Serum Substitute Medium for Bone Tissue Engineering Applications

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By

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PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Technische Universiteit Eindhoven, op gezag van de rector magnificus prof.dr.ir. F.P.T. Baaijens, voor een commissie aangewezen door het College voor Promoties, in het openbaar te verdedingen op woensdag 10 mei 2023 om 11:00 uur

door

Sana Ansari

Geboren te Tehran, Iran

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Het onderzoek of ontwerp dat in dit thesis wordt beschreven is uitgevoerd in overeenstemming met de TU/e Gedragscode Wetenschapsbeoefening.

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Chapter 1

General introduction

Preface

Fetal bovine serum (FBS) is a widely used supplement in cell culture media which provides cells with vital factors including growth factors, hormones, and vitamins essential for cell survival, growth, and division. However, the use of FBS in *in vitro* cell culture is controversial. FBS is a variable and undefined medium supplement with unknown and complex composition which can even change between batches. These unpredictable factors have an influence on cell responses and outcomes of the experiments. Thus, FBS containing medium should be avoided wherever possible. In tissue engineering studies, the influence of the unknown and complex composition of FBS should be avoided by formulating a defined and more controlled medium supplement. The aim of this thesis is to develop defined serum substitute media for bone tissue engineering studies. Such medium formulations should not only diminish the influence of unknown factors of FBS but should also enable the study of the effect of different soluble factors on cell behavior and *in vitro* bone tissue formation/resorption.

1.1 Bone

Within the musculoskeletal system, bones have a variety of functions such as providing structural support, facilitating movement, protecting internal organs, serving as a reservoir for minerals and growth factors, maintaining mineral homeostasis, and providing a site for hematopoiesis within the marrow space [1]. Bones are hierarchically organized over multiple length scales spanning from the macroscale to nanoscale. At the macroscopic level, two structural types of bone can be distinguished, namely, cortical (or compact) and trabecular (or cancellous) bone each with different structure, porosity, mechanical strength, and metabolic activity (Figure 1.1). Cortical bone constitutes 80% and trabecular bone 20% of the skeletal mass [2]. Cortical bone is highly organized and densely packed which forms the outer shell of bone. At the microscopic level, cortical bone consists of osteons, highly ordered cylindrical structures, with blood vessels running longitudinally through the center of each osteon within channels called Harversian canals. Trabecular bone is encapsulated beneath the cortical bone and forms a sponge-like network of trabecular plates with interconnecting spaces containing bone marrow and vascular system [1], [3]. At the nanoscale, by weight, the inorganic matrix accounts for 60% and the organic matrix makes up 30% of the bone tissue, while the remaining is water [4]. The organic matrix is primarily collagen type 1 molecules assembled into collagen fibrils and a small portion of non-collagenous proteins (NCPs), and inorganic matrix is mainly hydroxyapatite nanocrystals deposited within and along the collagen fibrils [5], [6].

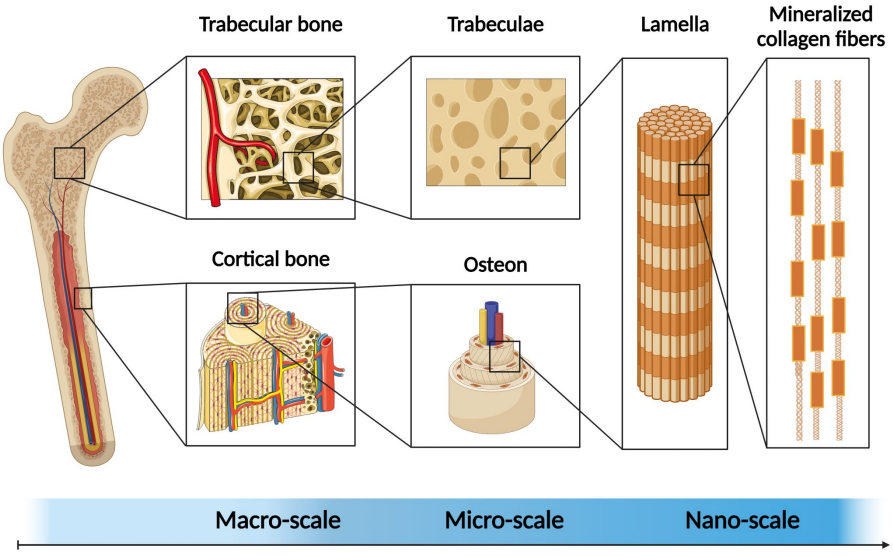

Figure 1.1 Multiscale structure of bone. Created with BioRender.com.

Bone maintains its integrity through a lifelong bone remodeling process which is essential for fracture healing, adaptation to mechanical loading, as well as maintaining mineral homeostasis. In this process old, micro-damaged bone is replaced by new, mechanically stronger bone. Bone remodeling is believed to be a cycle of four phases starting with recruitment of mononuclear osteoclast progenitor cells from circulation through biochemical or mechanical stimuli to the site of bone remodeling where they attract to the bone surface and form multinucleated osteoclasts. Then, these mature osteoclasts initiate organic and inorganic bone matrix resorption which is followed by the reversal phase. In this phase, bone resorption transitions to bone formation by osteoclasts leaving the bone surface, smoothing the resorbed surface by macrophages, and recruiting mesenchymal stromal cells (MSCs), as osteoblast progenitor cells, to the site of remodeling. Subsequently, MSCs differentiate towards osteoblasts which produce new bone by secreting a collagenous matrix, osteoid, and controlling its mineralization. Once the matrix gets mineralized, a large portion of osteoblasts embed within the mineralized matrix differentiate into osteocytes (Figure 1.2) [1], [7], [8]. During bone remodeling in the healthy situation, a balance between bone resorption and bone formation is maintained through biochemical factors and biomechanical stimulation. An imbalance in this process results in metabolic bone diseases such as osteoporosis or osteopetrosis [9]. They can change bone remodeling balance positively or negatively, resulting in excessive bone formation or resorption [10], [11].

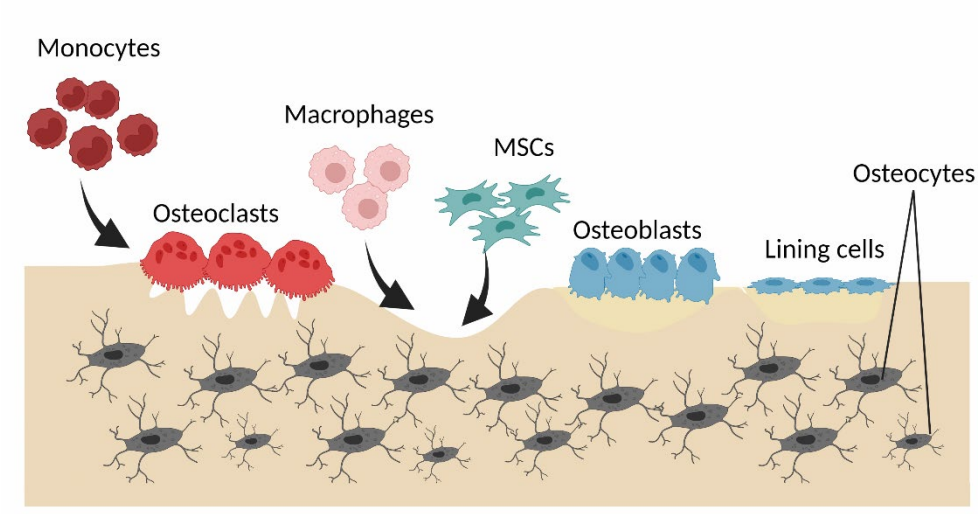

Figure 1.2 Bone remodeling process. Created with BioRender.com.

1.2 Bone tissue engineering

Tissue engineering is a discipline that integrates biology with engineering to repair, restore, or regeneration of living tissue using biomaterials, cells, and biochemical factors alone or in combination. Bone tissue engineering has emerged to facilitate the regeneration of large bone defects resulting from trauma, metabolic diseases, infection, or tumor removal [12], [13]. Specific scaffolds made of natural polymers, synthetic polymers, ceramics, metals, etc. have been designed and developed to induce *in situ* bone regeneration. These scaffolds, which could also be decorated with specific growth factors such as recombinant human bone morphogenic proteins 2 and 7 (rhBMP-2 and rhBMP-7) to induce bone formation, need to meet several criteria [14]. The developed scaffolds or grafts should be osteoconductive, osteoinductive, biocompatible, and mechanically stable [12], [15]. Besides this cell-free approach to induce bone regeneration, bone tissue engineering principles have been applied to develop functional engineered tissue *in vitro* for subsequent implantation into the bone defect [16]. In this approach, cells are seeded onto a three-dimensional (3D) scaffold with or without the presence of bioactive molecules to form an *in vitro* tissue. The use of such engineered tissue remained mainly experimental with limited clinical success for bone regeneration. However, the advances in development of such engineered tissue could be applied to create 3D *in vitro* human models. These tissue models could facilitate the investigation of complex physiological and pathological processes and evaluation of new therapeutic approaches [17]. Besides the use of sophisticated scaffolds (to mimic the 3D environment of the tissue), cell types, bioreactors (to apply mechanical loads needed for tissue growth), and biochemical factors (to control the cellular differentiation and activity) need to be considered in bone tissue engineering studies and developing engineered bone-like tissues [18].

1.2.1 Cell types

Different cell types such as immortalized cell lines, primary cells isolated directly from the tissue, induced pluripotent stem cells (iPSCs), and progenitor cells have been used in bone tissue engineering studies. In such studies and more importantly development of *in vitro* bone models, bone-specific progenitor cells, namely, MSCs and monocytes, are the most promising cell types due to their availability, relative ease of isolation, and their physiological relevance to the *in vivo* situation. MSCs, as osteoblast progenitor cells, can be isolated from wide variety of tissues such as bone marrow, adipose tissue, peripheral blood, dental pulp, umbilical cord, umbilical cord blood, and placenta [19]. Bone marrow and peripheral blood have also been the main sources to isolate monocytes, the osteoclast progenitor cells [20]. In the development of *in vitro* bone models using human cells, also the donor variability should be considered. The cell heterogeneity among patients and their different characteristics caused by diseases such as changes in cell receptors need to be considered when developing *in vitro* tissue models [21]. Development of *in vitro* personalized tissue models using each individuals' cells could potentially revolutionize the development of new treatments [22]. For instance, such personalized *in vitro* models could potentially reveal the genetic contribution to the development of specific diseases or even assist in formulating novel personalized drugs for these diseases [22], [23].

1.2.2 Mechanical stimuli

Mechanical forces are essential for bone homeostasis, formation, resorption, and adaptation. Loss of mechanical stimulation can result in weakening of the bone structure. These mechanical forces are applied to the load bearing bones through body movement and contractile activity of muscles [24]. Bone tissue engineering makes use of different types of bioreactors to provide mechanical stimuli *in vitro*. For instance, *in vivo*, shear stress is generated through movement of interstitial fluid in the narrow channels located within the mineralized bone matrix (where osteocytes are located) and on the surface of the bone matrix with bigger porosities (where osteoblasts and osteoclasts are located) [25]. Also, MSCs located in the periosteum and bone marrow are exposed to some shear stress generated by micro-deformation of bone due to external mechanical stimuli such as stretching and compression [26]. To induce such environments *in vitro*, several types of bioreactors have been developed such as spinner flasks, rotating wall bioreactors, and perfusion systems [27]. Using such bioreactors could improve the creation of physiologically relevant *in vitro* bone-like models [7].

1.2.3 Biochemical environment

The cellular behavior is greatly influenced by their biochemical environment, more specifically the culture medium composition. In bone tissue engineering, the cell culture medium is typically composed of a basal medium supplemented with fetal bovine serum (FBS) and osteoblast or osteoclast specific factors depending on the used cell type.

FBS

FBS is a widely used cell culture supplement for more than 60 years. It provides cells with a broad spectrum of macromolecules, proteins, lipids, trace elements, vitamins, and attachment factors essential for cell survival and growth [28]. The discovery of FBS dates back to 1958 when Theodore Puck tried to culture cells for a long period of time. Using a medium supplemented with 15% of FBS, he could establish a stable culture of fibroblasts isolated from human skin for up to 9 months [29]. After this discovery, the use of FBS increased significantly to the point that now it is considered as a standard cell culture supplement. Despite the common use of FBS in *in vitro* experiments, many disadvantages have been attributed to FBS [28], [30]. First, the ethical issue which is mainly focused on the methods of collecting FBS that may cause suffering to animals. Briefly, bovine fetuses are obtained from pregnant cows in slaughterhouses where the fetus is separated, and fetal blood is collected under aseptic conditions [31]. To maximize the collection of blood, it is collected by syringe from the beating heart [28]. This might induce pain or discomfort to the fetus until death. Second, FBS can be a potential source of microbial contaminants including fungi, bacteria, or viruses which might put laboratory personnel at the risk of being infected [28]. Moreover, this issue makes use of *in vitro* expanded cells in cell-therapy applications or implanting developed *in vitro* engineered tissues in patients challenging [32], [33]. Third, FBS is an undefined mixture of components displaying non-physiological levels of components with high batch-to-to batch variation. Moreover, the components of FBS could be different depending on the season or the location of harvesting serum [28]. These variations could impact the cells in culture and result in unreliable/unpredictable experimental outcomes. Thus, the use of FBS in tissue engineering studies needs to be reconsidered.

Development of serum substitute medium

The issues raised by using FBS in *in vitro* experiments were the reasons to move towards development of a defined serum free medium [34]. Over the past few decades, many attempts to recognize the role of serum in cell growth resulted in identifying the essential factors and nutrients that are required for cellular growth and function, and finally development of a broad selection of a serum substitute media [35]. In 1976, three different studies have shown that a trace element of selenium [36], a combination of transferrin and albumin [37], and a combination of several hormones and growth factors [38] could be used instead of FBS in cell cultures. Following these discoveries, various serum substitute media (Table 1.1) were developed, with each medium tailored to a specific cell type. The development of such serum substitute medium depends on many factors such as cell type, cell culture conditions, cell sources, application, and species. These variations indicate that a development of one universal defined medium would not be possible, and the defined medium needed to be developed for each specific cell type and/or application. To date, many of these medium types with different formulations are commercially available for specific cell types. However, the compositions of such media are often not provided due to for example commercial reasons, which limits the possibility of the investigating the impact of soluble factors or drugs on cellular behavior and function.

