DAPI (blue) (20x objective, scale 20 µm)

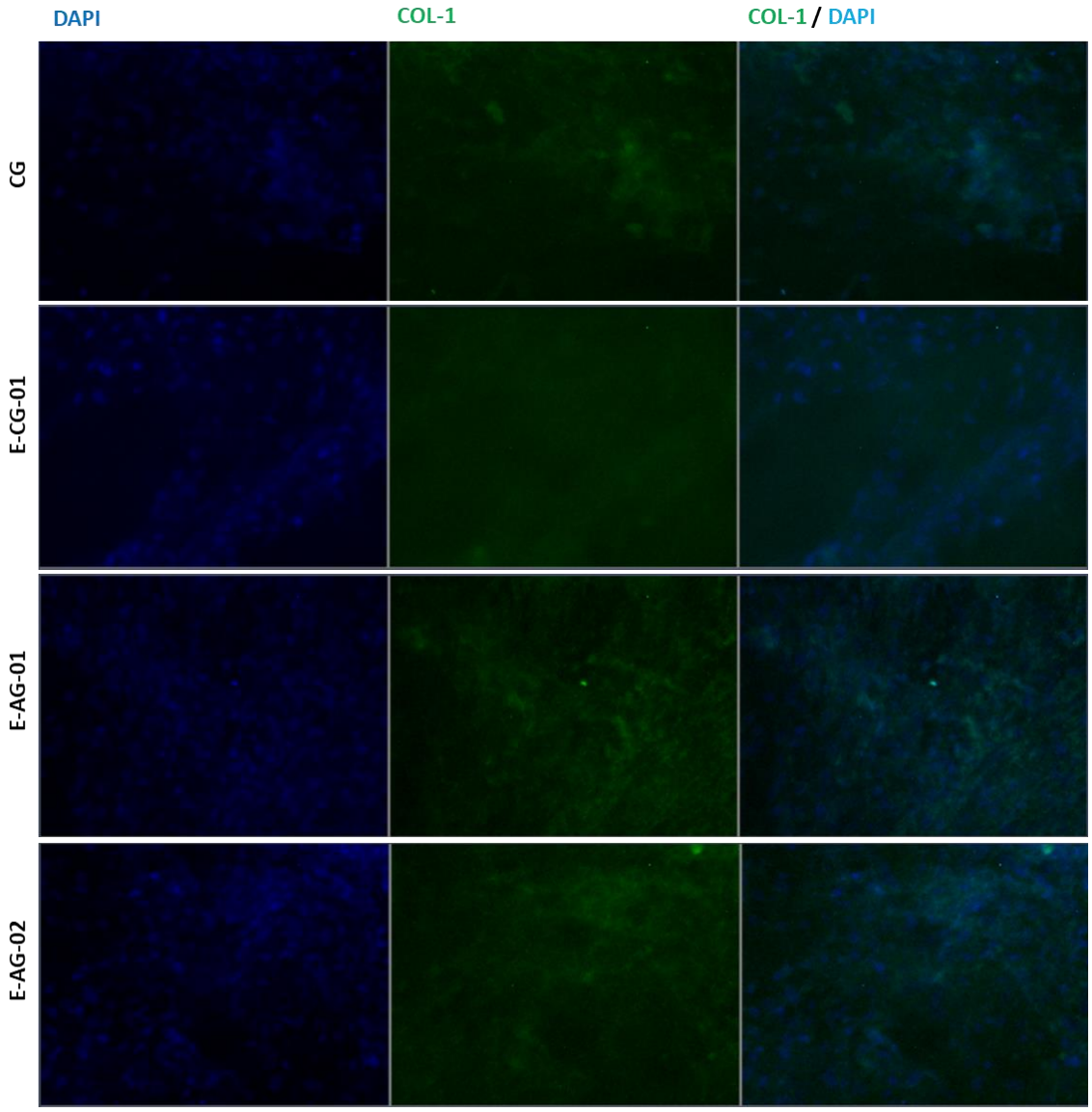

Figure 3.10. Immunofluorescence staining images from the third week of differentiation (100 µM treatment). COL-1 (green) and cell nuclei were stained with DAPI (blue) (20x objective, scale 50 µm)

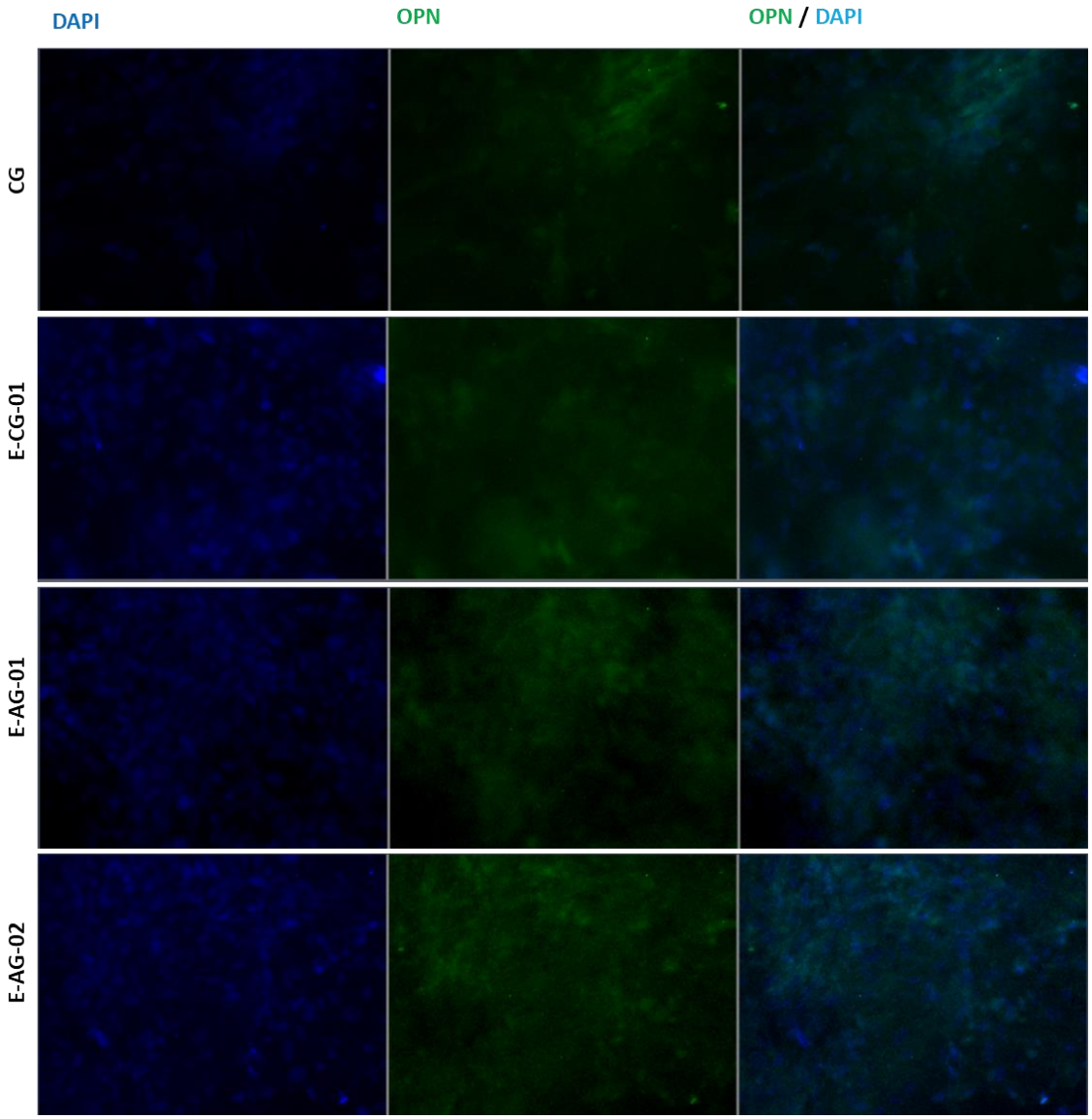

Figure 3.11. Immunofluorescence staining images from the third week of differentiation (100 µM treatment). OPN (green) and cell nuclei were stained with DAPI (blue) (20x objective, scale 50 µm)

