Tampere University

Roope Ohlsbom

# DIFFERENTIATING OSTEOCYTES FROM BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN COLLAGEN HYDROGEL

Faculty of Medicine and Health Technology
Master's Thesis
April 2022

# ABSTRACT

Roope Ohlsbom: Differentiating osteocytes from bone marrow-derived mesenchymal stem cells in collagen hydrogel
Master's thesis
Tampere University
Master's Programme in Biomedical Technology
Supervisors: Postdoctoral Researcher Arjen Gebraad and Professor Susanna Miettinen
Examiners: Professor Heli Skottman and Postdoctoral Researcher Arjen Gebraad
April 2022

---

Osteocytes are the most prominent cell type of bone tissue, comprising up to 95% of bone cells. They are terminally differentiated osteoblasts that reside in the mineral matrix of bone. Osteocytes have an important role in maintaining bone tissue homeostasis; they regulate the activities of bone producing osteoblast and bone resorbing osteoclasts. Osteocytes also produce proteins that are essential for bone matrix mineralization. In addition to their functions in healthy bone, osteocytes have central roles in various bone related diseases such as osteoporosis and different cancers of bone.

Due to their importance in bone structure and regulation of bone homeostasis, osteocytes should be included in bone tissue engineering solutions and in vitro bone models. Isolation of mature osteocytes, however, is challenging as they are embedded deep in the bone matrix. Differentiating osteocytes from human bone marrow mesenchymal stem cells (hBMSCs) could be a viable option for acquiring osteocytes; hBMSCs can be expanded in large quantities and differentiated to osteoblasts in vitro. Since osteocytes descent from osteoblasts, differentiating osteocytes from hBMSCs should be possible. Collagen I hydrogel could be an optimal environment for achieving osteocyte differentiation, as it resembles the collagen matrix where in vivo osteoblast-osteocyte differentiation takes place.

In the present work, various cell culture protocols were tested as conditions to differentiate hBMSCs towards osteocyte phenotype. hBMSCs were embedded in collagen I hydrogel directly or following an osteogenic pre-differentiation step on tissue culture plastic. Cell-laden hydrogels were cultured for 21 to 42 days in osteogenic culture conditions with and without S53P4 bioactive glass extract in the culture medium; S53P4 extract has shown to be a strong enhancer of osteogenic differentiation. As a reference condition, hBMSCs were cultured on collagen coatings, a condition which is known to induce osteoblast differentiation, rather than osteocyte differentiation. Confocal microscopy imaging was performed to assess cell morphology and presence of osteocyte marker dentin matrix protein (DMP1). Gene expression analysis and protein detection assays were performed for various osteoblast and osteocyte markers to determine the osteogenic differentiation state of the hBMSCs cultured in collagen hydrogels.

hBMSCs stained positive for DMP1 and had formed some dendric processes, that are characteristic for osteocytes. Gene expression analysis and protein detection assays, however, did not show signs of enhanced osteocyte differentiation in collagen gels; expression of osteocyte markers podoplanin and sclerostin were highest with hBMSCs cultured on collagen coatings.

In conclusion, some indications of osteocyte differentiation were seen in hBMSCs cultured in collagen gel, but mature osteocyte differentiation was not achieved in the present study. Based on the underlying biology and previous studies of osteocyte differentiation, culturing hBMSCs in collagen hydrogels is still a relevant set-up for acquiring osteocyte-like cells. Modifications to the collagen hydrogels and to other aspects of the cell culture environment could help to better mimic in vivo environment of differentiating osteocyte, and thus to achieve more mature osteocyte differentiation.

Keywords: osteocyte, collagen, hydrogel, human bone marrow-derived mesenchymal stem cells

# TIIVISTELMÄ

Roope Ohlsbom: Osteosyyttien erilaistaminen luuytimen mesenkymaalisista kantasoluista kollageeni hydrogeelissä
Pro Gradu -tutkielma
Tampereen yliopisto
Biolääketieteen tekniikan maisteriohjelma
Tampereen yliopisto
Ohjaajat: tutikjatohtori Arjen Gebraad ja professori Susanna Miettinen
Tarkastajat: professori Heli Skottman ja tutkijatohtori Arjen Gebraad
Huhtikuu 2022

---

Osteosyytit ovat luukudoksen yleisin solutyyppi, kattaen 95 % luun soluista. Ne ovat terminaalisesti erilaistuneita osteoblasteja, jotka sijaitsevat luun mineralisoituneessa väliaineessa. Osteosyyteillä on tärkeä rooli luun homeostasian ylläpitämisessä; ne säätelevät luuta tuottavien osteoblastien ja luuta hajottavien osteoklastien aktiivisuutta. Osteosyytit myös tuottavat proteiineja, jotka ovat olennaisia luun mineralisaation kannalta. Osteosyyteillä on myös keskeinen rooli luuhun liittyvissä taudeissa, kuten osteoporoosissa ja erilaisissa luun syövissä.

Koska osteosyytit ovat tärkeitä luun rakenteen ja homeostaasin säätelyn kannalta, luukudostekniikan ratkaisujen ja in vitro -luumallien tulisi sisältää osteosyyttejä. Osteosyyttien eristäminen on kuitenkin hankalaa, koska ne sijaitsevat syvällä luun väliaineessa. Osteosyyttien erilaistaminen ihmisen luuytimen mesenkymaalisista kantasoluista (LMKS) voisi olla toimiva vaihtoehto osteosyyttien hankkimiseksi; LMKS:ja voidaan monistaa suurissa määrin, ja ne kykenevät erilaistumaan osteoblasteiksi in vitro -olosuhteissa. Osteosyyttien polveutuessa osteoblasteista, osteosyyttien erilaistaminen LMKS:ista pitäisi olla mahdollista. Kollageeni I -hydrogeeli voisi olla optimaalinen ympäristö osteosyyttierilaistumisen saavuttamiseksi, sillä se muistuttaa kollageeniväliainetta, jossa osteoblastien osteosyyteiksi erilaistuminen luonnollisesti tapahtuu.

Tässä työssä testattiin erilaisia soluviljelyprotokollia osteosyyttien erilaistamiseksi LMKS:sta kollageeni I-hydrogeelissä. LMKS:t upotettiin kollageeni I-hydrogeeliin joko suoraan tai soluviljelymuovilla suoritetun osteogeenisen esierilaistamisvaiheen jälkeen. Soluja viljeltiin hydrogeeleissä 21–42 päivää osteogeenisissa viljelyolosuhteissa, S53P4 biolasiekstraktia sisältävässä sekä S53P4 biolasiekstraktia sisältämättömässä viljelymediumissa; S53P4 ekstraktin on aiemmin näytetty edistävän osteogeenista erilaistumista merkittävästi. Vertailuolosuhteena tutkimuksessa käytettiin kollageenipinnoitteilla viljeltyjä LMKS:ja. Kyseinen viljelyolosuhde indusoi osteoblastierilaistumista, osteosyyttierilaistumisen sijaan. Solujen morfologiaa ja osteosyyttimarkkeri dentiinimatriksiproteiini-1:den ekspressiota tutkittiin konfokaalimikroskooppikuvantamisella. Geeniekspressionanalyysi ja proteiinien havaitsemismäärityksiä suoritettiin eri osteoblasti- ja osteosyyttimarkkereille LMKS:jen osteogeenisen erilaistumisasteen määrittämiseksi.

LMKS:t värjäytyivät positiivisiksi DMP1:lle ja olivat muodostaneet jonkin verran osteosyyteille tyypillisiä dendriittisiä haarakkeita. Geeniekspressioanalyysi ja proteiinien havaitsemismääritykset eivät kuitenkaan osoittaneet merkkejä vahvistuneesta osteosyyttierilaistumisesta kollageenigeeleissä; osteosyyttimarkkerien podoplaniinin ja sklerostiinin ilmentyminen oli korkeinta kollageenipinnoitteilla viljellyillä LMKS:lla.

Yhteenvetona voidaan todeta, että kollageenigeelissä viljellyt LMKS:t osoittivat joitain merkkejä osteosyyttierilaistumisesta, mutta eivät täysin erilaistuneet osteosyyteiksi. Aiempien osteosyyttierilaistumisesta tehtyjen tutkimusten ja taustalla olevan biologian perusteella, LMKS:jen viljeleminen kollageenihydrogeeleissä on kuitenkin edelleen pätevä keino osteosyyttien hankkimiseksi. Modifikaatiot kollageenihydrogeeleihin ja muihin soluviljely-ympäristön oosuhteisiin voisivat auttaa paremmin jäljittelemään erilaistuvien osteosyyttien luonnollista ympäristöä ja siten johtaa kokonaisvaltaisempaan osteosyyttierilaistumiseen.

Avainsanat: osteosyytti, kollageeni, hydrogeeli, ihmisen luuytimen mesenkymaaliset kantasolut

# PREFACE

This is a Master's Thesis for the degree program in Biomedical technology at Tampere University. The thesis work was done in Professor Susanna Miettinen's Adult Stem Cell research group at Tampere University. I would like to thank the members of the Adult Stem Cell group for familiarizing me with the premises and resources, and the academic work in general. Special thanks to go to my thesis supervisor Postdoctoral Researcher Arjen Gebraad and Professor Susanna Miettinen for guiding me through this project.

# CONTENTS

# LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| 2D | Two-dimensional |
| 3D | Three-dimensional |
| ALP | Alkaline phosphatase |
| AMP | 2-amino-2-methyl-1-propanol |
| BAG | Bioactive glass |
| BGLAP | Bone gamma-carboxyglutamate protein |
| BM | Basic medium |
| BSA | Bovine serum albumin |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DMP1 | Dentin matrix protein 1 |
| DPBS | Dulbecco’s phosphate buffered saline |
| ECM | Extra cellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| FACIT | Fibril associated collagens with interrupted triple helixes |
| dex | Dexamethasone |
| hASC | Human adipose tissue derived mesenchymal stem cell |
| hBMSC | Human bone marrow-derived mesenchymal stem cell |
| hFGF2 | Human fibroblast growth factor 2 |
| HSC | Hematopoietic stem cell |
| LSCM | Laser scanning confocal microscopy |
| MM | Multiple myeloma |
| MSC | Mesenchymal stem cell |
| NPC | Non-collagenous protein |
| NPP | 4-Nitrophenyl phosphate |
| OCM | Osteocyte medium |
| OGM | Osteogenic medium |
| OOC | Organ-on-chip |
| PTH | Parathyroid hormone |
| PDPN | Podoplanin |
| RANKL | Receptor-activator of NF-kB ligand |
| RT | Room temperature |
| RUNX2 | Runt-related transcription factor 2 |
| SOST | Sclerostin |
| TCPS | Tissue culture polystyrene |
| TRICT | Tetramethylrhodamine |
| αMEM | Alpha Minimum Essential Medium |

# 1. INTRODUCTION

Bones support body, protect organs, and enable body movements together with skeletal muscles (Florencio-Silva et al., 2015). Additionally, bones also have an important role in calcium, phosphate, and magnesium metabolism (Iaquinta et al., 2019). Essentially, all bones consist of mineralized bone extracellular matrix (ECM) and different types of bone cells (Florencio-Silva et al., 2015). The properties of bones are mostly explained by the ECM; bone cells are important for bone integrity as they regulate bone tissue homeostasis (Florencio-Silva et al., 2015).

Osteocytes, the most abundant cells in bone tissue, have a central role in the maintenance of bone tissue homeostasis (Bonewald, 2011). They regulate the activity of bone forming osteoblasts and bone resorbing osteoclasts by secreting different signaling molecules, most notably sclerostin (SOST) and receptor-activator of NF-kB ligand (RANKL) (Divieti Pajevic & Krause, 2019). Secretion of these signaling molecules by osteocytes is regulated by parathyroid hormone (PTH) and mechanical loading of the bone tissue (Divieti Pajevic & Krause, 2019). In addition to regulating activity of osteoblasts and osteoclasts, osteocytes contribute to bone structure by secreting non-collagenous proteins (NPCs) that participate in mineralization of collagen (de Wildt et al., 2019). In certain situations, osteocyte also can themselves deposit or resorb bone matrix, mimicking the functions of osteoblasts and osteoclasts (Robling & Bonewald, 2020).

Osteocytes reside inside the bone ECM (Bernhardt et al., 2019). Osteocyte morphology is characterized by their rounded cell body and dendritic processes that connect them to other osteocytes, forming an extensive network in the bone ECM (Bonewald, 2011). The network reaches from outer surface of the bone to the of bone marrow, allowing the communication with other cells of bone tissue as well (Atkinson & Delgado-Calle, 2019). Osteocytes themselves reside in small chambers in the bone matrix and the dendritic processes travel in small ducts, called lacunae and canaliculi respectively (Bonewald, 2011; Reznikov et al., 2014). As these spaces cause discontinuations in bone ECM, there is also a structural relationship between osteocyte network and bone tissue (Kerschnitzki et al., 2011).

Importance of osteocytes on bone integrity is well demonstrated in different pathologies. Reduction in the number of osteocytes and abnormalities in the osteocyte network are associated with diseases, such as osteoporosis and osteomalacia, which can lead to bone fractures. (Dallas et al., 2013; Tiede-Lewis & Dallas, 2019; Zarrinkalam et al., 2012). Talking about pathologies, osteocytes also contribute to the progress of multiple

myeloma which is cancer of bone marrow and to bone metastasis of breast cancer and prostate cancer (Atkinson & Delgado-Calle, 2019; Delgado-Calle et al., 2016).

Since osteocytes are such an integral part of bone structure, play an important role in maintaining bone homeostasis, and are involved in different bone related pathogeneses, they should be included when modeling bone tissue function in vitro. So far, isolating primary osteocytes has proven quite challenging, as osteocytes lie deep in the bone ECM (Bernhardt et al., 2019). Osteocytes have been isolated from human trabecular bone samples with repeated collagenase II and ethylenediaminetetraacetic acid (EDTA) digestion procedure (Prideaux et al., 2016). The yield of osteocytes using the isolation protocol has also been relatively low (Bernhardt et al., 2019), which is a major downside as osteocytes are a non-proliferative cell type (Nasello et al., 2020), and hence cannot be acquired in large quantities from one bone sample.

To obtain osteocytes in large quantities, a viable option could be differentiating osteocytes in vitro from mesenchymal stem cells (MSCs). MSCs are multipotent stem cells that can be harvested relatively easily from many adult tissues, including bone marrow, adipose tissue, and dental pulp tissues (Andrzejewska et al., 2019; Han et al., 2019). MSCs also have a good self-renewal capacity and they can be expanded in vitro to obtain large numbers of cells (Han et al., 2019; Iaquinta et al., 2019). They can differentiate into multiple cell types including osteoblasts, adipocytes, and chondrocytes (Andrzejewska et al., 2019; Pittenger et al., 2019). Since osteocytes are descent from osteoblast (Bonewald, 2011), it should be possible acquire osteocytes from MSCs through osteogenic lineage differentiation.

There is quite clear consensus that the environment resembling the natural tissue can guide stem cells to differentiate towards the cell type of that tissue (Anderson et al., 2016; Bloom & Zaman, 2014; Salvatore et al., 2021). In vivo, osteocytes differentiation begins when osteoblasts get embedded in soft collagen matrix, which is later mineralized to produce mature bone ECM (Bonewald, 2011; de Wildt et al., 2019). Thus, culturing MSCs in collagen hydrogel is a logical set-up for differentiating osteocytes from MSCs. In addition to offering collagenous three-dimensional (3D) environment, hydrogels also mimic many other aspects of natural ECM (H. Liu et al., 2019; Tibbitt & Anseth, 2009). Ability of collagen I hydrogels to promote osteocyte differentiation is already shown in the context of human primary osteoblasts (Bernhardt et al., 2019; Skottke et al., 2019).

In the present study it was assessed whether human bone marrow-derived mesenchymal stem cells (hBMSCs) cultured in collagen I hydrogel in osteogenic culture medium would acquire osteocyte-like phenotype. Different cell culture conditions were

established to test the effect of different factors on osteocyte differentiation. hBMSCs were embedded into collagen hydrogels directly or after a 7-day osteogenic pre-differentiation in conventional two-dimensional (2D) culture to tests if initiating osteoblast differentiation prior the 3D culture would be beneficial for osteocyte differentiation. With hBMSCs directly embedded in collagen gels, two different culture periods were used to test if longer culture period leads to more osteocyte-like phenotype. All the forementioned cell culture conditions were cultured both with and without S53P4 bioactive glass (BAG) extract in the culture medium to test its effect on the osteocyte differentiation. S53P4 extract medium was generated by incubating S53P4 glass granules in the medium, resulting in release of ions from the S53P4 to the medium. S53P4 ions have previously shown to enhance osteoblast differentiation of MSCs (Ojansivu et al., 2015), which could mean that they can also help to achieve osteocyte phenotype as osteocytes descent from osteoblasts.

The osteocyte differentiation stage of hBMSCs was assessed with laser scanning confocal microscopy (LSCM), gene expression analysis, and protein detection assays. LSCM was utilized for analyzing the cell morphology and expression of osteocyte marker protein. Gene expression analysis and protein detection assays were performed for different osteoblast and osteocyte markers to determine the osteogenic differentiation state of the hBMSCs cultured in collagen hydrogels.

Before getting in more depth to the materials and methods used, and to the results, the background of the most important topics related to the present study are covered in detail in a literature review section. At the end, the results are discussed in the lights of earlier studies, and finally a brief conclusion of this study is drawn.

# 2. LITERATURE REVIEW

## 2.1 Mesenchymal stem cells

Stem cells are undifferentiated cells that possess two inherent features. First, they can self-renew, meaning that as they divide, they give rise to new stem cells. Second, they can differentiate to specialized cell types upon external and internal signals and cues. These features enable the simultaneous production of specialized cells found in mature tissues and maintenance, or even growth, of the stem cell pool. Stem cells play a key role during the development, tissue repair, and in maintenance of tissue homeostasis, but are also involved in development of some cancers. (Morrison & Kimble, 2006; Zakrzewski et al., 2019)

Stem cells are found in individuals during their whole life cycle, from the embryonic development to old age. While embryonic stem cells can differentiate to any cells of the organism, stem cells found in adult individuals have more limited differentiation capacity. For instance, hematopoietic stem cells (HSCs) can only differentiate to different blood cells and osteoclasts. (Charbord, 2010; Kursad, 2012; Zakrzewski et al., 2019)

Similarly with HSCs, mesenchymal stem cells (MSCs) are adult stem cells with multilineage differentiation capacity (Charbord, 2010; Pittenger et al., 2019). MSCs are perhaps the best known for their capacity to differentiate to osteoblasts, adipocytes, myocytes, and chondrocytes (Andrzejewska et al., 2019; Pittenger et al., 2019). These are the major cell types of bone, fat, muscle, and cartilage, respectively, which all are connective tissues derived from mesenchymal tissue of embryo. (Charbord, 2010; Pittenger et al., 2019; Robert et al., 2020). There are also reports of MSCs differentiating towards cells of non-mesenchymal origin, including neurons, hepatocytes, and endothelial cells (Charbord, 2010; Pittenger et al., 2019).

Besides differentiating to major cell types of different tissues, MSCs have important supporting functions as well. For instance, they can promote vascularization by differentiating into pericyte-like cells and by secreting pro-angiogenic factors (Mykuliak et al., 2022). In addition to pro-angiogenic factors, MSCs influence behavior and differentiation of other cells by secreting immunomodulatory factors and many other signaling molecules to their surroundings (Andrzejewska et al., 2019; Robert et al., 2020).

Due to their self-renewal potency, differentiation capacity, and paracrine effects, MSCs have been sought to use in regenerative medicine, where the aim is to replace the damaged tissues or their functions. (Iaquinta et al., 2019; Pittenger et al., 2019). MSCs

have been combined with biomaterial scaffolds to develop tissue engineering implants to treat major bone and cartilage injuries (Iaquinta et al., 2019; Oryan et al., 2017; Robert et al., 2020). The paracrine effects of MSCs have been utilized for example in treating heart tissue injuries; injection of MSCs to the site of an ischemic injury leads to a reduction in tissue injury and scar tissue formation, which is due to the paracrine and supporting effects of MSCs rather than differentiation of MSCs to cardiomyocytes (Pittenger et al., 2019).

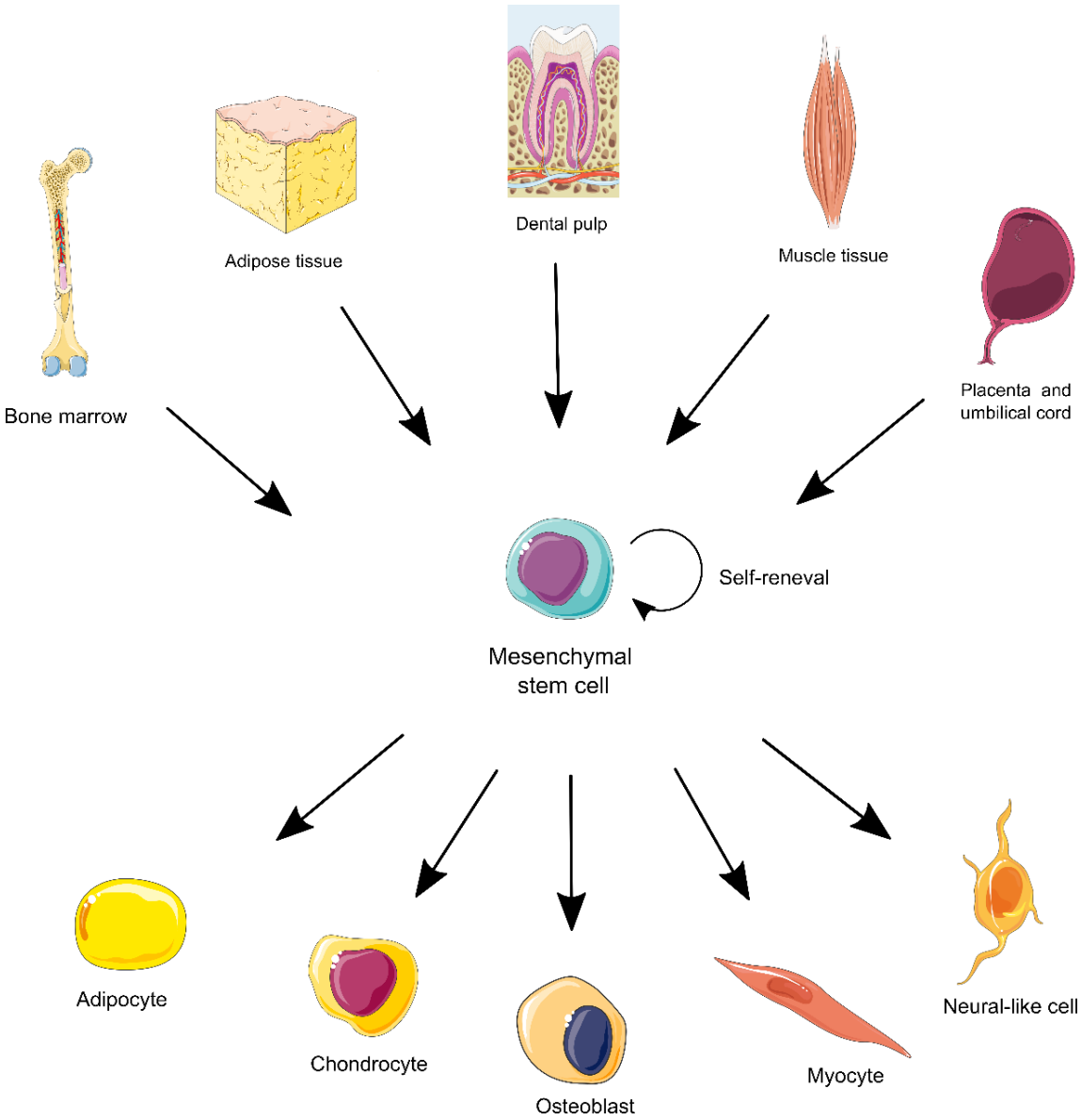


***Figure 1.*** *Mesenchymal stem cells are found in many tissues, and they possess multilineage differentiation capacity. Figure was created using images retrieved from Servier Medical Art.*

As MSCs and cells differentiated from MSCs have high abundance and central role in many tissues, MSCs are also used in microfluidic cell culture devices that aim to recreate

natural tissue environments to study normal tissue function, cell differentiation, disease progression, or efficacy of different drugs and substances in controlled and easily observable in vitro environment (Ahadian et al., 2018; Wnorowski et al., 2019; J. Zhang et al., 2017).

MSC can be isolated from many different tissues, including bone marrow, adipose tissue dental pulp, and placenta, among others (Andrzejewska et al., 2019; Han et al., 2019; Iaquinta et al., 2019). Most common sources for MSCs isolation have most likely been adipose tissue and bone marrow, due to their accessibility and renewability (Iaquinta et al., 2019; Pittenger et al., 2019)

Even though all MCSs are unified by their ability to differentiate to same cell types and by their shared cell surface markers, it is worth noting that MSC subtypes still show some differences regarding their differentiation potential (Andrzejewska et al., 2019; Mykuliak et al., 2022; Robert et al., 2020). BMSCs and adipose tissue derived MSCs (AMSCs) have superior osteogenic differentiation compared to umbilical cord stem cells and placental stem cells. (J. S. Heo et al., 2016). Some studies suggest that be osteogenic potential is higher in BMSCs than ASCs (Liao, 2014; Mohamed-Ahmed et al., 2018; Sakaguchi et al., 2005), which seems logical considering the origins of the BMSCs and ASCs. All and all BMSCs possess excellent osteogenic differentiation potential, and they are also most frequently used MSCs for acquiring osteoblasts (Han et al., 2019). Heterogeneity regarding differentiation capacity also exists between different donors and even between individual MSCs (Andrzejewska et al., 2019; Robert et al., 2020).

MSCs reside in stem cell niches, which are microenvironments that also contain extracellular matrix (ECM), vasculature, and supporting cells, including endothelial cells and adipocytes (Kurenkova et al., 2020; H. Lin et al., 2019). This microenvironment provides a set of physical and chemical cues that maintain stemness of the cells by regulating quiescence, self-renewal, proliferation, and differentiation of MSCs (H. Lin et al., 2019; Pittenger et al., 2019). When cells in the surrounding tissue need to be replaced due to injury, or natural cell turn-over, MSCs in the niche receive signals that promote their proliferation and migration(An et al., 2018; H. Lin et al., 2019; Walker et al., 2019). This results in some of the cells migrating away from the niche; as cells move away from the niche, they differentiate to specialized cell types (Anderson et al., 2016; Pittenger et al., 2019). Fate of MSC is determined by signals provided by their environment, which include growth factors and other small molecules, mechanical cues, and cell-cell interactions (Cheng et al., 2019). These signals alter the gene expression and epigenetic state of the MSCs, leading to their differentiation (Cheng et al., 2019).

Effects of soluble factors on MSC fate is well documented. Maintaining the stemness of MSCs in vitro has proven to be challenging, but self-renewal capacity of MSCs have been improved by supplementing cell culture media with growth factors, such as fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), and stem cell factor (SCF) (Pal & Das, 2017). It is well known that adding growth factors and other supplements to cell culture media can also be used to guide MSCs towards adipogenic, osteogenic, and chondrogenic differentiation on 2D surfaces (Andrzejewska et al., 2019; Cheng et al., 2019; Pittenger et al., 1999). Addition of ions found in bone ECM to culture media have also shown to promote osteogenic differentiation (Ojansivu et al., 2015). Besides the type of the soluble factors, their concentration and the timing of the exposure also seems to play a role in the fate determination of MSCs (Alm et al., 2012; Bhandi et al., 2021).

ECM was originally thought of as a passive scaffold for the cells, offering merely structural support (Salvatore et al., 2021). It is, however, much more as it contains multiple cell binding motifs and affects phenotype of cells through various cues (Salvatore et al., 2021). Increasing amount of evidence points to the conclusion that a microenvironment characteristic to specific tissue type causes MSCs to differentiate to the cell type of that tissue. Convincing evidence of this microenvironment guided cell differentiation was provided by a study where matrix elasticity was shown to affect MSC differentiation (Engler et al., 2006). The study showed that when MSCs are cultured on a substrate with stiffness corresponding to a natural tissue, MSCs started to differentiate towards the mature cells of that tissue, in an irreversible manner. After that, the effect of matrix elasticity on MSC, and cells in general has been much studied. Influence of substrate stiffness on MSC linage specification is explained by a phenomenon called mechanotransduction (B. Li et al., 2013; Martino et al., 2018; Naqvi & McNamara, 2020; Steward & Kelly, 2015). Briefly, mechanotransduction is conversion of mechanical signals to biochemical intracellular responses (Martino et al., 2018; Steward & Kelly, 2015). This can occur either via mechanoresponsive ion channels, or integrin mediated cell-ECM interactions (Steward & Kelly, 2015). Integrins are cell surface receptors that bind to ECM proteins. Binding of integrin to ECM leads to activation of intracellular signaling pathways that affect cellular behavior and differentiation (Martino et al., 2018; Naqvi & McNamara, 2020). Substrate stiffness increases the number of bound integrins and affects the signal that is generated via integrin-ECM binding; stiffer substrates lead to increased cytoskeletal tension, which can lead to changes in cell morphology, and furthermore affect the lineage specific differentiation of MSCs (Martino et al., 2018; Naqvi & McNamara, 2020; Steward & Kelly, 2015). Other factors than the bulk mechanical

properties of the substrate have also shown to affect cell differentiation and behavior (Chaudhuri et al., 2020; Doyle et al., 2015).

Also, other matrix properties such as surface topography, and surface chemistry of the material affect MSC fate (Anderson et al., 2016). Effect of the matrix chemistry on MSC differentiation is well demonstrated in the context of osteogenic differentiation. MSCs cultured on materials that contain elements found in natural bone have shown enhanced or even induced osteogenic differentiation (Viti et al., 2016). These materials include bioactive glasses, and calcium phosphates, and calcium silicates (Gao et al., 2017).

Culturing MSC in 3D microenvironment leads to enhanced osteogenic, adipogenic and hepatocytic differentiation (Bae et al., 2017; H. Kim et al., 2019), which is not surprising considering these cell types naturally reside in 3D environment. Differentiation of MSCs is also improved when other cell types, such as endothelial and hematopoietic, are present in the 3D environment, which is usually the situation in the natural tissue environment as well (H. Kim et al., 2019). Also, other external factors such as hypoxia and dynamic mechanical stimuli affect MSCs differentiation (Binder et al., 2015; Goetzke et al., 2018).