Table 1.1 Various types of serum substitute media.

Types of serum substitute medium	Characteristic
Serum-free medium	Contains protein fractions such as bovine serum albumin as supplements
Protein-free medium	Does not contain high molecular weight proteins or protein fractions, contains peptide fractions (protein hydrolysates) as supplements
Xeno-free medium	Contains no animal components, might contain plant extracts or human serum albumin
Chemically defined medium	Contains highly purified components such as recombinant proteins as supplements

Opposite to FBS, serum substitute media have several advantages including elimination of animal suffering, reduced variability in culture medium composition, and support of cell survival and growth in a more defined medium (Table 1.2). The known composition of serum substitute medium makes it feasible to systematically investigate the influence of soluble factors, drugs, and more complex biological components such as extracellular vesicles (EVs) on tissue development in a less variable environment.

Table 1.2 Comparison of FBS containing medium and serum substitute medium.

	FBS containing medium	Serum substitute medium
Complex composition	Unknown/complex	Known/defined
Animal welfare	Low	High
Reproducibility of experimental outcomes	Low	High
Risk of contamination	High	Low
Availability for all cell types	High	Low

Extracellular vesicles (EVs)

EVs are phospholipid-enclosed nanoparticles containing lipids, proteins, and nucleic acids produced by almost all cell types and involved in many biological processes. During bone remodeling, osteoblasts and osteoclasts can communicate through EVs. This communication can occur, for instance, via interaction of cells with ligands that are present on the surface of EVs or transferring EV cargoes [39]. A subset of EVs produced by osteoblasts are known as matrix vesicles (MtVs). The membrane of these vesicles is enriched with phosphatidylserine (PS)-binding annexin proteins such as annexin A5 and phosphatases such as alkaline phosphatase (ALP). These membrane proteins facilitate entry of calcium and phosphate ions into MtVs. These ions then form amorphous or crystalized minerals inside the MtVs which can rupture the membrane and form mineral nodules in the extracellular matrix (ECM) [40], [41]. MtVs have shown to target bone *in vivo* and induce bone formation *in vitro* and *in vivo* [42]–[45]. Considering the role of MtVs in the bone formation process, they could be used as natural delivery vehicles and biological components for bone regeneration and fracture healing through, for instance, integration with biomaterials to target bone formation locally.

1.3 Thesis outline

In this thesis, we aimed to develop osteoblast- and osteoclast- specialized and defined serum substitute media to replace the undefined and complex composition of FBS in bone tissue engineering studies.

Chapter 1 provides a general overview on bone tissue engineering and its potential in developing *in vitro* bone models, FBS and its use in *in vitro* studies, and the need to replace FBS with defined serum substitute medium.

The selection of cells in bone tissue engineering studies as a tool to develop human *in vitro* bone models is crucial. **Chapter 2** reviews the cell sources for human *in vitro* bone models. In this review, the importance of using osteoblast and osteoclast progenitor cells as the most promising cell types for development of *in vitro* bone models is discussed. Furthermore, osteoblast and osteoclast progenitor cells obtained from bone marrow and peripheral blood, the main two cell sources to obtain bone-specific progenitor cells, are compared in terms of their ease of cell isolation, proliferation capacity, and differentiation potential of progenitor cells.

In bone tissue engineering, the deposited mineralized matrix is one of the main outcome parameters, but using different brands/batches of FBS can result in great variations in mineralized matrix deposition [46]. In **Chapter 3**, we hypothesized that alkaline phosphatase (ALP), an enzyme produced by osteoblasts during bone formation, is present in FBS and contributes to changes in phosphate concentration of medium, mineral deposition within produced ECM *in vitro*, and osteogenic differentiation. The results of this study highlight the importance of development of specialized serum substitute medium for tissue engineering studies.

In **Chapter 4**, we aimed to develop an osteoblast-specific serum substitute medium in a stepwise approach. Essential components were added to the medium while human bone marrow MSCs (hBMSCs) were cultured in 2D well-plates or 3D scaffolds (in static and dynamic conditions). The developed serum substitute medium supported cell survival, osteoblast differentiation, and deposition of extracellular matrix.

In **Chapter 5**, we aimed to develop an osteoclast-specific serum substitute medium in a stepwise approach. Essential components were added to medium while peripheral blood monocytes were cultured in 2D well-plates. The developed serum substitute medium could support the differentiation of monocytes into resorbing TRAP-positive multinucleated osteoclasts.

Osteoblasts shed a subset of EVs known as matrix vesicles (MtVs), which contain phosphatases, calcium, and inorganic phosphate. These MtVs have a major role in matrix mineralization and feature innate bone-targeting and bone formation properties. In **Chapter 6**, we reviewed the EVs secreted by bone-specific cells, with a focus on MtVs, their biogenesis, characteristic, and contribution to bone mineralization. Finally, the potential therapeutic application of MtVs to treat bone related diseases or to support fracture healing was discussed.

Considering the role of MtVs in bone mineralization, in **Chapter 7**, we aimed at isolating the released EVs during osteogenic differentiation of hBMSCs in the human 3D *in vitro* woven bone constructs previously developed by our group. The EVs secreted into the culture medium were characterized based on their morphological, biological, and functional properties. These results indicated that a complex 3D environment mimicking bone development is favorable to stimulate MtV-producing cells to produce targeted MtVs *in vitro*.

In **Chapter 8**, the main findings of this thesis are presented and discussed, as well as potential future directions.

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Chapter 2

Cell sources for human *in vitro* bone models

This chapter is based on:

Sana Ansari, Keita Ito, Sandra Hofmann, Cell sources for human in vitro bone models, *Current osteoporosis reports.* 2021; 19(1): 88-100.
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Abstract

Purpose of Review One aim in bone tissue engineering is to develop human cell-based, 3D *in vitro* bone models to study bone physiology and pathology. Due to the heterogeneity of cells among patients, patient's own cells are needed to be obtained, ideally, from one single cell source. This review attempts to identify the appropriate cell sources for development of such models.

Recent Findings Bone marrow and peripheral blood are considered as suitable sources for extraction of osteoblast/osteocyte and osteoclast progenitor cells. Recent studies on these cell sources have shown no significant differences between isolated progenitor cells. However, various parameters such as medium composition affect the cell's proliferation and differentiation potential which could make the peripheral blood derived stem cells superior to the ones from bone marrow.

Summary Peripheral blood can be considered a suitable source for osteoblast/osteocyte and osteoclast progenitor cells, being less invasive for the patient. However, more investigations are needed focusing on extraction and differentiation of both cell types from the same donor sample of peripheral blood.

2.1 Introduction

Bone is a complex multifunctional organ that sustains the integrity of the vertebrate skeleton, provides mechanical support for locomotion, protects internal organs and acts as a mineral storage [1]. Throughout life, bone tissue continuously undergoes a physiological process called bone remodeling to adapt to environmental changes, repair old and damaged bone and maintain its shape and strength. Bone remodeling occurs via balanced activities of its specialized cells which are tightly regulated and controlled through biochemical pathways [2]. *In vivo,* bone remodeling is composed of four consecutive phases: recruitment and activation of mononuclear progenitor cells, resorption of the organic and inorganic matrix of bone by mature osteoclasts, preparation of the resorbed surface of matrix deposition, and deposition of new bone by osteoblasts [1], [3].

2.2 Bone cells

Osteoblasts

Osteoblasts are bone-forming cells derived from mesenchymal stromal cells (MSCs). MSCs differentiate into osteoblasts under appropriate mechanical and/or biochemical stimuli [4], [5]. Osteoblasts are responsible to produce the organic matrix of bone extracellular matrix composed of mainly collagen type 1 and a small percentage of non-collagenous proteins (NCPs) [6]. Moreover, they are involved in inorganic matrix deposition through mechanisms in which NCPs play important roles [7], [8]. At the end of the bone forming phase, osteoblasts can have one of the following fates: become embedded in the mineralized matrix and differentiate into osteocytes, transform into inactive bone lining cells, or undergo apoptosis (Figure 2.1) [9].

Osteocytes

Osteocytes as terminally differentiated osteoblasts form 95% of the cellular component of bone; thus, they are the most abundant bone cell type [10]. During bone formation, a large portion of osteoblasts becomes embedded in the mineralized matrix, decrease their cell body volume, and attain a stellar shape morphology with long processes which form a network with their neighboring cells and cells on the bone surface [10]. These cells are thought to orchestrate the activities of bone formation and resorption by translating mechanical loading into biochemical signals [11], [12].

Osteoclasts

Osteoclasts are bone resorbing cells that dissolve the inorganic matrix and enzymatically degrade extracellular matrix proteins by secreting acid and lytic enzymes [13]. These cells are large, multinucleated cells originating from the monocyte/macrophage lineage which differentiate from hematopoietic stem cells (HSCs) (Figure 2.1). These stem cells are situated in bone marrow and can be mobilized into the peripheral blood [14]. Osteoclast differentiation and activation is thought to be regulated by neighboring stromal cells and osteoblasts [15].

Other

Besides the cell types that are involved in bone remodeling process, bone consists of other cell types which are less known to have a direct role in the bone remodeling process; they will not be addressed

in this review. These cells are for example bone lining cells, which are inactive osteoblasts at the end of the bone formation phase, chondrocytes, endothelial, and perivascular cells due to the vascularized nature of bone tissue [10], [16], [17].

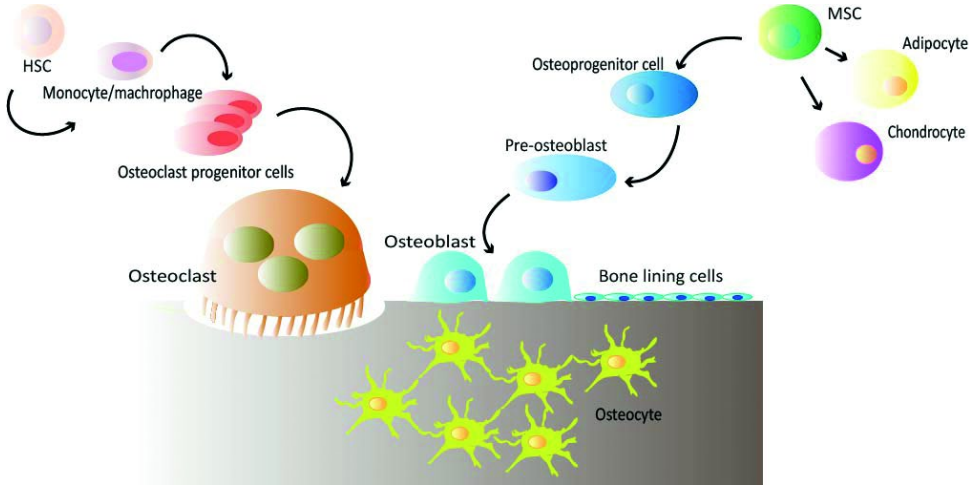

Figure 2.1 Bone progenitor cells and their differentiation into osteoblasts/osteocytes and osteoclasts.

2.3 Bone metabolic diseases

Disturbing the bone remodeling process results in the development of metabolic bone diseases including osteoporosis characterized by an altered bone turnover balance as a result of high osteoclast activity and impaired bone formation. Osteoporosis is the most common bone metabolic disease. It is characterized by decreased bone strength and increased bone fracture risk [18], [19]. Apart from osteoporosis, there are more diseases related to an impaired bone remodeling process including osteopetrosis, Paget's disease, renal osteodystrophy, and rickets. They are less prevalent, which limits our current knowledge on their pathology and their efficient treatment [20]. Thus, the development of *in vitro* models that mimic bone-related pathologies could enhance the understanding of these diseases and the design of more efficient treatments.

2.4 The need for personalized *in vitro* models

The current gold standard in developing novel treatments for bone pathologies and pre-clinical drug screening is using animal models. However, these often fail to represent human conditions due to interspecies differences in physiology [21], [22]. Moreover, the need for indicating the appropriate species to model a specific disease, ethical concerns due to genetic mutations and/or nutrient deficiency to induce the disease and high costs of maintenance limit the use of animals as models and thus our knowledge on specific bone metabolic diseases. Animal models often result in poor translation of pre-clinical studies to human clinical trials and promising new treatments might fail prior

to clinical testing [22]–[24]. The development of new therapies requires an in-depth and detailed understanding of bone physiology and pathology and how the different cells are affected in their interaction. Over the past few years, bone tissue engineering techniques have been applied to create 3D *in vitro* bone models based on human cells that can be used as an alternative to *in vivo* models [18], [23]. These *in vitro* bone models require the (co-)culture of the specific bone cells to work closely together under physiological conditions. Because there is a large cell heterogeneity among patients and their diverse characteristics caused by diseases include changes in cell receptors, there is a need to use patient-specific cells for personalized *in vitro* bone models [19], [25]. Thus, in order to represent the patient's bone biological system, representative *in vitro* models require the patient's own cells. The ideal and efficient way to achieve this approach is to isolate progenitor cells of osteoblasts and osteoclasts with high efficiency, to expand them *in vitro*, and to differentiate them towards osteoblasts/osteocytes and osteoclasts, respectively, ideally from one cell source with minimal invasiveness for the patient (i.e., either from peripheral blood or from bone marrow).

This review attempts the following: to (a) briefly identify what kind of cells can be used for bone-related studies, (b) explain the importance of progenitor cells as the most promising cell types for developing *in vitro* bone models, (c) discuss bone marrow and peripheral blood as sources to obtain both osteoblasts/osteocytes and osteoclasts progenitor cells, and (d) finally, the isolation method, proliferation capacity, and differentiation potential of progenitor cells from bone marrow and peripheral blood are discussed.