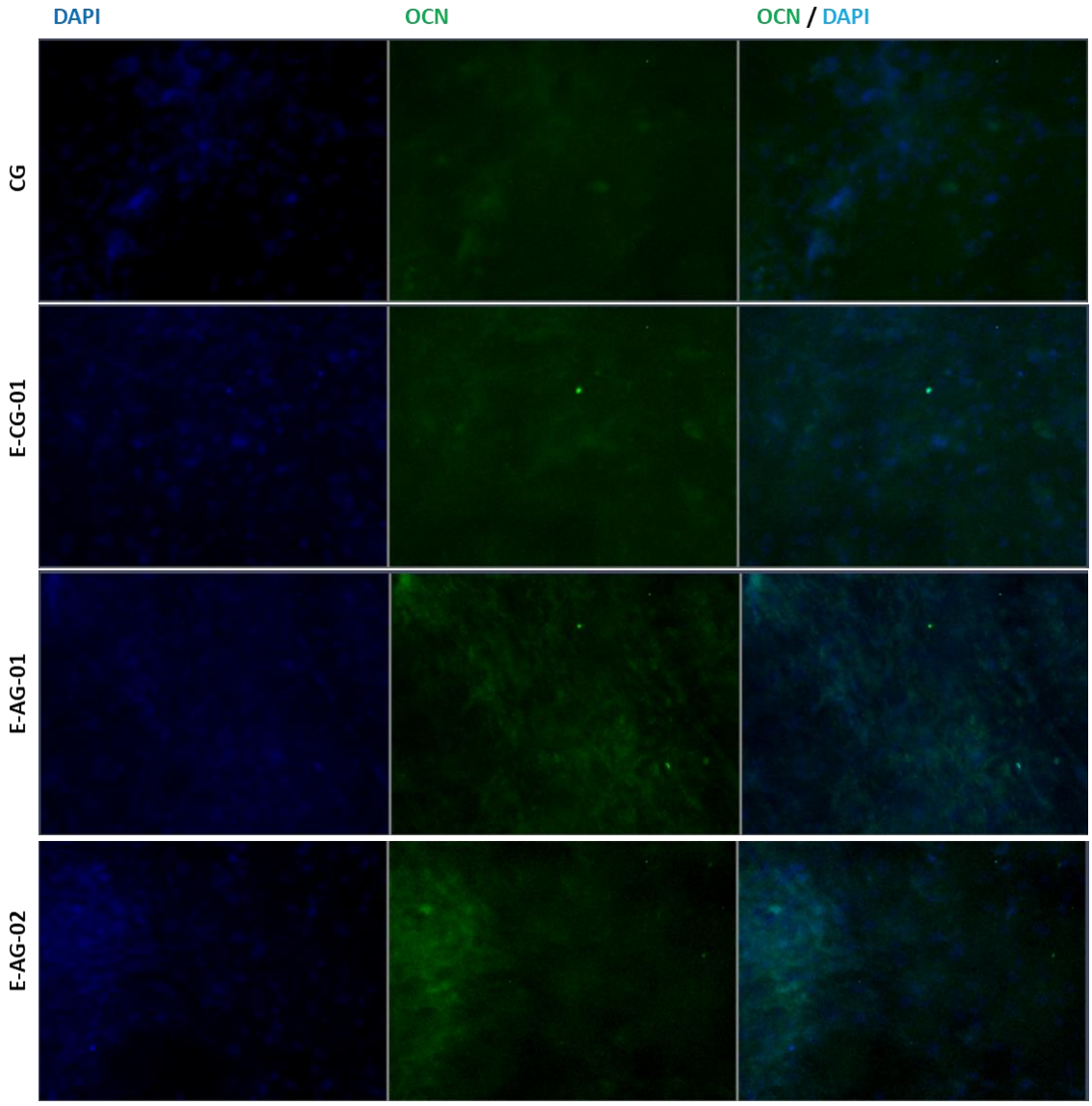

Figure 3.12. Immunofluorescence staining images from the third week of differentiation (100 µM treatment). OCN (green) and cell nuclei were stained with DAPI (blue) (20x objective, scale 50 µm)

3.3.4. Telomerase Activity

To examine the changes in telomerase activity during osteogenic differentiation, MHK (P8) was seeded in 12-well culture dishes, and after two days of incubation, differentiation was initiated by replacing the medium with an osteogenic differentiation medium. Simultaneously with the initiation of differentiation, the cells were treated with molecules, and the medium exchange and molecule treatment were repeated every three days. Cell pellets collected on days 7, 14 and 21 of differentiation were stored at -80°C for telomerase activity assay.

Telomerase activity was determined from the stored cell pellets using the Telotagg kit (Roche, 1203789001) according to the manufacturer's instructions and the change in activity was calculated and presented as a fold increase compared to the control group (Figure 3.13).