By now it is clear that MSC differentiation, and behavior of cells in general is greatly affected by their microenvironment. Therefore, the biomaterials, and the microenvironment in general, used for the purposes of regenerative medicine or for in vitro modelling of tissues should mimic the natural ECM and tissue environment as closely as possible (Ahadian et al., 2018; B. Li et al., 2013; Salvatore et al., 2021; Wu et al., 2020).

## 2.2 Collagen

As already stated, ECM surrounds cells and provides them with structural support, but also with biochemical and biomechanical cues that affect cell differentiation and behavior. Thus, the ECM largely determines the properties of each tissue and organ. ECM of each tissue is characteristic for the tissue type and reflects the functionality of the tissue. However, ECM in al tissues shares the basic structural components. ECM comprises from proteins, polysaccharides, and water. Fibrous proteins are the main structural component of ECM. They build fiber networks with various organizations and contribute to the mechanical properties of ECM. They also offer binding sites for cells and proteoglycans. Other proteins and polysaccharides also contribute to the mechanical properties and shape of ECM, as they bind water and different cations. They

also bind growth factors (GF) and GF receptors and thus contribute to cell behavior. (Frantz et al., 2010; Yue, 2014)

Collagen is the most abundant of the fibrous proteins in ECM, and altogether accounts about a third of the total protein content in human body (Frantz et al., 2010; Shoulders & Raines, 2009). In addition to bone, it is found in dentin, skin, tendons and ligaments, arteries, and cartilage. Collagen is synthesized by the cells in the corresponding tissues, most notably by fibroblasts and osteoblasts (Boraschi-Diaz et al., 2017; Frantz et al., 2010). Collagens greatly contributes to mechanical properties of these tissues, most notably to their tensile strength (Buehler, 2006; Fratzl, 2008; Roeder et al., extrac 2002). Collagen also affects cellular behavior as it offers numerous binding sites for integrins and other various cell surface receptors (Boraschi-Diaz et al., 2017). In addition to cell, collagens also associate with other ECM proteins and proteoglycans (Boraschi-Diaz et al., 2017).

High mechanical properties and multiple binding motifs for other ECM components make collagen an ideal ECM component. These properties are explained by a quaternary protein structure of collagen molecules, where three left-handed α-chains have tightly wound together to form a right a right-handed triple helix. This structure allows simultaneously maximal presentation of binding motifs on the surface of the structure and tight packing of the polypeptide chains resulting in high mechanical properties. There arre, in fact, as many as 28 different collagen types, some of which differ significantly from each other regarding their structure and function, but they all are considered to be part of the collagen protein family as they contain the triple helix structure described above (Fidler et al., 2018; Ricard-Blum, 2011).

The primary protein structure of α-chains in different collagen types also varies, but all α-chains comprise repeating sequence of three amino acids, where every third amino acid is glycine (Gly). This triplet of amino acids referred as Gly-X-Y, X and representing the two other amino acids. X and Y are most commonly proline (Pro) and hydroxyproline (Hyp), respectively, Pro occupying the X position with 28% frequency, and Hyp occupying the Y position with 38% frequency. Over 10% of all the triplets in collagen α-chains are Gly-Pro-Hyp (Ramshaw et al., 1998).

All these amino acids contribute to the formation of the triple helix structure. Proline residues induce polypeptide chains to the left-handed conformation seen in the α-chains. Glycine residues on the other hand provide the chains with flexibility and due to their small size can be buried inside the triple helix structure, allowing tight packing of the left-handed chains. Furthermore, the triple-helix is stabilized by hydrogen bonds formed

between the amine group of the glycine and carboxyl group of the second amino acid of the triplet in an adjacent chain. Post-translational modification of the prolines in the Y position to hydroxyprolines help to stabilize the triple helix structure through stereochemical effects and specific hydration. The important role of proline and hydroxyproline for the α-chain structure and triple helix stability is most likely explained by their pyrrolidine ring structure, which is not found in other amino acids (Fidler et al., 2018; Shoulders & Raines, 2009).

### 2.2.1 Higher order collagen structure

Collagen types (denoted with roman numerical) can be categorized based on their structural roles. These categories include fibril-forming collagens, network-forming collagens, fibril associated collagens with interrupted triple helixes (FACITs), membrane collagens and multiplexins. Structure of a single collagen molecule between these categories varies substantially. While in fibril-forming collagen I, the triple helix is a predominant structure (96%), in collagen XII (FACIT), its portion is less than 10% Also, fibril-forming collagens have just a single triple helical domain, whereas network forming collagens and FACITs have multiple shorter triple helix domains, separated by short non-helical segments. In addition to helical domains, different collagen types have a variable sets of other protein domains, that can interact with other collagen molecules and surrounding tissue. (Ricard-Blum, 2011)

Fibrillar collagens are the most abundant collagens in ECMs of connective tissues, such as bone, skin, and cartilage, and thus, they largely determine mechanical properties and structure of these tissues (Ricard-Blum, 2011; Shoulders & Raines, 2009). Collagen types belonging to other categories also have important roles in different tissue. Collagen IX and VII, two non-fibrillar collagens that comprise only a small portion of tissues, are known to be important for integrity of articular cartilage and skin, respectively (Ricard-Blum, 2011). From here on the focus is going to be on fibrilla collagens, as the fibrillar collagen I is the most abundant protein component in bone tissue, and commercial hydrogels used in tissue engineering applications consist of single type of fibrillar collagen (Antoine et al., 2014).

Fibrillar collagens form collagen fibrils in a process called fibrillogenesis. Once the triple helixes have formed, they have non-helical regions in their both ends. These non-helical propeptides are crucial for the triple helix formation but they must be cleaved by proteinases for the fibrillogenesis to occur. After the cleavage, only a very short non-helical telopeptides are left on both ends. Triple helical structures with telopeptides in both ends are referred to as tropocollagens, which in collagen I are 300 nm in length and

under 2 nm in diameter. Tropocollagen monomers form collagen microfibrils as illustrated in Figure 2. Collagen microfibrils consist of multiple sequent bundles of tropocollagen. In collagen I these bundles consist of five tropocollagen monomers that are twisted relative to the central axis, which results in right-handed twist in the microfibers. Aggregation of the tropocollagens to these bundles is driven by hydrophobic, hydrophilic, and electrostatic interactions (Raspanti et al., 2018). The tropocollagens in a single bundle are not completely in a same plane relative to each other. There is a small stagger, which results in short overlap of the tropocollagens in the subsequent bundles (Figure 2). The short overlap is important as it allows formation of covalent cross-links between the telopeptides of the subsequent tropocollagen bundles, in a reaction catalyzed by lysyl oxidase. Tropocollagens and microfibrils further aggregate with each other to form mature collagen fibrils, which can have diameter of 500 nm and length of 1 cm (Shoulders & Raines, 2009).

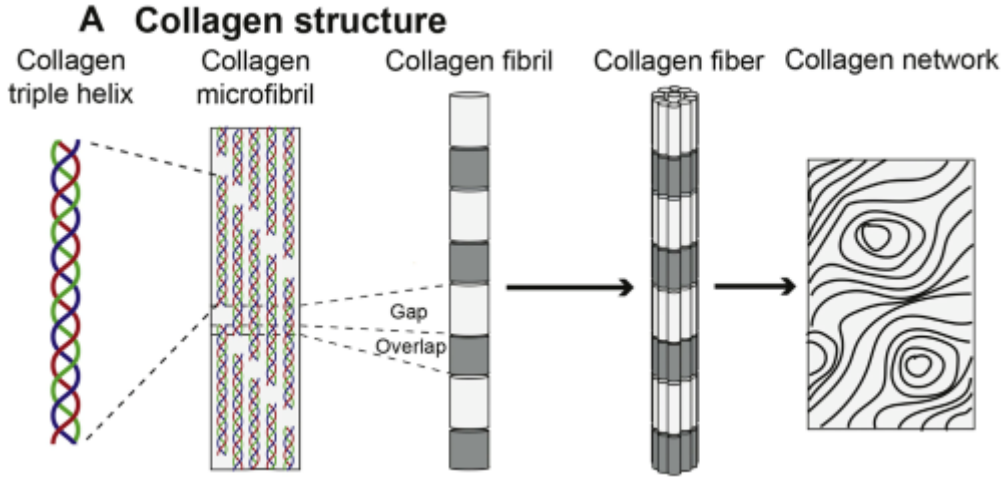


***Figure 2.*** *Assembly of fibrillar collagen from triple helix to higher collagen network. Adapted from* de Wildt et al., 2019.

Collagen fibrils form higher order structures that vary greatly in different collagenous tissues such as bone, skin, tendons, cartilage, and cornea; in some tissues the fibrils can be parallelly aligned, whereas in other tissues they form network-like structures. (J. Lin et al., 2020; Raspanti et al., 2018). These differences in orientation and alignment of collagen fibrils greatly contribute to differences in mechanical properties of these tissues (J. Lin et al., 2020; Revell et al., 2021). In addition to the organization of collagen fibrils, mechanical properties of collagenous tissues are also affected by covalent cross-links and weak interactions between collagen molecules (Depalle et al., 2015; Fratzl, 2008; Nam et al., 2016; Ricard-Blum, 2011; Shoulders & Raines, 2009; Sorushanova et al., 2019). It is highly likely that there is an interplay between all these aspects. There is

evidence that the collagen fibril organization and cross-linking are affected by each other (Herchenhan et al., 2015; Svensson et al., 1999; L. Wang et al., 2014). It is also highly likely that the weak interactions also contribute to the organization of the collagen fibrils, as weak interactions play a central role in any protein-protein interaction.

Collagen fibrils in natural tissue environment, or cell cultures, are heterotypic meaning that they contain combinations of different fibrillar collagens. Collagen I, which is the most prominent in all tissues except cartilage, co-exists with two other fibrillar collagens, collagen III and V. In cartilage, the most abundant collagen is collagen II, which forms the collagen fibrils together with collagen XI. (Raspanti et al., 2018). Proportion of these different collagens also appears to influence the organization and mechanical properties of collagen matrices (Piechocka et al., 2011; Raspanti et al., 2018).

It is good to keep in mind that other aspects than collagen also play a role in determining the properties of ECM. As mentioned at the beginning of this section, proteoglycans, different inorganic components, and hydration, also affect the properties of the ECM. (Frantz et al., 2010; Salvatore et al., 2021). This is perhaps the most evident in bone where inorganic mineral phase provides bone ECM its characteristic hardness (Koons et al., 2020; Salvatore et al., 2021).

### 2.2.2 Collagen self-assembly

In vivo other ECM components are required for proper assembly of most abundant collagens. Collagens V and XI, are crucial for in vivo collagen fibrillogenesis; their absence leads to impaired fibril formation of collagen I and II, both during development and tissue maturation (M. Sun et al., 2011, 2020). Formation of collagen I containing fibrils is also dependents on presence of fibronectin, an ECM glycoprotein (Kadler et al., 2008; Kubow et al., 2015). Collagen I co-localizes with fibronectin as the collagen fibrillogenesis occurs, and blocking the collagen binding sites of fibronectin prevents collagen fibrillogenesis (Kubow et al., 2015). As fibronectin polymerization is a cell-mediated process where fibronectin monomers interact with integrins on cell surface, cells of the ECM are also involved in the regulation of collagen fibrillogenesis (Kubow et al., 2015). However, in vitro, collagen I tropocollagens correctly self-assemble into fibrils without any assistance (Kadler et al., 2008). This points to the conclusion that collagen fibrillogenesis is a self-assembly process, which in vivo is disturbed by the large number of potential collagen binding partners present in natural tissue environment, and therefore needs to be regulated by other collagens and fibronectins (Kadler et al., 2008).

## 2.3 Collagen hydrogels

Being a natural polymer, highly abundant in many tissues, collagen possesses many desirable qualities for a biomaterial, including biocompatibility, biodegradation, and bioactivity (Delgado et al., 2017). These properties make collagen a promising scaffolding material for tissue engineering applications and for in vitro modelling of natural tissue functions (Salvatore et al., 2021; Terrell et al., 2020). Collagen I has already been used widely in the fields of skin, bone, cartilage, and tendon tissue engineering (X. Liu et al., 2019; Salvatore et al., 2021; Sarrigiannidis et al., 2021). Scaffold types used include hydrogels, porous sponges, nanofibers, and films (Irawan et al., 2018; Salvatore et al., 2021; Sorushanova et al., 2019).

Hydrogels are a popular scaffolding material as they mimic many aspects the natural tissue ECM (H. Liu et al., 2019; Thiele et al., 2014). Hydrogels are polymer networks with high water content (Terrell et al., 2020; Tibbitt & Anseth, 2009), a composition greatly resembling natural ECMs of connective tissues, which are essentially composed of fibrous proteins, water, proteoglycans, and glycoproteins (Frantz et al., 2010; Yue, 2014). Hydrogels also offer cells a 3D microenvironment, which is important as many cell types in natural tissues are surrounded by ECM, and cell matrix interactions play a central role in cell differentiation and behavior (Ahmed & ffrench-Constant, 2016; H. Liu et al., 2019; Yue, 2014). In addition, hydrogels allow the diffusion of gases, nutrients, waste, and soluble molecules, which is crucial for cell viability and for paracrine signaling (Antoine et al., 2014; Thiele et al., 2014; Tibbitt & Anseth, 2009).

Both synthetic and natural hydrogels are used for 3D cell cultures. Synthetic materials enable more control over the physical and chemical properties, and microstructure of the hydrogel compared to hydrogels derived from natural materials (Chaudhuri et al., 2016; Thiele et al., 2014). They, however, do not offer natural cell binding sites, which promote cell adhesion, survival, proliferation, and migration (Liaw et al., 2018; Thiele et al., 2014). In addition to these binding sites, natural hydrogels, possess other properties essential for many natural tissues; they can be reshaped and degraded by cells, which allows the cells to modify their surroundings, which is a common phenomenon in natural tissues (Chaudhuri et al., 2016; Creecy et al., 2021; Davidson et al., 2019; Shiflett et al., 2019; Thiele et al., 2014). Natural hydrogels are viscoelastic and show stress relaxation behavior, which is also the case in the natural tissues (Chaudhuri et al., 2016; Nam et al., 2016). This aspect of the natural environment is also important for the biomaterial as viscoelasticity and stress relaxation properties affect the behavior and differentiation of cells cultured in hydrogels (Chaudhuri et al., 2016).

Collagen hydrogels are easily generated in vitro, as solubilized collagen monomers self-assemble to form fibrillar networks, similarly as in vivo, but without the need for assistance by other collagen types or molecules of the ECM (Kadler et al., 2008; Kubow et al., 2015). Collagen I is the most popular collagen for hydrogel preparation (Antoine et al., 2014). It can be isolated from connective tissues of various animals including bovine, rat, porcine, equine, and fish (Antoine et al., 2014; Terzi et al., 2020). Collagen with low cross-linking density can be directly solubilized with acetic acid extraction; for collagen with higher cross-linking density a pepsin digestion is needed before it can be solubilized (Terrell et al., 2020; Terzi et al., 2020). Either way, the aim is to generate a monomeric collagen solution. Pepsin digested collagen, i.e., atelocollagen, lacks telopeptides, as they have been cleaved off to cut the cross-links between the collagen monomers; in the directly solubilized collagen the telopeptides are retained (Sarrigiannidis et al., 2021; Terzi et al., 2020). Thus, the collagen extracted by directly solubilizing to acetic acid is called telocollagen, basically consisting of tropocollagen molecules. Neither method produces completely pure monomeric solutions, but rather a mix of monomers and aggregates of still cross-linked molecules. Atelocollagen, however, usually has higher fraction of monomers when compared to telocollagen (Terzi et al., 2020). In vitro assembly of the solubilized collagen to hydrogel can be initiated by altering the temperature or pH of the solution (Antoine et al., 2014).

Even though collagen hydrogels capture many aspects of in collagen matrices, they still fail to mimic some aspects of in vivo collagen matrices. Hydrogels do not have similar level of covalent cross-linking between the collagen molecules as in vivo assembled collagen (Sarrigiannidis et al., 2021). They also fail to recapture the specific hierarchical organization of collagen fibrils seen in many tissues (Salvatore et al., 2021). Additionally, collagen hydrogels lack the other components present in tissue ECM as well. These shortcomings have two important implications. First, they lead to relative weak mechanical properties, which limits usage of hydrogel as a scaffold material for tissue regeneration in load-bearing tissues such as bone and cartilage, and also leads to shrinkage of cell-laden hydrogel (Salvatore et al., 2021; Sarrigiannidis et al., 2021). Second, they mean that the hydrogel does not recapture the exact structural organization, mechanical properties, and surface chemistry of in vivo tissues. This is problematic from the point of view of both regenerative medicine and in vitro modeling as all these aspects are known to affect cell differentiation and behavior (Anderson et al., 2016; Antoine et al., 2014; Liaw et al., 2018; Salvatore et al., 2021).

Another problem of collagen hydrogels is their batch-to-batch inconsistencies (Antoine et al., 2014; Dippold et al., 2019; Sorushanova et al., 2019; Walters & Stegemann, 2014),

a common issue with natural-derived biomaterials, which hampers their reproductivity (Dippold et al., 2019; Hussey et al., 2018; H. Liu et al., 2019; Stoppel et al., 2015).

Various approaches have been sought to modify the properties of collagen hydrogels, to better mimic in vivo collagenous tissues (Antoine et al., 2014; Sarrigiannidis et al., 2021; Walters & Stegemann, 2014). Various covalent cross-linking methods have been applied for collagen, including treatment with carboimides, glutaraldehyde, ultraviolet irradiation, and transglutaminase (Delgado et al., 2015; Sarrigiannidis et al., 2021). Increase in density and strength of cross-links leads to increased mechanical strength and affect the viscoelastic properties of hydrogels as the they prevent the collagen fibrils sliding past each other (Depalle et al., 2015; Sarrigiannidis et al., 2021).

Microstructure of collagen hydrogels have also been altered by different means. Adjusting hydrogel gelation parameters has shown to change the fibril size and network pore size of collagen hydrogels (Achilli & Mantovani, 2010; Antoine et al., 2015; Raub et al., 2008; Roeder et al., 2002). These adjustments also affect the mechanical properties of the hydrogels. For instance, increase in gelation pH and ionic strength correlate with smaller pore size, smaller collagen fiber diameter, and increased mechanical strength of collagen gels (Achilli & Mantovani, 2010; Antoine et al., 2015; Raub et al., 2008; Roeder et al., 2002). Change in the fibril diameter is caused by increased the bundling of the fibrils seen at the lower temperatures; fibril size has shown to alter cellular behavior via affecting the hydrogel stiffness on much smaller, focal adhesion, scale (Doyle et al., 2015). The pore size on the other hand affects passing of nutrients, gases, and waste products and migration of the cells in the hydrogel (Thiele et al., 2014).

Organization of collagen hydrogels have been altered also in more controlled manner than tuning the fabrication parameters. For example, magnetic flow, electrochemical fabrication, stretching, extrusion, and bioprinting have been utilized for tuning the microstructure of collagen hydrogels (Sarrigiannidis et al., 2021). These methods can offer more control over the orientation of collagen fibrils, which can be beneficial for mimicking natural tissues and thus guiding cell differentiation and behavior (Salvatore et al., 2021; Sarrigiannidis et al., 2021).

Collagen hydrogels have been combined with other materials to improve their properties. These materials include polymers and particulates, and they can be both natural and synthetic origin (Thoniyot et al., 2015; Walters & Stegemann, 2014). These composite materials usually come with improved mechanically properties, but they have many other advantages as well. Adding other natural polymer, such as fibrin, can be used to alter cell signaling and gene expression, whereas adding synthetic polymers gives more

control over the chemical and properties and microstructure of the resulting hydrogel (Liaw et al., 2018; H. Liu et al., 2019; Thiele et al., 2014; Walters & Stegemann, 2014; Zhao et al., 2020). Particles, including minerals, metals, and non-metals, on the other hand can functionalize the hydrogels in various ways (Thoniyot et al., 2015; Walters & Stegemann, 2014; Zhao et al., 2020). For instance, silver and gold nanoparticles have elicited antimicrobial effects (Thoniyot et al., 2015; Zhao et al., 2020), and hydroxyapatite and nanosilicates in collagen hydrogels have promoted osteoblastic differentiation and mineralization (Y. Li et al., 2021; Walters & Stegemann, 2014). In addition to adding other biomaterials, also growth factors have been added to collagen hydrogels to provide cells with functional cues (Liaw et al., 2018; Sarrigiannidis et al., 2021).

It is good to keep in mind that cells embedded in hydrogels also affect the properties of the hydrogels as they secrete extracellular matrix molecules and various enzymes that affect the properties of the hydrogel (Ahearne et al., 2010). Cells can also actively reshape their surrounding matrix (Shiflett et al., 2019). So, the hydrogel and cells have complementary effect on each other. Still, it is probable that the initial properties of the gel have substantial effect on the cells as it determines how the cells start to behave in their environment. Especially regarding stem cells, as their environment during early differentiation can impact the rest of the differentiation process, and after certain point the differentiation process cannot be reverted (Alm et al., 2012; Engler et al., 2006; Jaiswal et al., 1997).

## 2.4 Bone tissue

Bone is a connective tissue that supports the body, protects organs and together with skeletal muscles enables body movements (Florencio-Silva et al., 2015). Bone also holds bone marrow, and has an important role in calcium, phosphate, and magnesium metabolism (Iaquinta et al., 2019). How bone can do all this is explained when the structure of bone tissue is examined.

Basic structure of bone tissue is similar as any other tissue. It consists of cells surrounded by ECM (Koons et al., 2020). Bone ECM contains both organic (30-40%) and inorganic components (60-70%), proportion of which can vary depending on age, sex, and health conditions (de Wildt et al., 2019; X. Lin et al., 2020). The composite structure of the ECM explains the characteristic mechanical properties of bones, which enable the bone to serve as a structural support for body and organs (Koons et al., 2020). Organic components of bone provide bone with tensile strength and elasticity, whereas the inorganic mineral compounds contribute to the compression strength and rigidity of bone (de Wildt et al., 2019; Wittkowske et al., 2016).

Main structures of bone are illustrated in Figure 3. On a macroscopic level there are two distinct bone types: cortical and cancellous bone. Cortical bone forms the hard outer layer of bones, whereas cancellous bone usually is found in the bone interior (Koons et al., 2020; Morgan et al., 2018). Cortical bone accounts for 80% of bone weight and majorly contributes to mechanical properties of bones (Iaquinta et al., 2019; Morgan et al., 2018). Proportion of these bone types differ between bone types, which contributes to various anatomical roles of different bone types (Lopes et al., 2018). Trabecular bone is more minor contribution to the mechanical strength of bones, but nevertheless important, as it plays an important role in load transfer (Morgan et al., 2018; Oftadeh et al., 2015).

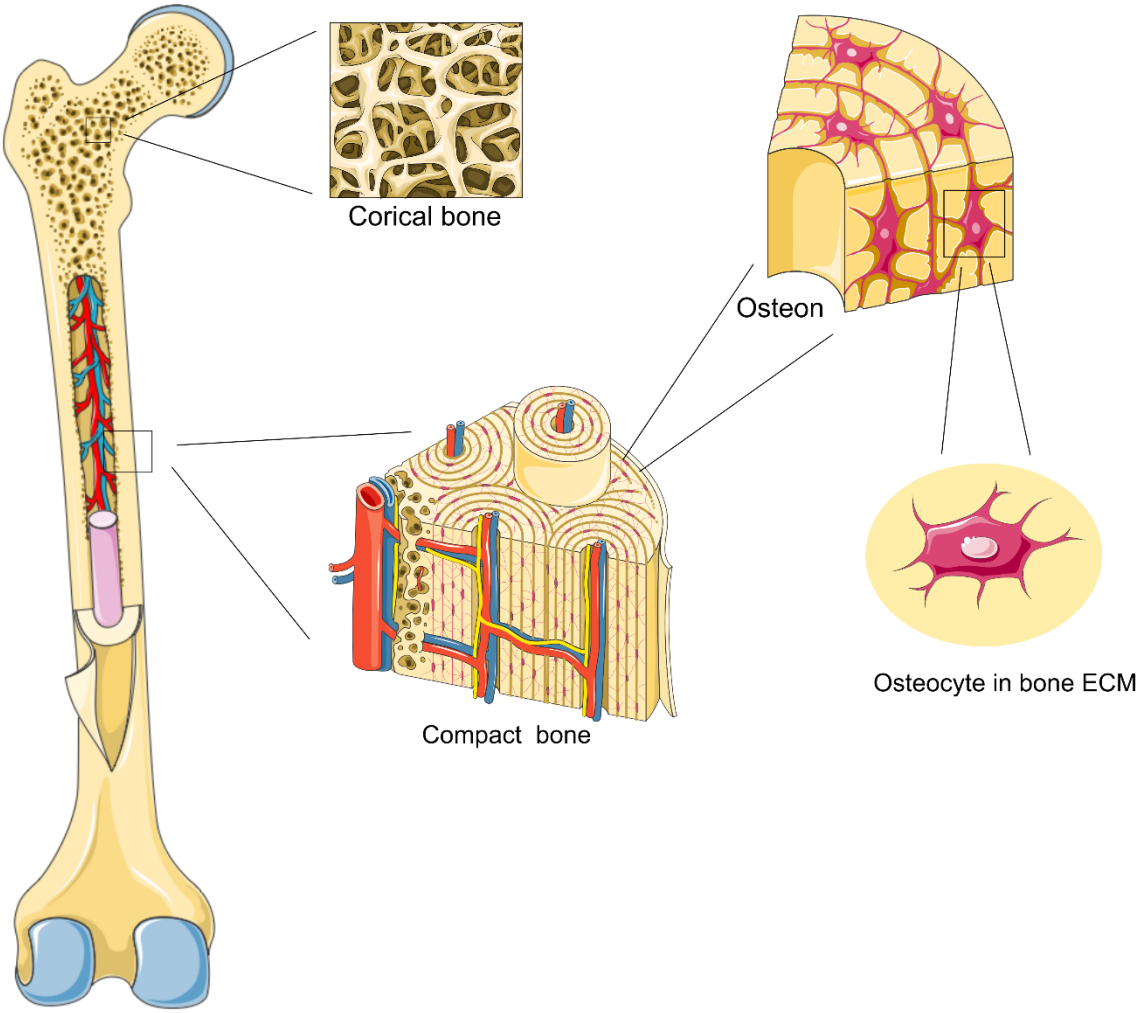


***Figure 3.*** *Organization of cortical and compact bone in long bones. Cortical bone consists of network of trabeculae. Compact bone consists of osteon, also called haversian system, where osteocytes reside inside highly organized bone ECM. Figure was created using images retrieved from Servier Medical Art.*

Cortical bone consists of functional units called Haversian systems (Reznikov et al., 2014). These cylindrical units, also called osteons, consist of 5 to 30 concentric lamellae, in middle of which travels Haversian canal, which supplies vasculature and innervation

to the bones (Lefèvre et al., 2019). In long bones multiple osteons are placed next to each other, so that their Haversian canals are aligned relative to each other and so that their direction is along the axis of the bone (Reznikov et al., 2014). Osteons in cortical bone are tightly packed next to each other, which results in low porosity (5-15%). Cancellous bone on the other hand has quite high porosity (40-95%) and low tissue density as it consists of a 3D network of intervening, differently oriented trabecular rods, and plates (Morgan et al., 2018; Oftadeh et al., 2015). Due to these structural differences, cortical bone resists compression and tension in longitudinal direction, whereas cancellous bone resists forces equally form all directions (Lopes et al., 2018; Morgan et al., 2018). Cortical and cancellous bone also differ in their relative composition and organization of the organic and inorganic matrix elements and level of hydration, which also greatly contribute their mechanical properties (Lefèvre et al., 2019; Morgan et al., 2018; Oftadeh et al., 2015).

Cancellous bone also serves other purposes than load transfer. It holds soft bone marrow in its intertrabecular space; bone marrow is home for hematopoietic stem cells and mesenchymal stem cells (Iaquinta et al., 2019; Oftadeh et al., 2015). It also has significantly higher turnover rate than cortical bone, which indicates that trabecular bone plays an important role on calcium and phosphate metabolism (Wittkowske et al., 2016). Calcium and phosphate contribute to the mechanical properties of bones, but also have important biological functions in other tissues (Blaine et al., 2015). Bone tissue can be utilized for regulating the concentration of calcium and phosphate in blood, and thus their supply for cells. If the amount of calcium and phosphate in the blood is low, bone can be resorbed to release calcium and phosphorus from it, and vice versa, if calcium and phosphate concentration is too high, more bone can be formed to take up calcium and phosphate from blood (Blaine et al., 2015). This regulation of calcium and phosphate metabolism is controlled by renal system, together with bone tissue (Blaine et al., 2015).

Bone is also remodeled for other reasons than to maintain calcium and phosphate homeostasis. Bone resorption and formation occurs during growth and after injuries, but also throughout life in response to changes in mechanical loading (García-Rodríguez & Martínez-Reina, 2017; Wittkowske et al., 2016). 2-3% of cortical bone is resorbed annually, which is sufficient for maintaining bone strength (Wittkowske et al., 2016). Disorders in the maintenance of bone homeostasis can lead to pathologies such as osteoporosis and osteopetrosis (García-Rodríguez & Martínez-Reina, 2017). Bone cells are responsible for maintaining bone homeostasis. Old bone is resorbed by osteoclasts, and new bone deposited osteoblasts, which both reside on the surface of the bone (García-Rodríguez & Martínez-Reina, 2017; Iaquinta et al., 2019). Osteoblasts have

three possible fates: they can go under apoptosis or differentiate to bone lining cells or osteocytes (Florencio-Silva et al., 2015). Osteocytes are embedded in the mineral matrix of bone and regulate the activity of osteoblasts and osteoclasts, in response to mechanical stimuli and hormonal regulation (Bonewald, 2011). Location of different bone cells in bone tissue are illustrated in in Figure 4.

Injuries and different pathologies can lead to large bone defects, which cannot be replaced by bone tissue's own regeneration capacity. Metal implants and bone transplants have been used to treat such conditions. They, however, include downsides such as need for surgical removal of the device, and possible disease transmission or unwanted immune reaction. To overcome these limitations and risks, development of tissue engineering applications to replace absent bone are under way. In general, tissue engineering combines stem cells and biomaterial scaffolds to recreate natural tissues. (Koons et al., 2020). In addition to replacing natural tissues, principle of tissue engineering has been utilized for developing 3D in vitro models. These models aim to mimic natural tissue environment to study natural tissue function, disease progression, and effect of different drugs and treatments (de Wildt et al., 2019). To engineer bone tissue, a microenvironment that resembles the natural bone as closely as possible should be created. That includes the components, structure, and different interactions contributing to the formation and maintenance of bone ECM (de Wildt et al., 2019).