2.5 Cells in development of *in vitro* bone models

Advancement in development of *in vitro* bone models requires the selection of suitable cell models which can behave similarly to the ones *in vivo*. Cells that have been used in bone-related studies might be originated from one of the following: immortalized cell lines, primary cells which are isolated directly from the tissue, induced pluripotent stem cells (iPSCs), and progenitor cells.

Immortalized cell lines such as MC3T3-E1, MLO-A5, and MG-63 have been used extensively in bone tissue engineering due to their ease of access, high expansion capacity, and reproducibility of outcomes [3]. However, these cell models do not always behave similarly to primary bone cells [26]. For instance, in murine calvarial cell line MC3T3-E1, the gene expression of specific transcripts coding for extracellular matrix proteins such as osteopontin may differ compared with primary osteoblastic cells [27], [28]. Besides, as immortalized cell lines are not patient-specific, it is clear that they cannot be considered as suitable candidates for personalized human *in vitro* bone models.

iPSCs, which are generated by transferring a mixture of nuclear transcriptional factors including Oct4, Sox2, Klf4, and c-Myc to human primary cells, exhibit high similarity to human embryonic stem cells [29]. Due to their robust proliferation capacity, differentiation potential into many cell types, and the ability to generate patient-specific stem cells, iPSCs gained high interest in disease modeling, drug screening, and transplantation therapies [29]. Several studies have shown the ability of iPSCs to differentiate into osteoblasts and osteoclasts, suggesting that iPSCs could be considered as a cell model for the generation of *in vitro* bone models [30]–[33]. However, approaches to generate iPSCs might be complex, expensive, and time consuming with low reprogramming efficiency and possible

alternations of gene expression profiles and pathways, which make iPSCs less appropriate for development human *in vitro* bone models, at least for the moment.

Primary osteoblasts and osteocytes can both be directly isolated from bone tissue and provide an alternative to cell lines for bone-related studies. Several protocols and methods are available for the isolation of human osteoblasts including enzymatic digestion and spontaneous outgrowth cultures from bone biopsies [34], [35]. Isolation of primary osteocytes is more challenging due to their location within the mineralized bone matrix which requires multiple digestion and decalcification steps [36]. As an alternative, human osteocytes can be obtained in culture through differentiation of isolated osteoblasts under osteogenic stimulation [37], [38]. For primary osteoclasts, it has been reported in early studies that they can be isolated from human bone tissue [39], [40]. However, isolation of primary osteoclasts from bone tissue requires multiple steps which might affect the number of extracted cells and their survival rate [41]. Primary cells have greatly enhanced the knowledge of bone biology; for instance, a recent study has shown development of an *in vitro* model to investigate the interaction of primary human osteoblasts and osteocytes [38]. But due to their need for a bone biopsy, slow proliferation rate, short lifespan, decreased doubling time after two or three passages, long isolation procedures, limited accessibility, restricted pool of potential donors (they are usually acquired during orthopedic surgery) [34], [42], their use for developing personalized human *in vitro* models is restricted.

The use of progenitor cells of the bone-specific cell types could be more promising to develop human *in vitro* bone models. MSCs are osteoblast/osteocyte progenitor cells which were primarily extracted from bone marrow and later from other tissues such as adipose tissue, muscle, peripheral blood, dental pulp as adult tissue sources and umbilical cord, umbilical cord blood, placenta, amniotic fluid as fetal and perinatal tissue sources [43]–[45]. MSCs can differentiate into various lineages such as adipogenic, chondrogenic, and osteogenic lineage under appropriate stimuli [46]–[48]. In addition to their multi-potency, their availability and relative ease of isolation and expansion have made them popular for use in many *in vitro* models. Bone marrow derived MSCs have shown significant roles in bone regeneration and fracture repair *in vivo*; furthermore, *in vitro* studies demonstrated a high osteogenic differentiation capacity under biochemical and/or mechanical stimuli [49]–[53]. In bone tissue engineering, bone marrow has so far probably gained the greatest attention as a source of MSCs, but due to the invasive and painful procedure of bone marrow aspirate collection which can also cause donor site morbidity, other adult and fetal tissue sources have been studied as the source of MSCs [54]. For instance, several studies have indicated the osteogenic differentiation and bone formation potential of adipose derived MSCs, which can be isolated from the tissue obtained during liposuction, lipoplasty, or lipectomy procedures with less discomfort and complications compared with bone marrow aspirate collection [55], [56]. Further, MSCs derived from umbilical cord blood and peripheral blood with less invasive cell collection methods have also shown their potential for bone defect repair [57]–[59].

Hematopoietic stem cells (HSCs) are multi-potent and self-renewing cells that can give rise to immune and blood cells [60], [61]. HSCs are primarily located in the bone marrow and can be mobilized into the bloodstream which makes bone marrow and peripheral blood the common tissue sources for HSC

extraction [62]–[64]. Moreover, it has been shown that these cells can also be isolated from umbilical cord blood [65], [66]. HSCs differentiate into the monocyte/macrophage lineage and further into osteoclasts under stimulation with receptor activator of nuclear factor kappa-B ligand (RANKL) and monocyte-colony-stimulating factors (MCSF), both of which are secreted *in vivo* by osteoblasts and osteocytes [67], [68].

Taken together, the most promising cell types for generation of personalized human *in vitro* bone models are progenitor cells. To develop these models, the patient's own progenitor cells should ideally be extracted from one source which makes the procedure more convenient for the patient, as well as results in less demanding clinical procedure. Among all adult tissue sources, due to the possibility to extract both MSCs and HSCs from bone marrow and peripheral blood, they can be considered being the most suitable sources for the isolation of osteoblast/osteocyte and osteoclast progenitor cells (Figure 2.2).

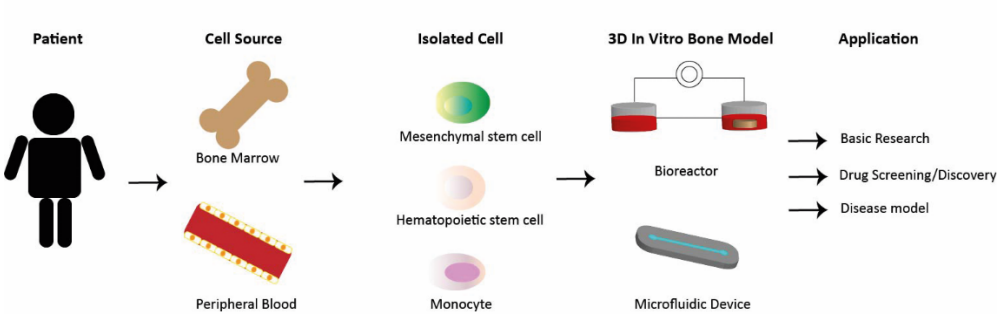

Figure 2.2 Cell sources for personalized in vitro bone models.

2.6 Bone marrow derived MSCs vs. peripheral blood derived MSCs

The frequency of MSCs derived from bone marrow and peripheral blood is very low, representing approximately 0.001– 0.01% and 0.000001% of isolated mononuclear cells, respectively [69], [70]. The number of isolated MSCs can be changed depending on the gender, donor age, health condition, and in case of bone marrow derived MSCs, skeletal site of isolation such as anterior or posterior iliac crest, vertebral body or femoral head [71]–[73]. It has been shown that the frequency of circulating MSCs in peripheral blood can be enhanced in response to pathological conditions such as bone fracture, osteoporosis, breast cancer, and bone sarcomas; for instance, a 9-fold increase in the number of MSCs has been reported in the bloodstream of patients with osteosarcoma compared with control subjects [74]–[78]. This could be as a result of released cytokines and chemical signals to recruit MSCs and mobilize them into the bloodstream. Several methods have been used to mimic these signals to increase the number of MSCs in blood circulation such as administration of granulocyte-colony stimulating factor (G-CSF) and activation of the sympathetic nervous system by electro-acupuncture [79]–[82]. These methods could result in elevated number of isolated peripheral blood derived MSCs which might be an advantage to develop patient-specific *in vitro* bone models; however, due to the

possible side effects of stimulating the migration of cells from bone marrow to peripheral blood, it might not be ethical for the donors and also not applicable for patients with specific diseases [83]. To isolate MSCs from bone marrow and peripheral blood, several protocols have been used such as direct plating based on the adherence property of MSCs to the plastic surface [45], [70], density gradient centrifugation, or hemolysis to separate mononuclear cells and remove erythrocytes prior to seeding cells on a plastic surface [63], [84]–[88], [89], [90], [91], using fibrin microbeads and fluorescence activated cell sorting (FACS) to increase the purity of extracted MSCs [79], [92]. Due to the low frequency of isolated MSCs, their applicability relies on their high *in vitro* proliferation capacity.

The proliferation capacity of MSCs can be evaluated by calculating population doubling time [93]. Various studies on MSCs derived from bone marrow and peripheral blood have shown different doubling times for MSCs; for instance, 80 and 27 h of doubling times have been reported for bone marrow derived MSCs and peripheral blood derived MSCs, respectively [44]. These differences could also be as a result of donor-to-donor variability, factors such as age and health condition of the donor, passage number of cells *in vitro* and the use of different protocols for cell isolation and culture [76], [87], [94]–[101]. For instance, isolation of peripheral blood derived MSCs based on positive expression of CD133 led to obtain MSCs with high proliferative potential in comparison with the peripheral blood derived MSCs based on their adherence capability to plastic surface [101]. This could be due to the heterogeneous population of cells in the plastic adherence method that might interfere with proliferation capacity. While an investigation on bone marrow- and peripheral blood derived MSCs obtained from same patients with the same isolation method and culture condition has reported no significant differences in their characteristics such as population doubling time [76], in another study, the quantity of obtained MSCs from bone marrow after two passages was 2 times higher than MSCs from peripheral blood [89]. These differences not only could be a result of donor variation, but also of the culture condition and most importantly the medium composition. Even though these observations could suggest that the use of peripheral blood derived MSCs in tissue engineering applications might be equally valuable as bone marrow derived MSCs, the chosen culture conditions need to be evaluated carefully.

The *in vitro* osteogenic differentiation of MSCs makes them highly interesting for the development of *in vitro* bone models [63], [76], [89], [102]–[106]. The general trend shows a beneficial osteogenic differentiation potential for bone marrow derived MSCs based on significantly increased expression of osteoblastic specific genes, such as alkaline phosphatase (ALP) and calcium deposition compared with other tissue sources [106], [107]. On the other hand, studies on MSCs extracted from bone marrow and peripheral blood of the same patients have shown no significant differences in quantitative measurements of ALP expression and calcium content; moreover, MSCs from both sources have demonstrated positive staining for calcium deposits [76], [89]. Besides the differentiation potential of MSCs towards the osteogenic lineage, their ability to promote bone formation after *in vivo* implantation has been shown in various studies. The majority of these studies has been conducted using bone marrow derived MSCs in human and animal models [108]–[112]. In a small number of studies, also peripheral blood derived MSCs have been used and shown to enhance bone regeneration in critical-sized bone defects in animal models [59], [113], [114]. Taken together,

peripheral blood derived MSCs seem to exhibit similar characteristics as bone marrow derived MSCs and can be used to develop patient-specific *in vitro* bone models.

The main challenge in developing *in vitro* bone models to represent bone remodeling is the formation of osteocytes embedded in the mineralized matrix. *In vitro*, human osteocytes have been obtained through the differentiation of primary osteoblasts, but so far, full differentiation of MSCs towards functional osteocytes has not been reported (Table 2.1). Further investigations will be needed to induce the formation of osteocytes that are embedded in their own matrix *in vitro*. This might be acquired for example through exposing cells to mechanical stimuli which are known to be involved in bone homeostasis and bone remodeling [3], [115].

Table 2.1 Current approaches to differentiate osteocytes in vitro.