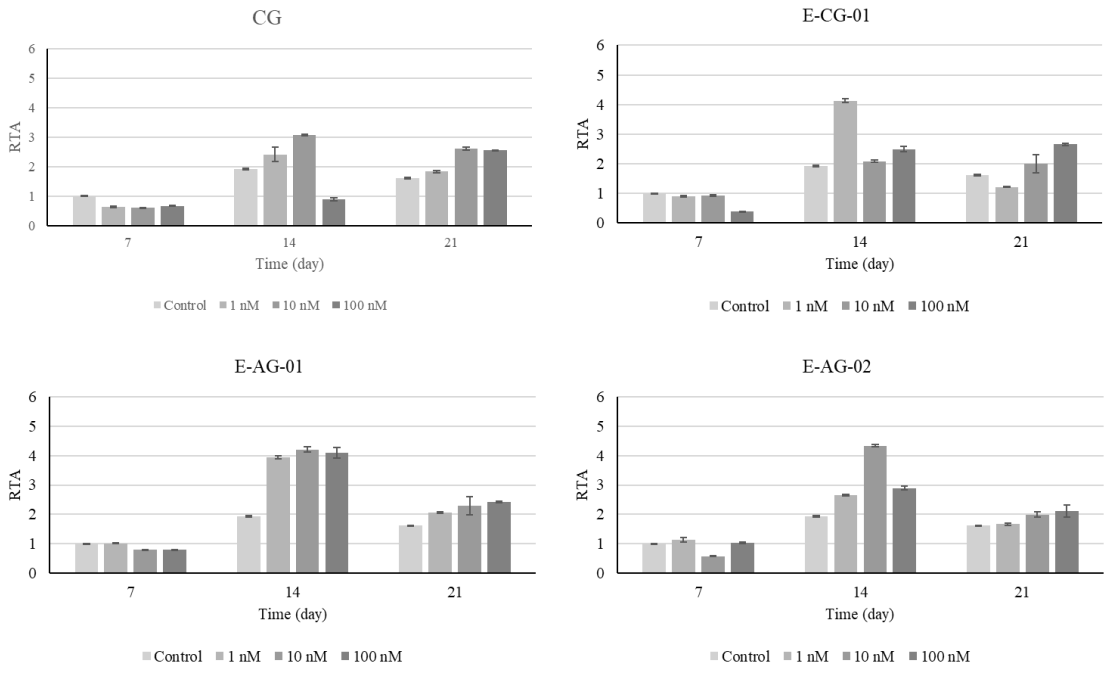

Figure 3.13. Effects of molecules on relative telomerase activity (RTA) during osteogenic differentiation. Error bars mean ± standard deviation (n = 3)

Table 3.2. Relative telomerase activity (RTA) during osteogenic differentiation

Molecules	**Conc., nM**	**Time, days**	**Activity/μg protein ml^{-1}**	**Activity/ng DNA ml^{-1}**
MSC at t_0	-	0	2.175 ± 0.116	2.089 ± 0.029
Control)	-	7	1.000 ± 0.011	1.000 ± 0.013
CG	1	7	0.680 ± 0.164	0.639 ± 0.025
CG	10	7	0.658 ± 0.009	0.619 ± 0.005
CG	100	7	0.735 ± 0.011	0.672 ± 0.009
E-CG-01	1	7	0.910 ± 0.035	0.802 ± 0.022
E-CG-01	10	7	1.003 ± 0.007	0.926 ± 0.034
E-CG-01	100	7	0.404 ± 0.013	0.389 ± 0.006
E-AG-01	1	7	1.015 ± 0.006	0.972 ± 0.009
E-AG-01	10	7	0.846 ± 0.036	0.804 ± 0.006
E-AG-01	100	7	0.874 ± 0.006	0.806 ± 0.005
E-AG-02	1	7	1.235 ± 0.012	1.135 ± 0.077
E-AG-02	10	7	0.636 ± 0.013	0.577 ± 0.011
E-AG-02	100	7	1.114 ± 0.017	1.028 ± 0.028
Control	-	14	2.133 ± 0.030	1.925 ± 0.025
CG	1	14	2.812 ± 0.065	2.424 ± 0.248
CG	10	14	3.468 ± 0.109	3.075 ± 0.020
CG	100	14	0.996 ± 0.043	0.897 ± 0.049
E-CG-01	1	14	4.127 ± 0.057	3.504 ± 0.062
E-CG-01	10	14	2.319 ± 0.099	2.087 ± 0.032
E-CG-01	100	14	2.764 ± 0.031	2.490 ± 0.091
E-AG-01	1	14	3.943 ± 0.114	3.290 ± 0.059
E-AG-01	10	14	4.778 ± 0.112	4.207 ± 0.085
E-AG-01	100	14	4.428 ± 0.113	4.102 ± 0.183
E-AG-02	1	14	3.101 ± 0.061	2.665 ± 0.024
E-AG-02	10	14	4.972 ± 0.223	4.335 ± 0.033
E-AG-02	100	14	3.105 ± 0.161	2.896 ± 0.064
Control	-	21	1.811 ± 0.034	1.615 ± 0.019
CG	1	21	2.016 ± 0.156	1.834 ± 0.031
CG	10	21	2.891 ± 0.046	2.612 ± 0.049
CG	100	21	3.006 ± 0.159	2.569 ± 0.006
E-CG-01	1	21	1.216 ± 0.032	1.154 ± 0.010
E-CG-01	10	21	2.299 ± 0.025	2.005 ± 0.303
E-CG-01	100	21	2.788 ± 0.242	2.653 ± 0.039
E-AG-01	1	21	2.052 ± 0.107	1.961 ± 0.025
E-AG-01	10	21	2.630 ± 0.037	2.299 ± 0.313
E-AG-01	100	21	2.697 ± 0.050	2.426 ± 0.030
E-AG-02	1	21	1.818 ± 0.050	1.667 ± 0.038
E-AG-02	10	21	2.084 ± 0.155	1.998 ± 0.084
E-AG-02	100	21	2.312 ± 0.065	2.114 ± 0.195

3.4. Discussion

It is noteworthy that there have been many studies in recent years to investigate/increase the application areas of telomerase activators, especially cycloastragenol (CG), in regenerative medicine. It is seen that these studies are primarily aimed at the treatment or prevention of diseases (Alzheimer's, Parkinson's, osteoarthritis, osteoporosis, etc.) that occur with aging and are associated with age-related telomere shortening. Szychlinska team carried out a critical analysis, pointing out that the loss of the ability to synthesize the ECM during long-term culture of the three-dimensional cartilage tissue created for the treatment of osteoarthritis (calcification), one of these diseases, is the biggest problem in stem cell treatments. In this study, the effect of CG was investigated, and it was reported that after a prolonged culture period (28 days), cells preserved their chondrocyte phenotypes and regenerative properties. In addition, mechanical properties were improved, and tissue integration was ameliorated [269-270]. In another study conducted by Yu *et al.*, it was shown that in both aging and chemical-induced bone loss models, CG application not only increases cell viability but also supports osteoblastic differentiation and suppresses osteoclast formation that leads to bone formation. The same study reported that CG increases osteo-activin secretion and mineralization, which resulted in improved biomechanical properties of bone tissue [271]. As a result of the studies conducted by Wu and his team examining the effect of CG on osteoporosis disease, it was reported that telomerase activation after CG administration showed a protective effect on glucocorticoid-induced osteogenic differentiation inhibition in rats [272].

In light of all this information, it is seen that telomerase activators have a high potential in the prevention/treatment of many diseases that occur due to aging, as they provide telomere homeostasis and delay aging. Apart from using naturally derived telomerase activators as food supplements, their high potential for clinical applications, primarily stem cell therapies, should be addressed.

In studies conducted to increase telomerase activity, it is concluded that in addition to increasing telomere length, telomerase also positively affects and accelerates stem cell differentiation. In the literature, it has been shown that telomerase activation

accelerates osteogenic differentiation both *in vitro* and in vivo by causing an increase in the expression of genes directly related to bone development, such as osterix and osteocalcin, accelerates the process by increasing calcium accumulation and provides 6 times more bone formation than the control group in the 2-6 week period after transplantation [245, 262]. In another study conducted by Yu and colleagues, it was shown that CG administration supported bone formation by suppressing osteoclast formation while promoting osteoblastic differentiation in addition to increasing cell viability in both aging-induced and chemical stimuli-induced bone loss models. In the same study, it was reported that CG increased osteoactivin secretion and mineralization and also improved the biomechanical properties of the formed bone tissue [273]. Similarly, our results obtained by alizarin staining showed that the molecules increased mineralization to much higher levels compared to the control group from the second week of osteogenic differentiation.