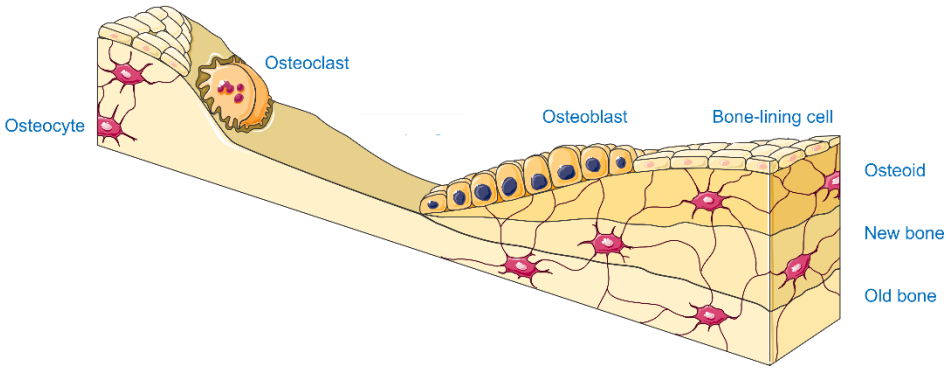


***Figure 4.*** *Different bone cell types in bone tissue. Image was retrieved from Servier Medical Art*

### 2.4.1 Bone ECM

Some aspects of bone ECM structure have already been discussed in the previous chapter. In this section different components of bone ECM and their roles in the formation and structure of bone ECM are discussed.

Organic phase of the ECM mainly consists of collagens but also contains non-collagenous proteins (NCPs), such as osteocalcin, osteopontin, and DMP1 (dentin matrix protein 1) (de Wildt et al., 2019; X. Lin et al., 2020). The inorganic, mineral phase on the other hand mainly consists of hydroxyapatite [HA; $Ca_{10}(PO_4)_6(OH)_2$] crystals, but contains also other inorganic components such as magnesium, potassium, copper, and ferrous ions (Florencio-Silva et al., 2015; Iaquinta et al., 2019).

There are two pathways of bone formation. Intramembranous ossification, where MSCs cluster and directly differentiate to osteoblast, which secrete bone ECM. Intramembranous ossification occurs after tissue injury and is responsible for the formation of the flat bones of skull and part of the clavicles. Endochondral ossification on the other hand happens through a cartilage intermediate, which later mineralizes and turns into bone tissue, as chondrocytes inside the cartilage matrix turn hypertrophic. Long bones are formed through endochondral ossification during fetal development. (Lopes et al., 2018)

Bone formation referred from now on refers to intramembranous ossification, as most of the in vitro and tissue engineering applications aiming to recreate bone tissue rely on direct differentiation of MSCs to osteoblasts, and their subsequent bone ECM deposition (Lopes et al., 2018). After the clustered MSCs have completely differentiated osteoblasts, they start to deposit collagen I and other components, which leads to formation of soft ECM, termed osteoid. Some osteoblasts get entrapped in the osteoid and start to differentiate to osteocytes (Lopes et al., 2018). Osteoblast and osteocytes then together contribute to the mineralization of the ECM (Barragan-Adjemian et al., 2006; Bonewald, 2011).

Collagen is the main organic component of the bone ECM, and a major contributor to mechanical properties of bone and an important support for bone cells. Collagen I is by far the most abundant individual protein of bone ECM, as collagens account 90% of the bone ECM proteins and collagen I accounts 90% of the total collagen; other collagens of bone include collagens III, V. Collagens assemble as already discussed under section 2.2. Briefly, triple helical collagen I tropocollagen molecules assemble to form collagen fibrils, which further aggregate to form collagen fibers. Other collagens regulate the assembly and organization of collagen I fibrils and contribute to the stability the collagen

network. Collagen fibers in freshly formed woven bone are randomly organized, whereas in mature, lamellar bone collagen fibers are oriented in arrays of parallel fibers (de Wildt et al., 2019; Wittkowske et al., 2016). Lamellae of osteons are organized so that the parallel fibers in successive lamellae are differently oriented (Wittkowske et al., 2016). Woven bone forms only when no bone matrix is already on the site, so during ontogenesis, or at the site of large bone defects (X. Lin et al., 2020; Wittkowske et al., 2016). Osteoblasts are responsible for collagen deposition, but it is not clear what leads to the alignment seen in lamellar bone (Wittkowske et al., 2016). It is postulated that alignment of collagen fibers seen in lamellar bone is driven by nanofibrillar topography of bone and dynamic mechanical forces, as they affect alignment of osteoblasts, which in turn is thought to affect collagen fibril organization (Kerschnitzki et al., 2011).

It is well known that degree of collagen cross-linking, and organization of collagen fibrils influence the mechanical properties of bone (Garnero, 2015). Organization of collagen itself contribute to the differences in the mechanical properties of tissues (J. Lin et al., 2020; Revell et al., 2021). In bone, collagen organization also influences the mineralization of the ECM as the collagen network serves as template and nucleation site for HA, affecting the size and distribution of the HA crystals (Alford et al., 2015; Tavafoghi & Cerruti, 2016; Wittkowske et al., 2016).

Osteoblasts also are responsible for the deposition of components required for collagen mineralization. They produce the components of HA, phosphate and calcium ions, but also various enzymes and NCPs essential for the mineralization process (Wittkowske et al., 2016). ALP is a membrane bound enzyme produced by osteoblast, which is thought to contribute to the mineralization process by increasing local phosphate concentration and by breaking down mineralization inhibitors (Wittkowske et al., 2016). NCPs contribute the mineralization by regulating the formation of collagen network, but also serving as nucleators for HA formation and facilitating the interactions between mineral and collagen (Alford et al., 2015; Tavafoghi & Cerruti, 2016). Some NCPs bind to collagen and attract phosphate and calcium ions to raise their local concentrations high enough for the nucleation to occur (Tavafoghi & Cerruti, 2016). For instance, NCPs such as osteocalcin, bone sialoprotein, asporin, and keratocan are known to contribute to the mineralization of bone (Alford et al., 2015; X. Lin et al., 2020). Some osteocyte secreted proteins such as DMP1 also are involved in bone mineralization process (T. Liu et al., 2019).

HA crystallization initiates inside, or at immediate proximity, of the gap zones of collagen fibrils as crystal plates, after which mineralization proceeds along collagen fibrils eventually filling all intrafibrillar space (D. Kim et al., 2016; Wittkowske et al., 2016). In

addition to this intrafibrillar mineral, mineral is also found in the spaces between the fibrils (D. Kim et al., 2018; Y. Liu et al., 2011). Extrafibrillar mineralization occurs in vitro through a spontaneous calcium phosphate aggregation which first results in formation on mineral spheres which later are deposited on the outer surface of collagen matrix, inhibiting intrafibrillar mineralization (D. Kim et al., 2016). Occurrence of intrafibrillar mineralization is probably important for bone integrity, as it significantly contributes to the mechanical properties of mineralized matrices (Y. Liu et al., 2011).

As discussed in previous chapters, bone ECM formation is a process where collagen, NCPs and inorganic components interact to form an organized, mineralized ECM, with high mechanical properties. All these components are deposited by osteogenic lineage cells (de Wildt et al., 2019; Wittkowske et al., 2016). It is, however, worth noting that the ECM also has various effects on the behavior and differentiation of osteogenic lineage cells (X. Lin et al., 2020). Also, other environmental factors likely affect the bone mineral formation; for example, modest fluid shear stress has shown to enhance the formation of hydroxyapatite (Niu et al., 2016). All in all, it can be said that the bone ECM formation is a dynamic process between the cells and their environment.

## 2.5 Osteocytes

Osteocytes are the most prominent cell type of bone tissue comprising up to 95% of bone cells (Skottke et al., 2019). They are embedded in the mineral matrix of bone (Bernhardt et al., 2019). Osteocyte morphology is characterized by their rounded cell body and dendritic processes that connect them to other osteocytes, forming an extensive network in the bone ECM (Figure 5) (Bonewald, 2011). The network reaches from outer surface of the bone to the surface of bone marrow, allowing osteocytes to forward messages to other cells found in bone tissue (Atkinson & Delgado-Calle, 2019). Osteocytes themselves reside in small chambers in ECM and the dendritic processes travel in small ducts, called lacunae and canaliculi, respectively (Bonewald, 2011; Reznikov et al., 2014). As these spaces cause discontinuations in bone ECM, there is a structural relationship between osteocyte network and bone tissue (Kerschnitzki et al., 2011).

Osteocytes also are essential for maintaining bone tissue homeostasis; they regulate the activity of bone forming osteoblasts and bone resorbing osteoclasts, mainly by secreting sclerostin and receptor-activator of NF-kB ligand (RANKL) (Divieti Pajevic & Krause, 2019). Sclerostin regulates bone homeostasis by affecting Wnt/β-catenin pathway (Skottke et al., 2019). It acts as a Wnt inhibitor and inhibits bone formation of osteoblasts. Decrease in mechanical stress upregulates RANKL expression, which leads to bone

resorption due to RANKL being an osteoclastogenesis promoting factor (Divieti Pajevic & Krause, 2019; Skottke et al., 2019).

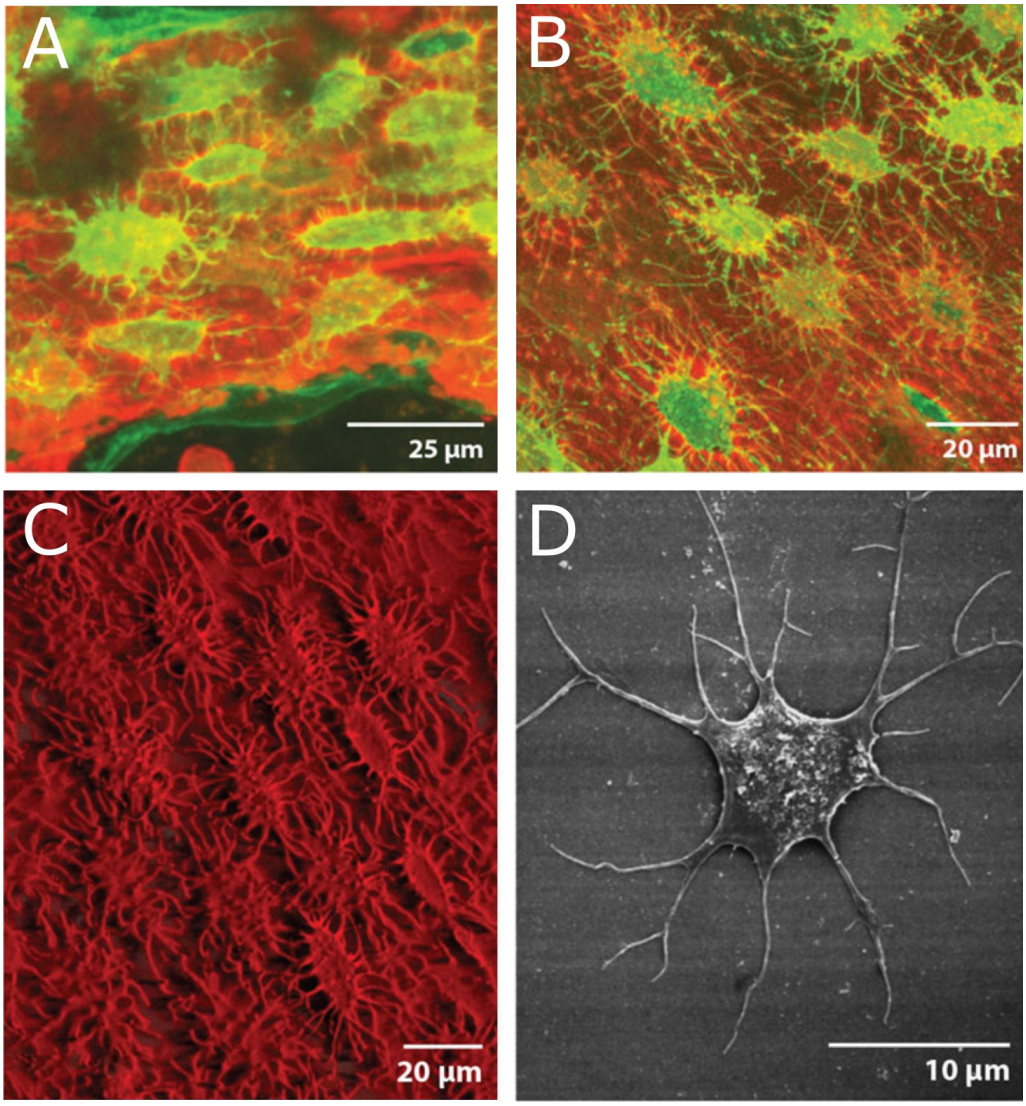


***Figure 5.*** *Natural osteocyte morphology. Fluorescent microscope images of osteogenic line OmGFP66 cells in bone bone-like structure (A) and osteocytes in 7-day-old mouse calvarium. Three-dimensional structure of avian osteocytes. Digital reconstruction of fluorescent microscope image and (C) field emission scanning electron microscope image of single osteocyte. Modified from Robling & Bonewald, 2020.*

Secretion of sclerostin and RANKL is regulated by mechanical cues detected by osteocytes. Increase in mechanical load suppresses sclerostin expression, whereas decrease in load upregulates it (Divieti Pajevic & Krause, 2019). Osteocytes sense mechanical stress via bending of the matrix microenvironment and through fluid flow on the in canaliculi; fluid shear stress on the surface of the bone is involved in the mechanosensory response of osteocytes as well (Wittkowske et al., 2016). In addition to

mechanical cues, both RANKL and sclerostin expression of osteocytes is molecularly regulated by parathyroid hormone (PTH) (Divieti Pajevic & Krause, 2019). Oxygen tension is also suggested to be important for osteocyte function. In vivo osteocytes are in low oxygen tension environment and hypoxic environment has shown to increase osteocyte mediated bone formation through HIF-1α signaling (Stegen et al., 2018).

Besides regulating activity of osteoblasts and osteoclasts, osteocytes contribute to bone structure by secreting NPCs that participate in mineralization of collagen (de Wildt et al., 2019). Osteocytes also can themselves deposit or resorb bone matrix in certain situations (Robling & Bonewald, 2020). This process, termed perilacunar, remolding also has important implications regarding bone homeostasis (Yee et al., 2019).

Irregularities concerning osteocytes are associated with different pathologies.For instance, increase in osteocyte apoptosis and necrosis, reduction in the osteocyte density, and abnormalities in the osteocyte network are associated pathological bone loss (Andreev et al., 2020; Ru & Wang, 2020; Tiede-Lewis & Dallas, 2019; Zarrinkalam et al., 2012). Loss of osteocytes can be caused by changes related to aging, excess of glucocorticoids, microfractures, inflammation, unloading, sex hormone deficiency, and by multiple myeloma (MM), a cancer of the bone marrow (Atkinson & Delgado-Calle, 2019; Giuliani et al., 2012; Ru & Wang, 2020). Relationship between MM and osteocytes extend beyond MM induced osteocyte death. Presence of MM cells also causes increased RANKL and sclerostin expression by osteocytes, which together with the osteocyte apoptosis leads to increased bone resorption and thus contribute to the formation of myeloma-induced bone disease (Atkinson & Delgado-Calle, 2019). The apoptotic effect of MM cells on osteocytes is inflicted through Notch signaling, which is a common type of cell-cell signaling (Delgado-Calle et al., 2016). The Notch signaling also leads to increased MM cell proliferation, which means that osteocyte-MM cell interactions also contribute to the progression of MM. Osteocytes are also involved in the progression of breast and prostate cancer, as they play a role in the bone remodeling needed for their bone metastasis (Atkinson & Delgado-Calle, 2019).

### 2.5.1 Osteocyte differentiation

Transformation to osteocyte phenotype is characterized by decreased cell motility and proliferation, and changes in cell morphology and gene expression profile (Dallas et al., 2013; Nasello et al., 2020; Shiflett et al., 2019). It has been already established that osteocytes descent from osteoblasts that get embedded in the bone ECM (Bonewald, 2011). There is, however, no clear consensus on whether the embedding is a passive or an active process (Bonewald, 2011; Robling & Bonewald, 2020). There are indications

of multiple different ways the cells can get entrapped in the collagen matrix of developing bone (Shiflett et al., 2019).

Anyhow, after the cells get entrapped in the collagen matrix, they start to acquire osteocyte phenotype and to modify their surrounding matrix. One of the first changes to occur is the formation of dendritic processes, which is thought to be dependent on the expression of podoplanin (PDPN), also called E11 and gp38 (Bonewald, 2011; Staines et al., 2017). At the same time, the osteoblasts going through osteocyte differentiation participate on the matrix mineralization process (Bonewald, 2011). Prior to the mineralization, differentiating osteocytes also have shown to modify the collagen matrix (Shiflett et al., 2019). Matrix proteins, such as osteocalcin, also known as bone gamma-carboxyglutamate protein (BGLAP), and DMP1 are known to play an important role in the bone ECM mineralization (de Wildt et al., 2019; Lu et al., 2011; Zoch et al., 2016). As osteoblasts mature and begin to differentiate to early osteocytes, the expression of early osteoblast markers, such as RUNX2 and ALP is downregulated (Bonewald, 2011; Dallas et al., 2013; Komori, 2019). Osteocalcin is already expressed by late osteoblasts, whereas expression of PDPN and DMP1 is considered to start during early osteocyte phenotype (Bonewald, 2011; Komori, 2019). Osteocalcin and PDPN continue to be expressed by differentiating osteocytes but are not expressed by mature osteocytes embedded in mineralized bone ECM (Dallas et al., 2013). DMP1 on the other hand is expressed in the mature osteocytes as well (Dallas et al., 2013). Phosphate regulating neutral endopeptidase on the chromosome X (PHEX) expression is also turned on during the earlier stages of osteocyte differentiation process and is expressed in matures osteocytes as well. Matrix extracellular phosphoglycoprotein (MEPE), fibroblast growth factor 23 (FGF23), RANKL, and sclerostin are considered to be expressed exclusively by mature osteocytes (Robling & Bonewald, 2020). In the previous sections it was established that the environment of the cells plays an important part in the cell differentiation process. After this chapter it is obvious that osteoblasts and differentiating osteocytes also actively modify their environment. Thus, it seems that the osteocyte differentiation is a quite dynamic process between the differentiating cells and their environment.

### 2.5.2 Acquiring osteocytes

Since osteocytes are the most prominent cell type in bone, contribute to structure of bone, play a key role in maintaining bone homeostasis, and are involved in many bone-related pathologies, they should be included when modeling bone tissue in vitro. Presence of osteocytes would also be beneficial from the viewpoint of regenerative

medicine; osteogenic differentiation of MSCs and bone formation have been showed to enhanced when osteocytes are in the proximity (Birmingham et al., 2012; Woo et al., 2011). So far, osteocytes used in in vitro studies have been mostly derived from osteocyte-like murine cell lines (MLO-Y4 and IDG-SW3) (Bernhardt et al., 2019; Prideaux et al., 2016; Vazquez et al., 2014). Using immortalized cell lines has various downsides (Prideaux et al., 2016) and mouse bone tissue does not completely recapitulate all structures of human bone (T. Liu et al., 2019). Therefore, using human osteocytes would be more preferrable for modeling bone tissue function.

Primary osteocytes have been isolated from human and mouse bone, using repeated collagenase II and EDTA digestions (Prideaux et al., 2016; Stern et al., 2012). Osteocyte phenotype of the isolated cells were characterized by their osteocyte morphology and expression of osteocyte marker genes; isolated human primary osteocytes also were shown to respond to PTH similarly as osteocyte in natural bone ECM (Prideaux et al., 2016; Stern et al., 2012). Isolated primary osteocytes tend to dedifferentiate and lose their osteocyte phenotype after the isolation (Bernhardt et al., 2019; Honma et al., 2015; Skottke et al., 2019). Therefore, isolated primary osteocytes have been embedded in collagen gels to maintain their osteocytic phenotype (Bernhardt et al., 2019; Honma et al., 2013, 2015; Skottke et al., 2019). Collagen hydrogels are considered a logical choice platform for osteocyte cultures as they resemble the natural environment of osteocytes (Bernhardt et al., 2019; Honma et al., 2013; Nasello et al., 2020; Skottke et al., 2019; Vazquez et al., 2014). Mouse primary osteocytes embedded in collagen I, expressed osteocyte marker genes after 9 days of cultivation in osteogenic media, but the expression of mature osteocyte markers *SOST* and *Fgf23* were constantly downregulated during the culture period (Honma et al., 2015). The forementioned study found that using composite hydrogel of collagen and Matrigel, instead of pure collagen gel, and using lower serum concentration in the media diminished the downregulation of *SOST* and *Fgf23.* Addition of Matrigel to the hydrogel composition also led to more osteocytic cell morphology. Human primary osteocytes embedded in collagen hydrogels have shown osteocytic morphology and positive staining for osteocyte marker DMP1 (Bernhardt et al., 2019; Skottke et al., 2019). Unlike with mouse primary osteocytes, no downregulation of late osteocyte marker genes was seen. Vice versa, expression of osteocyte marker genes *SOST* and *MEPE*, had increased after 7 days of culture in collagen gels (Bernhardt et al., 2019; Skottke et al., 2019). Even though these results seem promising, there is downsides for using isolated primary osteocytes as a main source for osteocytes. Because osteocytes lie in the mineral matrix of the bone, isolating osteocytes from bone samples is a time-consuming multistep process (Bernhardt et al.,

2019). The yield of osteocytes using the isolation protocol has also been relatively low (Bernhardt et al., 2019), which is a major downside as osteocytes are a non-proliferative cell type (Nasello et al., 2020). Hence, primary osteocyte cannot be acquired in large quantities from one bone sample.

Osteocyte-like cells have also been acquired by differentiating from osteoblastic cells. When cultured in collagen I hydrogels, primary human pre-osteoblast and mature osteoblasts have shown to acquire osteocytic features, including osteocyte-like morphology, positive immunostaining for osteocyte proteins, and expression of osteocyte marker genes (Bernhardt et al., 2019; Nasello et al., 2020; Skottke et al., 2019). The relative expression levels of osteocyte marker genes in the osteoblasts cultured in collagen I gels were similar with primary human osteocytes isolated with the protocol described by Prideaux et al. and cultured in collagen gels (Skottke et al., 2019). Additionally, osteoblasts cultured on top collagen hydrogels have shown to invade the hydrogels and differentiate towards osteocyte phenotype (Uchihashi et al., 2013). These results show that in addition to maintaining osteocytic phenotype, 3D collagen I environment can also promote osteocytic differentiation. One study also showed a comparison of collagen hydrogel and 2D culture, where it was found that collagen gel supported osteocyte differentiation of osteoblast better than conventional 2D culture (Sawa et al., 2019). The positive effect of 3D environment and collagen on osteocyte differentiation is demonstrated individually as well (Boukhechba et al., 2009; Q. Sun et al., 2017; Yang et al., 2020).

Other approaches than using pure collagen gels have also been shown to promote osteocytic differentiation of osteoblastic cells. The importance of 3D environment for osteocyte differentiation has been demonstrated by studies culturing human and mouse primary osteoblast within biphasic calcium phosphate particles (Boukhechba et al., 2009; Q. Sun et al., 2017). These culture set-ups allowed to control the distance between the cells and prevented proliferation of the cells, as the cells were entrapped in the spaces between the microbeads. These set-ups led to enhanced osteocyte differentiation compared to conventional 2D cultures. Another study showed that culturing mouse pre-osteoblastic cells in microbial transglutaminase (mtgase) gelatin hydrogels in media absent of osteogenic supplements induces their osteogenic differentiation (McGarrigle et al., 2016). Even though these other culture set-ups have shown to promote osteocyte differentiation, using collagen hydrogels still possesses some notable benefits. Especially if considering modeling osteocyte function in wider, tissue-wide context, or modeling the function of bone tissue, collagen hydrogel is a superior option as collagen offers cells natural binding sites and also otherwise an environment that resembles the

natural tissue ECM. For instance, modification of collagen network and mineral deposition inside the collagen fibers are integral for bone ECM development and occur simultaneously and interdependently with osteocyte differentiation (de Wildt et al., 2019; Robling & Bonewald, 2020; Shiflett et al., 2019). Both these processes can, at least in theory, occur in collagen hydrogels, as they consist of similar collagen networks as native tissues (Antoine et al., 2014).

As MSCs can differentiate to osteoblasts in vitro and osteoblasts can be differentiated to osteocyte-like cells in vitro, in vitro differentiation of osteocytes from MSCs should also be possible. MSCs would be a convenient source for osteocytes, as MSCs can be isolated from multiple tissues, and they can be expanded in large quantities. There are no reports of osteocyte differentiation of MSCs in hydrogel or microbead set-ups described above. Spheroid culture of MSCs, however have shown to result in upregulation of mature osteocyte marker genes (J. Kim & Adachi, 2021).

# 3. OBJECTIVES

The aim of the study was to develop effective environment for osteocyte differentiation from hBMSC. To achieve this, hBMSCs were cultured in collagen I hydrogel in various culture conditions. Collagen I hydrogel was considered an optimal environment for osteocyte differentiation due to its resemblance to natural environment of differentiating osteocytes, and as collagen I hydrogel has shown to promote osteocyte differentiation of osteoblasts (Bernhardt et al., 2019; Nasello et al., 2020; Sawa et al., 2019; Skottke et al., 2019).

It was studied if longer culture period or osteogenic pre-differentiation step on 2D surface would enhance osteocyte differentiation of hBMSCs cultured in collagen I hydrogel. Also, the effect of adding S53P4 bioactive glass extract to the culture medium was studied. S53P4 extract wad hypothesized to promote osteocyte differentiation of hBMSCs, as it has previously shown to enhance osteogenic differentiation of MSCs (Ojansivu et al., 2015).

# 4. MATERIALS AND METHODS

## 3.1 Medium compositions

Four medium compositions were used in this study. These included basic medium (BM), osteogenic medium (OGM), osteocyte medium (OCM), and S53P4 BAG extract OCM. BM consisted of Alpha Minimum Essential Medium (αMEM, Gibco™, Thermo Fisher Scientific, USA), 5% human serum (HS), and 100 U/mL penicillin and 100 µg/mL streptomycin (P/S, Lonza / Biowhittaker, Thermo Fisher Scientific, USA). OGM was prepared by supplementing BM with 200 µM ascorbic acid (Sigma Aldrich, USA), 10 mM β-glycerophosphate (Sigma Aldrich, USA), and 5 nM dexamethasone (dex, Sigma Aldrich, USA). OCM had a similar composition as OGM with the exceptions of omitting dex and using 2% HS instead of 5%, as osteocytes cultures have shown to benefit from lower serum concentrations (Honma et al., 2015; Prideaux et al., 2016).

S53P4 extract OCM was prepared using commercially available SP53P4 granules (0.5-0.8 mm, Bonalive Biomaterials, Finland). The granules first were disinfected in absolute ethanol (Altia Oyj, Finland) for 10 min and then in 70% ethanol for 10 min, after which they were left to dry for 2 hours. The 70% ethanol was diluted from absolute ethanol using sterile $H_2O$. Disinfected granules were incubated in αMEM containing 1 % P/S for 24 h at concentration of 87.5 mg/ml. S53P4 comprises oxides of silica, sodium, phosphorus, and calcium and it has shown to release ions of these metals to the medium using the extraction protocol described above (Ojansivu et al., 2015). After the iextraction, the medium was sterile filtered and human serum, ascorbic acid, and β-glycerophosphate were added to generate OCM. The S53P4 extract OCM was stored for maximum of 14 days (4 ˚C).

## 3.2 hBMSC isolation and culture

hBMSCs used in this study were isolated from a trabecular bone sample obtained from a male patient, aged 59, during a surgical procedure performed at Coxa Hospital for Joint Replacement. The sample was obtained with patient's written consent and under approval of the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R15174).

hBMSCs were isolated as previously described (X. Wang et al., 2019). Trabecular bone sample was rinsed in Dulbecco's phosphate buffered saline (DPBS, Gibco™, Thermo Fisher Scientific, USA) to obtain the bone marrow. Volume ratio of bone to DPBS was approximately 1:3. The solution containing the bone marrow in DPBS was rinsed through

a 100 µl cell strainer. The strainer was rinsed further with DPBS, which was then added to the bone marrow aspirate dilution. Volume of the DBPS used for the rinsing was equal to the volume of the bone sample. Mononuclear cells were separated by creating a density gradient, using Histopaque-1077. 2.6 ml of Histopaque-1077 (Sigma-Aldrich, USA) per 1 ml of the bone sample volume was added to a 50 ml centrifuge tube. The diluted bone marrow was layered on top of the Histopaque-1077 and centrifuged at 800 g for 20 min. After the centrifugation, mononuclear cells were collected from the liquid interface and washed twice with αMEM (centrifugation at 400 g for 5 min). After the second wash, cell pellet was resuspended into BM.

Isolated cells were plated on tissue culture polystyrene (TCPS) and expanded in BM supplemented with 5 µg/ml human fibroblast growth factor 2 (hFGF2, Miltenyi Biotec, Germany) at 37°C and 5% $CO_2$. Medium was changed twice a week. After reaching 80% confluency, cells were detached using TrypLE (Gibco™, Thermo Fisher Scientific, USA) and cryopreserved in vapor phase nitrogen. Surface marker expression of the cells was characterized to confirm the stemness and mesenchymal origin of the cells.

After the cryopreserved hBMSCs were thawed, they were expanded for one week in BM with 5 µg/ml hFGF2 on TCPS, after which they were detached using TrypLE and resuspended in BM. hBMSCs suspended in BM were then cultured in various conditions, in an incubator at 37°C and 5% $CO_2$. Detailed descriptions of the cell culture conditions used in this study are given in the following sections and on table 1.

## 3.3 Osteogenic pre-differentiation of hBMSCs

Osteogenically pre-differentiated hBMSC were acquired by culturing hBMSC on TCPS for one week in osteogenic medium (OGM). After the pre-differentiation period, the hBMSCs were incubated in collagenase I (Gibco™, Thermo Fisher Scientific, USA) solution (1.5 mg/ml) for 15 min to degrade the collagen matrix formed by the cells, after which the cells were detached using TrypLE, and resuspended in BM.