Cell	Osteoblast source	Culture substrate	Outcome	Reference
Human primary osteoblasts	Purchased from LONZA	Biphasic calcium phosphate particles	Expression of CX43, DMP1, E11, MEPE, SOST, PHEX Embedded osteocyte-like cells in collagenous matrix	[116]
Human primary osteoblasts	Femoral trabecular bone tissue from the knee region	Mineralized collagen matrix	Expression of DMP1 and FGF23 Formation of lacunae around the cell	[117]
Human primary pre-osteoblasts and mature osteoblasts	Spongious bone fragment of human femoral head	Collagen gel	Expression of E11, osteocalcin, PHEX, MEPE, RANKL Acquire stellar shape of osteocyte	[38]
		Mineralized collagen gel	Expression of ALP, PDPN, PHEX Acquire stellar shape of osteocyte	[37]
Human primary osteoblasts	Intertrochanteric bone	2D on tissue culture plastic	Expression of E11, DMP1, SOST, OOCN, BSP1, PHEX	[118]

Human primary osteoblasts	Knee cortical	3D microfluidic perfusion device	Expression of SOST and FGF23 Form 3D cellular network Inhibit cell proliferation	[119]
Mouse primary osteoblasts	Long bone	3D microfluidic perfusion device	Expression SOST, FGF23 Form 3D cellular network Inhibit cell proliferation	[120]
Mouse primary osteoblasts	Calvarial tissue	2D culture on poly-L-lysine-coated 2-well chamber slide	Expression of ALP, DMP1, sclerostin Formation of mineralized nodules Acquire stellar shape of osteocyte	[121]
Rat primary periosteal cells	Femur bone	Fibrin hydrogel with calcium phosphate ceramic anchors	Deposition of ordered matrix containing collagen and hydroxyapatite Expression of sclerostin and PDPN Embedded cell with osteocyte morphology in the mineralized matrix	[122]
Mouse mesenchymal stem cell	Bone marrow	2D culture on tissue culture plastic	Formation of mineralized nodules Expression of E11, DMP1, PHEX, SOST, FGF23, RANKL, OPG. Acquire stellar shape of osteocyte	[123]

2.7 Bone marrow derived HSCs vs. peripheral blood derived HSCs

HSCs represent a rare a population of cells in bone marrow and peripheral blood, representing less than 0.01% and less than 0.000001% of total nucleated cells, respectively [124]–[127]. However, the population of cells could be influenced by the age and health condition of patients and the method of cell isolation [128]–[131]. HSCs are primarily located in the bone marrow, but, just like MSCs, they display dynamic behavior by moving out of the bone marrow and entering into the general circulation [68], [132]. The mobilization process could be enhanced by administration various factors and depending on the type of used pharmacological agent such as G-CSF and CXCR4 receptor antagonist AMD3100, the frequency of HSCs in peripheral blood could be elevated up to 100 times [68], [132]–[135]. However, the possible side effects of exposing donors to these pharmacological agents might not be ethical for the donors [83]. Luckily, monocytes that are derived from HSCs and comprise 10-20% of peripheral blood mononuclear cells can be directly isolated from peripheral blood and have been used as osteoclast precursor cells in *in vitro* studies [136]–[142]. To isolate HSCs from bone marrow and peripheral blood, several protocols have been developed including direct plating of bone marrow aspirates and blood samples on plastic surface and collecting the non-adherent cells as it has been shown that HSCs are less likely to attach to the plastic substrate compared to MSCs. The molecular and biochemical analyses on the non-adherent cells of bone marrow and peripheral blood mononuclear cells revealed that they are positive for HSC markers such as SLAM F1 [63]. Culturing non-adherent cells in osteoclastogenesis promoting medium resulted in the differentiation of functional osteoclasts which was associated with expression of the tartrate-resistant acid phosphatase (TRAP) gene and an increased TRAP enzyme activity [62], [63], [143], [144]. Another method is culturing mononuclear cells separated via density gradient centrifugation under osteoclastogenesis culture condition which led to the generation of osteoclasts in culture [145], [146]. Moreover, HSCs and monocytes can be isolated and purified based on the expression of their own specific surface marker such as CD34 and CD14 using techniques including an automated magnetic purification system and FACS [124], [125], [136]–[138], [147]–[150].

Unlike MSCs which exhibit a high proliferation capacity and can be expanded *in vitro* to obtain a high number of cells for *in vitro* studies, the proliferation of HSCs and monocytes remains challenging. For the generation of *in vitro* bone models and osteoclast related studies, freshly isolated osteoclast progenitor cells have been used in most studies [137], [151]–[153]. The time-consuming procedure of cell isolation might cause difficulties in obtaining enough number of cells; as a result, large volume of bone marrow aspirates or peripheral blood would be required. Attempts to increase the number of osteoclast precursor cells *in vitro* resulted in the development and use of several components and factors in culture [126]. It has been shown that combinations of growth factors and cytokines such as interleukin 6 (IL-6), interleukin 3 (IL-3), thrombopoietin (TPO), and stem cell factor (SCF) with additional molecules such as Prostaglandin E2 (PGE2), Stemregenin 1 (SR1), and UM171 could support the proliferation of HSCs *in vitro* [126], [154]–[156]. Monocytes have also showed an increased proliferation potential *in vitro* in response to macrophage-colony stimulating factor (M-CSF), 1α,25-dihydroxyvitamin D3, and lymphokines [157]–[161]. However, the influence of these components on the subsequent osteoclastogenesis potential needs further investigation. Cryopreservation of osteoclast progenitor cells seems feasible as it has not affected monocyte viability and function in

response to various factors [162]–[165]. However, further exploration is required on the osteoclast differentiation ability of cryopreserved osteoclast progenitor cells.

Osteoclast differentiation of both HSCs and monocytes derived from bone marrow and peripheral blood has been shown in several *in vitro* studies [67], [152], [153]. Variations in donor age, health condition and osteoclastogenesis protocols have resulted in mixed outcomes regarding osteoclast differentiation capacity [143], [166], [167]**.** For instance, a mixture of growth factors such as RANKL, M-CSF, transforming growth factor beta (TGF-β), and dexamethasone has led to the generation of multinuclear cells with higher number of nuclei and an increased expression of osteoclast specific genes such as tartrate resistant acid phosphatase (TRACP) 5a and 5b in peripheral blood-derived monocytes compared to bone marrow-derived monocytes (Figure 2.3) [143]. However, no significant differences were reported in bone resorption activity between the used cell types and growth factor combinations [143]. This study highlighted the importance of carefully considering the combination of chosen growth factors for the osteoclastogenesis of osteoclast precursor cells.

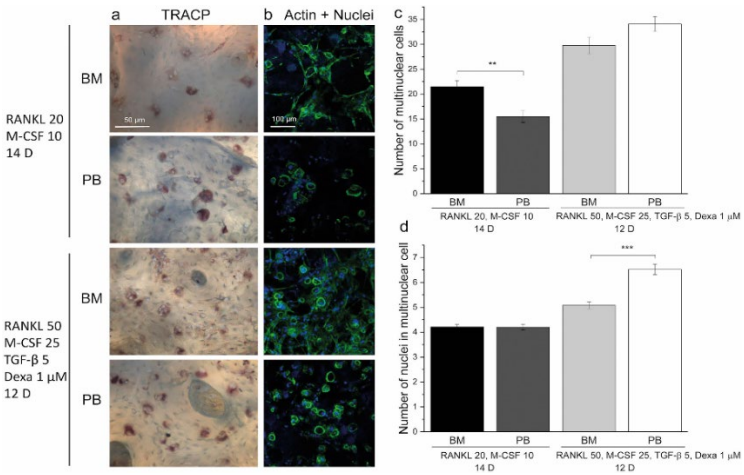

Figure 2.3 Osteoclast differentiated from bone marrow and peripheral blood cultures under different combination of growth factors. The multinuclear TRACP-positive cells are shown in (a) and the actin rings are illustrated in (b). Higher number of multinuclear cells in bone marrow derived cultures were obtained in the presence of RANKL and M-CSF (c). The number of nuclei in multinuclear cell was similar in bone marrow and peripheral blood derived osteoclasts when only RANKL and M-CSF were used, but in the presence of dexamethasone the peripheral blood derived osteoclasts contained significantly more nuclei (d). 12D and 14D: 12 and 14 days of culture, respectively [143]*. Reprinted from Heliyon, Vol 4, Elina Kylmäoja et al., Peripheral blood monocytes show increased osteoclast differentiation potential compared to bone marrow monocytes, Copyright (2018) with permission from Elsevier.*

2.8 Conclusion

In vitro bone models provide a platform to study bone physiology including bone remodeling, bone-related diseases, and potential treatments. These models require all three types of cells in bone; namely osteoblasts, osteocytes, and osteoclasts, ideally from individuals to account for donor-specific differences and disease-related cell reactions. To achieve that, it is required to collect progenitor cells

from one patient and ideally from one cell source for patient convenience. MSCs and HSCs are progenitor cells of osteoblasts/osteocytes and osteoclasts, respectively, and can be extracted from both bone marrow and peripheral blood as reviewed here. Limited studies directly comparing bone marrow-derived and peripheral blood derived MSCs and HSCs have shown no significant differences between osteogenesis and osteoclastogeneis of the progenitor cells from both sources. However, many parameters such as medium composition have been reported to affect cell proliferation and their differentiation potential which could make the peripheral blood derived stem cells superior to the ones from bone marrow. Thus, as both sources have their advantages and disadvantages (Table 2.3), yet, peripheral blood could be considered as a suitable source for both osteoblast/osteocyte and osteoclast progenitor cells, being less invasive for the patient. In this case, more investigations are needed focusing on extraction and differentiation of both cell types from the same sample of peripheral blood.

Table 2.3 Comparison between MSCs and HSCs/monocytes derived from bone marrow and peripheral blood for in vitro studies.

cell	source	Collection invasiveness	Frequency of cells	Proliferation capacity	Differentiation potential*
Mesenchymal stem cells (MSCs)	Bone marrow	+++	++	+++	+++
	Peripheral blood	+	+	+++	+++
Hematopoietic stem cells (HSCs)/monocytes	Bone marrow	+++	+	+	+++
	Peripheral blood	+	+++	+	+++

* The differentiation potential of MSCs and HSCs/monocytes strongly depends on the culture condition.

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Chapter 3

Alkaline phosphatase activity of serum affects osteogenic differentiation cultures

This chapter is based on:

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Abstract

Fetal bovine serum (FBS) is a widely used supplement in cell culture medium, despite its known variability in composition which greatly affects cellular function and consequently the outcome of studies. In bone tissue engineering, the deposited mineralized matrix is one of the main outcome parameters, but using different brands of FBS can result in large variations. Alkaline phosphatase (ALP) is present in FBS. Not only is ALP used to judge the osteogenic differentiation of bone cells, it may affect deposition of mineralized matrix. The present study focused on the enzymatic activity of ALP in FBS of different suppliers and its contribution to mineralization in osteogenic differentiation cultures. It was hypothesized that culturing cells in a medium with high intrinsic ALP activity of FBS will lead to higher mineral deposition compared to medium with lower ALP activity. The used FBS types were shown to have significant differences in enzymatic ALP activity. Our results indicate that the ALP activity of the medium not only affected the deposited mineralized matrix but also the osteogenic differentiation of cells as measured by a changed cellular ALP activity of human bone marrow derived mesenchymal stromal cells (hBMSC). In media with low inherent ALP activity, the cellular ALP activity was increased and played the major role in the mineralization process; while, in media with high intrinsic ALP activity contribution from the serum, less cellular ALP activity was measured and the ALP activity of the medium also contributed to mineral formation substantially. Our results highlight the diverse effects of ALP activity intrinsic to FBS on osteogenic differentiation and matrix mineralization and how FBS can determine the experimental outcomes, in particular for studies investigating matrix mineralization. Once again, the need to replace FBS with more controlled and known additives is highlighted.

3.1 Introduction

Fetal bovine serum (FBS) is a widely known supplement in cell culture media, used at concentrations up to 20% (v/v) [1]. FBS provides cells with vital factors including growth factors, hormones, and vitamins essential for cell survival, growth, and division [1], [2]. However, the use of FBS in *in vitro* cell culture is controversial due to a number of reasons, including ethical concerns, shortage in global supply and most importantly its undefined, complex composition and variability which could lead to unexpected and/or unreliable experimental outcomes [1], [3], [4]. Thus, either complete avoidance of FBS or at least awareness of the effects that some components of FBS might have on experimental outcomes should be considered [2], [5], [6].

FBS has previously been described having various effects on mineral deposition. It was shown being able to hydrolyze phosphate sources and by that increasing the concentration of free phosphate in the culture medium, which further resulted in mineralization of fibrous proteins such as collagen and silk fibroin even without the presence of cells [7], [8]. Moreover, the deposited calcium content on the fibrous scaffolds was significantly affected by the variation in the chemical composition of FBS [8]. Since the exact chemical composition of FBS is not provided and is known to differ even between batches within the same brand, it remains unknown which component(s) contributes to the mineralization process. Knowledge on which and how FBS component(s) contribute to mineralization could be beneficial for *in vitro* studies where mineralization of extracellular matrix is needed (e.g., bone tissue engineering) but also where mineralization should be avoided (e.g., cardiac tissue engineering).

Alkaline phosphatase (ALP) is a potential component of FBS affecting mineralization. ALP is an abundant membrane-bound glycoprotein [9]. It exists as four isozymes, depending on the tissue where it is expressed: placental ALP, germ cell ALP, intestinal ALP, and liver/bone/kidney ALP [10]. ALP enzymes expressed in the placenta, germinal and intestine tissue are tissue-specific meaning that under physiological conditions, they are found exclusively in the tissues where they are expressed, whereas the ones expressed in liver, bone, and kidney are known as tissue-nonspecific ALP because they can also be found in blood circulation [11]–[14].

In bone, ALP is expressed by osteoblasts, the bone forming cells, and either anchored to the cell membrane or to matrix vesicles generated by osteoblasts through glycosylphosphatidylinositol (GPI) linkage attached to the carboxyl-terminal of the enzyme [15]. ALP can be released into serum through matrix vesicles or after its cleavage from the osteoblast surface by circulating GPI-specific phospholipase D [14], [16], [17]. Thus, serum contains ALP which is used for example as a biomarker in the clinics to assess chronic kidney diseases or bone disorders [17].

During the osteogenic differentiation process, the presence and activity of ALP indicates the differentiation of mesenchymal stromal cells (MSCs) towards osteoblasts [18]. The activity of ALP can be measured thorough colorimetric assays where p-nitrophenyl phosphate, a phosphate substrate, is dephosphorylated by ALP [19], [20]. Besides the activity of ALP, the expression of ALP can be measured through techniques such as quantitative polymerase chain reaction (qPCR), western blot,

and immunofluorescence imaging [21], [22]. The latter can determine the location of expressed ALP with respect to the cell.

ALP expressed by osteoblasts is an important enzyme in the process of bio-mineralization [12]. This enzyme can hydrolyze extracellular inorganic pyrophosphate, generated by the hydrolysis of adenosine triphosphate (ATP), which leads to an increase in the local concentration of inorganic phosphate (Pi) [23]–[26]. Pi and calcium ions are thought to accumulate inside matrix vesicles to form amorphous calcium phosphate or hydroxyapatite crystals which is believed to be the initial stage of extracellular matrix mineralization during bone formation [27].

In *in vitro* bone studies, to avoid spontaneous mineralization, β-glycerophosphate (β-GP) has been used as the phosphate source that is believed to be cleaved through the ALP activity of osteoblasts, making Pi available for matrix mineralization [28]. However, hydrolyzing β-GP under cell-free conditions and in the presence of FBS indicated that serum ALP activity has its contribution in making Pi available in culture medium for subsequent calcium phosphate deposition [7], [29]. This effect resulted in non-physiological and uncontrollable mineralization prior to osteoblast differentiation *in vitro* which needs to be avoided in many research lines, for instance, the development of *in vitro* bone models [30].