In a study conducted by Szychlinska and colleagues, the effect of CG on stem cell morphology was examined, and it was reported that the cells retained stable chondrocyte phenotypes and regenerative properties as a result of chondrogenic differentiation at the end of a long culture period (28 days), in addition to improved mechanical properties and better tissue integration [269-270]. As a result of the studies conducted by Wu and colleagues examining the effect of CG on osteoporosis disease, it was reported that rats with telomerase activation following CG administration showed a protective effect against glucocorticoid-induced osteogenic differentiation inhibition [272]. As a result of the osteogenic differentiation studies conducted within the scope of the project, it was observed that molecule administration increased ALP activity in a statistically significant manner. It was determined that the mineralization expected to occur in the extracellular matrix due to increased ALP activity also increased with molecule application as expected.

As a result of the analyses, the relative telomerase activity was calculated as 5.34 in the MSCs (P8) obtained before osteogenic differentiation was initiated (t=0). In the first week of differentiation, this value decreased to 2.39 (Table 3.1). In other words, at the beginning of differentiation, MSCs lost almost half of their telomerase activity. It has been reported that telomerase activity is effective in maintaining self-renewal and pluripotency in stem cells but gradually decreases with differentiation [274-275]. In our studies, in the first week of differentiation, E-AG-01 caused a 1.2 and 1.1-fold increase

in telomerase activity at 1 nM and 100 nM concentrations compared to the control group, respectively. In contrast, other molecules did not cause any increase in telomerase activity. In this respect, the fact that telomerase activity remained at lower levels in the first week compared to the control group leads us to conclude that the molecules may have accelerated differentiation. In the second week of differentiation, our origin molecule, CG, increased telomerase activity by 2.4 and 3-fold at concentrations of 1 and 10 nM, respectively; E-CG-01 increased telomerase activity by 3. 5, 2 and 2.5-fold; E-AG-01, 3.29, 4.2 and 4.1-fold at 1, 10 and 100 nM concentrations, respectively; and E-AG-02, 2.6, 4.3 and 2.9-fold at 1, 10 and 100 nM concentrations, respectively. In the third week of differentiation, all molecules increased activity by 1.1 to 2.6-fold over the applied concentration range (Figure 3.13). While a gradual decrease in telomerase activity was expected in the later days of differentiation, fluctuations in telomerase activity were observed in both the control group and the other experimental groups. When the literature was examined, it was found that there were similar increases and decreases in telomerase activity in a study conducted by Zhang et al. In this study, it was reported that telomerase activity increased on the 14th day of differentiation and again in the following days in parallel with the results we obtained within the scope of this project [276]. As a result of the studies conducted within the scope of the project, it was concluded that the molecules improve osteogenic differentiation by increasing ALP activity, as mentioned before. Similarly, the observation of a high increase in telomerase activity, especially on day 14 with molecule administration, indicates that there may be a positive interaction between telomerase and ALP, which peaks on day 14. As a matter of fact, studies in telomerase overexpressing mice have shown that many components in the insulin-like growth factor (IGF) pathway are synthesized in high amounts in these mice, and IGF-induced AKT phosphorylation and ALP activity are significantly increased [249, 277]. Although the effect of the molecules used in the project on osteogenic differentiation has been partially elucidated, further studies are required to fully elucidate the mechanism of action of the molecules at the molecular level.

It was observed that all molecules used in the applied concentration range caused an increase in the relevant mRNA levels compared to the controls; ALP expression reached the maximum level on day 14 in accordance with the ALP activity result, as well as the expression of structural proteins at the mRNA level continued during

differentiation. On the other hand, structural proteins were visually examined by immunofluorescent staining and the results obtained as a result of differentiation studies were visually supported. When all the results obtained are evaluated together, it can be concluded that all four molecules used improve osteogenic differentiation at the applied concentrations. When all the results obtained are evaluated together, considering the effects of the molecules, especially on ALP activity and mineralization, it can be concluded that E-CG-01 and E-AG-01 molecules support osteogenic differentiation at lower concentrations (100 nM) compared to our starting molecule CG.

3.5. Conclusion

Stem cell therapy is promising for the elimination/prevention of degenerative bone diseases or accidental bone tissue damage that reduces quality of life. However, in most applications that require the cultivation of stem cells in vitro, stem cells experience a loss of chromosomal stability and lose their proliferation and differentiation potential [259]. At this point, the use of telomerase activator small molecules has come to the fore in recent years for the treatment of degenerative diseases associated with telomere shortening or telomere dysfunction, especially aging-related degenerative diseases (e.g., osteoporosis) [200, 278]. Previous studies emphasized that the accumulation of senescent cells in tissues with age due to the disruption in senescent cell clearance mechanisms causes degenerative diseases, and degeneration spreads progressively to surrounding tissue due to the senescence-associated secretory phenotype factors. Therefore, maintaining TL is essential for cellular integrity, and a specific enzyme called telomerase is primarily responsible for providing TL. Therefore, possible therapeutic effects of telomerase reactivation have been evaluated in different cellular backgrounds to cure various diseases that are found to be linked to short telomeres or telomere deficiencies. The treatment approaches providing telomere homeostasis, and natural small compounds are some of the available approaches to increase telomerase activity, which might be effective candidates for treating and preventing degenerative diseases.

CHAPTER 4

EVALUATION OF THE EFFECT OF CYCLOASTRAGENOL ON THE PRODUCTIVITY OF MONOCLONAL ANTIBODY PRODUCING CHINESE HAMSTER OVARY CELLS

4.1. Background

Immortalized Chinese Hamster ovary cell (CHO) lineages are the most preferred mammalian host cells to produce biopharmaceuticals owing to their ability to perform post-translational modifications like those in human cells. Indeed, CHOs can be adapted to serum-free and suspension culture conditions, which are more effective and advantageous for large-scale production processes. Suspension culture is required for large-scale commercial bioreactor culture, and serum-free conditions guaranties to avoid of serum-originated infectious agents [279].

Until today, significant improvements have been made in the production, characterization, and stabilization of mAbs after the successful production of the first therapeutic protein, Activase, by the subclone CHO-DG44 isolated in 1983 [149] in terms of genetic engineering approaches for new cell lines, effective expression systems, decreased immunogenicity, optimized bioprocess etc. [279]. As a result of these improvements, production techniques have evolved over the past few decades from the use of mouse ascites fluid to large-scale manufacturing. Today, the majority of large-scale applications are developed in disposable stirred tank bioreactors, maintaining a sterile environment for both manufacturing and downstream processing. However, current manufacturing and purification processes have limitations and thus need to be improved by decreasing overall production costs and increasing productivity [152-154].

One of the drawbacks of mAb production is the chromosomal instability of CHOs. Thus, they undergo genomic instability and senescence due to their chromosomal instability, which leads to shortened lifespan and decreased production in prolonged culture periods [155, 280]. Normal Chinese hamster cells have telomere repeats sequences (TTAGGG) at the end of chromosomes and pericentromeric regions [281]. The study by Slijepcevic and Bryant exerted that the telomeric sequences are shorter than 1 kb in chromosomes of immortal CHO lines, including CHO-K1 subtypes CHO-K1a, CHO-K1b, CHO-K1c, and also human-hamster hybrid cell line R342A4 carrying human chromosome 10 [156].