## 3.4 hBMSCs on collagen I coated TCPS

Rat tail-derived collagen I solubilized in acetic acid (3mg/ml, Gibco™, Thermo Fisher Scientific, USA) was further diluted with acetic acid to concentration of 50 µg/ml. Standard 6-well plates (Nunc™, Thermo Fisher Scientific, USA) were coated with collagen by adding the 50 µg/ml solution into 6-well plate wells at a concentration of 5 µg/cm$^2$. After 1 hour incubation, the solution was removed from the wells and hBMSCs suspended in BM (section 3.2) were plated on the plated collagen I coated 6-well plates

at a density of 2500 cells/cm$^2$. Cells were cultured for 21 days in BM or OGM. 3 ml of medium per well were added; medium was changed twice a week.

## 3.5 hBMSCs in collagen I hydrogels

Collagen gels were prepared by mixing the rat tail-derived collagen I solubilized in acetic acid (3 mg/ml) with 10X DPBS (Gibco™, Thermo Fisher Scientific, USA) and sterile $H_2O$, to obtain 2.5 mg/ml collagen solutions in 1X DPBS. The resulting solutions was neutralized with NaOH to obtain collagen solution with physiological pH (6.5-7.5). hBMSCs and osteogenically pre-differentiated cells suspended in BM (sections 3.2 and 3.3, respectively) were centrifuged and resuspended in the collagen solution at a concentration of 2.5 x $10^4$ cells/ml. Cell-laden solutions were plated into µ-Slide 8 Well plates (ibidi, Germany) and standard 24-well plates (Nunc™, Thermo Fisher Scientific, USA); volumes plated were 100 µl and 1000 µl, respectively. The cell-collagen solutions were incubated for 30 min at 37°C and 5% $CO_2$ for the gelation to occur, after which OCM or S53P4 extract OCM was added on top of the cell-laden hydrogels. hBMSCs directly embedded in collagen hydrogel were cultured for 21 and 42 days and osteogenically pre-differentiated hBMSCs were cultured for 21 days. Cells in collagen gels were cultured in OCM, both with and without S53P4 extract in the culture medium. For the µ-Slide 8 Well plate wells 200 µl well medium per well and for the 24-well plate wells 1 ml medium per well were added; in all conditions, medium was changed twice a week.

***Table 1.*** *List of cell culture conditions used in the study. In all conditions, cells were cultured at 37°C and 5% $CO_2$*

| Cell type | Pre-differentiation | Culture platform | Cell density | Culture period | Medium | S53P4 in medium |
|---|---|---|---|---|---|---|
| hBMSC | - | On collagen I coated TCPS | 2500 cells/cm$^2$ | 21 days | BM | - |
| hBMSC | - | On collagen I coated TCPS | 2500 cells/cm$^2$ | 21 days | OGM | - |
| hBMSC | - | In collagen I hydrogel | 2.5 x $10^4$ cells /ml | 21 and 42 days | OGM | - |
| hBMSC | - | In collagen I hydrogel | 2.5 x $10^4$ cells /ml | 21 and 42 days | OGM | + |
| hBMSC | 7-days on TCPS in OGM | In collagen I hydrogel | 2.5 x $10^4$ cells /ml | 21 days | OGM | - |
| hBMSC | 7-days on TCPS in OGM | In collagen I hydrogel | 2.5 x $10^4$ cells /ml | 21 days | OGM | + |

## 3.6 Isolation of primary osteocytes

Isolation of human primary osteocytes was done according to a previously described protocol (Prideaux et al., 2016). In total, six digestions were performed, using collagenase II solution (2mg/ml in αMEM, 1% P/S) and EDTA (Sigma-Aldrich, USA) solution (0.005M in Hanks′ Balanced Salt solution, (Gibco™, Thermo Fisher Scientific, USA), 1 % P/S, 0,1% bovine serum albumin (BSA, Sigma-Aldrich, USA)). First, three collagenase II digestions were performed (digestions 1, 2, and 3), after which EDTA digestion was performed (4), followed by another collagenase digestion (5) and a final EDTA digestion (6). All the digestions are performed for 25 min at 37˚C with mild agitation. Digests were collected to three fractions (digests 1&2, 3&4, and 5&6) and pelleted (centrifugation 1000 rpm, 5 mins) after which RNA isolation was performed as described in the section 3.8.

## 3.7 Immunocytochemistry

hBMSCs in collagen gels in µ-Slide 8 Well plates were fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) for 1 h at room temperature (RT). After the fixing, the constructs were washed twice with DPBS for 10 min and permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, USA) in DPBS for 10 min at RT. Permeabilized gels were blocked with a solution of 1% BSA and 0.1% Triton-X in DPBS for 2 h at RT. After the blocking, samples were washed with the blocking solution for 5 min at RT. Cells were stained with 2 µg/ml rabbit monoclonal anti-DMP1 (TaKaRa Bio, Japan) antibody in the blocking solution for 70 h at 4°C.

After the incubation one short wash and three long washes (2-3 h) were performed with 1% BSA in DPBS at RT. Secondary antibody staining was done by incubating with 5 µg/ml donkey anti-rabbit Alexa Fluor 488 antibody in 1% BSA in DPBS for 20 h at 4°C.

After the secondary antibody staining, the samples were washed multiple times with DPBS at RT. To stain the cell nuclei and the actin cytoskeleton, the samples were incubated with 1:1500 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) and 1µg/ml tetramethylrhodamine (TRICT) conjugated phalloidin (Sigma-Aldrich, USA) in DPBS for 2 h at RT. Finally, samples were washed three times (30 min and 1h at RT, and overnight at 4°C) with DPBS. Fresh DPBS was added, and cells were imaged with LSM 780 confocal laser scanning microscope (Zeiss, Germany), using a 10x air objective lens.

## 3.8 Gene expression analysis

Relative expression of *RUNX2*, *BGLAP* and *PDPN*, were quantified by a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR or qPCR). First, cells for the RNA isolation were harvested from the collagen cell-laden hydrogels cultured in 24-well plates, with a collagenase II digestion procedure described in Bernhardt et al. 2019. Hydrogels were incubated in collagenase II (Gibco™, Thermo Fisher Scientific, USA) solution (3 mg/ml in αMEM, 10% fetal bovine serum, 1% P/S), for 90 min, after which the digests were transferred to centrifuge tubes and the cells were pelleted by centrifugation (1000 rpm, 5mins); digest from two 24-well plate wells were combined for one sample. Cell pellets were washed twice with DPBS (centrifugation 1000 rpm, 5 mins), after which the RNA isolation was done using Nucleospin™ RNA isolation kit (Macherey-Nagel, Germany) according to the kit instructions. For the hBMSCs cultured on the collagen coated TCPS and osteocytes isolated from trabecular bone sample, RNA was isolated according to Nucleospin RNA isolation kit instruction. RNA concentration and purity was measured using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Isolated RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific, USA). A maximum of 100 ng/µl RNA was transcribed. PCR reaction mixtures contained SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA), 300nM forward and reverse primers (metabion, Germany) and at most 50 ng of cDNA. qPCR reaction was run with QuantStudio™ 12K Flex Real-Time PCR System -instrument (Thermo Fisher Scientific,USA) with initial enzyme activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and anneal and extend at 60°C for 60 seconds. Primers sequences used in the PCR reactions are listed on table 2. Relative

***Table 2.*** *Primer sequences used for qPCR reaction*

| Gene | Sequence | Product size (bp) | Accession number |
|---|---|---|---|
| RPLP0 | Forward: AATCTCCAGGGGCACCATT<br>Reverse: CGCTGGCTCCCACTTTGT | 74 | NM_001002 |
| RUNX2 | Forward: CCCGTGGCCTTCAAGGT<br>Reverse: CGTTACCCGCCATGACAGTA | 73 | NM_001024630.4 |
| BGLAP | Forward: CCACCGAGACACCATGAGAGCC<br>Reverse: TTGCTGGACTCTGCACCGC | 108 | NM_199173.6 |
| PDPN | Forward: AACGTGGCCACCAGTCACTC<br>Reverse: GGGCGAGTACCTTCCCGAC | 180 | NM_006474.5 |

gene expression was calculated using Pfaffl method (Pfaffl, 2001); RPLP0 was used as a normalizer gene.

## 3.9 Protein detection assays

Conditioned medium from each well of 24 well-plate and 6-well plate was collected into 2 ml Eppendorf tubes and centrifuged for at 1000 g for 10 min at 4°C. The supernatant was stored at -80°C before analysis.

For detection of ALP activity, the conditioned medium samples were mixed with 4-Nitrophenyl phosphate (NPP, Sigma-Aldrich, USA) and 2-amino-2-methyl-1-propanol (AMP, Sigma-Aldrich, USA), and incubated for 10 min at 37 °C, after which the reaction was stopped with NaOH. Mixing ALP with AMP and NPP results in colorimetric reaction (Sabokbar et al., 1994). Resulting absorbance was measured using Wallac 1420 Victor$^2$ Microplate Reader (Perkin Elmer, USA), at 405 nm.

The concentration of sclerostin in the conditioned medium samples was measured with commercial sclerostin ELISA kit (Invitrogen$^{TM}$, Thermo Fisher Scientific, USA), according to the kit's instructions. Briefly, the conditioned medium samples were added to 96-well plate wells, with antibodies for sclerostin attached to the well bottom. Also, sclerostin stock solution was diluted to different concentrations, and the sclerostin dilutions were added into the 96-well plate wells to generate a standard curve for quantifying the sclerostin amount in the samples. After 2.5 hours incubation at RT, samples and sclerostin dilutions were removed and biotin conjugate solution was added to the wells and incubated in the wells for 1 hour at RT. During this time the biotin conjugates bind to the sclerostin antigens that were bound to the antibodies presented on the well bottoms. Next biotin conjugate solution was removed, and Streptavidin-HRP solution was added to the wells and incubated in the wells for 45 min at RT, followed by removal of the Streptavidin-HRP and addition of TMB substrate. Streptavidin-HRP binds to the biotin conjugates, and its reaction with the TMB substrate leads to a colorimetric reaction. After 30 min incubation, stop solution was added to the wells, and absorbance value of each well was measured using Wallac 1420 Victor$^2$ Microplate Reader, at 405 nm.

In both assays, the intensity of the absorbance is in relation to the enzyme activity or protein concentration, which allows to determine the enzyme activity of ALP and concentration of sclerostin based on the measured absorbance. In both assays the mediums used for culturing the cells were used as blank samples and their values were deducted from the measured absorbances to remove the effect of the background absorbance.

## 3.10 Data handle and statistical analysis

Confocal microscope images were processed using Imaris image analysis software (9.0), and Fiji ImageJ software (2.1.0). Image panels and illustrative figures were created using Inkscape vector graphics editor (1.0.2.2)

Results of the gene expression analysis, and the ALP and sclerostin detection assays are reported as bar charts, where the bar represents mean value of the three biological replicates from each cell culture condition, and error bars represent standard deviation of the three replicates. All the charts were generated using Microsoft® Excel® for Microsoft 365 MSO (16.0.14326.20850).

With each analysis, two-tailed Welch's t-test was used to assess whether there was a statistically significant difference in the mean values of BMSCs cultured on collagen coated TCPS in OGM., and the other cell culture condition. P-values $< 0.05$ were considered statistically significant. It was assumed that the values of each analysis come from population following standard distribution. The test was performed using R Statistical software (4.0.3).

# 5. RESULTS

Following abbreviations are used for the culture conditions used in the study from here on. CC-BM: hBMSCs cultured on collagen coated TCPS in BM; CC-OGM: hBMSCs cultured on collagen coated TCPS in OGM; D21: cultured 21 days in collagen hydrogel in OCM; PD: osteogenically pre-differentiated hBMSC cultured 21 days in OCM; D42: hBMSCs cultured 42 days in collagen hydrogel in OCM. With hBMSCs cultured in collagen gels (D21, PD, D42), +/- after the condition indicates whether S53P4 extract was added to the culture media. With CC-BM and CC-OGM, S53P4 extract media was not used.

When the effect of the pre-differentiation, longer culture period, and S53P4 extract on the protein expression and cell morphology are discussed in sections 4.2., 4.3, and 4.4., D21- is used as a reference condition. D21- can be considered as a baseline condition for the hBMSCs cultured in collagen hydrogels, as in all the conditions BMSCs are cultured embedded in collagen at least for 21 days.

## 4.1 hBMSCs in collagen at higher cell density

Originally BMSCs were cultured in collagen hydrogels at a density of $10^5$ cells/ml. This resulted as a similar shrinkage of the collagen gels as with primary osteocytes embedded in collagen gels shown by Bernhardt et.al, 2019 (Figure 6). Due to the shrinkage, cell concentration in subsequent experiment were lowered to 2.5 x $10^4$ cells/ml, which prevented the shrinkage of the collagen hydrogels. All the results presented in the following sections are from the experiment done with cell density of 2.5 x $10^4$ cells/ml.

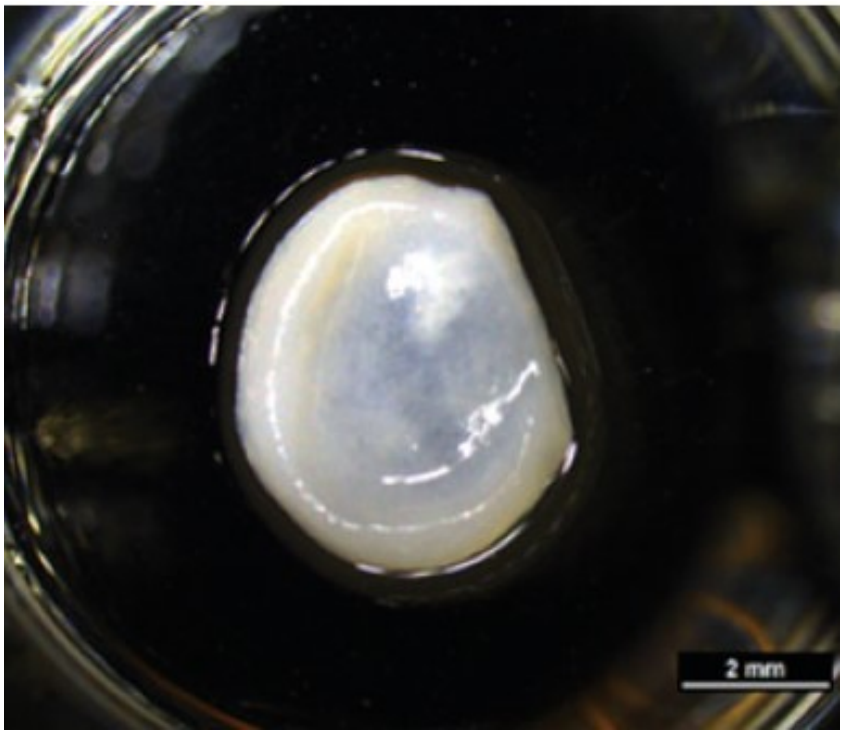


***Figure 6.*** *Collagen hydrogel shrunken, as a result of cultivating $10^5$ osteocytes/ml embedded in the gel, demonstrated by Bernhardt et al. Similar shrinkage was seen in this study as well. Image modified from Bernhardt et al., 2019.*

## 4.2 Confocal microscope imaging

Results of confocal microscope imaging are presented in Figures 7 and 8. Figure 7 shows hBMSCs cultured in collagen hydrogel without S53P4 extract in OCM, and Figure 8 shows BMSCs cultured in collagen hydrogel with S53P4 extract in OCM. hBMSCs cultured in collagen I hydrogels show positive staining for osteocytic marker DMP1. Cell morphology on the other hand does not show clear indications of osteocyte differentiation. Some of the cells have extended dendritic processes, which is characteristic for osteocytes, but the phenotype is still quite far from a natural osteocyte (Figure 6), and virtually no interconnections have formed between the cells.

Cell culture conditions seem to affect the DMP1 expression. D21- and D42- show quite weak DMP1 staining. Addition of the osteogenic pre-differentiation step and SP53P4 extract seem to lead to higher DMP1 expression levels as the staining for DMP1 is the strongest with PD-, D21+, and D42+. Counterintuitively to the results mentioned so far, the combined effect of pre-differentiation step and S53P4 does not lead to increased DMP1 expression as the staining in PD+ is relatively weak.

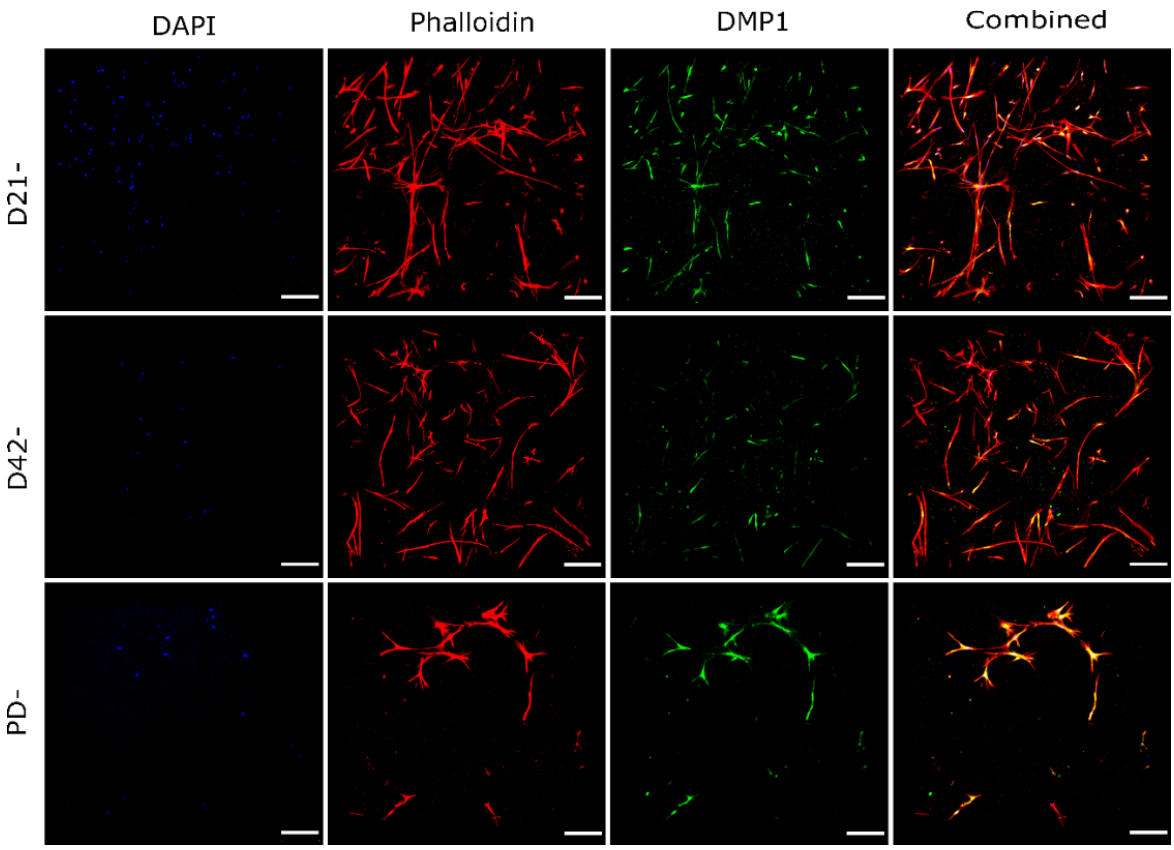


***Figure 7.*** *Confocal images of hBMSCs cultured in collagen gel, without S53P4 extract in the culture medium. Cell nuclei were stained with DAPI (blue) and actin cytoskeletons with phalloidin-TRICT (red). Also, immunostaining for osteocyte specific marker DMP1 (green) was performed. Scale bar represents 200 µm.*

Differences in the cell morphology are less pronounced than differences in DMP1 staining. Formation of dendritic processes has occurred at least to some degree in all culture conditions. Some of the cells in D21- and D42- have formed dendritic processes, but at the same time many of the cells have not formed any processes and most of the cells are quite narrow and elongated, which is not characteristic for osteocytes. Longer culture period alone has not affected the cell morphology as there are no notable difference between the D21- and D42- conditions. Pre-differentiation step and S53P4 extract on the other hand seem to influence the morphology. With PD-, cells are less elongated and have formed dendritic processes. Addition of the S53P4 extract has resulted in thicker cell bodies and also seems to result in higher relative number of dendritic processes. This was not quantified, but the differences can be seen when D21+ and D42+ are compared to D21- and D42-. Although longer culture period itself did not lead to any changes, combination with S53P4 and longer culture period seem to influence morphology, as the individual cells with highest number of dendritic processes are found with D42+. Similar to DMP1 staining, the pre-differentiation step and addition

of S53P4 extract do not seem to have a cumulative effect. PD+ are quite have quite narrow cell bodies and they have formed proportionally lower number dendritic processes compared to PD-. However, the processes of PD+ are generally longer than the processes of PD-.

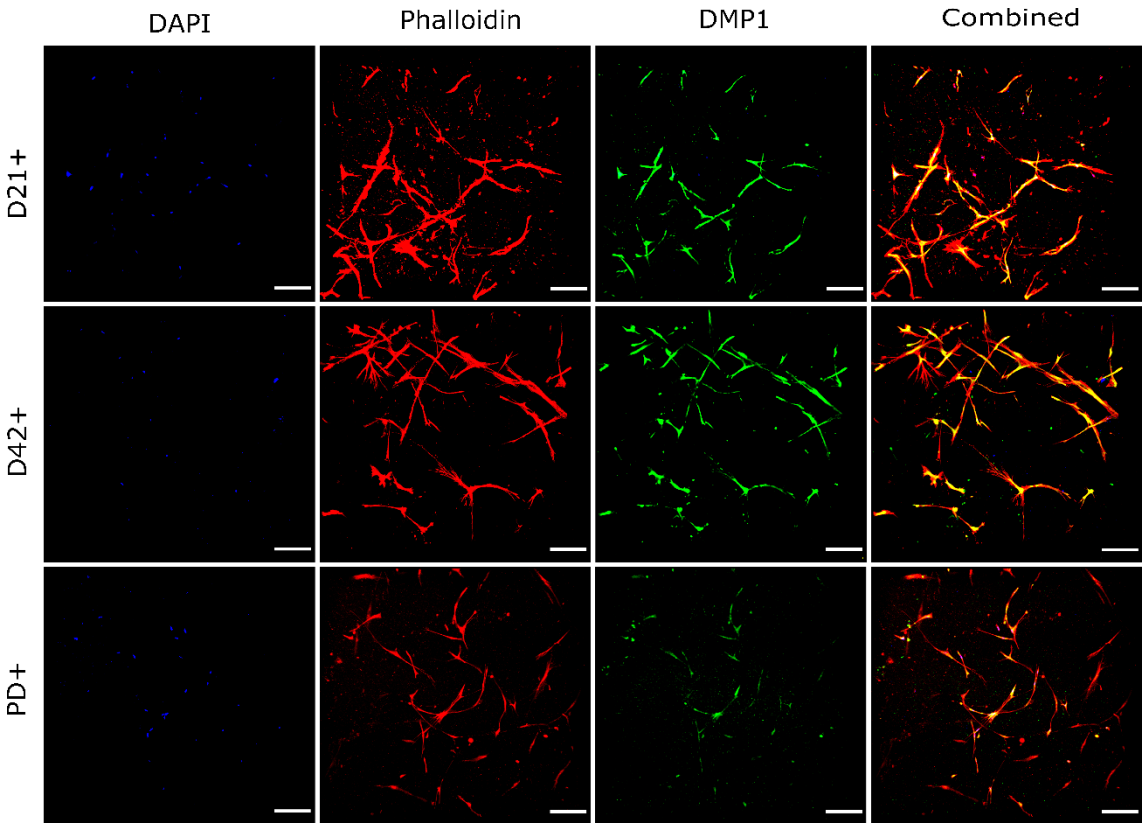


***Figure 8.*** *Confocal images of hBMSCs cultured in collagen gel, without S53P4 extract in the culture medium. Cell nuclei were stained with DAPI (blue) and actin cytoskeletons with phalloidin-TRICT (red). Also, immunostaining for osteocyte specific marker DMP1 (green) was performed. Scale bar represents 200 µm.*

## 4.3 Gene expression analysis

In gene expression analysis, CC-OGM was set as a reference condition and expression of genes in different conditions are reported as a relative expression compared to the reference condition. Relative gene expression of *RUNX2*, *BGLAP*, and *PDPN* are reported in Figures 9, 10, and 11, respectively

Based on the qPCR results, expression of early osteoblastic marker *RUNX2* was substantially higher in the hBMSCs directly embedded into collagen I hydrogels compared to the hBMSC cultured on the collagen coated surfaces in OGM, as the relative expression values for D21- and D42- conditions were in ten-folds compared to the OGM. With these conditions, addition of S53P4 further upregulated *RUNX2*

expression, as the relative expression values for D21+ and D42+ were in hundred folds. Much more modest upregulation of *RUNX2* was seen with PD- condition and both fractions of isolated primary osteocytes, slightly over 2-folds in each condition. PD+ and CC-BM on the other hand showed lower relative expression compared to CC-OGM. There were notable differences between the three biological replicates in each condition where hBMSCs were cultured in collagen gels, which can be seen as high standard deviations in these conditions.

Expression of the late osteoblast marker *BGLAP* was notably lower in all the cell culture conditions except D21-, when compared to CC-OGM With D21-, triplicate samples yielded widely different values from each other as evident by the high standard deviation value. Both cell fractions from the osteocyte isolation procedure showed increase in the BGLAP expression. Fraction from digestions 3-4 showing approximately 10-fold expression and fraction from digestions 5-6 showing approximately 2-fold expression.

Compared to the CC-OGM, most of the cell culture conditions, and in the cells obtained with the osteocyte isolation showed lower expression of the osteocyte marker gene *PDPN*. Two conditions showed higher *PDPN* relative expression than CC-OGM, D21- showing approximately 2-fold relative expression and D42+ a more modest, approximately 1.2-foldrelative expression.

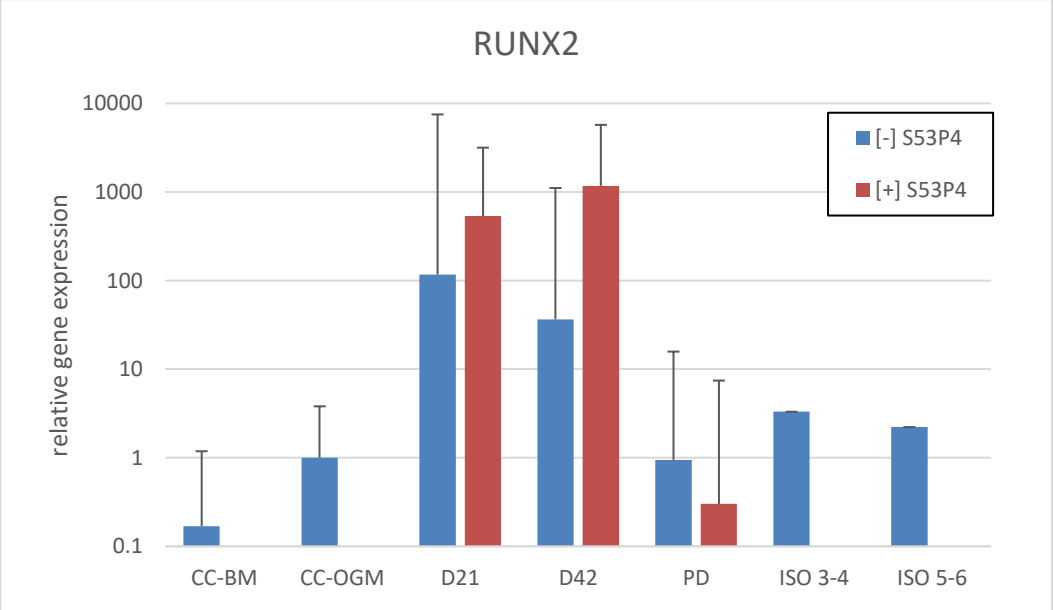


***Figure 9.*** *Relative expression of early osteoblast marker gene RUNX2 in the different culture conditions and in cells acquired with osteocyte isolation protocol.*

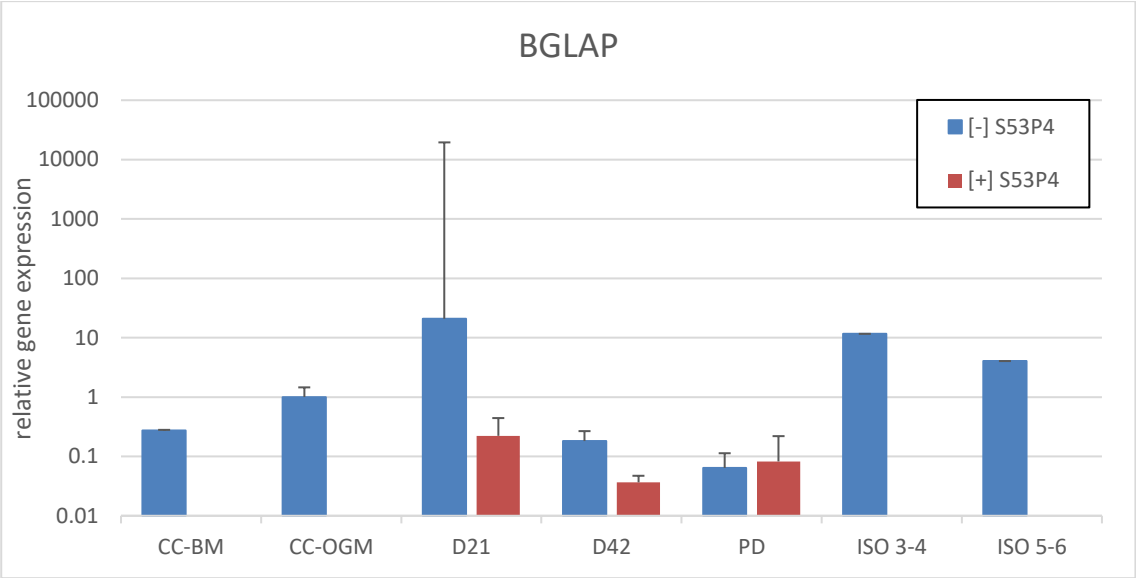


***Figure 10.*** *Relative expression of early osteoblast marker gene BGLAP in the different culture conditions and in cells acquired with osteocyte isolation protocol.*

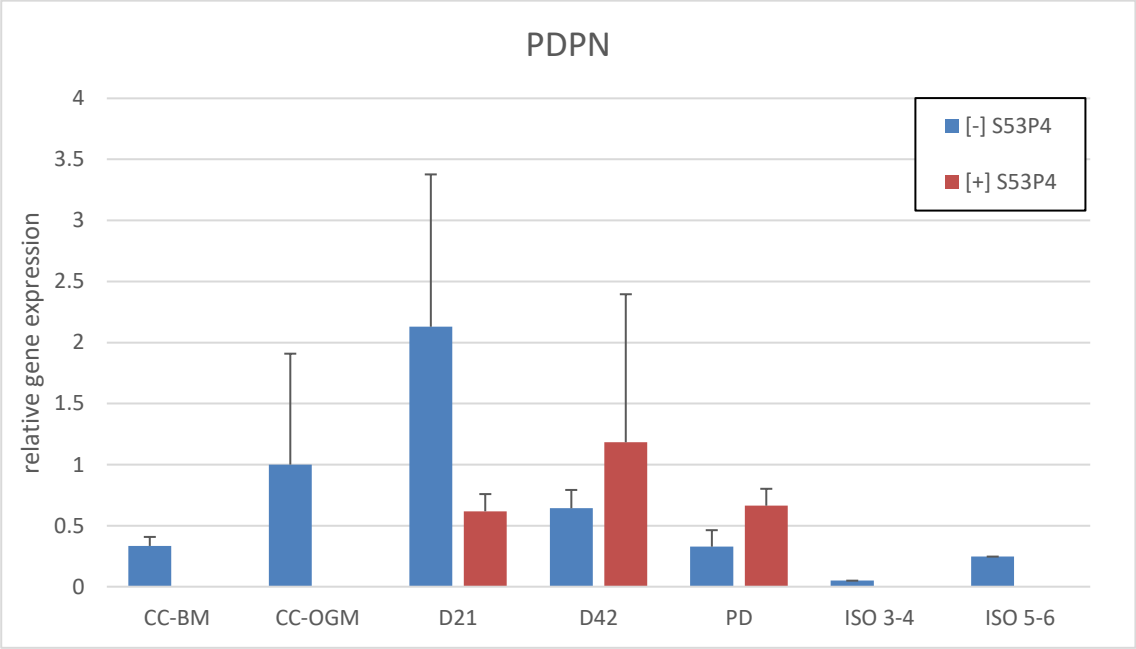


***Figure 11.*** *Relative expression of early osteoblast marker gene PDPN in the different culture conditions and in cells acquired with osteocyte isolation protocol.*

## 4.4 Protein detection assays

Results from the sclerostin ELISA assay and ALP activity assay described in the section 2.4 are represented on Figures 12 and 13, respectively.