In this study, four different types of FBS with different intrinsic ALP activity were investigated with the aim to investigate the influence and contribution of medium (provided by FBS) and cellular ALP activity on mineralized tissue formation. For this, silk fibroin scaffolds were either left acellular or were seeded with human bone marrow derived mesenchymal stromal cells (hBMSCs) and cultivated *in vitro*. We hypothesized that the ALP activity of medium containing FBS not only affects calcium phosphate deposition in the presence and absence of cells, but that it also has an influence on the cellular ALP activity. We further investigated whether heat inactivation of FBS, a process which is commonly used to destroy complement activity in serum, also can eradicate the effects of FBS ALP. Knowledge on the influence of ALP activity of FBS, as one of the many components in FBS that could be responsible for the high variation in experimental outcomes, can shed a light on the necessity of developing serum-free medium with clearly defined components.

3.2 Materials and methods

3.2.1 Materials

Dulbecco's modified eagle medium (DMEM high glucose, Cat. No. 41966 and low glucose Cat. No. 22320), antibiotic/antimycotic (Anti-Anti, Cat. No. 15240062), non-essential amino acids (NEAA, Cat. No. 11140050), and trypsin-EDTA (0.5%, Cat. No. 2530054) were from Life Technologies (The Netherlands). FBS types were from Bovogen (Cat. No. SFBS), Sigma (Cat. No. F7524), Hyclone (South American research grade FBS, Cat. No. SV30160.02), and U.S. Origin FetalClone III serum (Fetalclone III, Cat. No. SH30109.03). Silkworm cocoons were purchased from Tajima Shoji Co., LTD. (Japan). Unless noted otherwise, all other substances were of analytical or pharmaceutical grade and obtained from Sigma Aldrich (The Netherlands).

3.2.2 Measurement of ALP activity of serum and medium supplemented with FBS

The ALP activity of four types of FBS and the resulting control medium containing DMEM low glucose, 1% Anti-Anti and 10% FBS (Table 3.1) was measured as follows: In a 96-well plate, 80 µL of each serum sample or medium sample was mixed with 20 µL of 0.75 M 2-amino-2-methyl-1-propanol buffer and 100 µL 10 mM p-nitrophenylphosphate solution and incubated until colour developed, before 0.2 M NaOH was added to stop the conversion of p-nitrophenylphosphate to p-nitrophenol. Absorbance was measured in a spectrophotometer at 405 nm and ALP activity was calculated by comparison to standards of known p-nitrophenol concentration.

Table 3.1 List of abbreviations of FBS types and the medium containing each type of FBS

Serum brand	LOT number	Abbreviation	Medium	Abbreviation
Bovogen	51113	B	Control medium containing 10% Bovogen	DMEM%10B
Sigma	7611	S	Control medium containing 10% Sigma	DMEM%10S
Hyclone	RE00000004	H	Control medium containing 10% Hyclone	DMEM%10H
Fetalclone III	AD19958305	F	Control medium containing 10% Fetalclone III	DMEM%10F

3.2.3 Heat inactivation of FBS

Five mL of each serum type was placed in a water bath at 56°C for 30 minutes. After 30 minutes, the sera samples were removed from the water bath and transferred into an ice bath for rapid cooling. The ALP activity of heat inactivated (HI) FBS and the media containing 10% of HI FBS (Table 3.2) was measured according to section 2.

Table 3.2 List of abbreviations of heat inactivated (HI) FBS and the medium containing each type of HI FBS.

Serum	Abbreviation	Medium	Abbreviation
HI Bovogen	HI-B	Control medium containing 10% HI Bovogen	DMEM%10HI-B
HI Sigma	HI-S	Control medium containing 10% HI Sigma	DMEM%10HI-S
HI Hyclone	HI-H	Control medium containing 10% HI Hyclone	DMEM%10HI-H
HI Fetalclone III	HI-F	Control medium containing 10% HI Fetalclone III	DMEM%10HI-F

3.2.4 Measurement of Pi concentration in medium supplemented with FBS

Concentration measurements of free phosphate Pi in control medium containing DMEM low glucose, 1% Anti-Anti and 10% FBS or HI FBS (Table 3.1 and 3.2) with and without the addition of 10 mM β-glycerophosphate (β-GP) after 48 hours of incubation at 37°C were performed according to the manufacturer's instruction (Malachite Green Phosphate Assay Kit, Sigma-Aldrich, The Netherlands).Briefly, 80 µL of 1:200 (v/v) diluted samples in ultra-pure water (UPW) were mixed with 20 µL of working reagent and incubated for 30 minutes at room temperature. In this assay, a green

complex is formed between molybdate and Pi. Colour formation from the reaction was measured spectrophotometrically at 620 nm and phosphate concentration was calculated by comparison to a phosphate standard provided in the kit.

3.2.5 Silk fibroin scaffold fabrication

To prepare silk fibroin scaffolds, 3.2 grams of cut and cleaned Bombyx mori L. silkworm cocoons were degummed by boiling in 1.5 L UPW containing 0.02 M Na_2CO_3 for 1 hour, whereafter it was rinsed with 10 L cold UPW to extract sericin. Dried purified silk fibroin was dissolved in 9 M lithium bromide (LiBr) solution in UPW at 55°C for 1 hour and dialyzed against UPW for 36 hours using SnakeSkin Dialysis tubing (molecular weight cutoff: 3.5 kDa, Thermo Fisher Scientific, The Netherlands). The silk fibroin solution was frozen at -80°C for at least 2 hours and lyophilized (Freezone 2.5, Labconco, USA) for 4 days. lyophilized silk fibroin (1.7 grams) was then dissolved in 10 mL 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) at room temperature for 5 hours resulting in a 17% (w/v) solution. One mL of silk-HFIP solution was added to a Teflon container containing 2.5 grams NaCl with a granule size between 250-300 µm. After 3 hours, HFIP was allowed to evaporate for 4 days. Silk fibroin-NaCl blocks were immersed in 90% (v/v) methanol (Merck, The Netherlands) in UPW for 30 minutes to induce the protein conformational transition to β-sheets [31]. Scaffolds were cut into disks of 3 mm height with an Accutom-5 (Struer, Type 04946133, Ser.No. 4945193), followed by immersion in UPW for 2 days to extract NaCl. Disc-shaped scaffolds were made with a 5 mm diameter biopsy punch (KAI medical, Japan) and autoclaved in phosphate buffered saline (PBS) at 121°C for 20 minutes.

3.2.6 Cellular and acellular scaffold preparation

Human bone marrow mesenchymal stromal cells (hBMSCs) were isolated from human bone marrow (Lonza, USA) and characterized as previously described [32]. Passage 3 hBMSCs were expanded in expansion medium (DMEM high glucose with 10% FBS Sigma, 1% Anti-Anti, 1% NEAA, and 1 ng/ml bFGF) for 7 days. At day 7, cells were 80% confluent and trypsinized. 16 scaffolds were dynamically seeded with $1*10^6$ cells per scaffold as previously described [33]. Briefly, each scaffold was incubated with a cell suspension ($1*10^6$ cells/4 mL control medium (DMEM, 10% FBS respective of each group, 1% Anti-Anti)) in 50 mL tubes placed on an orbital shaker at 150 rpm for 6 hours in an incubator at 37°C [33]. The remaining scaffolds were left acellular and incubated in the control medium as described above. All scaffolds were incubated in 24-well plates at 37°C and 5% CO_2 for 4 weeks. Each well was filled with 1 mL osteogenic medium (control medium from Table 3.1 supplemented with 50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, 10 mM β-GP). The medium was refreshed 3 days a week.

3.2.7 Measurement of ALP activity of cells

After 4 weeks of culture, scaffolds (n=3 per group) were washed with PBS and each disintegrated in 500 µL of 0.2% (v/v) Triton X-100 and 5 mM $MgCl_2$ solution using steel balls and a minibeadbeater (Biospec, USA). The solids were separated by centrifugation (3000g, 10 minutes). The measurement of ALP activity in the supernatant was performed as described in section 3.2.2. In a 96-well plate, 80

µL of the supernatant was mixed with 20 µL of 0.75 M 2-amino-2-methyl-1-propanol buffer and 100 µL 10 mM p-nitrophenylphosphate solution and incubated until colour developed, before 0.2 M NaOH was added to stop the conversion of p-nitrophenylphosphate to p-nitrophenol. Absorbance was measured spectrophotometrically at 405 nm and ALP activity was calculated by comparison to standards of known p-nitrophenol concentration.

3.2.8 Measurement of (soluble) calcium concentration in medium supplemented with FBS

The calcium concentration was performed on control medium and osteogenic medium in the presence and absence of cells after 48 hours of incubation at 37°C. Five µL of each medium condition was mixed with 95 µL of working solution (Stanbio Calcium (CPC) LiquiColor Test, Stanbio Laboratories) and incubated at room temperature for at least 1 minute. In this assay, the calcium ion concentration is measured by the chromogenic complex formed between calcium ions and o-cresolphthalein. Absorbance at 550 nm was measured and calcium concentration was calculated by comparison to standards of known calcium chloride concentrations.

3.2.9 Measurement of (deposited/precipitated) calcium and phosphate on cell-seeded and acellular scaffolds

After 4 weeks of culture, scaffolds (n=3 per group) were washed with PBS and each disintegrated in 500 µL of 5% Trichloroacetic acid (TCA) in UPW using steel balls and a minibeadbeater (Biospec, USA). After 48 hours of incubation at room temperature, the solids were separated by centrifugation (3000g, 10 minutes). Calcium and phosphate assays were performed on each sample as described below.

Measurement of (deposited/precipitated) calcium on scaffolds

Five µL of samples were mixed with 95 µL of working solution (Stanbio Calcium (CPC) LiquiColor Test, Stanbio Laboratories) and incubated at room temperature for at least 1 minute. In this assay, the calcium ion concentration is measured by the chromogenic complex formed between calcium ions and o-cresolphthalein. Absorbance at 550 nm was measured and calcium concentration was calculated by comparison to standards of known calcium chloride concentrations.

Measurement of (deposited/precipitated) phosphate on scaffolds

Phosphate assay was performed according to the manufacturer's instruction (Malachite Green Phosphate Assay Kit, Sigma-Aldrich, The Netherlands). Briefly, 80 µL of 1:200 (v/v) diluted samples in UPW were mixed with 20 µL of working reagent and incubated at room temperature for 30 minutes. Absorbance was measured spectrophotometrically at 620 nm and phosphate concentration was calculated by comparison to the phosphate standard provided in the kit.

3.2.10 Histology

After 4 weeks of culture, scaffolds were washed with PBS and immersed first in 5% and then in 35% sucrose solution in PBS at room temperature for 10 minutes each. The scaffolds were embedded in cryomold containing Tissue-Tek OCT compound (Sakura, The Netherlands), frozen on dry ice, cut into 5 µm thick sections using a cryostat cryotome (Fisher Scientific, The Netherlands) and mounted on

Superfrost Plus microscope slides (Thermo Fisher Scientific, The Netherlands). Sections were washed with PBS, fixed in 10% neutral buffered formalin for 10 minutes at room temperature, washed again with PBS and stained with Alizarin Red to identify mineralization.

3.2.11 Micro-computed tomography imaging (µCT)

µCT measurements were executed on a µCT100 imaging system (Scanco Medical, Brüttisellen Switzerland) after 4 weeks of culture (n=4 per group). Scanning was performed at an isotropic nominal resolution of 17.2 µm, an energy level of 55 kVp, and an intensity of 200 µA. Integration time was set to 300 ms and two-fold frame averaging was performed. To reduce part of the noise, a constrained Gaussian filter was applied. Filter support was set to 1.0 and filter width sigma to 0.8 voxel. To distinguish mineralized tissue from non-mineralized tissue, segmentation was performed. A global threshold range was set to 148-1970 after visual judgment of the grey images to identify mineralized structures compared to histologically stained samples. Unconnected objects smaller than 50 voxels were removed through component labelling and neglected for further analysis. Quantitative morphometry was performed to assess the mineralized volume of the entire construct [34].

3.2.12 Statistics

GraphPad Prism 9.0.2 (GraphPad Software, USA) was used to perform statistical analysis and to make graphs. For ALP activity of serum (Figure 3.1A-B), ALP activity of HI serum (Figure 3.6A-B), and ALP activity of cells and medium (Figure 3.2B, blue and dark yellow dots) Kruskal-Wallis test with Dunn post-hoc testing was performed. For comparing ALP activity of cell and medium within each medium group (Figure 3.2B), multiple Mann-Whitney test was performed. For Pi concentration (Figure 3.1C and Figure 3.6C), multiple Mann-Whitney tests have been performed between control medium and control medium containing β-GP. Deposited calcium and phosphate (Figure 3.3A-B) and mineralized volume (Figure 3.4I) between cell-seeded and acellular scaffolds were analysed by multiple Mann-Whitney test. Deposited calcium and phosphate and mineralized volume on either cell-seeded (Figure 3.3A-B and 3.4I, blue dots) or acellular scaffolds (Figure 3.3A-B and 3.4I, dark yellow dots) were analysed by Kruskal-Wallis test with Dunn post-hoc testing. To compare medium calcium concentration (Figure 3.3C), within each category (control group, cell-seeded scaffolds, and acellular scaffolds), or between control group, cell-seeded, and acellular scaffolds of each medium type (F, B, H, or S) a Kruskal-Wallis test with Dunn post-hoc testing was performed. Differences between groups were considered statistically significant at a level of $p<0.05$. Histological figures show representative images per group of all the samples assessed.