Therefore, telomerase activation may have some advantages in mAb production by CHO-K1 line that has extremely short telomeres [156, 282], in terms of increased productivity by providing chromosomal stability which may lead to improved cell growth and physiology. Indeed, it has been demonstrated that hTERT over-expression in CHO cells increased cell proliferation and protected cells from stress-induced apoptosis during recombinant protein production, which resulted in higher productivity [283-284].

The expected benefits of telomerase activation in mAb productions primarily include (i) enhancing cell line development of robust CHO cell lines by enabling the selection of high-producing clones that maintain their productivity over extended culture periods [280], (ii) maintaining telomere length, which is crucial for genomic stability, may reduce the rate of mutations and chromosomal aberrations that might occur during the extensive cell culture periods required for biopharmaceuticals, thereby maintaining the integrity of the generated cell line [285], (iii) extending the lifespan of CHOs by delaying senescence and allowing for longer production runs, (iv) reducing the frequency of developing new production cell lines [286-287], and (v) providing a healthier state of the cells can support optimal metabolic and biosynthetic activities necessary for the efficient production of monoclonal antibodies [288].

On the other hand, various media compositions, including some of the natural small molecules, have been tested in order to protect mammalian cells against various stress factors during cultivation and recombinant protein production and have been shown to exert higher protein titer in CHOs. In general, it was observed that the studies used natural small molecules owing to their antioxidant properties. For example, polyphenolic flavonoids (e.g., catechin, resveratrol, and rosmarinic acid) with high antioxidant

properties were used as additives in CHO media composition [289]. They resulted in increased viability and productivity of cells without causing any change in product quality and functionality [290-291]. For example, resveratrol was patented with many other polyphenolic compounds (catechin, chlorogenic acid, pelargonidin, quercetin, rosmarinic acid and silibinin) in 2016 as a successful additive for recombinant protein production in CHO cells (Tian 2016, US20230002800A1) [290]. However, none of these studies examines whether this enhancement is related to telomerase activation or not. Thus, we aimed to use cycloastragenol (CG) as an additive in mAb production for the first time since CG is a natural telomerase activator compound, exerting various health benefits with its tremendous protective and therapeutic properties.

4.2. Materials and Methods

4.2.1. Cell line and Culture

Humanized recombinant mAb (HUMIRA® biosimilar) producing stable CHO-K1 cells with a glutamine synthetase gene expression system was purchased from Creative Dynamic Inc., USA. CHO-K1 cells were grown in T-75 tissue culture flasks with chemically defined CD Opti-CHO medium (Gibco, Invitrogen, ABD) in a humidified incubator at 37°C with %5 CO_2.

4.2.2. Growth Kinetics of CHO-K1

CHO-K1 cells were seeded ($3x10^5$ cells/ml) in 50ml Erlenmeyer Flasks with 10 ml of the working volume and cultured for 10 days at 37ºC for 24 hours in the 5% CO_2 atmosphere with 150 rpm shaking speed. After constructing the growth curve, the

exponential growth phase and kinetic parameters were calculated by using the standard curve obtained from the exponential growth phase.

Cell Proliferation Reagent WST-1 (Roche, Cat# 11644807001) was used to determine the cell viability of the cells cultured in 96-well plates at different time points. 100 µl/well (1:10 final dilution). The assay was performed according to the manufacturer's instructions. Luminescence was read using a multi-scan plate reader (Fisher, Waltham, MA).

4.2.3. Monoclonal Antibody Production

For monoclonal antibody production, CHO-K1 cells were grown in OPTİ-CHO medium (GİBCO) at $3x10^5$ cell/ml starting concentration. Both cell growth and mAb production were examined during 11 days of cultivation.

4.2.4. The enzyme-linked immunosorbent assay (ELISA)

Wells of 96-well plates (Thermo Maxisorp NC-442404) were coated with Fc-specific anti-human IgG capture antibody (Sigma, I2136), then blocked with a 2% solution of bovine serum albumin (BSA, Sigma, A2153) and washed between steps with wash buffer (1x DPBS supplemented with 0.5% Tween 20). Assay buffer (1x DPBS supplemented with 1% BSA, 0.5% Tween 20) was used for diluting the samples. Captured protein from samples was detected with Fc-specific anti-human IgG–peroxidase detection antibody (Sigma, A0170), and TMB (3,3′,5,5′-Tetramethylbenzidine, Sigma T0440) was used as a substrate. Stopping the reaction with 2N sulfuric acid solution resulted in the formation of a yellow reaction product. Absorbance could then be measured with Tecan Ultra 384 Multimode Microplate Reader at 450 nm. Product concentration in each sample was calculated based on the standard curve generated with the original assayed protein or human IgG (Sigma, I2511).

Phosphate Buffer Saline (PBS), 1 L (0.01 M, pH 7.2-7.4, w/o Ca^{2+} - Mg^{2+}) 8.5 g NaCl (Merck, Germany), 1.44 g Na_2HPO_4 (Merck, Germany), 0.2 g K_2PO_4 (Merck, Germany) and 0.2 g KCl (Merck, Germany). **Carbonate Coating Buffer, 1 L** (pH 9.5) 3.56 g Na_2CO_3 (Merck, Germany) and 8.4 g $NaHCO_3$ (Merck, Germany) **Wash Buffer (T-PBS)** 0.05% Tween-20 (Merck, Germany) in PBS (1X) **Blocking Solution** 10% FBS (Gibco, ABD) or 1% BSA (Sigma, ABD) in T-PBS (1X). 0.5% (w/v) BSA (Sigma-Aldrich A9647-100G, Germany) in T-PBS **Conjugate Solution, 0.01% HRP,** conjugated anti-human IgG (Thermo, ABD) in PBS (1X). **Stop Solution** 3 M H_2SO_4 (97-98%) (Merck, Germany)

4.2.5. Statistical Analysis

All experiments were conducted at least in triplicates, and GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) was used for statistical analyses. Data were presented as mean ± standard deviation. Statistical significance was determined by Student's t-test (two-tail) or two-way analysis of variance (ANOVA) with Sidak's post hoc correction through GraphPad Prism version 6.0 (GraphPad Software), and $p < 0.05$ was considered statistically significant. Significance on the graphs was indicated with *, where $p < 0.05$; **, where $p < 0.01$; ***, where $p < 0.001$; ****, where $p < 0.0001$. Non-significant correlations ($p > 0.05$) were not indicated on the graphs.

4.3. Results

4.3.1. Growth Kinetics of CHO-K1

In static culture experiments, it was observed that CHO-K1 cells tend to attach to the tissue culture plate surface. For equal sampling every other day, submerged cultivation

in Erlenmeyer flasks was used for growth kinetics studies. Based on the results, specific growth rate (μ) and doubling time (td) were calculated via the data from the exponential growth phase (Figure 4.1). Thus, μ and td were calculated as 0.63±0.14 d^{-1} and 1.15±0.14 d^{-1}, respectively.