Concentrations in all conditions except CC-BM falls below the reported range of the assay (40.96 pg/ml to 10 000 pg/ml). Nevertheless, differences seen between the cell

culture conditions are discussed below. Differences seen are relatively small as the measured values ranged from 23 to 46 pg/ml. Highest concentrations for sclerostin are seen with CC-BM and CC-OGM. Regarding BMSCs cultured in collagen gels, results show that osteogenic pre-differentiation of the BMSCs and combined effect of longer culture period and S53P4 led to higher in sclerostin concentration. Longer culture period alone did not affect the sclerostin concentration, whereas S53P4 extract seemed to decrease it. No trend in the effect of the SP53P4 extract on the sclerostin concentration was seen. Effect was different with each culture condition. With D21 it resulted in decrease, but with D42 it led to increase, resulting in highest concentration of the BMSCs cultured in collagen hydrogels. With PD conditions it had no effect on detected the sclerostin concentration.

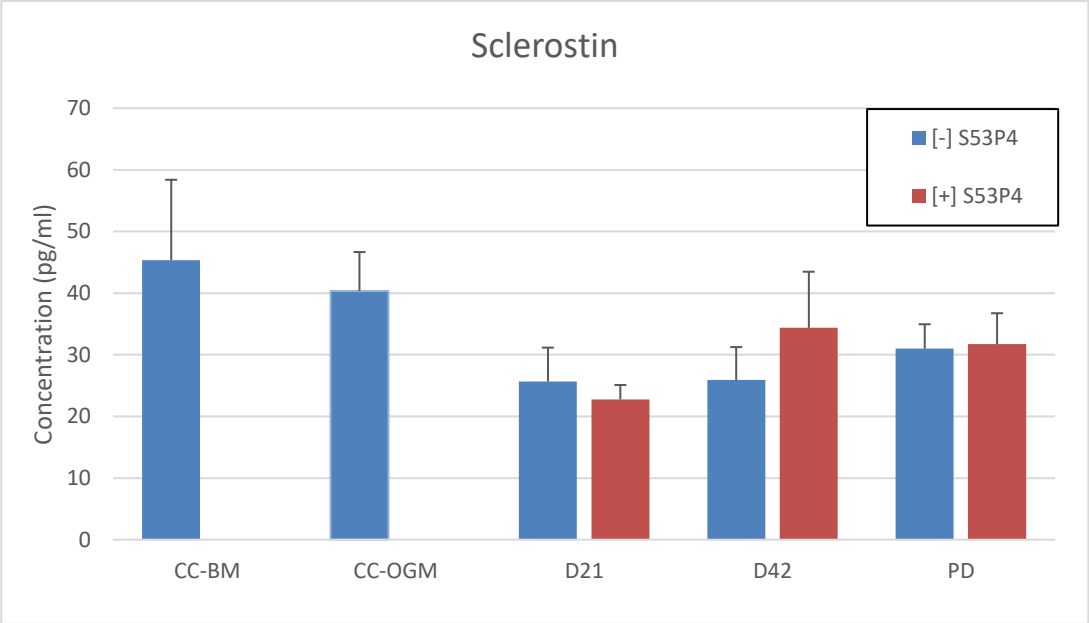


***Figure 12.*** *Measured sclerostin concentration in each cell culture conditions used in the present study.*

Compared to the changes in sclerostin concentration, relative changes were greater in ALP activity. The ALP activity in different cell culture conditions is reported as a relative activity compared to ALP activity seen with CC-OGM. All the cells embedded in collagen show notably lowered ALP activity compared to CC-OGM. ALP activity was quite low with CC-BM as well. Regarding the BMSCs culture in collagen gels, pre-differentiation and longer culture period seemed to lead to lower ALP expression as D42 and PD show lower ALP activity than D21. S53P4extract in the culture medium, led to lower in ALP activity across the culture conditions. With PD+ and D42+ conditions measured ALP activity was virtually non-existent.

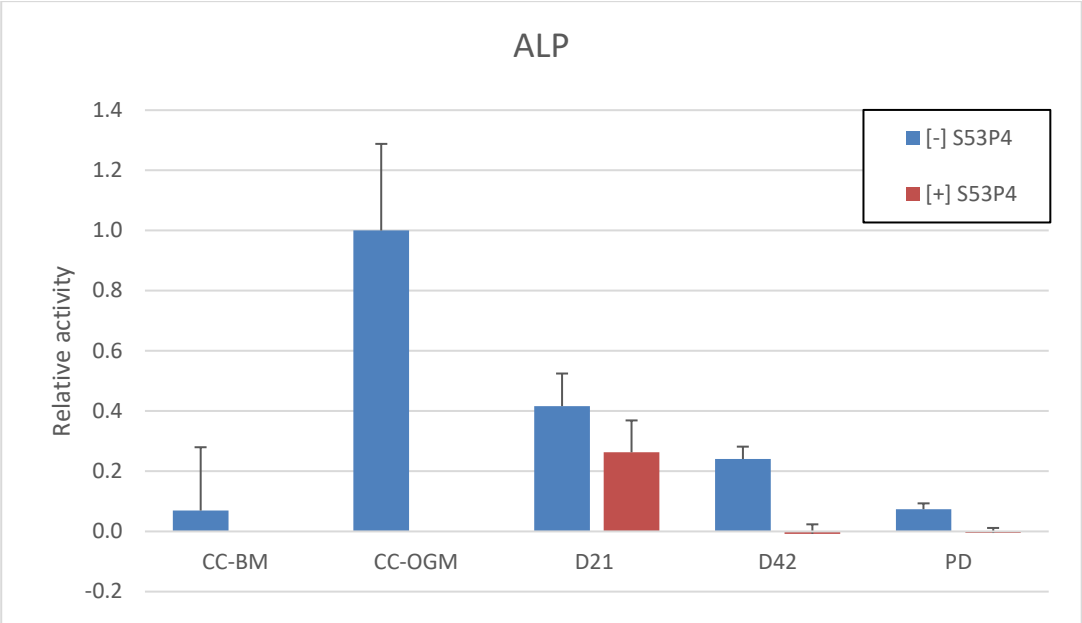


***Figure 13.*** *Relative ALP activity of each culture conditions used in the present study, with CC-OGM used as a reference condition.*

## 4.5 Statistical analysis

Differences between the reference condition (CC-OGM) and the other cell culture conditions in the gene expression analysis were found to be statistically non-significant ($p > 0.05$), with all the studied genes. ALP activity analysis showed some significant differences from the reference condition. Significant p-values were seen with BM, D42+, PD-, and PD+. With sclerostin ELISA assay, D21+ was the only condition to significantly differ from CC-OGM.

# 6. DISCUSSION

This study aimed to differentiate osteocyte-like cells from hBMSCs by culturing them in collagen I hydrogels. This experimental set-up seemed a potential way for acquiring osteocytes, as osteocytes are known to descent from BMSCs via osteoblast differentiation (Bonewald, 2011), and collagenous 3D microenvironment has shown to be a promising platform for osteocytic differentiation, likely due to its resemblance of natural osteocyte environment (Bernhardt et al., 2019; Nasello et al., 2020; Skottke et al., 2019). In the current study, some indications of osteocytic differentiation were seen in the confocal microscope images but based on the gene expression analysis and sclerostin detection assay, the 3D environment provided by collagen hydrogel did not promote osteocytic differentiation any more than 2D environment provided by collagen coated TCPS.

It is good to note that most of the measured differences between CC-OGM and the hBMSCs cultured in collagen hydrogels were found to be statistically non-significant. The data set generated in this study was relatively small, which can partly explain the lack of non-significant results. Higher number of biological replicates would be needed for meaningful statistical analysis of the results.

Anyhow, in the following sections, the effect of different cell culture conditions of this study on the osteocyte differentiation are discussed. Also, other parameters that possibly could influence osteocyte differentiation are briefly gone through.

## 5.1 3D environment

Confocal images of the BMSCs cultured in collagen gels showed some indications of osteocytic differentiation, but gene expression data and results from the protein detection assays do not show enhanced osteocytic differentiation with BMSC cultured in collagen gels compared to BMSCs cultured on collagen coated TCPS in OGM.

Dendritic processes are characteristic for osteocyte morphology and essential for their function (Dallas et al., 2013; Prideaux et al., 2016). Therefore, the formation of dendritic processes can be considered a positive indication for osteocytic differentiation. In this study, some of the hBMSCs cultured in collagen gels had formed dendritic processes, but overall, the cell morphology is quite far from natural osteocyte morphology. In vivo, osteocytes have rounded cell body, and extend far greater number of dendritic processes than the cells imaged in this study (Figure 5). Here the number of dendritic processes branching from the cells also is lower when compared to studies, where primary human osteocytes, pre-osteoblasts, and mature osteoblasts were cultured in collagen I

hydrogels in similar osteogenic conditions (Bernhardt et al., 2019; Skottke et al., 2019). Cells have neither formed interconnections with each other; in natural tissues osteocytes form great number of interconnections, creating networks which allow them to communicate with each other and other cells of bone tissue (Atkinson & Delgado-Calle, 2019; McGarrigle et al., 2016). Lack of cell interconnectivity is also seen in other studies where cells have been cultured in collagen hydrogels for osteocytic differentiation (Bernhardt et al., 2019; Nasello et al., 2020; Skottke et al., 2019). These results evoke a question whether collagen hydrogel is a suitable culture environment for generating a model or engineering a tissue, that can replicate the natural function and morphology of osteocytes. The lack of interconnectivity seen in these studies could be caused by the relatively low cell density used in all these studies. The effect of the cell density on osteocytic differentiation is covered in more detail in a coming section.

Positive staining for DMP1 seen in this study can be considered an indication for osteocytic differentiation as DMP1 is known to play key role in osteocyte maturation (Bonewald, 2011; Lu et al., 2011). Positive DMP1 staining has also been seen in osteocytes and osteoblasts cultured in collagen hydrogels, with similar intracellular localization as seen in this study (Bernhardt et al., 2019; Nasello et al., 2020). This goes to show that BMSCs cultured in collagen gels have acquired some of the same features as the more mature osteogenic lineage cells cultured in collagen hydrogels.

Gene expression analysis did not show enhanced osteocytic differentiation in the 3D environment. Of the cells embedded in collagen gels, D21- and D42+ were the only conditions showing higher relative expression of early osteocyte marker *PDPN* than CC-OGM. D21- also was the only condition which, showed higher relative expression of late osteoblast marker *BGLAP* than CC-OGM. However, with both D21 and D42, clearly the highest relative expression value is seen with *RUNX2*. *RUNX2* is essential for the pre-osteoblast differentiation, regulating activity of other osteoblast lineage genes, but during later stages of osteogenic differentiation *RUNX2* expression is downregulated, as *RUNX2* inhibits mature osteoblast and osteocyte differentiation (Komori, 2002, 2019). As the high *RUNX2* expression levels in this study are coupled with low *BGLAP* expression, it seems that these cells have not reached later stages of osteoblast differentiation. If this is the case, osteocyte differentiation is also unlikely to occur as osteocyte differentiate from mature osteoblasts and proteins secreted by osteoblasts are known to play key roles in bone ECM mineralization, which is an integral step in the osteocyte differentiation (Robling & Bonewald, 2020; Wittkowske et al., 2016). Expression of other osteoblast markers, in addition to *BGLAP*, could be studied to verify the state of the osteoblast differentiation of BMSCs in this study. Based on the literature,

the low *BGLAP* expression and diminished osteogenic differentiation should not be due the 3D environment. Comparative studies between MSC cultured on collagen coated TCPS and MSCs cultured in collagen hydrogels have shown the 3D environment to enhance osteogenic differentiation (Lund et al., 2009; Naito et al., 2013). These studies also showed upregulation of *RUNX2*, although not to similar extent as seen here. In addition to *RUNX2*, more mature osteoblast markers including *Osterix* and *BGLAP*, and its protein product osteocalcin, were also upregulated in the 3D environment. Also, a more recent study has shown strong positive staining for osteocalcin with MSCs cultured in collagen gels (Vuornos et al., 2019). These results indicate that the 3D collagen environment enhances osteogenic differentiation to some degree but not enough to generate mature osteoblasts or osteocytes where *RUNX2* is downregulated. Absence of similar enhanced osteogenic differentiation in the present study could be explained by slight variations in the cell culture conditions in relation to these earlier studies, which are discussed briefly in the coming sections.

Low relative expression of *PDPN* in BMSCs cultured in collagen gels compared to CC-OGM could simply be explained by the more mature state of osteogenic differentiation of CC-OGM, evidenced by the higher relative *BGLAP* expression; as the BMSCs have not differentiated to mature osteoblasts, they cannot differentiate to osteocytes either and therefore do not express the early osteocyte marker. Relative expression values, however, are generally higher in *PDPN* compared to *BGLAP*, which indicates that upregulation of osteocyte marker genes can occur even though the cells have not achieved late osteoblast phenotype. With *BGLAP* and *PDPN* this is a quite reasonable assumption as their gene products serve completely different purposes. Whereas osteocalcin has a role in the mineralization of bone tissues, podoplanin is involved in dendrite formation of osteocytes, and is also expressed in other tissues during development and in adults (Astarita et al., 2012; Staines et al., 2017; Zoch et al., 2016). Therefore, it is possible that expression of these genes is not dependent on each other, even though they are both involved in osteocyte differentiation. Previous study has shown that the 3D microenvironment provided by collagen hydrogel to support *PDPN* expression better than *BGLAP* expression. Primary human osteocytes and osteoblast cultured in collagen hydrogel showed upregulation of *PDPN* and downregulation of *BGLAP* during 7-day and 28-day culture period, respectively (Bernhardt et al., 2019). It is good to note that the results of the study are not completely comparable to this study, as the osteogenic differentiation state of the cells was different at the moment when the cell were embedded in the collagen hydrogels.

Positive DMP1 staining is contradictory to the gene expression data as it is considered a more mature osteocyte marker than *BGLAP* and *PDPN*, which showed low relative expression values compared to CC-OGM. Seeing positive DMP1 counterintuitively to the gene expression is not something extraordinary, as Bernhard et al. showed positive staining for DMP1, when no *DMP1* gene expression was detected (Bernhardt et al., 2019). They speculated that even a low *DMP1* expression could lead to the positive signal, which would explain the simultaneous positive immunostaining and low gene expression. Here *DMP1* expression was not analyzed, but it could be expressed, even when relative expression values of *BGLAP* and *PDPN* are low. As was already discussed in the previous chapter, different proteins have different functions in the osteogenic differentiation process, and therefore it could be possible that they are expressed separately from each other. *DMP1* and *PDPN* expression are related to different cellular processes. DMP1 has been associated with bone ECM mineralization (Ling et al., 2005; T. Liu et al., 2019; Y. Sun et al., 2015), whereas PDPN has a role in the dendritic formation of osteocytes (Staines et al., 2017; K. Zhang et al., 2006). It is possible that the BMSCs embedded in collagen gels have acquired some aspects of osteocytic phenotype, and thus express *DMP1*, but not *PDPN.* That could be the situation here as cells in the gels showed visible mineral deposition (no data) but did not form dendritic processes to same extent as osteocytes in natural tissues. Situation regarding low *BGLAP* expression and positive DMP1 staining is somewhat more puzzling as they are both involved in mineralization of bone (T. Liu et al., 2019; Zoch et al., 2016). Even though osteocalcin and DMP1 are both NCPs of bone and involved in the mineralization process, seem to have different role in the mineralization. Their exact functions are not known, but absence of these proteins have very different impacts on the mineralization. Depletion of DMP1 leads to reduced mineralization, whereas osteocalcin knockout leads to increase in bone formation rate and bone mass (Ducy et al., 1996; Ling et al., 2005). DMP1 is also thought to be possibly involved in other cellular processes as it has also shown to localize inside the nucleus of osteoblast lineage cells (Ravindran & George, 2015). Considering these facts, it does not sound impossible that DMP1 would be expressed simultaneously with low *BGLAP* expression, even though the cells have not differentiated to late osteoblasts or osteocytes. Study of bone mineralization using osteoid-like collagen model showed that this can be the case (Silvent et al., 2013). They studied the expression of both *BGLAP* and *DMP1* at five time points (7, 14, 21, 28, and 60 days). Initial upregulation of *BGLAP* was seen was at day 14, and highest *BGLAP* expression was seen on day 60. On days 21 and 28 *BGLAP* expression was notably lower than on day 14. Initial *DMP1* upregulation on the other hand was seen on day 21, so during the period of low *BGLAP* expression (Silvent et al., 2013).

As the gene expression data has been discussed in the previous chapters, it is good to note at this point that some aspects of the gene expression data raise some questions on the validity of the gene expression values seen in this study. It seems relatively unlikely that D21- surpasses the relative expression of *BGLAP* and *PDPN* of all the other conditions, since it does not show signs of osteocytic differentiation in the other assays and has high relative expression of *RUNX2.* High *RUNX2* expression values of the BMSCs directly embedded in collagen gels could also be questioned. As already discussed, upregulation of *RUNX2* in 3D environment is seen in previous studies in much more moderate scale (Lund et al., 2009; Naito et al., 2013). The gene expression data itself gives even greater reason to doubt its validity. All these high expression values (*RUNX2*: D21-, D21+, D42-, D42+; BGLAP: D21-) showed substantial variation between biological replicates as evident by the high standard deviation values. Some of the replicates showed reduced relative expression, whereas others showed over thousand-fold relative expression. This great variance between the parallel samples indicates that some of the expression values could be due to technical errors rather than actual gene expression differences. It is unclear what could cause this error, but one possibility is a protein contamination from the gel which has disturbed the RNA or DNA sample, or the qPCR reaction. Other issues concerning the RNA isolation protocol resulting in a sample that does not represent the real mRNA content in the cell cannot be excluded either, as the protocol have not been validated in our research group. Studies previously using the protocol, however, have not reported issues with extracting RNA from pure collagen I gels (Bernhardt et al., 2019; Skottke et al., 2019). Anyhow, whether the variance is caused by a technical error or not, it is obvious that clear conclusions cannot be drawn based on these results due to the high variance. The experiment should be repeated, possibly with more parallel samples, to get clarity regarding the *RUNX2* expression seen with hBMSCs directly embedded in collagen gels.

It has so far been established that osteogenic differentiation is quite complex process, and has different aspects, and therefore it is hard to draw conclusions based on the expression of three genes and the immunostainings of the cells, especially considering the uncertainties regarding some of the gene expression values. As discussed earlier, analysis of additional osteoblast and osteocyte genes could offer more insights on the differentiation state of the cells. It is, however, quite clear that hBMSCs do not show strong differentiation towards osteocyte phenotype, evident by the high *RUNX2* expression and lack of osteocytic morphology. This conclusion is supported by the results of the sclerostin detection assay. Sclerostin is protein expressed by mature osteocytes (Stegen et al., 2018), and the results of this study indicate that no sclerostin

expression was seen in any of the cell culture conditions. Overall, the sclerostin concentrations across all the samples (23-45 pg/ml) can be considered quite low as the sclerostin concentration in serum of healthy humans varies between 200-1000 pg/ml (Delanaye et al., 2014; Drake et al., 2018). Furthermore, all the values, except the highest, fall below the reported range of the assay (40.96 - 10 000 pg/ml), which indicates that the modest sclerostin expression seen here could be explained by an error margin or caused by technical aspects or other substances present in the medium, rather than actual sclerostin expression. This is supported by the fact that the highest sclerostin concentration was seen with CC-BM, a condition that should be least likely to promote osteoblast or osteocyte differentiation. Studies that have aimed to differentiate osteocytes by embedding pre-osteoblasts or mature osteoblasts in 3D matrices have not presented results regarding sclerostin protein expression (Bernhardt et al., 2019; Nasello et al., 2020; Skottke et al., 2019). Reason for this is not revealed but could be simply because no sclerostin have been detected. If that is the case it indicates that embedding cells capable of osteocytic differentiation in 3D matrix and culturing them in static conditions might not be enough for differentiating mature, sclerostin secreting osteocytes. Murine primary osteocytes cultured in collagen hydrogels have showed higher sclerostin expression when compared to osteoblasts, and primary mouse osteoblast cultured on 2D surface (Honma et al., 2013, 2015). Sclerostin concentration was measured with and ELISA assay from cell culture media. This goes to show that if cells express sclerostin, it should be possible to quantify the expression of sclerostin in cell culture.

Results of the ALP activity measurement do not give a clear indication on how the osteogenic differentiation state of the hBMSCs cultured in collagen gels. Some consider low ALP activity to be a positive indication of osteocytic differentiation, as ALP expression is more characteristic for osteoblast than osteocytes (Bernhardt et al., 2019; Skottke et al., 2019). As ALP is well known osteoblast marker (Sabokbar et al., 1994), lowered ALP activity could mean that the cells have differentiate past the osteoblast stage and towards osteocyte phenotype. Conclusions on the osteocytic differentiation based on the ALP expression alone, however, cannot be drawn as lack of osteoblast phenotype does not automatically indicate osteocytic differentiation. Reduced ALP expression can offer further confirmation on the osteocytic differentiation detected in other assays, that have showed signs of osteocytic differentiation. Here the sclerostin expression assay or the gene expression data, however, do not show indication on osteocytic differentiation to support the findings of ALP activity assay. Thus, lowered ALP activity could be as well

seen as an indication of diminished osteogenic differentiation, signs of which are seen with the gene expression data as well.

Some cell culture conditions show basically non-existent ALP activity. It is still possible that these cells express ALP. The experimental protocol used here might not be optimal for measuring lower ALP amounts. As ALP is a membrane bound enzyme, the optimal way of measuring ALP activity is to solubilize ALP from cell samples with combination of Triton-X treatment and freeze-thaw cycle (Sabokbar et al., 1994). Here the ALP activity was measured from the conditioned medium, which only includes the ALP enzymes released to their surrounding by cells, which could lead to lower signal, compared to the ALP activity measured from cell samples. Even though the protocol most likely is not optimal for measuring ALP activity, there is still reason to believe that the ALP activity detected in this study is due to actual ALP expression, as the differences in the activities do not seem to occur by chance. Most notably, the difference in the activity between CC-OGM and CC-BM shows that addition to osteogenic supplements leads to increased ALP activity, which is expected as ALP is a well-known osteoblast marker and these supplements are known to induce osteogenic differentiation (Birmingham et al., 2012; Jaiswal et al., 1997; Sabokbar et al., 1994; Vater et al., 2011).

It is already established that results of the ALP activity do not offer definitive information on the state of osteogenic differentiation of the hBMSCs embedded in collagen hydrogels. The situation is worsened by an incomplete experimental set-up the protein detection assays; detected sclerostin and ALP were not normalized in relation to the cell amount. Without the normalization there is no certainty whether the differences in the protein amount or activity are caused by differences in the expression of these proteins by the cells or by the differences in number of viable cells. For the normalization, amount of DNA or total protein content for each sample should have been measured. Quantifying cell amounts could also offer other insights beyond the normalization of protein concentrations, as it is known that transition to non-proliferative state is an integral step in the osteoblast osteocyte differentiation (Nasello et al., 2020). As ALP upregulation is also known to occur simultaneously with the transition to non-proliferative state, Nasello et al., postulated that higher ALP expression could indicate that osteoblasts have started their differentiation towards osteocytes. In addition to ALP activity, they assessed the presence of other osteoblastic and osteocytic markers, and measured cell proliferation over a 21-day culture period. It was indeed found that higher ALP activity correlated with transfer to quiescent cell type and increase in expression of osteocyte markers and decrease in other osteoblast markers (Nasello et al., 2020). This further highlights the complexity of ALP as marker of osteocyte differentiation.

It has been established that the 3D environment set-up established in this study did not lead to mature osteocyte phenotype. There is still a good reason to believe that the 3D environment provided by collagen I hydrogel is a viable option for differentiating osteocytes from hBMSCs, as collagen I hydrogel can capture such many aspects of natural environment of differentiating osteocytes, and it is known that hBMSCs possess the capability to differentiate to osteocytes. Osteocyte features acquired by osteoblasts in collagen I hydrogel also speaks for the collagen hydrogels as a platform for osteocyte differentiation (Bernhardt et al., 2019; Sawa et al., 2019; Skottke et al., 2019). Modifications to the culture set-up could help to promote osteocyte differentiation of hBMSCs in collagen hydrogel. Effect of the other culture parameters of this study are discussed below, as the information gathered here can possibly be utilized in future studies aiming for osteocyte differentiation.

## 5.2 Osteocyte medium composition

Osteocyte medium (OCM), which was used in all collagen gel cultures, diverged from the classical osteogenic medium composition (OGM) in two ways. Serum concentration of OCM was 2% instead of the 5% used with OGM and dex was omitted from the OCM. 2% serum concentration was used, as lowering serum concentration has shown to be beneficial for maintaining osteocyte phenotype in vitro (Honma et al., 2015). Lower serum concentration has also been shown to benefit osteogenic differentiation of MSCs (Bhandi et al., 2021; Binder et al., 2015), and therefore is most likely an optimal choice for differentiating osteocytes from BMSCs as well.

Dex was omitted as previous study aiming to differentiate osteocytes from primary osteoblasts suggested that omitting dex could be beneficial for osteocyte differentiation (Bernhardt et al., 2019). They did not show data regarding this, but they considered an upregulation of ALP seen with the osteoblasts cultured in collagen gels to be a negative indicator of osteocyte differentiation, and as dex is known to upregulate ALP, they suggested that omitting dex could lead to more osteocyte like phenotype. The logic is somewhat solid when aiming to differentiate osteocyte from mature osteoblasts, but when differentiating from BMSCs the omitting of dex should be reconsidered. Dex is known to promote osteogenic differentiation by enhancing *RUNX2* transcription but also by activating RUNX2 via transcriptional co-activator with PDZ-binding motif (TAZ) and mitogen-activated protein kinase (MAPK) phosphatase (MKP-1) (Langenbach & Handschel, 2013). As osteocyte differentiation is in the end the results of osteogenic differentiation and RUNX2 is early master regulator of osteogenic differentiation, promoting expression of multiple osteogenic marker genes, dex should be included in

the medium composition for differentiating osteocytes from BMSCs, at least during the first stages of culture to promote the beginning of osteogenic differentiation. Lower *BGLAP* expression in 3D environment compared to 2D environment, which was contradictory to earlier studies, could be explained by the absence of dex in the 3D cultures of this study (Lund et al., 2009; Naito et al., 2013). Absence of dex could lead to low *BGLAP* expression directly through reduced *RUNX2* transcription as RUNX2 is known to promote *BGLAP* expression. Here D21 and 42, however, showed relatively high *RUNX2* expression. As already stated, the *RUNX2* expression data exhibited high variance, which gives a reason to suspect the validity of the *RUNX2* expression values seen in this study. But even if the other cell culture conditions had caused D21 and D42 express *RUNX2* in higher levels than CC-OGM, it is still possible that absence of dex has caused the low *BGLAP* expression shown in BMSCs cultured in collagen gels. As already stated, dex also regulates the activity of RUNX2; it has been shown that RUNX2 mediated mineral formation and *BGLAP* expression is notably enhanced in vitro (Mikami et al., 2007; Phillips et al., 2006). To confirm the effect of omitting dex, the study done here ought to be repeated with dex in the culture medium.

If absence of dex indeed affects RUNX2 activity, it could also explain the simultaneous positive DMP1 staining and very low *BGLAP* expression. When effect of RUNX2 ablation on different osteogenic marker genes, including *BGLAP* and *DMP1* was studied, it was seen that *DMP1* was the only gene that could be expressed independently from RUNX2 (Nakamura et al., 2020).