3.3 Results

3.3.1 The ALP activity of FBS elevated the Pi concentration in the medium

Four different FBS types and their corresponding control media containing 10% FBS were analysed for their intrinsic ALP activity. The ALP activity varied between the different brands in both concentrated (Figure 3.1A) and diluted state (Figure 3.1B). As the enzymatic activity is influenced by enzyme concentration, high ALP activity corresponds to high concentration of ALP in FBS [35]. The ALP activity

in the diluted state decreased significantly compared to concentrated FBS which was not necessarily 10 times less. To investigate whether this enzymatic activity contributes to the supply of Pi in the medium, control media containing 10% FBS were supplemented with 10 mM β-GP. β-GP is generally used as the phosphate source for *in vitro* osteogenic differentiation processes. The enzymatic activity of ALP was able to convert β-GP into Pi, resulting in an increased Pi level in the medium. The concentration of Pi in the medium was elevated by a factor of 1.51, 4.54, 4.69, and 5.03-fold in medium supplemented with 10% Fetalclone III, Bovogen, Hyclone, and Sigma FBS, respectively, within 48 hours of incubation compared to respective control medium (Figure 3.1C). This indicates that ALP present in FBS is capable to cleave β-GP regardless of the presence of cells in the system. Moreover, the increase in the concentration of Pi after 48 hours was correlated to the intrinsic ALP activity of medium containing FBS.

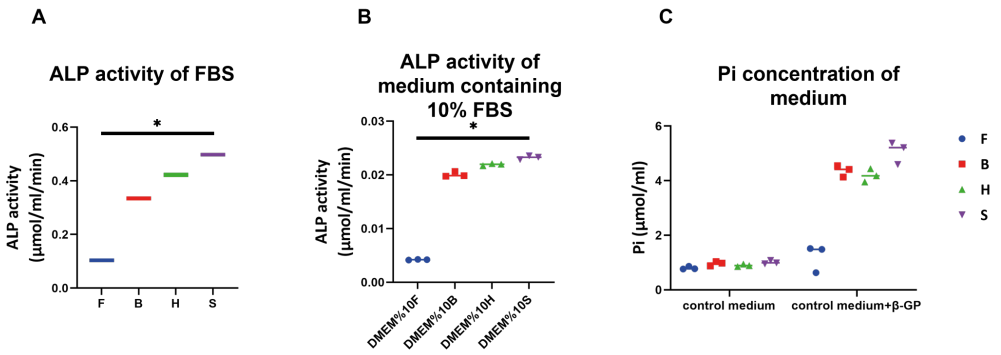

*Figure 3.1 ALP is present in FBS and its activity was different between the four different FBS types tested (A). Control medium supplemented with 10% FBS showed differences in ALP activity with the same trend (B). 48 hours incubation of control medium supplemented with FBS and 10 mM β-GP resulted in an increase in Pi concentration in the medium (C). The increase in Pi seems to be correlated with the ALP activity in the medium, it showed that the FBS with lowest ALP activity (Fetal clone III) led to lowest increase in Pi concentration of the medium. *p<0.05 (a Kruskal-Wallis test with Dunn post-hoc has been done on all groups but only groups F and S were statically different in figure A and B).*

3.3.2 Cellular and medium ALP activity was negatively correlated

Two types of ALP activity were measured after 4 weeks of culture since they can both contribute to the overall amount of available Pi. First, the activity of membrane-bound ALP, expressed by osteoblasts during osteogenic differentiation (Figure 3.2A, blue stars). Second, the ALP activity present within the different media containing 10% FBS (Figure 3.2A, dark yellow stars). The measured ALP activity was normalized to the time of incubation. The cellular enzymatic activity of ALP in the groups of medium containing Fetalclone III and Bovogen was higher than that of cells grown in media containing Hyclone and Sigma FBS. This was in contrast to the activity of ALP in medium containing FBS. There seemed to be a negative correlation between the cellular and medium ALP activity; in

medium with low inherent ALP activity, the cells have a higher ALP activity (Fetalclone III) compared to the medium with high inherent ALP activity (Sigma) (Figure 3.2B). Notably, after four weeks, the total ALP activity in the construct was roughly equal in all four groups and did not show any significant differences (Figure 3.2C).

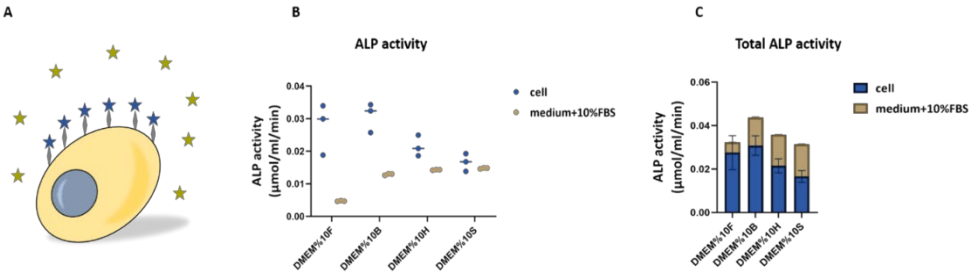

Figure 3.2 Osteoblasts express ALP, a membrane-bound protein (A-blue stars) and the medium containing FBS has shown to have active ALP (A-dark yellow stars). The cellular ALP activity seems negatively correlated to the medium ALP activity; in the groups with low medium ALP activity, the cells expressed higher ALP activity compared to the groups with high ALP activity (B). Multiple Mann-Whitney tests between "cell" and "medium+10%FBS" within each group and a Kruskal-Wallis test with Dunn post-hoc between the groups in either "cell" or "medium+10%FBS" did not show any significant differences. The total ALP activity in all groups was equal with no significant differences (C).

3.3.3 Cellular and medium ALP activity both contribute to calcium phosphate deposition

The amount of calcium and phosphate deposited within the constructs was measured both in the presence and absence of cells after 4 weeks. Incubation of acellular scaffolds in medium containing FBS indicated the contribution of medium ALP activity on calcium phosphate deposition. The ALP activity inherent to the media enabled the deposition of calcium phosphate even when no cells were present. As expected, the amount of calcium and phosphate per construct varied in different culture media used (Figure 3.3). The deposited calcium phosphate per acellular scaffold (Figure 3.3A and B, dark yellow dots) which indicates the contribution of medium ALP activity showed the same trend as the ALP activity of the medium (Figure 3.2B, dark yellow dots): Sigma > Hyclone > Bovogen > Fetalclone III FBS. As hypothesized, even in the absence of cells, a high ALP activity in medium resulted in more calcium phosphate deposition compared to the medium with low ALP activity. The presence of cells and their differentiation towards osteoblasts increased the calcium phosphate deposition further, which indicated the contribution of cellular ALP activity (Figure 3.3, blue dots) next to the medium ALP activity. The cellular ALP activity resulted in increasing Pi and thus calcium phosphate deposition. The calcium and phosphate content of cell-seeded scaffolds followed the following pattern: Sigma > Bovogen > Hyclone > Fetalcone III FBS. This pattern is not, however, consistent with the cellular ALP activity which was Bovogen > Fetalclone III > Hyclone > Sigma FBS.

The calcium concentration of control medium in all groups was similar, as expected. When the medium was supplemented with osteogenic factors containing dexamethasone, ascorbic acid, and β-GP in the absence and presence of cells, this concentration decreased in the medium (Figure 3.3C).

The medium with high ALP activity (Sigma) showed a larger decrease of calcium concentration in the medium compared to medium with low ALP activity (Fetalclone III). The decrease of calcium concentration in the medium indicated the deposited calcium phosphate on the scaffolds.

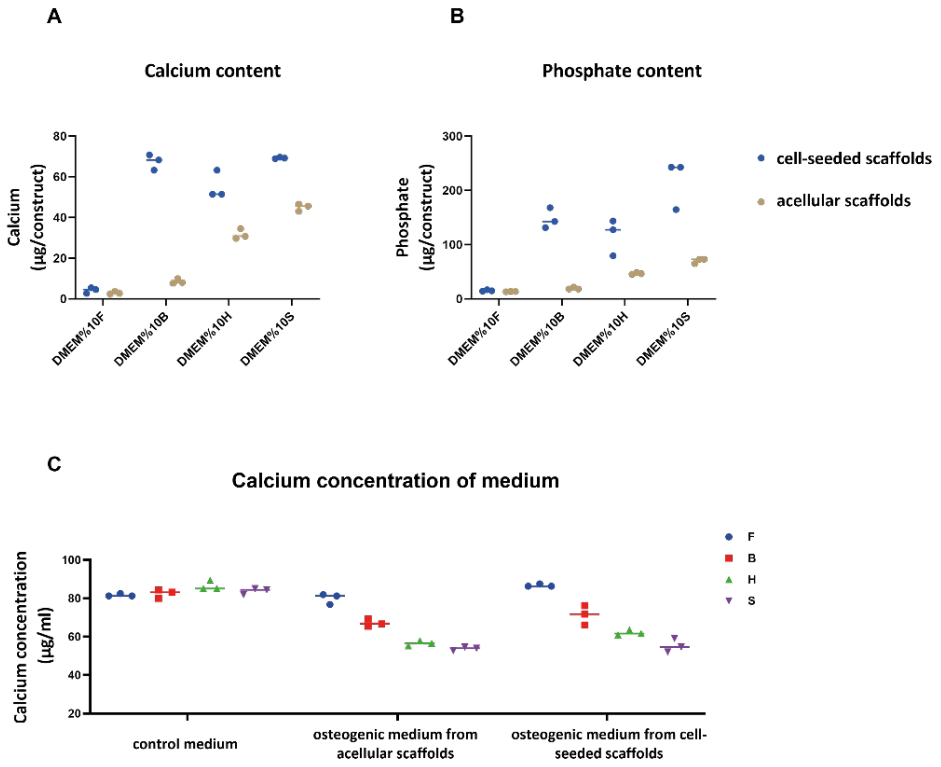

Figure 3.3 Deposited calcium (A) and phosphate (B) after 4 weeks of culture either without cells (contribution of medium ALP activity, dark yellow dots) or with cells (contribution of cellular ALP activity, blue dots) on 3D silk fibroin scaffolds. Multiple Mann-Whitney tests between "cell-seeded scaffolds" and "acellular scaffolds" within each group and a Kruskal-Wallis test with Dunn post-hoc between the groups in either "cell-seeded scaffolds" or "acellular scaffolds" did not show any significant differences The calcium concentration in the medium decreased at higher ALP activities, probably because it was deposited in the form of calcium phosphate (C). No significant differences were detected between the groups (control medium, osteogenic medium from acellular scaffolds or cell-seeded scaffolds) or within each group between different medium types (F, B, H, and S).

3.3.4 µCT analysis and Alizarin red staining detected calcium phosphate deposition on both acellular and cell-seeded scaffolds

µCT imaging (Figure 3.4) and histology (Figure 3.5) of the samples after 4 weeks of culture confirmed the deposition of a mineralized matrix either within the silk fibroin scaffold and/or in the extracellular space. Incubation of acellular scaffolds in media containing Hyclone and Sigma with high medium ALP activity led to mineral deposition within the silk fibroin scaffold. The resulting mineral volume was significantly higher than that on scaffolds incubated in media containing Fetalclone III and Bovogen

FBS with low medium ALP activity. In the presence of cells, the mineralized volume changed significantly in all groups compared to acellular constructs, most likely as a result of the cellular ALP activity. With µCT, the mineralized volume in the medium containing Fetalclone III and Bovogen FBS was visible only in the presence of cells; while the media containing Hyclone and Sigma FBS showed large, mineralized volumes even on acellular scaffolds which indicates the contribution of their medium ALP activity (Figure 3.4I). As the scaffolds are made of silk fibroin, which is a protein similar to collagen, in the acellular groups, the minerals are expected to be found in and/or on the scaffolds. In the cell-seeded groups, the minerals could be found both in/on the scaffolds and in the extracellular matrix (ECM) formed by cells. On acellular scaffolds cultured in the medium containing Hyclone and Sigma FBS, minerals were detected on the scaffolds, most likely showing mineral precipitations (Figure 3.5C and D). The cell-seeded scaffolds cultured in medium containing Bovogen, Hyclone, and Sigma FBS showed mineralization both in the ECM and within the scaffolds (Figure 3.5F, G and H).

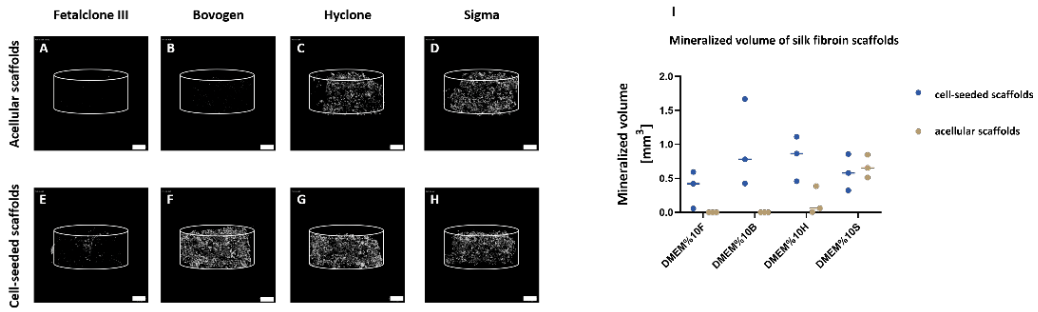

Figure 3.4 µCT analysis of mineralized volume within acellular and cell-seeded silk fibroin scaffolds after 4 weeks of culture. In the medium containing Fetalclone III (A and E) and Bovogen (B and F) with low medium ALP activity, the mineral deposition happened exclusively in the presence of cells. In the Hyclone and Sigma containing medium groups with high medium ALP activity, substantial amounts of mineral deposition happened even if cells were not present (C, G, D, and H). The mineralized volume in the medium containing Fetalclone III and Bovogen FBS was detected only in the presence of cells, while the media containing Hyclone and Sigma FBS showed large, mineralized volumes even on acellular scaffolds (I). Multiple Mann-Whitney tests between "cell-seeded scaffolds" and "acellular scaffolds" within each group and a Kruskal-Wallis test with Dunn post-hoc between the groups in either "cell-seeded scaffolds" or "acellular scaffolds" did not show any significant differences. Scale bar: 1mm.