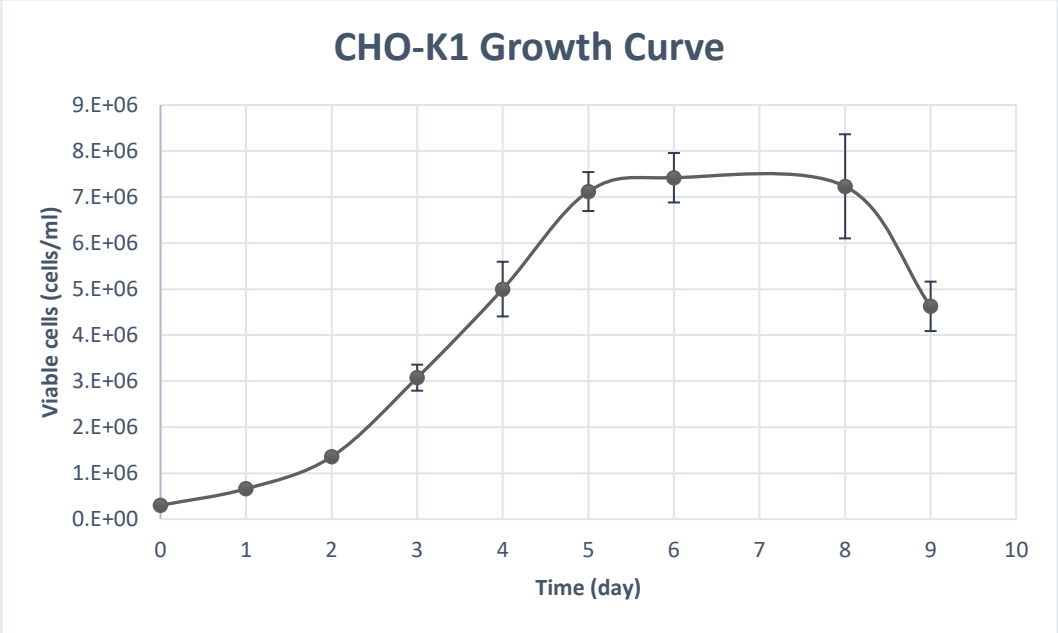

Figure 4.1. The growth curve of CHO-K1 cells

4.3.2. mAb Production

Monoclonal antibody production during 11 days of static cultivation of CHO-K1 cells was determined via the indirect ELISA method, and the results are given in Figure 4.2. The results indicate that mAb production increased at 5 days of incubation, where cells go through the stationary phase and continue to increase exponentially until day 7, then reach the maximum level.

Batch and fed-batch productions were constructed for 7 days of production in order to determine the effects of CG on mAb production. For this purpose, CG (0.5 to 5000 nM) was applied at the beginning of the production and added to the fed-batch

operation every other day, together with a 10% culture medium. The samples were collected and subtracted to ELISA for the quantification of the produced mAb amount for each condition (Figure 4.3 - 4.4). There are no significant differences were observed between batch and fed-batch operation modes (Figure 4.5).

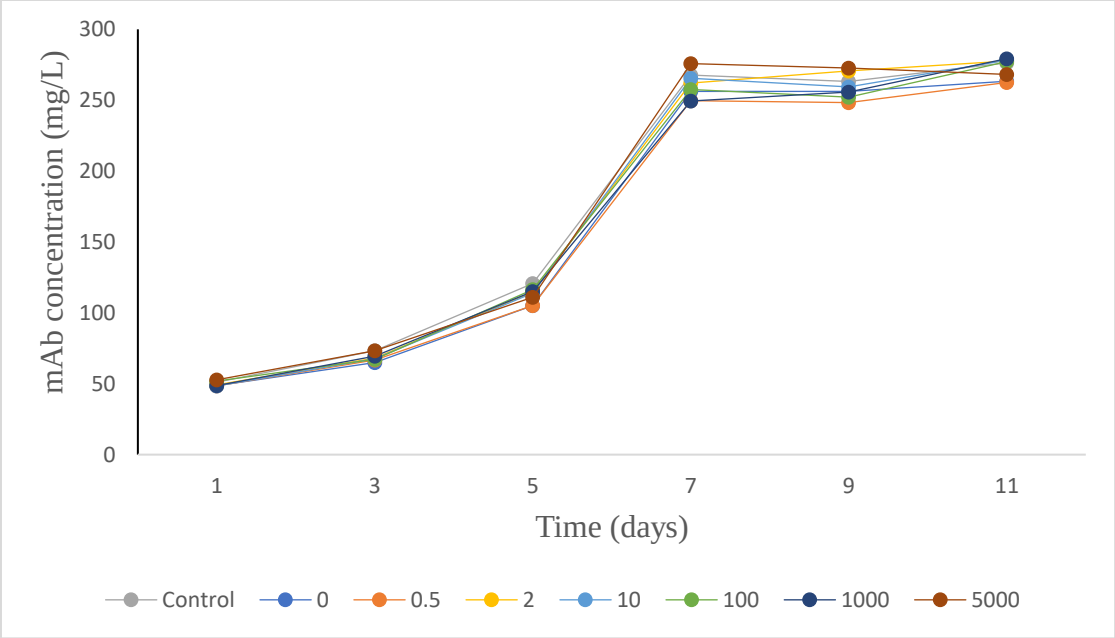

Figure 4.2. mAb production of CHO-K1 cells during batch cultivation with different concentrations of CG (0.5 to 5000 nM)

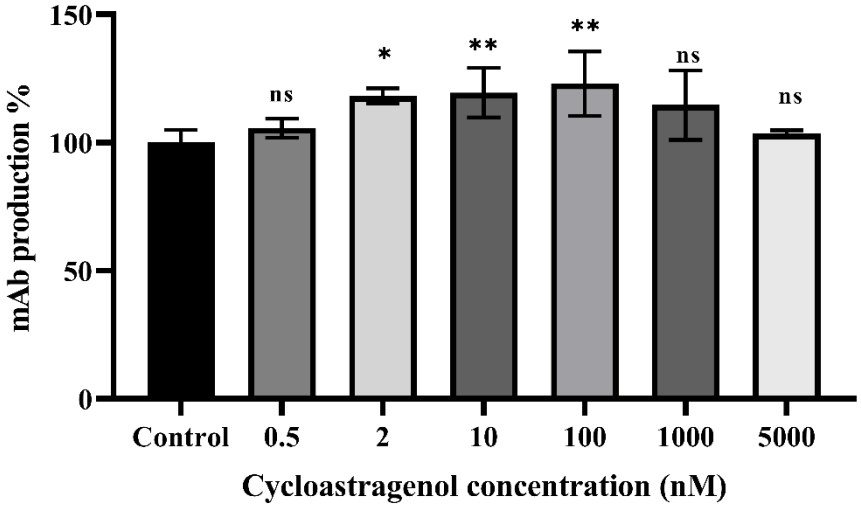

Figure 4.3. Production Batch operation: CG applied once simultaneously with cell seeding. Data represented by mean ± standard deviation (n = 3)