## 5.3 Osteogenic pre-differentiation

In addition to directly embedding hBMSCs into collagen gels, hBMSCs were also embedded into collagen gels after 7-day pre-differentiation step. Since osteocyte decent from hBMSC through osteoblast interphase and primary osteoblasts have shown to acquire osteocyte features when cultured in collagen gels (Andrzejewska et al., 2019; Bernhardt et al., 2019; Skottke et al., 2019), it was studied if initiating the hBMSCs differentiation in culture conditions that are known to promote osteogenic differentiation would be beneficial for differentiating osteocytes from hBMSCs. Based on the confocal microscope images the osteogenic pre-differentiation step is beneficial for osteocyte differentiation as PD- showed stronger DMP1 signal and had formed proportionally more primary dendritic processes compared to D21-.

Gene expression data does not support the findings of confocal images. It indicates that the pre-differentiation step has not resulted in enhanced osteoblast or osteocyte differentiation, as the relative expression of all the genes studied here is lower with PD-

compared to D21-. This could, however, be caused by the possible faulty results with D21- gene expression data, discussed earlier.

ALP activity with PD- is lower compared to D21-, which could indicate either diminished osteoblast differentiation or enhanced osteocyte differentiation. As other analyses do not show conclusive evidence of neither, no conclusions based on the ALP activity data cannot be drawn. Furthermore, the reduced ALP activity could be caused by differences in number of viable cells between PD- and D21-, and therefore renders the ALP activity data unsuitable for comparing differences between PD- and D21-.

This study did not show indisputable evidence in favor for differentiating osteocytes from osteogenically pre-differentiated BMSCs compared to undifferentiated BMSC, but as the osteocytes are known to decent from osteoblast, osteogenic pre-differentiation prior to culturing hBMSCs in osteocyte differentiation supporting conditions should be still considered as a valid option for aiming to differentiate osteocytes from hBMSCs. Effect of longer pre-differentiation step could be studied to see if differentiation to more mature osteoblasts prior to embedding hBMSCs into collagen gel would benefit osteocyte differentiation.

Including cells at the different stages of osteogenic differentiation simultaneously in the cell culture set-up should also be considered. It would help to mimic the natural environment of differentiating osteocyte; in the bone microenvironment all the osteoblasts do not differentiate to osteocytes, and therefore it is likely that there are cells in the different state of osteogenic differentiation present in the tissue environment (Florencio-Silva et al., 2015). Biochemical signals from osteoblasts and osteocytes have shown to promote osteogenic differentiation of MSCs (Birmingham et al., 2012). It is not completely unthinkable that hBMSCs or osteoblasts not differentiating to osteocytes could play a role in the regulation of osteocyte differentiation, as they are both integral part of bone tissue environment and MSCs are already known to have different regulatory functions (Andrzejewska et al., 2019; Robert et al., 2020), and they have shown to affect the differentiation of other cell types in vitro (Mykuliak et al., 2022). Therefore, it should be tested if adding osteoblasts in the culture set-up at the beginning of the culture or adding more hBMSCs later during the culture would enhance the osteocyte differentiation.

## 5.4 Cell culture period

hBMSCs directly embedded into collagen were analyzed after 21 days and 42 days of culture to assess if longer culture period would promote osteocyte differentiation.

Based on the results seen here D42- did not show enhanced osteocyte differentiation, or osteogenic differentiation in general, compared to D21-. In confocal images, no differences are seen between the two conditions, whereas the gene expression data indicated enhanced osteogenic differentiation with D21- compared to D42- as the expression of all the genes analyzed here were higher with D21-. Again, as already discussed in the previous section, the difference could be caused by the possibly faulty expression data of D21-. The results seen here could be seen as a support of this, as it seems rather unlikely that longer culture period in osteogenic conditions would lead to diminished osteogenic differentiation.

The situation regarding ALP activity data is also similar as with the pre-differentiation results, meaning that it is not known if the reduce in activity as the result of longer culture period is caused by reduced ALP activity or reduced number of viable cells. As the only difference between the culture is the culture duration, the reduction in the cell number would be caused by the transformation to less proliferative and more apoptotic cell type, which would be characteristic for osteocytes (Nasello et al., 2020). And as discussed with the gene expression data, it seems rather unlikely that the longer culture period would lead to diminished osteogenic differentiation. So based on the ALP activity data it could be argued that that the longer culture period has led to more osteocytic phenotype, but since the other analyses do not show indications of osteocyte differentiation, no conclusions can be drawn.

Studies that have examined murine osteoblasts cultured in mtgase gelatin hydrogels and migrating to collagen I hydrogels have shown longer culture periods to benefit osteocyte differentiation (McGarrigle et al., 2016; Uchihashi et al., 2013). MC3T3-E1 cells were cultured in the mtgase gelatin hydrogels up to 56 days and on the collagen gels up to 35 days. Expression of osteocyte marker genes *PDPN* and *PHEX* have also been shown to increase during 28 day of culturing primary osteoblasts in collagen gel, when gene expression was assessed on day 0, 14, and 28. Skottke et al cultured pre-osteoblasts in collagen gels for 6 weeks, which resulted in more osteocyte-like morphology compared to this study (Skottke et al., 2019). This offers probably the best reference point for the present study regarding osteocyte differentiation, as the osteogenically pre-differentiated hBMSCs most likely pay quite close resemblance to pre-osteoblasts. Culture periods in this study can be considered shorter as the maximum culture periods in collagen gels for pre-differentiated cells and for non-differentiated hBMSCs here were 21 days and 42 days, respectively. Therefore, even longer culture periods could have been applied for hBMSCs embedded in collagen gels to see whether it would result in more osteocytic phenotype. In general, it is unlikely that longer culture period would have negative effect

on the osteocyte differentiation, as osteocytes are terminally differentiated osteoblasts and single osteocyte can reside inside the bone matrix for decades (Atkinson & Delgado-Calle, 2019). However, if the culture conditions are not otherwise sufficient to promote osteocyte differentiation, prolonging the culture is most likely not a solution for achieving more osteocyte-like phenotype.

## 5.5 S53P3 extract

S53P4 extract has shown to be a potent enhancer of osteogenic differentiation (Ojansivu et al., 2015). In this study it was assessed whether it would promote osteocyte differentiation as well. Effect of S53P4 extract was studied only in 2D environment, but impact of another BAG extract on the osteogenic differentiation of MSCs in collagen hydrogels has previously been studied (Vuornos et al., 2019). The study used an experimental glass (2-06) which releases potassium and boron ions in the medium, in addition to the ions released by S53P4. In 2D cell cultures, influence S53P4 and 2-06 extracts on osteogenic differentiation were similar with each other; compared to non-BAG control, both showed downregulation of *RUNX2*, upregulation of late osteoblast markers, *Osterix* and *DLX5*, and increased mineralization (Ojansivu et al., 2015). As S53P4 and 2-06 share many of their components and their effect in osteogenic 2D environment has shown to be similar, it can be considered that they quite likely have similar effects in osteogenic 3D environment as well. Therefore, the MSCs cultured in collagen hydrogels with 2-06 extract medium can also be considered as a good reference point for the hBMSCs directly embedded to collagen hydrogel in this study. 2-06 extract was shown to enhance osteogenic differentiation in the 3D environment similarly as in the 2D environment; early osteoblast markers *ALPL* and *RUNX2* were downregulated, whereas other osteoblast markers *Osterix* and *DLX5* were upregulated, and mineral deposited by the MSCs was increased (Ojansivu et al., 2015; Vuornos et al., 2019).

### 5.5.1 hBMSCs directly embedded into collagen gels

Based on the confocal images, S53P4 extract influenced the phenotype of D21 and D42, possibly promoting osteocyte differentiation. Changes seen in cell morphology do not offer clear indication of enhanced osteocytic differentiation. Implication of the thicker cell body, seen with D21+ and D42+, on osteocyte differentiation is not known, but it does not resemble the natural osteocyte morphology notably closer than the thinner cell morphology seen with D21- and D42-. The increased dendritic formation seen with D42+ compared to D42-, is mostly due to secondary or tertiary dendrite formation, meaning that the dendrites do not extend from the cell body but from the existing dendrites. As

osteocytes are characterized by their primary dendrites extending directly from the cell body (Figure 5) (Bonewald, 2011), the increased dendritic formation with D42+ compared to D42- cannot necessarily be considered as a sign of enhanced osteocytic differentiation.

Stronger DMP1 signal in the presence of S53P4 extract on the other can be considered as an indication for enhanced osteocytic differentiation, as DMP1 is a known to be expressed by osteocytes (Bonewald, 2011). Increase in the DMP1 expression appears a logical consequence for addition of S53P4, considering the role of DMP1 in the bone mineralization process and the mineralization promoting effects of S53P4 and 2-06 extracts in 2D and 3D, respectively (Ling et al., 2005; T. Liu et al., 2019; Ojansivu et al., 2015; Vuornos et al., 2019). Mineral deposition was not quantified in this study, but a notable increase in the mineral deposition was seen with D42+ compared to D42-, when cells were examined under phase contrast microscope. In contrary to the DMP1 expression and mineral deposition, gene expression and ALP activity data did not show signs of enhanced osteogenic differentiation. The gene expression data was also contrary to the earlier results considering S53P4 and 2-06 extracts, as here *RUNX2* expression was upregulated and more mature osteoblast marker *BGLAP* was downregulated. (Ojansivu et al., 2015; Vuornos et al., 2019). Downregulation of *BGLAP* was also contrary to the increased osteocalcin deposition in the presence of S53P4 extract in seen in 2D cultures (Ojansivu et al., 2015). The situation seen in this study is, however, completely possible, as osteocalcin is not similarly essential for mineral formation and nucleation as DMP1 (Ducy et al., 1996; G. He et al., 2003). The divergence from the studies done by Ojansivu et al. and Vuornos et al. remains unclear. The most notable difference in the cell culture conditions regarding osteogenic differentiation potential between this study and the forementioned studies are the absence of dex from the medium composition in this study, and the source of MSCs. Vuornos et al. and Ojansivu et al. used human adipose tissue derived MSCs (hAMSCs), which do not have as high osteogenic differentiation potential as hBMSCs, even though they can differentiate undergo osteogenic differentiation (Liao, 2014; Mohamed-Ahmed et al., 2018). There is, however, not an obvious scenario which would explain the differences between this and he previous studies. As the cells in the earlier study show enhanced osteogenic differentiation compared to this study. It is unlikely that the different osteogenic differentiation potential of hAMSCs and hBMSCs would explain the detected differences. One explanation could be that the absence of dex has prevented the RUNX2 mediated upregulation of *BGLAP* but not *DMP1,* as discussed in the previous section. This however still does not explain the upregulation of *RUNX2* or downregulation of

*BGLAP.* Even if *RUNX2* is somehow upregulated by the S53P4 extract, it does not explain the downregulation of *BGLAP*; absence of dex has shown to diminish or prevent RUNX2 mediated upregulation of other osteoblast marker genes, not lead to their downregulation. In conclusion, it is still quite unclear why the results of the gene expression data seen here wre not in line with previous studies. As *PDPN* was not measured in the previous studies, and here the effect on the D21 and D42 varied, it is difficult to draw conclusion on the impact of the S53P4 regarding the early osteocyte marker. The upregulation of *PDPN* seen with D42, is in the line with the cell morphology as *PDPN* is involved with the dendrite formation of cells (Staines et al., 2017; K. Zhang et al., 2006), but as stated in the previous chapter, the dendritic process formation seen with D42+ cannot necessary be considered an indication of osteocytic differentiation. With D21 the downregulation may be due a technical error, since the gene expression results from D21- do not necessarily accurately represent the real gene expression profile, as discussed previously. There are altogether relatively small number of studies concentrating on the effect of BAG extracts on osteogenic differentiation, which makes it somewhat difficult to assess the validity of results seen here.

Reduced ALP activity in all the conditions from S53P4 extract medium on the other hand was in line with previous studies. As discussed previously, ALP activity was not normalized to the cell amount in this study, which means that the reduction seen here may have been due to lower number viable cells in the conditions containing S53P4 extract. Based on the previous studies this does not, however, seem probable as 2-06 extract was shown to increase the number of viable MSCs cultured in collagen gel after 21 days (Vuornos et al., 2019). Low *ALPL* expression seen with 2-06 extract has been suggested to be caused by more advanced stage of mineralization, as decrease in *ALPL* was associated with increased mineral formation (Vuornos et al., 2019). The situation could be same here, as D42+ showed the lowest ALP activity of hBMSCs directly embedded in collagen gels and was the condition where an increase in mineral deposition was clearly visible under phase microscope inspection. This could mean that S53P4 addition is beneficial for osteocyte differentiation as the matrix mineralization is an integral part of the osteocyte differentiation process (Dallas et al., 2013; Robling & Bonewald, 2020).

### 5.5.2 Osteogenically pre-differentiated hBMSCs

Regarding the ALP activity assay, PD showed to react similarly to S53P4 extract as hBMSCs directly embedded into collagen hydrogel. Based on the gene expression data, it seems that addition of S53P4 had somewhat positive effect on the osteocyte

differentiation, as it resulted in downregulation of *RUNX2,* and in modest upregulation of *BGLAP* and *PDPN*.

Confocal microscope images on the other hand do not offer as clear indications of osteocytic differentiation. Number of dendritic processes did not increase as a result S53P4 extract, but the dendritic processes appear longer in PD+ compared to PD-, which some studies consider a sign of enhanced osteocyte differentiation (Nasello et al., 2020). Lower DMP1 signal seen with PD+ could be considered a negative indicator of osteocyte differentiation. One potential explanation for the lowered signal could be that PD+ have already differentiated past the DMP1 expressing osteocyte state. This is, however, not a likely scenario as the relative expression of osteocyte marker genes is low compared to other conditions, and there is no sclerostin expressed by the cells. Instead, different differentiation state of cells could explain the different effects seen with PD and directly embedded hBMSCs. This theory is, however, hard to confirm as there are no studies regarding the effect of S53P4 extract on osteoblasts. The timing of S53P3 addition to the cell culture could also explain the detected difference between PD and hBMSCs directly embedded into collagen. PD is cultured without the S53P4 extract for the first week of their differentiation, meaning the osteogenic pre-differentiation step. This could explain the difference in the reaction to S53P4 extract, as the conditions during the early stages of the differentiation are known to affect the whole osteogenic differentiation process of MSCs (Jaiswal et al., 1997). For instance, dex supplementation during the first week induces osteogenic differentiation similarly as dex supplementation for the whole four-week culture period, whereas dex supplementation during second or third week of culture leads to substantially diminished osteogenic differentiation (Alm et al., 2012).

### 5.5.3 Mechanism of action

S53P4 extract clearly influenced the differentiation of hBMSCs, also affecting some aspects of osteocyte differentiation. Effects of S53P4 extract on the differentiation can be simply due the enhanced osteogenic differentiation that the ions released from S53P4 have shown to have on MSCs (Ojansivu et al., 2015). As osteocytes descent from BMSCs through osteoblast interphase, it could be that increase in osteoblast differentiation directly leads to increase in osteocyte differentiation, when cultured in an environment that supports osteocytic differentiation.

Other mechanisms could also explain the impact of the S53P4 extract on the differentiation of hBMSCs. 2-06 BAG has shown to affect the mechanical properties of acellular collagen hydrogels after a 21-day incubation period, most likely due to increased ionic cross-linking between collagen fibrils (Vuornos et al., 2019). 2-06 BAG

also resulted in increased mineralization inside collagen hydrogels, which also possibly further modify the properties of hydrogels, as the mineral phase of bone is known to significantly contribute to its mechanical properties (de Wildt et al., 2019; Wittkowske et al., 2016). Mineralization of the collagen gels or the mechanical properties of the gels were not quantified in this study, but there is no reason to believe that the S53P4 would have substantially different effect on the properties of collagen hydrogels compared to 2-06 extract. As the mechanical properties of the environment are known to affect cell differentiation of MSCs (Anderson et al., 2016; Engler et al., 2006), the S53P4 extract could also have affected the differentiation through altering the mechanical properties of the gels. Increased mineral deposition could also help to better mimic the natural environment of osteocytes and therefore enhance osteocyte differentiation.

Bernhardt et al. did not found mineralization of collagen to be beneficial for osteocyte differentiation (Bernhardt et al., 2019). It might be that the mineralized collagen did not mimic the environment of early osteoid, and thus did not benefit osteocyte differentiation. BAG extract mediated mineralization could be better opinion as the gel is mineralized efficiently as the BMSC differentiate, but initially offers softer environment that resembles the early osteoid better. There is, however, no certainty if the in vitro mineral deposition results in intrafibrillar mineralization, which is the situation in vivo. Extrafibrillar mineralization does not result in as strong mechanical properties and may fail to mimic the natural bone ECM structure (D. Kim et al., 2016; Y. Liu et al., 2011).

Bioactive ions are known to have diverse effects on cell differentiation and behavior in general (F. He et al., 2021; Saghiri et al., 2015). Hence, it is not unthinkable that in addition to their known effects they promote the later stages of osteogenic differentiation as well, thus promoting osteocyte differentiation. At least phosphate ions have already demonstrated to enhance osteocyte differentiation (Sai et al., 2018).

## 5.6 Cell density and collagen shrinkage

Cell density is well known to affect the osteogenic differentiation of MSCs (Bitar et al., 2008; Noda et al., 2019). Impact of cell density on osteocyte differentiation was not studied here. Two different cell densities, however, were tested. Analyses of osteogenic differentiation were performed for hBMSCs embedded in collagen gels at a density of $2.5 \times 10^4$ cell/ml due shrinkage of collagen hydrogel seen with higher cell density ($10^5$ cell/ml). The phenomenon seen here is not an unusual one as rapid contraction of collagen in 3D cultures has been reported by others, with MSCs and osteoblasts, and it has been shown that collagen gel contraction becomes more rapid as cell density is raised (Bernhardt et al., 2019; Chieh et al., 2010; Lund et al., 2009). Collagen shrinkage

has also been reported with fibroblasts and chondrocytes cultured in collagen I (Jin & Kim, 2017; Zhu et al., 2001). It is commonly accepted that the collagen hydrogel shrinkage in cell cultures is due to the cell mediated collagen contraction (Chieh et al., 2010; Zhu et al., 2001).

The relatively low cell density used in this study could be one explanation for the lack of osteocytic phenotype. The cell density used here (2.5 x $10^4$ cell/ml) was significantly lower than osteocyte density reported in natural bone tissue (Mullender, Meer, Huiskes, & Lips, 1996). Thus, the 3D microenvironment created here does not mimic the natural microenvironment of osteocytes in this regard. Raising cell density would probably help to solve the problem of lacking cell interconnectivity seen in this study, and other studies that aimed to differentiate osteocytes from osteoblasts (Bernhardt et al., 2019; Skottke et al., 2019); at least intuitively, closer proximity of cells with each other should help to establish cell-to-cell connections.

Using higher cell densities has, indeed, shown to increase cell interconnectivity, and led to more osteocytic phenotype overall, when culturing pre-osteoblasts or osteoblasts in hydrogels. McGarrigle et al. compared three cell densities (0.25 x $10^6$, 1.0 x $10^6$, 2.0 x $10^6$ cells/ml) in mtgase gelatin hydrogels; the highest cell density supported osteocytic phenotype the best, both regarding dendrite formation and cell interconnectivity (McGarrigle et al., 2016). Nasello et al. embedded cells in collagen gel at two different densities: 2.5 x $10^5$ and 2 x $10^6$ cellls/ml. The higher cell density supported osteocyte differentiation better during 21-day culture period. Compared to the lower cell density, the cells had more dendritic cell morphology, and showed increased expression of DMP1 and decreased expression bone sialoprotein 2 (BSP2), an osteoblast marker protein (Nasello et al., 2020). Also, with the higher density, the cells were non-proliferative which is characteristic for osteocytes. Spheroid culture of murine pre-osteoblast cell line also speaks for importance of high cell density for osteocyte differentiation (J. Kim & Adachi, 2019). It was found that the cells in the of the spheroids had acquired osteocytic phenotype. The authors postulated that the osteocyte phenotype was due to the cell condensation that occurred in the spheroid culture (J. Kim & Adachi, 2019).; as raising high cell density appears to positively influence osteocyte differentiation with osteoblasts and pre-osteoblasts, the effect of higher cell density should be tested with hBMSCs as well.

Nasello et al. cultured primary osteoblasts in collagen with quite high cell density (2 x $10^6$ cellls/ml) for 21 days and did not report of hydrogel shrinkage. This might be due to higher collagen concentration used (6mg/ml) compared to this study. This is in line other studies; with MSCs, fibroblasts, and chondrocytes the shrinkage of collagen has been

less pronounced when the collagen concentration has been raised (Chieh et al., 2010; Jin & Kim, 2017; Zhu et al., 2001). Increase in collagen I concentration has also been demonstrated to increase shear modulus and compressive modulus of collagen I gels (Antoine et al., 2015; Valero et al., 2018). These facts point to the conclusion that cell mediated collagen shrinkage is affected by the mechanical properties of the collagen hydrogel, which are known to be relatively weak (J. Heo et al., 2016). Thus, improving mechanical properties of collagen gel could help to culture BMSCs in collagen I at higher cell density for longer time periods without gel shrinkage. There are multiple means for improving the mechanical properties of collagen hydrogels, including covalent cross-linking, alteration of collagen gelation parameters, and altering the orientation of collagen fibrils through different means (Sarrigiannidis et al., 2021). This could, however, be disadvantageous for osteocyte differentiation as lowering the substrate stiffness has shown to promote osteocyte differentiation (McGarrigle et al., 2016; Mullen et al., 2013).

Shrinkage of collagen hydrogel was considered a problem in this study. There is, however, some evidence showing that the shrinkage of the hydrogel could actually be beneficial for osteocyte differentiation. For instance, hydrogels that permit cell mediated degradation have shown to promote osteogenesis, whereas covalently cross-linked hydrogel with similar stiffnesses that do not allow matrix degradation promoted adipogenesis (Khetan et al., 2013). Faster stress relaxation rate in collagen I hydrogels also has shown to induce osteogenesis (Chaudhuri et al., 2016). Also, areas with different arrangement of collagen in cell contracted collagen hydrogel have shown different stages of osteogenic differentiation and matrix mineralization (Klumpers et al., 2013). These results imply that ability of cells to deform or reshape their surrounding matrix is important for osteogenic differentiation of MSCs. Collagen reorganization has been shown to occur during osteocyte differentiation as well. Cell mediated tissue level movement of collagen fibrils have been shown during the osteocyte differentiation (Shiflett et al., 2019). The same study showed condensation of collagen at the site of the mineralization. Ability of the cells to reorganize their surrounding matrix has also been shown to be important for the formation of cellular networks in the context of endothelial cells (Davidson et al., 2019). These results imply that matrix reorganization by cells could play a role in osteocyte differentiation and formation of osteocyte networks as well. Knowing this, cell mediated gel shrinkage could be seen as a solution rather than problem, regarding osteocyte differentiation. The shrinkage of collagen hydrogel would also lead to increase in cell density, and possibly to cell condensation, which are both shown to benefit osteocyte differentiation (J. Kim & Adachi, 2019; McGarrigle et al., 2016; Nasello et al., 2020; Shiflett et al., 2019). Allowing the hydrogel shrinkage would also

enable the usage of higher cell density with softer substrates. The hydrogel shrinkage would, of course, be problematic if the original size and shape of the construct must be maintained. But if the goal is merely to achieve osteocyte differentiation, the shrinkage itself would not be an issue. Many in vitro culture set-ups would also allow subsequent addition of cell-laden collagen to replace the shrank area of the construct.

## 5.7 Future considerations

The analyses used in this study were not sufficient to determine the exact differentiation state of the BMSCs embedded in collagen gels, due to limitation of the study, possible faulty data, and just simply due to the narrowness of the gathered data, both regarding quality and quantity. To confirm some of the conclusions drawn here, further analyses should be conducted. First, the number of viable cells should be quantified to normalize the data from protein detection assays, and to gain information about the proliferation state of the BMSCs embedded in collagen gels. Second, expression of more genes could be analyzed to get better picture of the differentiation state of the BMSCs; most comprehensive picture would be provided by RNA-seq, which could even give new insights on the genes involved in the different aspects of osteocyte differentiation. In addition to the new analyses, higher number of biological replicates should be included so that statistically significant results could be obtained. Also, hBMSCs from different donors should be analyzed, as there can be donor variability in the differentiation potential of MSCs (Andrzejewska et al., 2019; Robert et al., 2020).

Even though the results in this study are not conclusive, it is obvious that the experimental set-up established was not sufficient to differentiate hBMSCs to osteocyte-like-cells. Therefore, in addition to the further analyses, some modifications to the experimental set-up should be made in future. As discussed already, longer pre-differentiation period, longer culture period, addition of dex to the medium composition, using higher cell density, and including cells at different stage of osteocyte differentiation to the culture set-up could be viable options to promote osteocyte differentiation.

An important aspect that has not been yet discussed is the collagen hydrogel itself. In the sections 2.3, it was established that even though collagen hydrogels recapture many aspects collagenous tissues, but at the same time lacks some properties of natural collagenous tissues. There is growing evidence that close mimicry of collagen orientation in the natural tissues is beneficial for guiding the stem cells to differentiate towards the mature cell type of the respective tissue (Salvatore et al., 2021). In mature lamellar bone, collagen is highly organized, but in woven bone, that develops at the site of large fractures, collagen is randomly organized, and woven bone still includes osteocytes

(Kerschnitzki et al., 2011). This points to the conclusion that no highly organized collagen fibril network is required for osteocyte differentiation. As stated in the previous sections, a hydrogel resembling more mature bone does not either appear to promote osteocyte differentiation any better than a relatively soft, unmineralized hydrogel (Bernhardt et al., 2019; McGarrigle et al., 2016). Modification of collagen hydrogel properties for achieving enhanced osteocyte differentiation should still be considered, as multiple aspects of collagen hydrogels, that extend to viscoelastic properties and local, cell adhesion scale, stiffness, have shown to affect cell differentiation and behavior (Chaudhuri et al., 2016; Doyle et al., 2015). Optimization of the collagen hydrogel properties can, however, be challenging in by due to the batch-to-batch variability seen with collagen hydrogels (Antoine et al., 2014).

There is the possibility that in vitro assembled collagen matrix is not sufficient to mimic the environment of differentiating osteocyte, as collagen hydrogel initially lacks the NCPs of bone ECM that are known to regulate the collagen mineralization (de Wildt et al., 2019; Wittkowske et al., 2016). Correct mineralization of bone could be important for osteocyte differentiation as the matrix mineralization occurs simultaneously with osteocyte differentiation (Dallas et al., 2013; Robling & Bonewald, 2020). One approach to acquire collagen matrix that resembles the natural osteocyte environment could be to rely on cells' own collagen deposition. Cell deposited collagen would most likely mimic the natural collagen better than in vitro assembled solubilized collagen. Collagen matrix deposition rate by cells in vitro, unfortunately, is quite low, which hampers the production of cell generated 3D matrices (Kumar et al., 2015). Macromolecular crowding (MMC) of cell culture media has shown to enhance collagen deposition by MSCs, osteoblasts, and fibroblasts (Kumar et al., 2015; Satyam et al., 2014; Zeiger et al., 2012). MMC led to increased deposition of different collagens and various NCPs as well (Kumar et al., 2015; Satyam et al., 2014), which goes that the cell deposited collagen matrix mimics the natural ECM more closely than in vitro assembled collagen hydrogels. Even if the ECM deposition enhanced by MMC would not be enough to create sufficient amount of ECM for osteocyte differentiation, it could be used as a combination with other approaches; for instance, to create newly synthesized matrix to replace some of the area lost due to cell mediated gel shrinkage. This sort of set-up where collagen is reorganized, and new collagen is similarly synthesized would capture the dynamicity of the natural tissue environment much more closely than a set-up where cells are cultured in hydrogel that does not go through any major reorganization.

Another major aspect that was missing from this experimental set-up did were dynamic external cues that are normally present in natural tissues environment. For instance,

mechanical forces are known to affect cell differentiation, and external mechanical stimuli has shown to positively affect MSC differentiation to different cell types in vitro (Goetzke et al., 2018; B. Li et al., 2013). It would seem logical that this would apply for osteocyte differentiation as well, since osteocytes are known to sense mechanical stimuli in vivo. Mechanical loading has shown to upregulate earlier osteocyte markers *PDPN* and *DMP1* expression by osteocytes in vivo (Gluhak-Heinrich et al., 2003; K. Zhang et al., 2006). Similar upregulation was not seen with osteoblasts, so there is no certainty if mechanical loading would stimulate osteocyte differentiation of BMSCs or osteoblast.

Compared to direct mechanical loading, applying fluid flow could be more applicable way to mechanically stimulate cells in vitro. It might also mimic the natural situation more closely, since osteocytes are proposed to sense the mechanical loading via fluid flow on the bone surface and in the canaliculi (Qin et al., 2020; Wittkowske et al., 2016). Fluid flow has already been utilized in cultures aiming for osteocyte differentiation from osteoblasts in 3D microbead system, and in organ-on-chip (OOC) device where primary osteoblasts were cultured in collagen gels (Nasello et al., 2020; Q. Sun et al., 2015, 2017). Both platforms showed indication of osteocyte differentiation but neither studied the effect of the flow. Osteoblast differentiation and bone formation on the other hand are known to be affected by fluid flow (Wittkowske et al., 2016; Yu et al., 2017), which gives a reason to assume that osteocyte differentiation could also benefit from fluid flow. OOC devices allow the formation of fluidic flow that can both brush the surface of the hydrogel or the cells, but also to flow through the hydrogel at the same time (Mykuliak et al., 2022; J. Zhang et al., 2017). Thus, an OOC device can capture both ways by which the osteocytes sense fluid flow in vivo. OOC device would also allow co-culture of multiple cell types, either together in the same hydrogel, or in different compartments of the device (Wu et al., 2020), which could also help to mimic in vivo bone environment and possibly help to promote osteocyte differentiation, as discussed in the section 5.3.

# 7. CONCLUSIONS

This study assessed osteocyte differentiation of hBMSCs cultured in collagen I hydrogels. Some indications of osteocyte differentiation were seen; the cells showed positive staining for osteocyte marker DMP1, and had formed some dendritic processes, that are characteristic for osteocyte phenotype. However, the experimental set-up established in the present study was not sufficient for differentiating mature osteocytes from hBMSCs, as evident by the lack of sclerostin expression and osteocyte morphology, and a gene expression profile that is uncharacteristic for osteocytes.