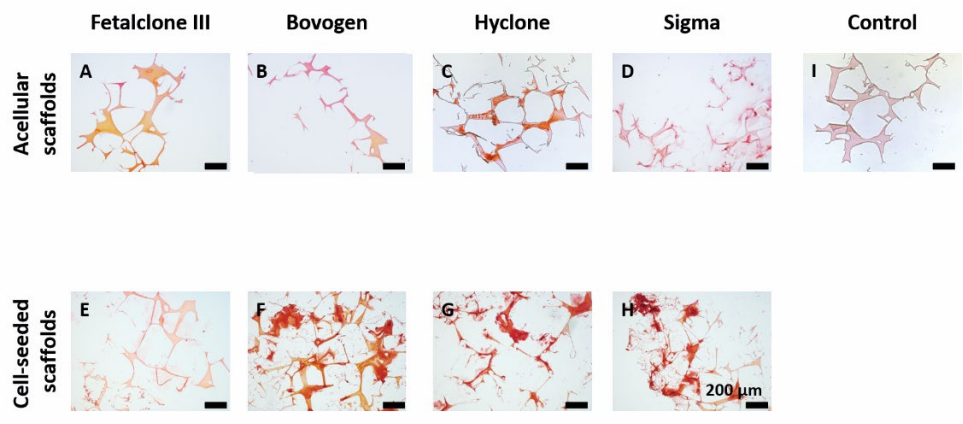

Figure 3.5 All constructs show mineral deposition with Alizarin Red staining. Acellular (A-D) and cell-seeded (E-H) 3D silk fibroin scaffolds after 4 weeks of culture in media containing different FBS types. In the medium containing Fetalclone III (A and E) and Bovogen (B and F) with low medium ALP activity, the mineral deposition happened exclusively in the presence of cells. In the Hyclone and Sigma containing medium groups with high medium ALP activity, substantial amounts of mineral deposition happened even if cells were not present (C, G, D, and H). The silk fibroin scaffolds without cells and minerals used as a control (I). Scale bar: 200 µm.

3.3.5 Enzymatic activity of ALP is declined in heat inactivated (HI) FBS

The ALP activity of HI FBS types and their corresponding control media containing 10% HI FBS was analysed. The heat inactivation process was able to decrease the ALP activity of FBS (Figure 3.6A) compared to the non-HI FBS (Figure 3.1A) by 88.05% (Fetalclone III), 94.77% (Bovogen), 95.61% (Hyclone), and 95.54% (Sigma), respectively. The ALP activity of 10% HI FBS in medium decreased further compared to concentrated FBS (Figure 3.6B). To investigate the contribution of ALP activity of HI FBS in concentration of Pi in the medium, control media containing 10% HI FBS were supplemented with 10 mM β-GP. The concentration of Pi did not change in control medium supplemented with β-GP and 10% HI FBS after 48 hours of incubation compared to respective control medium (Figure 3.6C). This result demonstrated that the ALP present in FBS can be deactivated through the HI process.

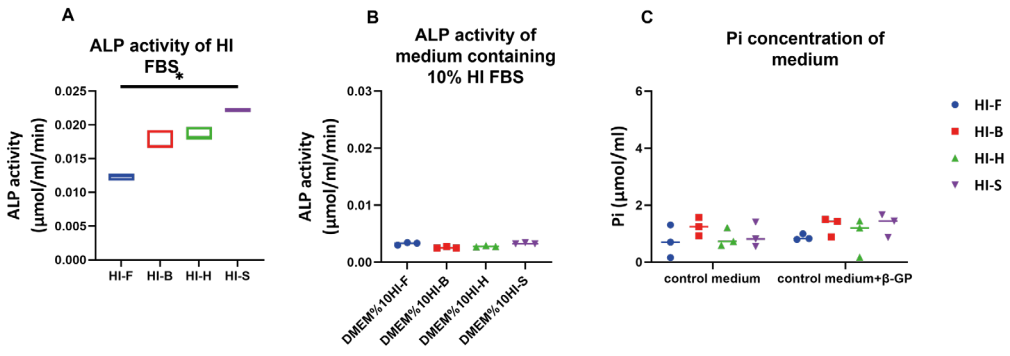

*Figure 3.6 ALP activity of FBS decreased through HI process (A). A 10% dilution of FBS in media decreased ALP activity further and eliminated differences between the groups (B). 48 hours incubation of control medium supplemented with FBS and 10 mM β-GP resulted in no changes in Pi concentration in the medium which indicated the deactivation of ALP in HI FBS (C) *p<0.05 (a Kruskal-Wallis test with Dunn post-hoc has been done on all groups but only groups F and S were statically different in figures A and B).*

3.4 Discussion

FBS was introduced more than 50 years ago as cell culture supplement for cellular growth as it contains crucial components for cell proliferation and maintenance including hormones, growth factors, vitamins, trace elements, and transport proteins [1], [2], [36]. However, the composition of FBS is not defined and consistent which could provoke significant differences in experimental outcomes and contribute to a low reproducibility of data [4], [8], [37]. Due to the disadvantages of using FBS in cell culture, it should be replaced by defined and more controlled media supplements. However, owing to time consuming and costly process of serum-free medium development, FBS is still a common cell culture supplement in cell culture practice. As such, researchers should at least be aware of a potential influence of FBS on their study outcomes, and, if needed, identify the influence of crucial factors.

Bone tissue engineering has been known as a promising approach to develop tissue-engineered grafts for patients with large osseous defects [38]. In the past few years, bone tissue engineering has been applied to create three-dimensional (3D) *in vitro* human bone models [30]. These models can be used as a platform to study the bone physiology/pathology, cell-cell- or cell-material-interaction, and drug discovery/testing [39]. However, in such models, using FBS does not necessarily represent the physiological condition and can influence the cellular behavior and function [1], [3]. It has previously been shown that FBS can affect the mineralization process in bone tissue engineering studies [7], [8]. In the present study, ALP was investigated as a component present in FBS affecting in the mineralization process during *in vitro* bone-like tissue formation. We showed that the inherent ALP activity of FBS could lead to significantly different conclusions about the osteogenic differentiation capability of cells and in particular about the amount of mineralized ECM deposition when performed with different FBS brands.

Bone tissue forms through two different procedures: endochondral ossification, which is a multistep process that requires the formation of cartilage template and its replacement with bone tissue, and intramembranous ossification through which bone tissue develops by the concentration of mesenchymal stromal cells (MSCs) that directly undergo osteogenic differentiation [40]–[42]. During intramembranous ossification, osteoblasts originating from MSCs deposit bone matrix through production of collagen type 1 fibrils and regulation of deposited minerals within the collagenous matrix [40]. To regulate the mineralization of collagenous matrix, osteoblasts express proteins including ALP which provides the phosphate required for mineralization process [12]. In bone tissue engineering and more precisely development of *in vitro* bone models, the aim is to differentiate MSCs towards osteoblasts which produce the collagenous matrix and control matrix mineralization through the expression of non-collagenous proteins (NCPs) [30]. However, the presence of ALP - and possibly

NCPs too - in FBS influences the whole osteogenic differentiation and mineralization process as we have shown here.

The presence of phosphatases in FBS was suggested in previous studies, as FBS showed the capability to hydrolyze β-GP and increase the phosphate concentration of medium in the absence of cells [43], [44]. Among the proteins and phosphatases, ALP is a well-known one that is present in FBS and provides the cell culture media with free phosphate [29]. To the best of our knowledge, there is no evidence of the presence of other types of phosphatases that could hydrolyze β-GP. The four different brands of FBS tested in this study showed to differ in ALP activity in both concentrated and diluted state. Differences in the ALP activity of each FBS brand resulted in differences in the concentration of phosphate in the medium after 48 hours of incubation of FBS containing medium supplemented with β-GP; in the medium with low ALP activity (Fetalclone III), the lowest increased in phosphate concentration was detected. The amount of spontaneous mineralization depends on the ion concentration of the solution surrounding the substrate [45]. With the same basal medium being used, the initial calcium concentration of control medium was the same in all groups. As expected, differences in the ALP activity of FBS containing medium resulted in variation in Pi concentration in medium which further influences the calcium phosphate deposition.

The ALP activity of FBS containing medium also affected the cellular ALP activity. Though cells from the same vial (same donor, same passage) were used for the experiment, it seems that in the medium with high inherent ALP activity (Sigma), the cells showed lower ALP activity compared to the cells cultured in the medium with low ALP activity (Fetalclone III). This effect could be due to the calcium phosphate deposition because of the ALP activity of medium prior to expression of ALP from cells. In bone and calcifying cartilage, ALP is expressed early in the development, and is localized on the cell surface and on matrix vesicles. As the mineralized tissue matures, ALP expression and activity decrease [12], [24]. Thus, the presence of calcium phosphate deposition prior to osteogenic differentiation of MSCs could have influenced the cellular ALP activity. Due to the complex and unknown composition of FBS, there is always a possibility of the presence of a component that influences the calcium phosphate deposition on cell-seeded/acellular scaffolds in addition to the cellular ALP activity. But a high inherent ALP activity of FBS could be a sign of how the cells might react or how the calcium phosphate deposits might develop in osteogenic differentiation cultures.

One possible way to avoid the influence of the ALP activity inherent to the various FBS brands is through the HI process. It is mostly used to destroy complement activity in serum through protein denaturation by heat and was also effective in reducing the ALP activity down to a base level that no longer was able to cleave substantial amounts of phosphate from β-GP. The HI process at the same time also influences the structural configuration of other heat-sensitive proteins which results in changes in their activity too [46]. These changes in turn can potentially affect the cellular behavior including metabolic activity, proliferation and colony-forming units of hBMSCs [47]. An increase in cellular ALP activity has been reported when cells were cultured under HI serum supplemented medium [48]. This might be related to the reduction of the medium ALP activity but will need further investigation.

The present study was limited to four different FBS types and batch variation for each FBS types was not investigated here. But it can be expected that differences in ALP activity can be detected in other batches of the same FBS type and even other serum types, including human serum. Moreover, silk fibroin was the only biomaterial substrate tested in this study. The chemical structure of silk fibroin is similar to collagen type 1 which makes it an ideal environment for spontaneous mineralization, similarly as the collagen within the bone matrix. The effect of ALP activity of FBS on other substrates might be different.

To avoid the effect of medium ALP activity and the other known/unknown components of FBS, development of defined and more controlled medium supplements is recommended. The information on existing defined media supplements is already available in databases, which could facilitate the process of formulating new media supplements [2]. So far, no general formula that suits all needs has been found. It seems as if these formulations are specific to the cell type, thus studying the factors impacting the specific cell behavior is needed to develop such medium supplements.

3.5 Conclusion

In this study, we have demonstrated that the ALP activity inherent to FBS influences both the cellular differentiation and the mineralization process, the two most important output parameters in bone tissue engineering. FBS types with differences in inherent ALP activity affected the calcium phosphate deposition in the presence and absence of cells. In media with high ALP activity, the amount of deposited calcium phosphate was higher compared to media with lower ALP activity. Moreover, the ALP activity of the medium affected the ALP activity of the cells; in media with higher ALP activity, the cellular ALP activity was reduced. Our results highlight the importance of considering the components present in FBS in tissue engineering studies. Generally, it is suggested that the development and optimization of specialized serum-free medium for tissue engineering applications should be advanced further.

Acknowledgement

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Chapter 4

Development of serum substitute medium for bone tissue engineering

This chapter is based on:

Sana Ansari, Keita Ito, Sandra Hofmann, Development of serum substitute medium for bone tissue engineering, Journal of Biomedical Materials Research: Part A, Accepted.

Abstract

In tissue engineering, cells are grown often on scaffolds and subjected to chemical/mechanical stimuli. Most such cultures still use fetal bovine serum (FBS) despite its known disadvantages including ethical concerns, safety issues, and variability in composition, which greatly influences the experimental outcomes. To overcome the disadvantages of using FBS, defined serum substitute medium needs to be developed. Development of such medium depends on cell type and application - which makes it impossible to define one universal serum substitute medium for all cells in any application. Here, we developed a serum substitute medium for bone tissue engineering (BTE) in a step-by-step process. Essential components were added to the medium while human bone marrow mesenchymal stromal cells (hBMSCs, osteoblast progenitor cells) were cultured in two-dimensional (2D) and three-dimensional (3D) substrates. In a 3-week culture, the developed serum substitute medium worked equally well as FBS containing medium in term of cell attachment to the substrate, cell survival, osteoblast differentiation, and deposition of extracellular matrix. In the next step, the use of serum substitute medium was evaluated when culturing cells under mechanical loading in the form of shear stress. The outcomes showed that the application of shear stress is essential to improve extracellular matrix formation while using serum substitute medium. The developed serum substitute medium could pave the way in replacing FBS for BTE studies eliminating the use of controversial FBS and providing a better-defined chemical environment for BTE studies.

4.1 Introduction

In vitro tissue engineering approaches include progenitor cells grown on scaffolds under chemical and/or mechanical stimuli [1]. Many of these *in vitro* methods still make use of fetal bovine serum (FBS). FBS contains hormones, growth factors, attachment factors, protease inhibitors, vitamins, proteins, etc., essential for cell growth and maintenance [2], [3]. The exact components of FBS are not known. There have been some reports on certain components within FBS and their concentrations, however, these components/concentrations might differ per FBS batches/brands (Table 4.1) [3], [4]. The variation in composition between different FBS brands and batches impacts the experimental outcomes [5], [6]. For instance, an investigation on the elemental component of FBS showed significant elemental variations in different FBS batches which influenced the protein expression of human umbilical vein endothelial cells *in vitro* [6]. Besides having an undefined and complex composition, FBS holds other disadvantages such as safety and ethical issues and shortage in global supply which makes the use of FBS in culture controversial [2]. To overcome the disadvantages of using FBS in culture, defined serum substitute media with known components need to be developed and replace FBS [7].