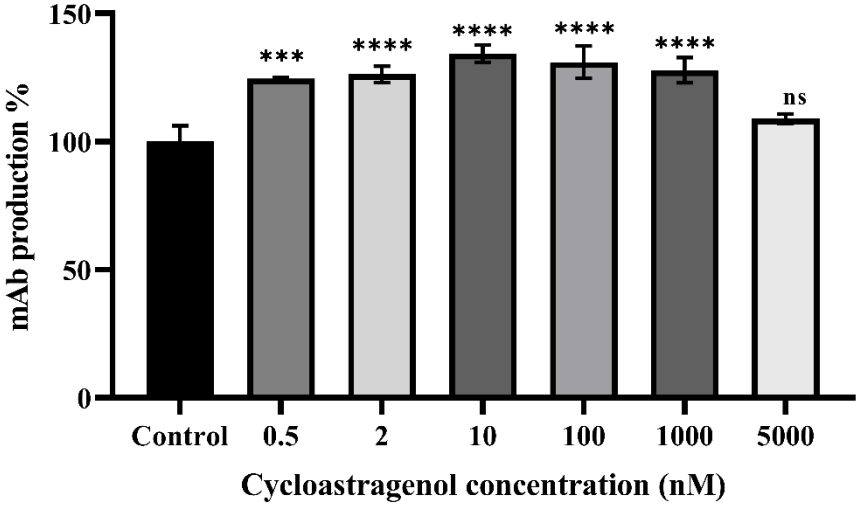

Figure 4.4. Production Fed-Batch operation: CG applied every other day with fresh medium. Data represented by mean ± standard deviation (n = 3)

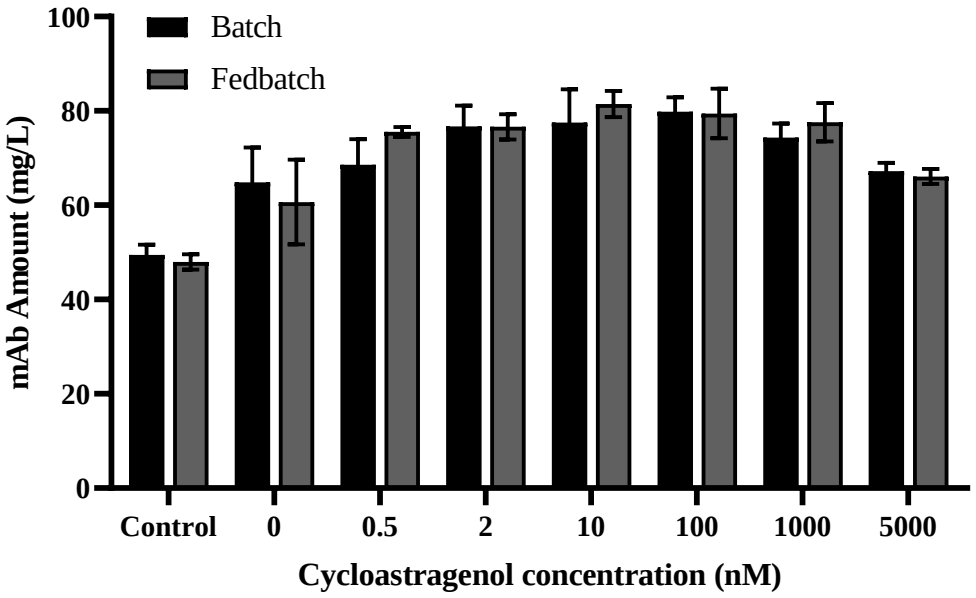

Figure 4.5. The comparison of mAb production in batch and fed-batch operation modes. Data represented by mean ± standard deviation (n = 3)

Based on the results from batch and fed-batch operation studies, we decided to continue mAb production in batch operation; in other words, static culture cultivation was used in further investigations. For this purpose, CHO cells were seeded in 24-well plates in 1 ml medium supplemented with CG (the concentration range between 1 to 1000 nM) and incubated for 5 days. However, the mAb production yields were not affected by either DMSO or CG exposure in terms of productivity since a significant effect was not observed in both calculations as mAb amount in percentages or specific productivity (pg mAb/cell.day) compared to control groups (Figure 4.6).

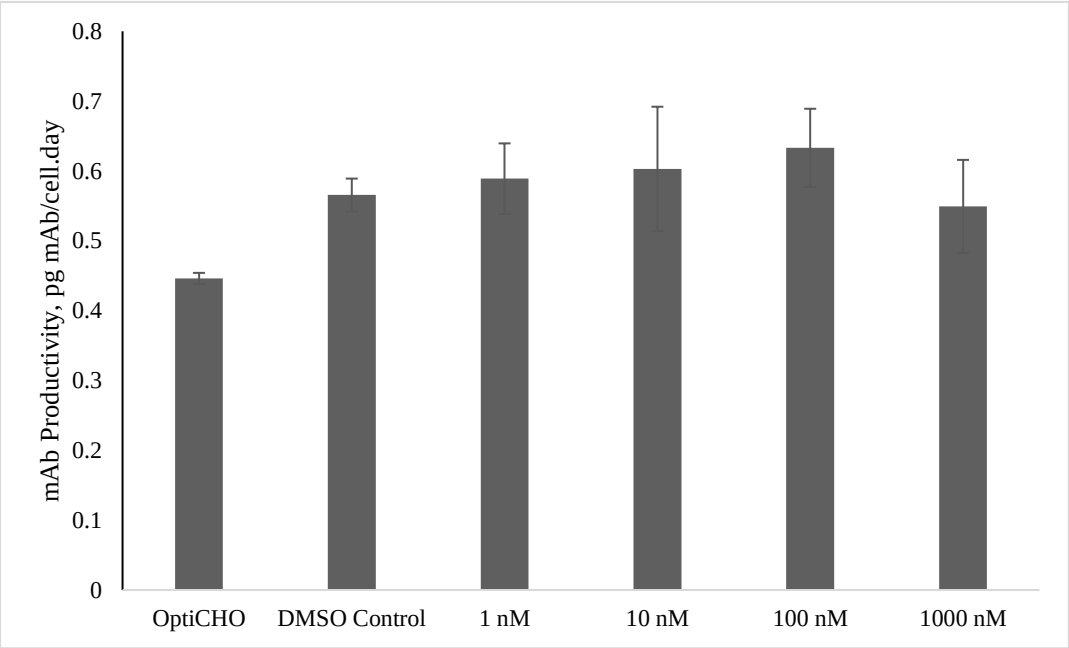

Figure 4.6. The productivity of mAb production. Data represented by mean ± standard deviation (n = 3)

4.4. Discussion

Recombinant proteins are widely used for therapeutic and diagnostic purposes. Despite the fact that there are several alternatives, both in vitro and in vivo production methods, including using *E. coli* as host cells to produce recombinant protein fragments and plant cells [292], the CHO lineages are still the most common mammalian cells for the large-scale production of high-valued biopharmaceuticals. In 2021, excepting drug-conjugated or multi-specific antibodies, a total of 93 therapeutic mAbs among 1437 candidates have been approved by the FDA 296; a steady rise continues in the number of new antibody product companies. Based on successful trends in previous years, it can be concluded that mAbs are more successful in achieving FDA approval compared to the other pharmaceutical products [293]. The mAb production scale mainly depends on the area that will be used; while micrograms to grams would be enough for experimental or diagnostic purposes, grams to kilogram production capacities are needed for approved

and licensed manufacturing. As a result of their success and high market share, mAb production techniques have improved over the past few decades. However, current manufacturing and purification processes have limitations and thus need to be enhanced by decreasing overall production costs and increasing productivity [294].