Based on the underlying biology and earlier studies of osteocyte differentiation, culturing hBMSCs in collagen I hydrogel is still a potential way for acquiring osteocyte-like cells. Modifications to the current culture set-up could help to enhance the osteocyte differentiation. Based on the present study, osteogenic pre-differentiation step in 2D culture, and addition of S53P4 extract in the culture medium potentially enhance osteocyte differentiation. Based on literature, higher cell density could also benefit osteocyte differentiation. It is also obvious that the environment provided by in vitro assembled collagen I does not capture the environment of differentiating osteocyte; in vitro collagen may differ regarding the fibril organization and most likely does not have the same degree of covalent cross-linking as in vivo assembled collagen I. Also, other components essential for bone ECM organization, such as NCPs and other collagen types, are missing from collagen I hydrogels. Therefore, more effort should be put on developing a collagen matrix that mimics the environment of differentiating osteocyte. Ability of cells to reorganize their surroundings and to produce new ECM could also be utilized for achieving natural environment for osteocyte differentiation. This approach could also better recapture the dynamic interactions between the differentiating osteocytes and their environment.

Including other cell types and external mechanical stimuli to the culture set-up should also be considered as they are an integral part of osteocyte environment and have shown to affect differentiation of MSCs. Culturing hBMSCs in collagen hydrogel in an OOC device could be a potential platform for future osteocyte differentiation studies, as it would allow the incorporation of the forementioned elements to the cell culture set-up.

# REFERENCES


Achilli, M., & Mantovani, D. (2010). Tailoring mechanical properties of collagen-based scaffolds for vascular tissue engineering: The effects of pH, temperature and ionic strength on gelation. *Polymers*, *2*(4), 664–680. https://doi.org/10.3390/polym2040664

Ahadian, S., Civitarese, R., Bannerman, D., Mohammadi, M. H., Lu, R., Wang, E., Davenport-Huyer, L., Lai, B., Zhang, B., Zhao, Y., Mandla, S., Korolj, A., & Radisic, M. (2018). Organ-On-A-Chip Platforms: A Convergence of Advanced Materials, Cells, and Microscale Technologies. In *Advanced Healthcare Materials* (Vol. 7, Issue 2). Wiley-VCH Verlag. https://doi.org/10.1002/adhm.201700506

Ahearne, M., Liu, K.-K., Haj, A. J. el, Then, K. Y., Rauz, S., & Yang, Y. (2010). *Online Monitoring of the Mechanical Behavior of Collagen Hydrogels: Influence of Corneal Fibroblasts on Elastic Modulus*. www.liebertpub.com

Ahmed, M., & ffrench-Constant, C. (2016). Extracellular Matrix Regulation of Stem Cell Behavior. In *Current Stem Cell Reports* (Vol. 2, Issue 3, pp. 197–206). Springer International Publishing. https://doi.org/10.1007/s40778-016-0056-2

Alford, A. I., Kozloff, K. M., & Hankenson, K. D. (2015). Extracellular matrix networks in bone remodeling. In *International Journal of Biochemistry and Cell Biology* (Vol. 65, pp. 20–31). Elsevier Ltd. https://doi.org/10.1016/j.biocel.2015.05.008

Alm, J. J., Heino, T. J., Hentunen, T. A., Väänänen, H. K., & Aro, H. T. (2012). Transient 100nM dexamethasone treatment reduces inter-and intraindividual variations in osteoblastic differentiation of bone marrow-derived human mesenchymal stem cells. *Tissue Engineering - Part C: Methods*, *18*(9), 658–666. https://doi.org/10.1089/ten.tec.2011.0675

An, Z., Akily, B., Sabalic, M., Zong, G., Chai, Y., & Sharpe, P. T. (2018). Regulation of Mesenchymal Stem to Transit-Amplifying Cell Transition in the Continuously Growing Mouse Incisor [Article]. *Cell Reports (Cambridge)*, *23*(10), 3102–3111. https://doi.org/10.1016/j.celrep.2018.05.001

Anderson, H. J., Sahoo, J. K., Ulijn, R. v., & Dalby, M. J. (2016). Mesenchymal stem cell fate: Applying biomaterials for control of stem cell behavior. In *Frontiers in Bioengineering and Biotechnology* (Vol. 4, Issue MAY). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2016.00038

Andreev, D., Liu, M., Weidner, D., Kachler, K., Faas, M., Grüneboom, A., Schlötzer-Schrehardt, U., Muñoz, L. E., Steffen, U., Grötsch, B., Killy, B., Krönke, G., Luebke, A. M., Niemeier, A., Wehrhan, F., Lang, R., Schett, G., & Bozec, A. (2020). Osteocyte necrosis triggers osteoclast-mediated bone loss through macrophage-inducible C-type lectin. *Journal of Clinical Investigation*, *130*(9), 4811–4830. https://doi.org/10.1172/JCI134214

Andrzejewska, A., Lukomska, B., & Janowski, M. (2019). Concise Review: Mesenchymal Stem Cells: From Roots to Boost. In *Stem Cells* (Vol. 37, Issue 7, pp. 855–864). Wiley-Blackwell. https://doi.org/10.1002/stem.3016

Antoine, E. E., Vlachos, P. P., & Rylander, M. N. (2014). Review of collagen i hydrogels for bioengineered tissue microenvironments: Characterization of mechanics, structure, and transport. In *Tissue Engineering - Part B: Reviews* (Vol. 20, Issue 6, pp. 683–696). Mary Ann Liebert Inc. https://doi.org/10.1089/ten.teb.2014.0086

Antoine, E. E., Vlachos, P. P., & Rylander, M. N. (2015). Tunable collagen I hydrogels for engineered physiological tissue micro-environments. *PLoS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0122500

Astarita, J. L., Acton, S. E., & Turley, S. J. (2012). Podoplanin: Emerging functions in development, the immune system, and cancer. In *Frontiers in Immunology* (Vol. 3, Issue SEP). https://doi.org/10.3389/fimmu.2012.00283

Atkinson, E. G., & Delgado-Calle, J. (2019). The Emerging Role of Osteocytes in Cancer in Bone. In *JBMR Plus* (Vol. 3, Issue 3). Blackwell Publishing Ltd. https://doi.org/10.1002/jbm4.10186

Bae, Y. J., Kwon, Y. R., Kim, H. J., Lee, S., & Kim, Y. J. (2017). Enhanced differentiation of mesenchymal stromal cells by three-dimensional culture and azacitidine. *Blood Research*, *52*(1), 18–24. https://doi.org/10.5045/br.2017.52.1.18

Barragan-Adjemian, C., Nicolella, D., Dusevich, V., Dallas, M. R., Eick, J. D., & Bonewald, L. F. (2006). Mechanism by which MLO-A5 Late Osteoblasts/Early Osteocytes Mineralize in Culture: Similarities with Mineralization of Lamellar Bone [Article]. *Calcified Tissue International*, *79*(5), 340–353. https://doi.org/10.1007/s00223-006-0107-2

Bernhardt, A., Weiser, E., Wolf, S., Vater, C., & Gelinsky, M. (2019). Primary Human Osteocyte Networks in Pure and Modified Collagen Gels. *Tissue Engineering - Part A*, *25*(19–20), 1347–1355. https://doi.org/10.1089/ten.tea.2018.0338

Bhandi, S., Alkahtani, A., Mashyakhy, M., Ali Baeshen, H., Mustafa, M., Chohan, H., Boreak, N., & Patil, S. (2021). Study of optimal conditions for growth and osteogenic differentiation of dental pulp stem cells based on glucose and serum content. *Saudi Journal of Biological Sciences*, *28*(11), 6359–6364. https://doi.org/10.1016/j.sjbs.2021.06.101

Binder, B. Y. K., Sagun, J. E., & Leach, J. K. (2015). Reduced Serum and Hypoxic Culture Conditions Enhance the Osteogenic Potential of Human Mesenchymal Stem Cells. *Stem Cell Reviews and Reports*, *11*(3), 387–393. https://doi.org/10.1007/s12015-014-9555-7

Birmingham, E., Niebur, G. L., Mchugh, P. E., Shaw, G., Barry, F. P., & McNamara, L. M. (2012). Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *European Cells and Materials*, *23*, 13–27. https://doi.org/10.22203/eCM.v023a02

Bitar, M., Brown, R. A., Salih, V., Kidane, A. G., Knowles, J. C., & Nazhat, S. N. (2008). Effect of cell density on osteoblastic differentiation and matrix degradation of biomimetic dense collagen scaffolds. *Biomacromolecules*, *9*(1), 129–135. https://doi.org/10.1021/bm701112w

Blaine, J., Chonchol, M., & Levi, M. (2015). Renal control of calcium, phosphate, and magnesium homeostasis. *Clinical Journal of the American Society of Nephrology*, *10*(7), 1257–1272. https://doi.org/10.2215/CJN.09750913

Bloom, A. B., & Zaman, M. H. (2014). Influence of the microenvironment on cell fate determination and migration [Article]. *Physiological Genomics*, *46*(9), 309–314. https://doi.org/10.1152/physiolgenomics.00170.2013

Bonewald, L. F. (2011). The amazing osteocyte. *Journal of Bone and Mineral Research*, *26*(2), 229–238. https://doi.org/10.1002/jbmr.320

Boraschi-Diaz, I., Wang, J., Mort, J. S., & Komarova, S. v. (2017). Collagen type i as a ligand for receptor-mediated signaling. In *Frontiers in Physics* (Vol. 5, Issue MAY). Frontiers Media S.A. https://doi.org/10.3389/fphy.2017.00012

Boukhechba, F., Balaguer, T., Michiels, J. F., Ackermann, K., Quincey, D., Bouler, J. M., Pyerin, W., Carle, G. F., & Rochet, N. (2009). Human primary osteocyte differentiation in a 3D culture system. *Journal of Bone and Mineral Research*, *24*(11), 1927–1935. https://doi.org/10.1359/jbmr.090517

Buehler, M. J. (2006). *Nature designs tough collagen: Explaining the nanostructure of collagen fibrils*. www.pnas.orgcgidoi10.1073pnas.0603216103

Charbord, P. (2010). Bone marrow mesenchymal stem cells: Historical overview and concepts. In *Human Gene Therapy* (Vol. 21, Issue 9, pp. 1045–1056). https://doi.org/10.1089/hum.2010.115

Chaudhuri, O., Cooper-White, J., Janmey, P. A., Mooney, D. J., & Shenoy, V. B. (2020). Effects of extracellular matrix viscoelasticity on cellular behaviour. In *Nature* (Vol. 584, Issue 7822, pp. 535–546). Nature Research. https://doi.org/10.1038/s41586-020-2612-2

Chaudhuri, O., Gu, L., Klumpers, D., Darnell, M., Bencherif, S. A., Weaver, J. C., Huebsch, N., Lee, H. P., Lippens, E., Duda, G. N., & Mooney, D. J. (2016). Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nature Materials*, *15*(3), 326–334. https://doi.org/10.1038/nmat4489

Cheng, Y. H., Dong, J. C., & Bian, Q. (2019). Small molecules for mesenchymal stem cell fate determination. In *World Journal of Stem Cells* (Vol. 11, Issue 12, pp. 1084–1103). Baishideng Publishing Group Co. https://doi.org/10.4252/wjsc.v11.i12.1084

Chieh, H. F., Sun, Y., Liao, J. der, Su, F. C., Zhao, C., Amadio, P. C., & An, K. N. (2010). Effects of cell concentration and collagen concentration on contraction kinetics and mechanical properties in a bone marrow stromal cell-collagen construct. *Journal of Biomedical Materials Research - Part A*, *93*(3), 1132–1139. https://doi.org/10.1002/jbm.a.32606

Creecy, A., Damrath, J. G., & Wallace, J. M. (2021). Control of Bone Matrix Properties by Osteocytes. In *Frontiers in Endocrinology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fendo.2020.578477

Dallas, S. L., Prideaux, M., & Bonewald, L. F. (2013). The osteocyte: An endocrine cell . . . and more. In *Endocrine Reviews* (Vol. 34, Issue 5, pp. 658–690). https://doi.org/10.1210/er.2012-1026

Davidson, C. D., Wang, W. Y., Zaimi, I., Jayco, D. K. P., & Baker, B. M. (2019). Cell force-mediated matrix reorganization underlies multicellular network assembly. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-018-37044-1

de Wildt, B. W. M., Ansari, S., Sommerdijk, N. A. J. M., Ito, K., Akiva, A., & Hofmann, S. (2019). From bone regeneration to three-dimensional in vitro models: tissue engineering of organized bone extracellular matrix. In *Current Opinion in Biomedical Engineering* (Vol. 10, pp. 107–115). Elsevier B.V. https://doi.org/10.1016/j.cobme.2019.05.005

Delanaye, P., Krzesinski, J. M., Warling, X., Moonen, M., Smelten, N., Médart, L., Bruyère, O., Reginster, J. Y., Pottel, H., & Cavalier, E. (2014). Clinical and biological determinants of sclerostin plasma concentration in hemodialysis patients. *Nephron - Clinical Practice*, *128*(1–2), 127–134. https://doi.org/10.1159/000366449

Delgado, L. M., Bayon, Y., Pandit, A., & Zeugolis, D. I. (2015). To Cross-Link or Not to Cross-Link? Cross-Linking Associated Foreign Body Response of Collagen-Based Devices. *Tissue Engineering - Part B: Reviews*, *21*(3), 298–313. https://doi.org/10.1089/ten.teb.2014.0290

Delgado, L. M., Fuller, K., & Zeugolis, D. I. (2017). Collagen Cross-Linking: Biophysical, Biochemical, and Biological Response Analysis. *Tissue Engineering - Part A*, *23*(19–20), 1064–1077. https://doi.org/10.1089/ten.tea.2016.0415

Delgado-Calle, J., Anderson, J., Cregor, M. D., Hiasa, M., Chirgwin, J. M., Carlesso, N., Yoneda, T., Mohammad, K. S., Plotkin, L. I., Roodman, G. D., & Bellido, T. (2016). Bidirectional notch signaling and osteocyte-derived factors in the bone marrow microenvironment promote tumor cell proliferation and bone destruction in multiple myeloma. *Cancer Research*, *76*(5), 1089–1100. https://doi.org/10.1158/0008-5472.CAN-15-1703

Depalle, B., Qin, Z., Shefelbine, S. J., & Buehler, M. J. (2015). Influence of cross-link structure, density and mechanical properties in the mesoscale deformation mechanisms of collagen fibrils. *Journal of the Mechanical Behavior of Biomedical Materials*, *52*, 1–13. https://doi.org/10.1016/j.jmbbm.2014.07.008

Dippold, D., Cai, A., Hardt, M., Boccaccini, A. R., Horch, R. E., Beier, J. P., & Schubert, D. W. (2019). Investigation of the batch-to-batch inconsistencies of Collagen in PCL-Collagen nanofibers. *Materials Science and Engineering C*, *95*, 217–225. https://doi.org/10.1016/j.msec.2018.10.057

Divieti Pajevic, P., & Krause, D. S. (2019). Osteocyte regulation of bone and blood. In *Bone* (Vol. 119, pp. 13–18). Elsevier Inc. https://doi.org/10.1016/j.bone.2018.02.012

Doyle, A. D., Carvajal, N., Jin, A., Matsumoto, K., & Yamada, K. M. (2015). Local 3D matrix microenvironment regulates cell migration through spatiotemporal dynamics of contractility-dependent adhesions. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms9720

Drake, M. T., Fenske, J. S., Blocki, F. A., Zierold, C., Appelman-Dijkstra, N., Papapoulos, S., & Khosla, S. (2018). Validation of a novel, rapid, high precision sclerostin assay not confounded by sclerostin fragments. *Bone*, *111*, 36–43. https://doi.org/10.1016/j.bone.2018.03.013

Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., & Karsenty, G. (1996). Increased bone formation in osteocalcin-deficient mice [Article]. *Nature (London)*, *382*(6590), 448–452. https://doi.org/10.1038/382448a0

Engler, A. J., Sen, S., Sweeney, H. L., & Discher, D. E. (2006). Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell*, *126*(4), 677–689. https://doi.org/10.1016/j.cell.2006.06.044

Fidler, A. L., Boudko, S. P., Rokas, A., & Hudson, B. G. (2018). The triple helix of collagens - An ancient protein structure that enabled animal multicellularity and tissue evolution. In *Journal of Cell Science* (Vol. 131, Issue 7). Company of Biologists Ltd. https://doi.org/10.1242/jcs.203950

Florencio-Silva, R., Sasso, G. R. D. S., Sasso-Cerri, E., Simões, M. J., & Cerri, P. S. (2015). Biology of Bone Tissue: Structure, Function, and Factors That Influence

Bone Cells. In *BioMed Research International* (Vol. 2015). Hindawi Publishing Corporation. https://doi.org/10.1155/2015/421746

Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. In *Journal of Cell Science* (Vol. 123, Issue 24, pp. 4195–4200). https://doi.org/10.1242/jcs.023820

Fratzl, P. (2008). *Collagen* (1. Aufl.) [Book]. Springer-Verlag. https://doi.org/10.1007/978-0-387-73906-9

Gao, C., Peng, S., Feng, P., & Shuai, C. (2017). Bone biomaterials and interactions with stem cells. In *Bone Research* (Vol. 5). Sichuan University. https://doi.org/10.1038/boneres.2017.59

García-Rodríguez, J., & Martínez-Reina, J. (2017). Elastic properties of woven bone: effect of mineral content and collagen fibrils orientation. *Biomechanics and Modeling in Mechanobiology*, *16*(1), 159–172. https://doi.org/10.1007/s10237-016-0808-z

Garnero, P. (2015). The Role of Collagen Organization on the Properties of Bone. *Calcified Tissue International*, *97*(3), 229–240. https://doi.org/10.1007/s00223-015-9996-2

Giuliani, N., Ferretti, M., Bolzoni, M., Storti, P., Lazzaretti, M., Dalla Palma, B., Bonomini, S., Martella, E., Agnelli, L., Neri, A., Ceccarelli, F., & Palumbo, C. (2012). Increased osteocyte death in multiple myeloma patients: Role in myeloma-induced osteoclast formation. *Leukemia*, *26*(6), 1391–1401. https://doi.org/10.1038/leu.2011.381

Gluhak-Heinrich, J., Ye, L., Bonewald, L. F., Feng, J. Q., Macdougall, M., Harris, S. E., & Pavlin, D. (2003). *Mechanical Loading Stimulates Dentin Matrix Protein 1 (DMP1) Expression in Osteocytes In Vivo*.

Goetzke, R., Sechi, A., de Laporte, L., Neuss, S., & Wagner, W. (2018). Why the impact of mechanical stimuli on stem cells remains a challenge. In *Cellular and Molecular Life Sciences* (Vol. 75, Issue 18, pp. 3297–3312). Birkhauser Verlag AG. https://doi.org/10.1007/s00018-018-2830-z

Han, Y., Li, X., Zhang, Y., Han, Y., Chang, F., & Ding, J. (2019). Mesenchymal Stem Cells for Regenerative Medicine. *Cells*, *8*(8), 886. https://doi.org/10.3390/cells8080886

He, F., Cao, J., Qi, J., Liu, Z., Liu, G., & Deng, W. (2021). Regulation of Stem Cell Differentiation by Inorganic Nanomaterials: Recent Advances in Regenerative Medicine. In *Frontiers in Bioengineering and Biotechnology* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.721581

He, G., Dahl, T., Veis, A., & George, A. (2003). Nucleation of apatite crystals in vitro by self-assembled dentin matrix protein 1. *Nature Materials*, *2*(8), 552–558. https://doi.org/10.1038/nmat945

Heo, J., Koh, R. H., Shim, W., Kim, H. D., Yim, H. G., & Hwang, N. S. (2016). Riboflavin-induced photo-crosslinking of collagen hydrogel and its application in meniscus tissue engineering. *Drug Delivery and Translational Research*, *6*(2), 148–158. https://doi.org/10.1007/s13346-015-0224-4

Heo, J. S., Choi, Y., Kim, H. S., & Kim, H. O. (2016). Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. *International Journal of Molecular Medicine*, *37*(1), 115–125. https://doi.org/10.3892/ijmm.2015.2413

Herchenhan, A., Uhlenbrock, F., Eliasson, P., Weis, M., Eyre, D., Kadler, K. E., Magnusson, S. P., & Kjaer, M. (2015). Lysyl oxidase activity is required for ordered collagen fibrillogenesis by tendon cells. *Journal of Biological Chemistry*, *290*(26), 16440–16450. https://doi.org/10.1074/jbc.M115.641670

Honma, M., Ikebuchi, Y., Kariya, Y., Hayashi, M., Hayashi, N., Aoki, S., & Suzuki, H. (2013). RANKL subcellular trafficking and regulatory mechanisms in osteocytes. *Journal of Bone and Mineral Research*, *28*(9), 1936–1949. https://doi.org/10.1002/jbmr.1941

Honma, M., Ikebuchi, Y., Kariya, Y., & Suzuki, H. (2015). Establishment of optimized in vitro assay methods for evaluating osteocyte functions. *Journal of Bone and Mineral Metabolism*, *33*(1), 73–84. https://doi.org/10.1007/s00774-013-0555-5

Hussey, G. S., Dziki, J. L., & Badylak, S. F. (2018). Extracellular matrix-based materials for regenerative medicine. In *Nature Reviews Materials* (Vol. 3, Issue 7, pp. 159–173). Nature Publishing Group. https://doi.org/10.1038/s41578-018-0023-x

Iaquinta, M. R., Mazzoni, E., Bononi, I., Rotondo, J. C., Mazziotta, C., Montesi, M., Sprio, S., Tampieri, A., Tognon, M., & Martini, F. (2019). Adult Stem Cells for Bone Regeneration and Repair. In *Frontiers in Cell and Developmental Biology* (Vol. 7). Frontiers Media S.A. https://doi.org/10.3389/fcell.2019.00268

Irawan, V., Sung, T. C., Higuchi, A., & Ikoma, T. (2018). Collagen Scaffolds in Cartilage Tissue Engineering and Relevant Approaches for Future Development. In *Tissue Engineering and Regenerative Medicine* (Vol. 15, Issue 6, pp. 673–697). Korean Tissue Engineering and Regenerative Medicine Society. https://doi.org/10.1007/s13770-018-0135-9

Jaiswal, N., Haynesworth, S. E., Caplan, A. I., & Bruder, S. P. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro [Article]. *Journal of Cellular Biochemistry*, *64*(2), 295–312. https://doi.org/10.1002/(SICI)1097-4644(199702)64:2<295::AID-JCB12>3.0.CO;2-I

Jin, G. Z., & Kim, H. W. (2017). Effects of Type I Collagen Concentration in Hydrogel on the Growth and Phenotypic Expression of Rat Chondrocytes. *Tissue Engineering and Regenerative Medicine*, *14*(4), 383–391. https://doi.org/10.1007/s13770-017-0060-3

Kadler, K. E., Hill, A., & Canty-Laird, E. G. (2008). Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. In *Current Opinion in Cell Biology* (Vol. 20, Issue 5, pp. 495–501). https://doi.org/10.1016/j.ceb.2008.06.008

Kerschnitzki, M., Wagermaier, W., Roschger, P., Seto, J., Shahar, R., Duda, G. N., Mundlos, S., & Fratzl, P. (2011). The organization of the osteocyte network mirrors the extracellular matrix orientation in bone. *Journal of Structural Biology*, *173*(2), 303–311. https://doi.org/10.1016/j.jsb.2010.11.014

Khetan, S., Guvendiren, M., Legant, W. R., Cohen, D. M., Chen, C. S., & Burdick, J. A. (2013). Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nature Materials*, *12*(5), 458–465. https://doi.org/10.1038/nmat3586

Kim, D., Lee, B., Thomopoulos, S., & Jun, Y. S. (2016). In situ evaluation of calcium phosphate nucleation kinetics and pathways during intra- and extrafibrillar

mineralization of collagen matrices. *Crystal Growth and Design*, *16*(9), 5359–5366. https://doi.org/10.1021/acs.cgd.6b00864

Kim, D., Lee, B., Thomopoulos, S., & Jun, Y. S. (2018). The role of confined collagen geometry in decreasing nucleation energy barriers to intrafibrillar mineralization. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-03041-1

Kim, H., Bae, C., Kook, Y. M., Koh, W. G., Lee, K., & Park, M. H. (2019). Mesenchymal stem cell 3D encapsulation technologies for biomimetic microenvironment in tissue regeneration. In *Stem Cell Research and Therapy* (Vol. 10, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13287-018-1130-8

Kim, J., & Adachi, T. (2019). Cell Condensation Triggers the Differentiation of Osteoblast Precursor Cells to Osteocyte-Like Cells. *Frontiers in Bioengineering and Biotechnology*, *7*. https://doi.org/10.3389/fbioe.2019.00288

Kim, J., & Adachi, T. (2021). Cell-fate decision of mesenchymal stem cells toward osteocyte differentiation is committed by spheroid culture. *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-92607-z

Klumpers, D. D., Zhao, X., Mooney, D. J., & Smit, T. H. (2013). Cell mediated contraction in 3D cell-matrix constructs leads to spatially regulated osteogenic differentiation. *Integrative Biology (United Kingdom)*, *5*(9), 1174–1183. https://doi.org/10.1039/c3ib40038g

Komori, T. (2002). Runx2, a multifunctional transcription factor in skeletal development. In *Journal of Cellular Biochemistry* (Vol. 87, Issue 1, pp. 1–8). https://doi.org/10.1002/jcb.10276

Komori, T. (2019). Regulation of proliferation, differentiation and functions of osteoblasts by runx2. In *International Journal of Molecular Sciences* (Vol. 20, Issue 7). MDPI AG. https://doi.org/10.3390/ijms20071694

Koons, G. L., Diba, M., & Mikos, A. G. (2020). Materials design for bone-tissue engineering. In *Nature Reviews Materials* (Vol. 5, Issue 8, pp. 584–603). Nature Research. https://doi.org/10.1038/s41578-020-0204-2

Kubow, K. E., Vukmirovic, R., Zhe, L., Klotzsch, E., Smith, M. L., Gourdon, D., Luna, S., & Vogel, V. (2015). Mechanical forces regulate the interactions of fibronectin and collagen i in extracellular matrix. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms9026

Kumar, P., Satyam, A., Fan, X., Collin, E., Rochev, Y., Rodriguez, B. J., Gorelov, A., Dillon, S., Joshi, L., Raghunath, M., Pandit, A., & Zeugolis, D. I. (2015). Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. *Scientific Reports*, *5*. https://doi.org/10.1038/srep08729

Kurenkova, A. D., Medvedeva, E. v., Newton, P. T., & Chagin, A. S. (2020). Niches for Skeletal Stem Cells of Mesenchymal Origin. In *Frontiers in Cell and Developmental Biology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fcell.2020.00592

Kursad, T. (2012). *Stem Cell Biology and Regenerative Medicine*. http://www.springer.com/series/7896

Langenbach, F., & Handschel, J. (2013). Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro [Article]. *Stem Cell Research & Therapy*, *4*(5), 117–117. https://doi.org/10.1186/scrt328

Lefèvre, E., Farlay, D., Bala, Y., Subtil, F., Wolfram, U., Rizzo, S., Baron, C., Zysset, P., Pithioux, M., & Follet, H. (2019). Compositional and mechanical properties of growing cortical bone tissue: a study of the human fibula. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-54016-1

Li, B., Moshfegh, C., Lin, Z., Albuschies, J., & Vogel, V. (2013). Mesenchymal stem cells exploit extracellular matrix as mechanotransducer. *Scientific Reports*, *3*. https://doi.org/10.1038/srep02425

Li, Y., Liu, Y., Li, R., Bai, H., Zhu, Z., Zhu, L., Zhu, C., Che, Z., Liu, H., Wang, J., & Huang, L. (2021). Collagen-based biomaterials for bone tissue engineering. *Materials and Design*, *210*. https://doi.org/10.1016/j.matdes.2021.110049

Liao, H.-T. (2014). Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells. *World Journal of Stem Cells*, *6*(3), 288. https://doi.org/10.4252/wjsc.v6.i3.288

Liaw, C. Y., Ji, S., & Guvendiren, M. (2018). Engineering 3D Hydrogels for Personalized In Vitro Human Tissue Models. In *Advanced Healthcare Materials* (Vol. 7, Issue 4). Wiley-VCH Verlag. https://doi.org/10.1002/adhm.201701165

Lin, H., Sohn, J., Shen, H., Langhans, M. T., & Tuan, R. S. (2019). Bone marrow mesenchymal stem cells: Aging and tissue engineering applications to enhance bone healing. *Biomaterials*, *203*, 96–110. https://doi.org/10.1016/j.biomaterials.2018.06.026

Lin, J., Shi, Y., Men, Y., Wang, X., Ye, J., & Zhang, C. (2020). Mechanical roles in formation of oriented collagen fibers. In *Tissue Engineering - Part B: Reviews* (Vol. 26, Issue 2, pp. 116–128). Mary Ann Liebert Inc. https://doi.org/10.1089/ten.teb.2019.0243

Lin, X., Patil, S., Gao, Y. G., & Qian, A. (2020). The Bone Extracellular Matrix in Bone Formation and Regeneration. In *Frontiers in Pharmacology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fphar.2020.00757

Ling, Y., Rios, H. F., Myers, E. R., Lu, Y., Feng, J. Q., & Boskey, A. L. (2005). DMP1 Depletion Decreases Bone Mineralization In Vivo: An FTIR Imaging Analysis [Article]. *Journal of Bone and Mineral Research*, *20*(12), 2169–2177. https://doi.org/10.1359/JBMR.050815

Liu, H., Wang, Y., Cui, K., Guo, Y., Zhang, X., & Qin, J. (2019). Advances in Hydrogels in Organoids and Organs-on-a-Chip. *Advanced Materials*, *31*(50). https://doi.org/10.1002/adma.201902042

Liu, T., Wang, J., Xie, X., Wang, K., Sui, T., Liu, D., Lai, L., Zhao, H., Li, Z., & Feng, J. Q. (2019). DMP1 Ablation in the Rabbit Results in Mineralization Defects and Abnormalities in Haversian Canal/Osteon Microarchitecture. *Journal of Bone and Mineral Research*, *34*(6), 1115–1128. https://doi.org/10.1002/jbmr.3683

Liu, X., Zheng, C., Luo, X., Wang, X., & Jiang, H. (2019). Recent advances of collagen-based biomaterials: Multi-hierarchical structure, modification and biomedical applications. In *Materials Science and Engineering C* (Vol. 99, pp. 1509–1522). Elsevier Ltd. https://doi.org/10.1016/j.msec.2019.02.070

Liu, Y., Mai, S., Li, N., Yiu, C. K. Y., Mao, J., Pashley, D. H., & Tay, F. R. (2011). Differences between top-down and bottom-up approaches in mineralizing thick, partially demineralized collagen scaffolds. *Acta Biomaterialia*, *7*(4), 1742–1751. https://doi.org/10.1016/j.actbio.2010.11.028

Lopes, D., Martins-Cruz, C., Oliveira, M. B., & Mano, J. F. (2018). Bone physiology as inspiration for tissue regenerative therapies. In *Biomaterials* (Vol. 185, pp. 240–275). Elsevier Ltd. https://doi.org/10.1016/j.biomaterials.2018.09.028

Lu, Y., Yuan, B., Qin, C., Cao, Z., Xie, Y., Dallas, S. L., McKee, M. D., Drezner, M. K., Bonewald, L. F., & Feng, J. Q. (2011). The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa c-terminal fragment. *Journal of Bone and Mineral Research*, *26*(2), 331–340. https://doi.org/10.1002/jbmr.226

Lund, A. W., Stegemann, J. P., & Plopper, G. E. (2009). Inhibition of ERK promotes collagen gel compaction and fibrillogenesis to amplify the osteogenesis of human mesenchymal stem cells in three-dimensional collagen I culture. *Stem Cells and Development*, *18*(2), 331–341. https://doi.org/10.1089/scd.2008.0075

Martino, F., Perestrelo, A. R., Vinarský, V., Pagliari, S., & Forte, G. (2018). Cellular mechanotransduction: From tension to function. In *Frontiers in Physiology* (Vol. 9, Issue JUL). Frontiers Media S.A. https://doi.org/10.3389/fphys.2018.00824

McGarrigle, M. J., Mullen, C. A., Haugh, M. G., Voisin, M. C., & McNamara, L. M. (2016). Osteocyte differentiation and the formation of an interconnected cellular network in vitro. *European Cells and Materials*, *31*, 323–340. https://doi.org/10.22203/eCM.v031a21

Mikami, Y., Omoteyama, K., Kato, S., & Takagi, M. (2007). Inductive effects of dexamethasone on the mineralization and the osteoblastic gene expressions in mature osteoblast-like ROS17/2.8 cells. *Biochemical and Biophysical Research Communications*, *362*(2), 368–373. https://doi.org/10.1016/j.bbrc.2007.07.192

Mohamed-Ahmed, S., Fristad, I., Lie, S. A., Suliman, S., Mustafa, K., Vindenes, H., & Idris, S. B. (2018). Adipose-derived and bone marrow mesenchymal stem cells: A donor-matched comparison. *Stem Cell Research and Therapy*, *9*(1). https://doi.org/10.1186/s13287-018-0914-1

Morgan, E. F., Unnikrisnan, G. U., & Hussein, A. I. (2018). Bone Mechanical Properties in Healthy and Diseased States. In *Annual Review of Biomedical Engineering* (Vol. 20, pp. 119–143). Annual Reviews Inc. https://doi.org/10.1146/annurev-bioeng-062117-121139

Morrison, S. J., & Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer [Article]. *Nature (London)*, *441*(7097), 1068–1074. https://doi.org/10.1038/nature04956

Mullen, C. A., Haugh, M. G., Schaffler, M. B., Majeska, R. J., & McNamara, L. M. (2013). Osteocyte differentiation is regulated by extracellular matrix stiffness and intercellular separation. *Journal of the Mechanical Behavior of Biomedical Materials*, *28*, 183–194. https://doi.org/10.1016/j.jmbbm.2013.06.013

Mykuliak, A., Yrjänäinen, A., Mäki, A.-J., Gebraad, A., Lampela, E., Kääriäinen, M., Pakarinen, T.-K., Kallio, P., Miettinen, S., & Vuorenpää, H. (2022). Vasculogenic Potency of Bone Marrow- and Adipose Tissue-Derived Mesenchymal Stem/Stromal Cells Results in Differing Vascular Network Phenotypes in a Microfluidic Chip. *Frontiers in Bioengineering and Biotechnology*, *10*. https://doi.org/10.3389/fbioe.2022.764237

Naito, H., Yoshimura, M., Mizuno, T., Takasawa, S., Tojo, T., & Taniguchi, S. (2013). The advantages of three-dimensional culture in a collagen hydrogel for stem cell

differentiation. *Journal of Biomedical Materials Research - Part A*, *101*(10), 2838–2845. https://doi.org/10.1002/jbm.a.34578

Nakamura, T., Nakamura-Takahashi, A., Kasahara, M., Yamaguchi, A., & Azuma, T. (2020). Tissue-nonspecific alkaline phosphatase promotes the osteogenic differentiation of osteoprogenitor cells. *Biochemical and Biophysical Research Communications*, *524*(3), 702–709. https://doi.org/10.1016/j.bbrc.2020.01.136

Nam, S., Hu, K. H., Butte, M. J., & Chaudhuri, O. (2016). Strain-enhanced stress relaxation impacts nonlinear elasticity in collagen gels. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(20), 5492–5497. https://doi.org/10.1073/pnas.1523906113

Naqvi, S. M., & McNamara, L. M. (2020). Stem Cell Mechanobiology and the Role of Biomaterials in Governing Mechanotransduction and Matrix Production for Tissue Regeneration. In *Frontiers in Bioengineering and Biotechnology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2020.597661

Nasello, G., Alamán-Díez, P., Schiavi, J., Pérez, M. Á., McNamara, L., & García-Aznar, J. M. (2020). Primary Human Osteoblasts Cultured in a 3D Microenvironment Create a Unique Representative Model of Their Differentiation Into Osteocytes. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.00336

Niu, X., Wang, L., Tian, F., Wang, L., Li, P., Feng, Q., & Fan, Y. (2016). Shear-mediated crystallization from amorphous calcium phosphate to bone apatite. *Journal of the Mechanical Behavior of Biomedical Materials*, *54*, 131–140. https://doi.org/10.1016/j.jmbbm.2015.09.024

Noda, S., Kawashima, N., Yamamoto, M., Hashimoto, K., Nara, K., Sekiya, I., & Okiji, T. (2019). Effect of cell culture density on dental pulp-derived mesenchymal stem cells with reference to osteogenic differentiation. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-41741-w

Oftadeh, R., Perez-Viloria, M., Villa-Camacho, J. C., Vaziri, A., & Nazarian, A. (2015). Biomechanics and Mechanobiology of Trabecular Bone: A Review. In *Journal of Biomechanical Engineering* (Vol. 137, Issue 1). American Society of Mechanical Engineers (ASME). https://doi.org/10.1115/1.4029176

Ojansivu, M., Vanhatupa, S., Björkvik, L., Häkkänen, H., Kellomäki, M., Autio, R., Ihalainen, J. A., Hupa, L., & Miettinen, S. (2015). Bioactive glass ions as strong enhancers of osteogenic differentiation in human adipose stem cells. *Acta Biomaterialia*, *21*, 190–203. https://doi.org/10.1016/j.actbio.2015.04.017

Oryan, A., Kamali, A., Moshirib, A., & Eslaminejad, M. B. (2017). Role of Mesenchymal Stem Cells in Bone Regenerative Medicine: What Is the Evidence? In *Cells Tissues Organs* (Vol. 204, Issue 2, pp. 59–83). S. Karger AG. https://doi.org/10.1159/000469704

Pal, B., & Das, B. (2017). In vitro culture of naïve human bone marrow mesenchymal stem cells: A stemness based approach. In *Frontiers in Cell and Developmental Biology* (Vol. 5, Issue AUG). Frontiers Media S.A. https://doi.org/10.3389/fcell.2017.00069

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. In *Nucleic Acids Research* (Vol. 29, Issue 9).

Phillips, J. E., Gersbach, C. A., Wojtowicz, A. M., & García, A. J. (2006). Glucocorticoid-induced osteogenesis is negatively regulated by Runx2/Cbfa1 serine phosphorylation [Article]. *Journal of Cell Science*, *119*(Pt 3), 581–591. https://doi.org/10.1242/jcs.02758

Piechocka, I. K., van Oosten, A. S. G., Breuls, R. G. M., & Koenderink, G. H. (2011). Rheology of heterotypic collagen networks. *Biomacromolecules*, *12*(7), 2797–2805. https://doi.org/10.1021/bm200553x

Pittenger, M. F., Discher, D. E., Péault, B. M., Phinney, D. G., Hare, J. M., & Caplan, A. I. (2019). Mesenchymal stem cell perspective: cell biology to clinical progress. In *npj Regenerative Medicine* (Vol. 4, Issue 1). Nature Research. https://doi.org/10.1038/s41536-019-0083-6

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., & Marshak, D. R. (1999). Multilineage Potential of Adult Human Mesenchymal Stem Cells [Article]. *Science (American Association for the Advancement of Science)*, *284*(5411), 143–147. https://doi.org/10.1126/science.284.5411.143

Prideaux, M., Schutz, C., Wijenayaka, A. R., Findlay, D. M., Campbell, D. G., Solomon, L. B., & Atkins, G. J. (2016). Isolation of osteocytes from human trabecular bone. *Bone*, *88*, 64–72. https://doi.org/10.1016/j.bone.2016.04.017

Qin, L., Liu, W., Cao, H., & Xiao, G. (2020). Molecular mechanosensors in osteocytes. In *Bone Research* (Vol. 8, Issue 1). Springer Nature. https://doi.org/10.1038/s41413-020-0099-y

Ramshaw, J. A. M., Shah, N. K., & Brodsky, B. (1998). *Gly-X-Y Tripeptide Frequencies in Collagen: A Context for Host-Guest Triple-Helical Peptides*.

Raspanti, M., Reguzzoni, M., Protasoni, M., & Basso, P. (2018). Not only tendons: The other architecture of collagen fibrils. In *International Journal of Biological Macromolecules* (Vol. 107, pp. 1668–1674). Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2017.10.037

Raub, C. B., Unruh, J., Suresh, V., Krasieva, T., Lindmo, T., Gratton, E., Tromberg, B. J., & George, S. C. (2008). Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties. *Biophysical Journal*, *94*(6), 2361–2373. https://doi.org/10.1529/biophysj.107.120006

Ravindran, S., & George, A. (2015). Dentin matrix proteins in bone tissue engineering. In *Advances in Experimental Medicine and Biology* (Vol. 881, pp. 129–142). Springer New York LLC. https://doi.org/10.1007/978-3-319-22345-2_8

Revell, C. K., Jensen, O. E., Shearer, T., Lu, Y., Holmes, D. F., & Kadler, K. E. (2021). Collagen fibril assembly: New approaches to unanswered questions. *Matrix Biology Plus*, *12*. https://doi.org/10.1016/j.mbplus.2021.100079

Reznikov, N., Shahar, R., & Weiner, S. (2014). Bone hierarchical structure in three dimensions. *Acta Biomaterialia*, *10*(9), 3815–3826. https://doi.org/10.1016/j.actbio.2014.05.024

Ricard-Blum, S. (2011). The Collagen Family. *Cold Spring Harbor Perspectives in Biology*, *3*(1), 1–19. https://doi.org/10.1101/cshperspect.a004978

Robert, A. W., Marcon, B. H., Dallagiovanna, B., & Shigunov, P. (2020). Adipogenesis, Osteogenesis, and Chondrogenesis of Human Mesenchymal Stem/Stromal Cells:

A Comparative Transcriptome Approach. In *Frontiers in Cell and Developmental Biology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fcell.2020.00561

Robling, A. G., & Bonewald, L. F. (2020). The Osteocyte: New Insights. In *Annual Review of Physiology* (Vol. 82, pp. 485–506). Annual Reviews Inc. https://doi.org/10.1146/annurev-physiol-021119-034332

Roeder, B. A., Kokini, K., Sturgis, J. E., Robinson, J. P., & Voytik-Harbin, S. L. (2002). Tensile Mechanical Properties of Three-Dimensional Type I Collagen Extracellular Matrices With Varied Microstructure [Article]. *Journal of Biomechanical Engineering*, *124*(2), 214–222. https://doi.org/10.1115/1.1449904

Ru, J. ying, & Wang, Y. fen. (2020). Osteocyte apoptosis: the roles and key molecular mechanisms in resorption-related bone diseases. In *Cell Death and Disease* (Vol. 11, Issue 10). Springer Nature. https://doi.org/10.1038/s41419-020-03059-8

Sabokbar, A., Millett, P. J., Myer, B., & Rushton, N. (1994). *A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro*.

Saghiri, M. A., Asatourian, A., Orangi, J., Sorenson, C. M., & Sheibani, N. (2015). Functional role of inorganic trace elements in angiogenesis-Part I: N, Fe, Se, P, Au, and Ca. In *Critical Reviews in Oncology/Hematology* (Vol. 96, Issue 1, pp. 129–142). Elsevier Ireland Ltd. https://doi.org/10.1016/j.critrevonc.2015.05.010

Sai, Y., Shiwaku, Y., Anada, T., Tsuchiya, K., Takahashi, T., & Suzuki, O. (2018). Capacity of octacalcium phosphate to promote osteoblastic differentiation toward osteocytes in vitro. *Acta Biomaterialia*, *69*, 362–371. https://doi.org/10.1016/j.actbio.2018.01.026

Sakaguchi, Y., Sekiya, I., Yagishita, K., & Muneta, T. (2005). Comparison of human stem cells derived from various mesenchymal tissues: Superiority of synovium as a cell source. *Arthritis and Rheumatism*, *52*(8), 2521–2529. https://doi.org/10.1002/art.21212

Salvatore, L., Gallo, N., Natali, M. L., Terzi, A., Sannino, A., & Madaghiele, M. (2021). Mimicking the Hierarchical Organization of Natural Collagen: Toward the Development of Ideal Scaffolding Material for Tissue Regeneration. In *Frontiers in Bioengineering and Biotechnology* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.644595

Sarrigiannidis, S. O., Rey, J. M., Dobre, O., González-García, C., Dalby, M. J., & Salmeron-Sanchez, M. (2021). A tough act to follow: collagen hydrogel modifications to improve mechanical and growth factor loading capabilities. In *Materials Today Bio* (Vol. 10). Elsevier B.V. https://doi.org/10.1016/j.mtbio.2021.100098

Satyam, A., Kumar, P., Fan, X., Gorelov, A., Rochev, Y., Joshi, L., Peinado, H., Lyden, D., Thomas, B., Rodriguez, B., Raghunath, M., Pandit, A., & Zeugolis, D. (2014). Macromolecular crowding meets tissue engineering by self-assembly: A paradigm shift in regenerative medicine. *Advanced Materials*, *26*(19), 3024–3034. https://doi.org/10.1002/adma.201304428

Sawa, N., Fujimoto, H., Sawa, Y., & Yamashita, J. (2019). Alternating Differentiation and Dedifferentiation between Mature Osteoblasts and Osteocytes. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-50236-7

Shiflett, L. A., Tiede-Lewis, L. A. M., Xie, Y., Lu, Y., Ray, E. C., & Dallas, S. L. (2019). Collagen Dynamics During the Process of Osteocyte Embedding and

Mineralization. *Frontiers in Cell and Developmental Biology*, *7*. https://doi.org/10.3389/fcell.2019.00178

Shoulders, M. D., & Raines, R. T. (2009). Collagen structure and stability. In *Annual Review of Biochemistry* (Vol. 78, pp. 929–958). https://doi.org/10.1146/annurev.biochem.77.032207.120833

Silvent, J., Nassif, N., Helary, C., Azaïs, T., Sire, J. Y., & Guille, M. M. G. (2013). Collagen Osteoid-Like Model Allows Kinetic Gene Expression Studies of Non-Collagenous Proteins in Relation with Mineral Development to Understand Bone Biomineralization. *PLoS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0057344

Skottke, J., Gelinsky, M., & Bernhardt, A. (2019). In vitro co-culture model of primary human osteoblasts and osteocytes in collagen gels. *International Journal of Molecular Sciences*, *20*(8). https://doi.org/10.3390/ijms20081998

Sorushanova, A., Delgado, L. M., Wu, Z., Shologu, N., Kshirsagar, A., Raghunath, R., Mullen, A. M., Bayon, Y., Pandit, A., Raghunath, M., & Zeugolis, D. I. (2019). The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. In *Advanced Materials* (Vol. 31, Issue 1). Wiley-VCH Verlag. https://doi.org/10.1002/adma.201801651

Staines, K. A., Javaheri, B., Hohenstein, P., Fleming, R., Ikpegbu, E., Unger, E., Hopkinson, M., Buttle, D. J., Pitsillides, A. A., & Farquharson, C. (2017). Hypomorphic conditional deletion of E11/Podoplanin reveals a role in osteocyte dendrite elongation. *Journal of Cellular Physiology*, *232*(11), 3006–3019. https://doi.org/10.1002/jcp.25999

Stegen, S., Stockmans, I., Moermans, K., Thienpont, B., Maxwell, P. H., Carmeliet, P., & Carmeliet, G. (2018). Osteocytic oxygen sensing controls bone mass through epigenetic regulation of sclerostin. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-04679-7

Stern, A. R., Stern, M. M., van Dyke, M. E., Jähn, K., Prideaux, M., & Bonewald, L. F. (2012). Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice. *BioTechniques*, *52*(6), 361–373. https://doi.org/10.2144/0000113876

Steward, A. J., & Kelly, D. J. (2015). Mechanical regulation of mesenchymal stem cell differentiation. In *Journal of Anatomy* (Vol. 227, Issue 6, pp. 717–731). Blackwell Publishing Ltd. https://doi.org/10.1111/joa.12243

Stoppel, W. L., Ghezzi, C. E., McNamara, S. L., III, L. D. B., & Kaplan, D. L. (2015). Clinical Applications of Naturally Derived Biopolymer-Based Scaffolds for Regenerative Medicine. *Annals of Biomedical Engineering*, *43*(3), 657–680. https://doi.org/10.1007/s10439-014-1206-2

Sun, M., Chen, S., Adams, S. M., Florer, J. B., Liu, H., Kao, W. W. Y., Wenstrup, R. J., & Birk, D. E. (2011). Collagen V is a dominant regulator of collagen fibrillogenesis: Dysfunctional regulation of structure and function in a corneal-stroma-specific Col5a1-null mouse model. *Journal of Cell Science*, *124*(23), 4096–4105. https://doi.org/10.1242/jcs.091363

Sun, M., Luo, E. Y., Adams, S. M., Adams, T., Ye, Y., Shetye, S. S., Soslowsky, L. J., & Birk, D. E. (2020). Collagen XI regulates the acquisition of collagen fibril structure, organization and functional properties in tendon. *Matrix Biology*, *94*, 77–94. https://doi.org/10.1016/j.matbio.2020.09.001

Sun, Q., Choudhary, S., Mannion, C., Kissin, Y., Zilberberg, J., & Lee, W. Y. (2017). Ex vivo construction of human primary 3D–networked osteocytes. *Bone*, *105*, 245–252. https://doi.org/10.1016/j.bone.2017.09.012

Sun, Q., Gu, Y., Zhang, W., Dziopa, L., Zilberberg, J., & Lee, W. (2015). Ex vivo 3D osteocyte network construction with primary murine bone cells. *Bone Research*, *3*. https://doi.org/10.1038/boneres.2015.26

Sun, Y., Weng, Y., Zhang, C., Liu, Y., Kang, C., Liu, Z., Jing, B., Zhang, Q., & Wang, Z. (2015). Glycosylation of Dentin Matrix Protein 1 is critical for osteogenesis. *Scientific Reports*, *5*. https://doi.org/10.1038/srep17518

Svensson, L., Aszódi, A., Reinholt, F. P., Fässler, R., Heinegård, D., & Oldberg, Å. (1999). Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. *Journal of Biological Chemistry*, *274*(14), 9636–9647. https://doi.org/10.1074/jbc.274.14.9636

Tavafoghi, M., & Cerruti, M. (2016). The role of amino acids in hydroxyapatite mineralization. In *Journal of the Royal Society Interface* (Vol. 13, Issue 123). Royal Society of London. https://doi.org/10.1098/rsif.2016.0462

Terrell, J. A., Jones, C. G., Kabandana, G. K. M., & Chen, C. (2020). From cells-on-a-chip to organs-on-a-chip: Scaffolding materials for 3D cell culture in microfluidics. In *Journal of Materials Chemistry B* (Vol. 8, Issue 31, pp. 6667–6685). Royal Society of Chemistry. https://doi.org/10.1039/d0tb00718h

Terzi, A., Gallo, N., Bettini, S., Sibillano, T., Altamura, D., Madaghiele, M., de Caro, L., Valli, L., Salvatore, L., Sannino, A., & Giannini, C. (2020). Sub- and Supramolecular X-Ray Characterization of Engineered Tissues from Equine Tendon, Bovine Dermis, and Fish Skin Type-I Collagen. In *Macromolecular Bioscience* (Vol. 20, Issue 5). Wiley-VCH Verlag. https://doi.org/10.1002/mabi.202000017

Thiele, J., Ma, Y., Bruekers, S. M. C., Ma, S., & Huck, W. T. S. (2014). 25th anniversary article: Designer hydrogels for cell cultures: A materials selection guide. In *Advanced Materials* (Vol. 26, Issue 1, pp. 125–148). https://doi.org/10.1002/adma.201302958

Thoniyot, P., Tan, M. J., Karim, A. A., Young, D. J., & Loh, X. J. (2015). Nanoparticle–Hydrogel Composites: Concept, Design, and Applications of These Promising, Multi-Functional Materials. In *Advanced Science* (Vol. 2, Issues 1–2). Wiley-VCH Verlag. https://doi.org/10.1002/advs.201400010

Tibbitt, M. W., & Anseth, K. S. (2009). Hydrogels as extracellular matrix mimics for 3D cell culture. In *Biotechnology and Bioengineering* (Vol. 103, Issue 4, pp. 655–663). https://doi.org/10.1002/bit.22361

Tiede-Lewis, L. A. M., & Dallas, S. L. (2019). Changes in the osteocyte lacunocanalicular network with aging. *Bone*, *122*, 101–113. https://doi.org/10.1016/j.bone.2019.01.025

Uchihashi, K., Aoki, S., Matsunobu, A., & Toda, S. (2013). Osteoblast migration into type I collagen gel and differentiation to osteocyte-like cells within a self-produced mineralized matrix: A novel system for analyzing differentiation from osteoblast to osteocyte. *Bone*, *52*(1), 102–110. https://doi.org/10.1016/j.bone.2012.09.001

Valero, C., Amaveda, H., Mora, M., & García-Aznar, J. M. (2018). Combined experimental and computational characterization of crosslinked collagen-based hydrogels. *PLoS ONE*, *13*(4). https://doi.org/10.1371/journal.pone.0195820

Vater, C., Kasten, P., & Stiehler, M. (2011). Culture media for the differentiation of mesenchymal stromal cells [Article]. *Acta Biomaterialia*, *7*(2), 463–477. https://doi.org/10.1016/j.actbio.2010.07.037

Vazquez, M., Evans, B. A. J., Riccardi, D., Evans, S. L., Ralphs, J. R., Dillingham, C. M., & Mason, D. J. (2014). A new method to investigate how mechanical loading of osteocytes controls osteoblasts. *Frontiers in Endocrinology*, *5*(DEC). https://doi.org/10.3389/fendo.2014.00208

Viti, F., Landini, M., Mezzelani, A., Petecchia, L., Milanesi, L., & Scaglione, S. (2016). Osteogenic differentiation of MSC through calcium signaling activation: Transcriptomics and functional analysis. *PLoS ONE*, *11*(2). https://doi.org/10.1371/journal.pone.0148173

Vuornos, K., Ojansivu, M., Koivisto, J. T., Häkkänen, H., Belay, B., Montonen, T., Huhtala, H., Kääriäinen, M., Hupa, L., Kellomäki, M., Hyttinen, J., Ihalainen, J. A., & Miettinen, S. (2019). Bioactive glass ions induce efficient osteogenic differentiation of human adipose stem cells encapsulated in gellan gum and collagen type I hydrogels. *Materials Science and Engineering C*, *99*, 905–918. https://doi.org/10.1016/j.msec.2019.02.035

Walker, J. V., Zhuang, H., Singer, D., Illsley, C. S., Kok, W. L., Sivaraj, K. K., Gao, Y., Bolton, C., Liu, Y., Zhao, M., Grayson, P. R. C., Wang, S., Karbanová, J., Lee, T., Ardu, S., Lai, Q., Liu, J., Kassem, M., Chen, S., … Hu, B. (2019). Transit amplifying cells coordinate mouse incisor mesenchymal stem cell activation. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-11611-0

Walters, B. D., & Stegemann, J. P. (2014). Strategies for directing the structure and function of three-dimensional collagen biomaterials across length scales. In *Acta Biomaterialia* (Vol. 10, Issue 4, pp. 1488–1501). Elsevier Ltd. https://doi.org/10.1016/j.actbio.2013.08.038

Wang, L., Uhlig, P. C., Eikenberry, E. F., Robenek, H., Bruckner, P., & Hansen, U. (2014). Lateral growth limitation of corneal fibrils and their lamellar stacking depend on covalent collagen cross-linking by transglutaminase-2 and lysyl oxidases, respectively. *Journal of Biological Chemistry*, *289*(2), 921–929. https://doi.org/10.1074/jbc.M113.496364

Wang, X., Molino, B. Z., Pitkänen, S., Ojansivu, M., Xu, C., Hannula, M., Hyttinen, J., Miettinen, S., Hupa, L., & Wallace, G. (2019). 3D Scaffolds of Polycaprolactone/Copper-Doped Bioactive Glass: Architecture Engineering with Additive Manufacturing and Cellular Assessments in a Coculture of Bone Marrow Stem Cells and Endothelial Cells. *ACS Biomaterials Science and Engineering*, *5*(9), 4496–4510. https://doi.org/10.1021/acsbiomaterials.9b00105

Wittkowske, C., Reilly, G. C., Lacroix, D., & Perrault, C. M. (2016). In Vitro Bone Cell Models: Impact of Fluid Shear Stress on Bone Formation. In *Frontiers in Bioengineering and Biotechnology* (Vol. 4, Issue NOV). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2016.00087

Wnorowski, A., Yang, H., & Wu, J. C. (2019). Progress, obstacles, and limitations in the use of stem cells in organ-on-a-chip models. In *Advanced Drug Delivery Reviews* (Vol. 140, pp. 3–11). Elsevier B.V. https://doi.org/10.1016/j.addr.2018.06.001

Woo, S. M., Rosser, J., Dusevich, V., Kalajzic, I., & Bonewald, L. F. (2011). Cell line IDG-SW3 replicates osteoblast-to-late-osteocyte differentiation in vitro and accelerates

bone formation in vivo. *Journal of Bone and Mineral Research*, *26*(11), 2634–2646. https://doi.org/10.1002/jbmr.465

Wu, Q., Liu, J., Wang, X., Feng, L., Wu, J., Zhu, X., Wen, W., & Gong, X. (2020). Organ-on-a-chip: Recent breakthroughs and future prospects. In *BioMedical Engineering Online* (Vol. 19, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12938-020-0752-0

Yang, Y., Wang, M., Yang, S., Lin, Y., Zhou, Q., Li, H., & Tang, T. (2020). Bioprinting of an osteocyte network for biomimetic mineralization. *Biofabrication*, *12*(4). https://doi.org/10.1088/1758-5090/aba1d0

Yee, C. S., Schurman, C. A., White, C. R., & Alliston, T. (2019). Investigating Osteocytic Perilacunar/Canalicular Remodeling. In *Current Osteoporosis Reports* (Vol. 17, Issue 4, pp. 157–168). Current Medicine Group LLC 1. https://doi.org/10.1007/s11914-019-00514-0

Yu, L., Ma, X., Sun, J., Tong, J., Shi, L., Sun, L., & Zhang, J. (2017). Fluid shear stress induces osteoblast differentiation and arrests the cell cycle at the G0 phase via the ERK1/2 pathway. *Molecular Medicine Reports*, *16*(6), 8699–8708. https://doi.org/10.3892/mmr.2017.7720

Yue, B. (2014). Biology of the extracellular matrix: An overview. In *Journal of Glaucoma* (Vol. 23, Issue 8, pp. S20–S23). Lippincott Williams and Wilkins. https://doi.org/10.1097/IJG.0000000000000108

Zakrzewski, W., Dobrzyński, M., Szymonowicz, M., & Rybak, Z. (2019). Stem cells: Past, present, and future. In *Stem Cell Research and Therapy* (Vol. 10, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13287-019-1165-5

Zarrinkalam, M. R., Mulaibrahimovic, A., Atkins, G. J., & Moore, R. J. (2012). Changes in osteocyte density correspond with changes in osteoblast and osteoclast activity in an osteoporotic sheep model. *Osteoporosis International*, *23*(4), 1329–1336. https://doi.org/10.1007/s00198-011-1672-4

Zeiger, A. S., Loe, F. C., Li, R., Raghunath, M., & van Vliet, K. J. (2012). Macromolecular crowding directs extracellular matrix organization and mesenchymal stem cell behavior. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0037904

Zhang, J., Wei, X., Zeng, R., Xu, F., & Li, X. J. (2017). Stem cell culture and differentiation in microfluidic devices toward organ-on-a-chip. In *Future Science OA* (Vol. 3, Issue 2). Future Medicine Ltd. https://doi.org/10.4155/fsoa-2016-0091

Zhang, K., Barragan-Adjemian, C., Ye, L., Kotha, S., Dallas, M., Lu, Y., Zhao, S., Harris, M., Harris, S. E., Feng, J. Q., & Bonewald, L. F. (2006). E11/gp38 Selective Expression in Osteocytes: Regulation by Mechanical Strain and Role in Dendrite Elongation. *Molecular and Cellular Biology*, *26*(12), 4539–4552. https://doi.org/10.1128/mcb.02120-05

Zhao, Z., Vizetto-Duarte, C., Moay, Z. K., Setyawati, M. I., Rakshit, M., Kathawala, M. H., & Ng, K. W. (2020). Composite Hydrogels in Three-Dimensional in vitro Models. In *Frontiers in Bioengineering and Biotechnology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2020.00611

Zhu, Y. K., Umino, T., Liu, X. D., Wang, H. J., Romberger, D. J., Spurzem, J. R., & Rennard, S. I. (2001). Contraction of Fibroblast-Containing Collagen Gels: Initial Collagen Concentration Regulates the Degree of Contraction and Cell Survival. In *Source: In Vitro Cellular & Developmental Biology. Animal* (Vol. 37, Issue 1).

Zoch, M. L., Clemens, T. L., & Riddle, R. C. (2016). New insights into the biology of osteocalcin. In *Bone* (Vol. 82, pp. 42–49). Elsevier Inc. https://doi.org/10.1016/j.bone.2015.05.046