As mentioned previously, CHO cells need to be improved in terms of genomic stability during long-term production without losing their production capacity. Thus, telomerase activation may help to avoid apoptosis of the cells by protecting their chromosomes from further deterioration or fusion to neighbor chromosomes since they already have extremely short telomeres [156, 282]. It has been demonstrated that hTERT over-expression in CHO cells resulted in improved cell growth and physiology by protecting cells from stress-induced apoptosis during recombinant protein production [283-284]. The study by Crea *et al*. revealed evidence that telomerase has a relationship with cell attachment pathways since telomerase caused a 10-fold increase in the expression of collagen type III and V in CHO cells, which leads to reduced serum dependency of the cells. Also, they showed that telomerase activation caused decreased apoptosis [283].

On the other hand, another important frequent problem during mAb production is the accumulation of toxic and inhibitory metabolites such as ROS, lactate and ammonia, which are negatively affecting cell growth and production. To overcome this problem, several media combinations and supplements have been developed, including the ones taking advantage of the antioxidant properties of some natural small molecules, which are mostly phenolic compounds such as catechin, rosmarinic acid and resveratrol [291, 295-296].

From this point of view, in the scope of this thesis, we primarily suggested that cycloastragenol (CG) might be a promising supplement as a telomerase activator exerting antioxidant properties (see section 1.4.3) that could improve the mAb production via protecting both cells and the product from environmental stress, toxic byproducts during the production process. For this purpose, time course mAb production via CHO-K1 was constructed under static cultures in 24-well plates and cells treated with different concentrations of CG. However, there was a significant increase in mAb amounts in terms of percentages compared to the control group. Unfortunately, a significant effect neither on cell growth/viability nor specific mAb productivity during 11 days of cultivation was observed in our studies.

The data obtained from our studies, if CG increased telomerase activity in CHO-K1 cells, we could suggest that exogenous hTERT is advantageous in mAb production of CHO cell's endogenous telomerase activity. Based on the results from the study conducted by Akiyama *et al*., which revealed exogenous hTERT over-expression together with minimal endogenous activity results in robust chromosomal stability via stabilizing DNA break repair in damaged chromosomes [297].

Other possible problems could be raised from the disturbed balance between heavy and light chain amounts, over-expressed protein or over-growth cell toxicity. It is important to protect this balance during mAb production because such a condition can be toxic and might cause the production of dysfunctional chains [298]. If we had such a misbalance of the chains, this may have led to misinterpretation of the ELISA results.

Based on our latest literature review, as mentioned previously, polyphenolic flavonoids (e.g., catechin, resveratrol, and rosmarinic acid) with high antioxidant properties were used as additives in CHO media composition [289] and resulted in increased viability and productivity of cells without causing any change in product quality and functionality [290-291]. In 2016, resveratrol was patented with many other polyphenolic compounds (catechin, chlorogenic acid, pelargonidin, quercetin, rosmarinic acid and silibinin) as a successful additive for recombinant protein production in CHO cells (Tian 2016, US20230002800A1). Other studies showed that rosmarinic acid [291] and resveratrol [299] addition to the culture media during mAb production with CHOs prevented cells from apoptosis and resulted in approximately 2-fold and 1.44-fold increase in specific mAb productivities, respectively. In these studies, these compounds caused cells to get stuck in the S and G2/M phases, similar to the results in the case of increased telomerase activity [286]. Still, none of these research evaluated the change in the telomerase activity during cultivation.

The titer of the production is the overriding cost driver factor for mAb production 304. However, the usage of CG as an additive to culture media under our experimental conditions did not result in an increased titer. Therefore, further investigations are needed to clarify whether CG does not exert positive or negative effects on mAb production or not.

4.5. Conclusion

Antibodies, antibody fragments and antibody-conjugated products will be continuously developed for diagnostic or therapeutic purposes, many of them expected to move through and gain FDA approval, and a few of those will be manufactured as licensed high-valued biopharmaceuticals.

In the scope of this thesis, we made basic and cost-effective trials to evaluate the effects of CG in CHOs in terms of cell growth/viability and produced mAb amount. The wide range of therapeutic potential of CG is related to several biological interactions through numerous signaling pathways that need to be further investigated in CHO cells in order to enlighten possible effects on CHO cell metabolism and mAb production. We believe that CG should be considered and further studied for use in recombinant protein production, since our experimental design and analyzing methods were not enough to make a certain conclusion at this point.

Indeed, it is well known that small molecules exert their effects in a dose-dependent manner and can also be completely reversed due to small differences in the administration dose. Thus, the molecule-specific feeding regime should be taken into account when designing media additives, such as small molecules, in order to determine the production strategies. In addition to feeding strategies, the accumulation of proteolytic enzymes and other protein-degrading by-products or any other components that could affect post-translational modification of the product in the broth must be analyzed. Furthermore, cell debris removal methods should be designed to avoid additional host cell components.

CHAPTER 5

CONCLUDING REMARKS

In the last two decades, researchers have been focused on the relationship between TL and age-related diseases to develop novel prevention and treatment strategies. Based on the latest literature search, one can state that the growing evidence points out that short TL not only increases the risk of diseases but also accelerates their progression, especially in age-related diseases. On the other hand, in some cases, short TL can be observed as only a symptom because of disease pathology. Nevertheless, considering the relation between telomerase and complex cellular pathways, it is reasonable to expect telomerase activators to have enhanced survival effects on the course of the disease. Therefore, telomerase activators are substantial to cope with age-related diseases, which are a big burden on the economy and social life. In this regard, natural products have gathered attention as a source of novel telomerase activators. As a result of intensive studies, several natural products have been discovered to be potent telomerase activators, and many of them have already been known to be effective against age-related diseases. Although studies on age-related diseases investigate the effects of these molecules, we realized that their properties towards telomerase activation were ignored most of the time. In the thesis, we aimed to report the benefits of natural telomerase activators on aging-associated degenerative diseases, thus drawing attention to the important role of telomere/telomerase activator small molecules for further studies.

In this doctoral thesis, the main goal was to investigate the efficacy of the usage of the telomerase activator small molecules in stem cell research in terms of their biological characteristics and osteogenic differentiation potential. Furthermore, the possible usage of these molecules in mAb production via CHO-K1 cells was evaluated. In the first part of the study, the effects on the biological characteristics such as cell viability, proliferation/migration and senescence condition were determined for the first time. Additionally, the osteogenic differentiation capacity of MSCs was identified and

compared at high passage numbers and under serial sub-cultivation with continuous molecule administration. In the second part of the study, the efficacy of these selected molecules on osteogenic differentiation was evaluated for the first time, and the relation between osteogenic markers and telomerase activity was enlightened. In the third part of the study, the benefits of the molecules in mAb production via CHO-K1 cells were studied for the first time. Based on the outcomes of this thesis, novel telomerase activators deriving from natural resources of our country have significant potential in stem cell research, thus regenerative medicine, since promising results were obtained for the clinical use of these novel molecules. Our data also suggest that molecules simultaneously promote osteogenic differentiation and telomerase activation. In order to confirm these findings, parallel studies with higher budgets, wider dose ranges in mono/co-culture conditions and *in vivo* studies would be appropriate for further studies.

Lastly, within the scope of this thesis, strong evidence has shown that telomerase activators are useful agents for maintaining the self-renewal and differentiation capacity of stem cells during *in vitro* expansion. In the near future, it seems rational to utilize telomerase activators as adjuvants for stem cell transplantation in age-related diseases.

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