Table 4.1 Known components of FBS [3].

Components	**Examples of the components**	
Proteins	Serum protein	Albumin, Globulins, α1-Antitrypsin, α2-Macroglobulin
	Transport proteins	Transferrin, Transcortin, α1-Lipoprotein, β1-Lipoprotein
	Attachment and spreading factors	Fibronectin, Laminin, serum spreading factor
	Enzymes	Lactate dehydrogenase, Alkaline phosphatase, γ-Glutamyl Tranferase, Alanine Aminotransferase
Hormones	Insulin, Glucagon, Corticosteroids, Vasopressin, Thyroid Hormones, Parathyroid Hormone, Growth hormone, Pituitary glandotropic factors, Prostaglandins	
Growth factors	Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Nerve growth factor (NGF), Endothelial cell growth factor (ECGF), Platelet-derived growth factor (PDGF), Insulin-liked growth factors (IGFs), Interleukins, Interferons, Transforming growth factors (TGFs)	
Fatty acids and lipids	Free and protein-bound fatty acids, Triglycerides, Phospholipids, Cholesterol, Ethanolamine, Phosphatidylethanolamine	
Vitamins	Retinol/Retnoic acid (Vitamin A), Vitamin B-Group, Ascorbic acid (Vitamin C), α-Tocopherol (Vitamin E)	
Trace elements	Selenium, Iron, Zinc, Copper, Cobalt, Chromium, Iron, etc.	
Carbohydrates	Glucose, Galactose, Fructose, Mannose, Ribose, Glycolytic Metabolites	
Nonprotein Nitrogens	Urea, Purines/Pyrimidines, Polyamines, Creatinine, Amino acids	

Development of serum substitute medium offers several advantages including 1) avoiding the suffering of animals, 2) reducing variability in culture medium composition, 3) supporting cell survival, growth, and homeostasis in the physiological state of a specific tissue or 4) the possibility to recreate pathophysiological states of a specific tissue to investigate potential treatments. The formulation of serum substitute medium depends on many factors such as the cells (primary cells or cell lines), cell culture conditions (mono-culture or co-culture), cell sources (bone marrow, adipose tissue, etc.), applications (cell-based therapy, food industry, or cell-seeded implants), and species (human, canine, etc.) [8]–[18]. These variations in medium formulations indicate that one universal serum substitute medium might not exist but it should be developed/optimized for each specific cell type and specific application [7]. Many of these serum substitute medium formulations are already commercially available or can be found in few databases [3], [19]. A drawback of commercially available serum substitute media is that their composition is not provided which limits the possibility of studying the influence of soluble factors or drugs on cellular behavior and function.

Bone is a complex multifunctional organ that continuously undergoes a physiological process called bone remodeling to maintain bone strength and mineral homeostasis [20]. This process occurs via balanced activities of its specialized cells, namely, osteoclasts, osteoblasts, and osteocytes [21]. Disturbing the bone remodeling process leads to the development of metabolic bone diseases (e.g. osteoporosis and osteopetrosis) [22]. Bone tissue engineering (BTE) exploits the same tissue engineering principles to develop healthy or pathological human *in vitro* bone models to study bone physiology/pathology or assess drug effects in a pre-clinical setting. FBS has been routinely used to create such *in vitro* models, but to overcome the issues raised by using FBS, a serum substitute medium with known components needs to be formulated for BTE application. The development of a serum substitute medium with known components for BTE has not yet been reported and is the focus of the current study. In order to use the formulated medium in BTE studies, the newly developed serum substitute medium needs to support the attachment of cells to the substrate, the differentiation of hBMSCs towards osteoblasts/osteocytes, and the deposition of extracellular matrix (ECM). We started developing the serum substitute medium in a step-by-step process by adding the essential components to the medium while the cells were cultured in 2D well-plates and later on 3D silk fibroin scaffolds and evaluated their contribution to cell survival, attachment to the substrate, osteoblast/osteocyte differentiation, collagen production and mineral deposition after 3 weeks of culture (Table 4.2) [23]. Next, the potential of the newly developed serum substitute medium in supporting cells under application of mechanical loading was assessed. In brief, hBMSCs were seeded on 3D silk fibroin scaffolds in either FBS containing medium or serum substitute medium and placed inside the spinner flask bioreactors. The spinner flask bioreactor generates wall shear stress through a continuous flow of cell culture medium [24]. Cell attachment to the scaffold, hBMSCs differentiation into osteoblasts/osteocytes, and deposition of mineralized collagenous matrix were investigated after 3 weeks of culture. Such defined serum substitute medium provides the opportunity to study the bone formation process in a controlled environment without the influence of variable components of FBS.

Table 4.2 Serum substitute medium components.

Component	Impact of the components on cells	Reference
Vitronectin	Enhances attachment of cells to the substrate	[25]–[27]
Human Serum Albumin (HSA)	Acts as antioxidant to prevent oxidative damages to cells through binding to metal ions including copper or iron	[28]
	Binds to various compounds including fatty acids, amino acids, hormones, vitamins, and metal ions (such as Zinc), and transport them to the cells	
antibiotic/antimycotic	Controls the growth of bacterial and fungal contamination	[29]
Insulin/transferrin/Selenium	Insulin: enhances glucose and amino acid uptake by cells, intercellular transport, and synthesis of proteins	[15], [30]
	Transferrin: transports iron to cells which is a co-factor for enzymes involved in DNA synthesis and prevent extracellular oxidation	
	Selenium: protects cells from oxidative damages by reducing the production of free radical	
Glutamine	Stable form of glutamine which is an essential amino acid for the synthesis of proteins and nucleic acids	[7], [31]
	Acts as a secondary energy source for metabolism	
Non-essential amino acids	Increases collagen synthesis and stabilizes collagen structure	[32]–[35]
Chemically defined lipids	Serves as structural constitutes of cell membrane, energy stores, and signaling molecules	[7], [36]
Basic fibroblast growth factor	Enhances proliferation of mesenchymal stromal cells and stimulate osteoblast differentiation during early stages	[37]–[39]
Bone morphogenetic protein-2	Stimulates osteogenic differentiation of mesenchymal stromal cells	[37], [40], [41]

4.2 Materials and methods

4.2.1 Materials

Dulbecco's modified eagle medium (DMEM high glucose, 41966 and low glucose, 22320), antibiotic/antimycotic (Anti-Anti, 15240062), non-essential amino acids (NEAA, 11140050), and trypsin-EDTA (0.5%, 2530054), Insulin-Transferrin-Selenium (ITS-G, 41400045), GlutaMAX (35050061), chemically defined lipid concentrate (CDlipid, 11905031) were from Life Technologies (The Netherlands). For cell expansion, FBS (F7524) from Sigma-Aldrich (The Netherlands) and for osteogenic differentiation, FBS (SFBS, Lot. No. 51113) from Bovogen (Australia) were used. Basic fibroblast growth factor (b-FGF, 100-18B) was purchased from Peprotech (UK). Recombinant human bone morphogenetic protein-2 (rhBMP-2, 7510200) was purchased from Medtronic Sofamor Danek (USA). Human serum albumin (HSA, A1653) was purchased from Sigma-Aldrich (The Netherlands).

Unless noted otherwise, all other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich (The Netherlands).

4.2.2 Medium composition

Cells require nutrients such as amino acids, lipids, carbohydrates, vitamins, trace minerals and inorganic salts to grow, proliferate, and differentiate. Basal media such as Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI), and Eagle's Minimum Essential Medium (EMEM) provide cells with many of these nutrients [29]. To develop a serum substitute medium, the first step is basal medium selection. In this study, we have selected DMEM (low glucose Cat. No. 22320) as the basal medium. This basal medium supplemented with 10% FBS has been shown to be suitable in stimulating osteoblast differentiation of human bone marrow mesenchymal stromal cells (hBMSCs) [23], [24], [42]–[44]. DMEM contains a wide variety of amino acids, vitamins, inorganic salts, glucose, and sodium pyruvate (Table 4.3).

Table 4.3 The components of DMEM and their function on cells.

Components	Impact of the components on cells	References
Amino acids	Basic building blocks of proteins and essential for cell proliferation and viability	[45]
Vitamins	Essential for cell growth and proliferation, act as co-factor for enzymes	[7], [46]
Inorganic salts	Maintain the osmotic balance and regulate membrane potential which is necessary to transport ions through cell membrane	[47], [48]
Glucose	Main source of energy for cell metabolism	[29]
Sodium Pyruvate	By-product of glucose consumption in the glycolytic pathway and functions as a source of energy	[49]
HEPES	Increase buffering capacity of medium to maintain the pH of cultures	[50]
Phenol Red	pH indicator	[29]

4.2.2.1 FBS containing medium formulation

FBS containing medium consisted of, 10% FBS (Bovogen), 1% Anti-Anti. To induce osteogenic differentiation of the cells, this medium was supplemented with 50 µg/ml ascorbic-acid-2-phosphate (Sigma-Aldrich, A8960), 100 nM dexamethasone (Sigma-Aldrich, D4902), 10 mM β-glycerophosphate (Sigma-Aldrich, G9422).

4.2.2.2 Serum substitute medium formulation

The serum substitute medium formulation consisted of DMEM (low glucose, 22320) supplemented with components of Table 4.4. To induce osteogenic differentiation of the cells, this medium was supplemented with 50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, 10 mM β-glycerophosphate.

Table 4.4 Concentrations of serum substitute medium components.

Component	Impact of the components on cells	Concentration
HSA	Acts as antioxidant to prevent oxidative damages to cells through binding to metal ions including copper or iron	1% w/v
	Binds to various compounds including fatty acids, amino acids, hormones, vitamins, and metal ions (such as Zinc), and transport them to the cells	
Anti-Anti	Controls the growth of bacterial and fungal contamination	1% v/v
ITS-G	Insulin: enhances glucose and amino acid uptake by cells, intercellular transport, and synthesis of proteins	1% v/v
	Transferrin: transports iron to cells which is a co-factor for enzymes involved in DNA synthesis and prevent extracellular oxidation	
	Selenium: protects cells from oxidative damages by reducing the production of free radical	
GlutaMax	Stable form of glutamine which is an essential amino acid for the synthesis of proteins and nucleic acids	1% v/v
	Acts as a secondary energy source for metabolism	
NEAA	Increases collagen synthesis and stabilizes collagen structure	1% v/v
CDlipid	Serves as structural constitutes of cell membrane, energy stores, and signaling molecules	0.1% v/v
b-FGF	Enhances proliferation of mesenchymal stromal cells and stimulate osteoblast differentiation during early stages	10 ng/mL
rhBMP-2	Stimulates osteogenic differentiation of mesenchymal stromal cells	100 ng/mL

4.2.3 Silk fibroin scaffold fabrication

Silk fibroin scaffolds were prepared by cutting and cleaning 3.2 grams of Bombyx mori L. silkworm cocoons (Tajima Shoki., Ltd. Japan) and then degumming them by boiling in 1.5 L ultra-pure water (UPW) containing 0.02 M Na_2CO_3 (Sigma-Aldrich, S7795) for 1 hour, whereafter it was rinsed with 10 L cold UPW to extract sericin. Dried purified silk fibroin was dissolved in 9 M lithium bromide (LiBr, Acros organics, 199870025) solution in UPW at 55°C for 1 hour and dialyzed against UPW for 36 hours using SnakeSkin Dialysis tubing (molecular weight cut-off: 3.5 kDa, Thermo Fisher Scientific, The Netherlands). The silk fibroin solution was frozen at -80°C for at least 2 hours and lyophilized (Freezone 2.5, Labconco, USA) for 4 days. Lyophilized silk fibroin (1.7 grams) was then dissolved in 10 mL 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, Fluorochem, 003409) at room temperature for 5 hours resulting in a 17% (w/v) solution. One mL of silk-HFIP solution was added to a Teflon container containing 2.5 grams NaCl with a granule size between 250-300 µm. After 3 hours, HFIP was allowed to evaporate for 4 days. Silk fibroin-NaCl blocks were immersed in 90% (v/v) methanol (Merck, The Netherlands) in UPW for 30 minutes to induce the protein conformational transition to β-sheets and let dry overnight [51]. Scaffolds were cut into disks of 3 mm height with an Accutom-5 (Struer, Type 04946133, Ser. No. 4945193), followed by immersion in UPW for 2 days to extract NaCl. Disc-shaped scaffolds were made with a 5 mm diameter biopsy punch (KAI medical, Japan) and autoclaved in phosphate buffered saline (PBS, Sigma-Aldrich, P4417) at 121°C for 20 minutes.

4.2.4 Cell isolation, expansion, and subsequent seeding

hBMSCs were isolated from 2 donors of human bone marrow (Lonza, USA) and characterized as previously described [52]. Passage 4 hBMSCs were expanded (2500 cell/cm^2) in expansion medium (DMEM high glucose containing 10% FBS Sigma, 1% Anti-Anti, 1% NEAA, and 1 ng/ml b-FGF) for 9 days, the medium was replaced 3 times per week. At day 9, cells were 80% confluent and trypsinized and proceed as follow.

4.2.4.1 Cells seeded in well-plates (2D set-up)

The wells of a 48- well-plate (Greiner bio-one, CELLSTAR, 677-180) were coated with 5 µg/ml vitronectin (Peprotech, 140-09) diluted in PBS. Briefly, 100 µL of vitronectin solution was added to each well and incubated in an incubator (37°C, 5% CO_2) for 2 hours and then at 4°C for 16 hours. The following day, the vitronectin solution was aspirated, the wells were rinsed 3 times with PBS, and the well-plate was pre-warmed in an incubator before seeding cells in wells. 2500 cells were seeded per well resulting in 2500 cells/cm^2, cultured in either **FBS containing medium** or **Serum substitute medium**, both supplemented with osteogenic differentiation factors (50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, 10 mM β- glycerophosphate). The medium was refreshed 3 days a week for 3 weeks.

4.2.4.1.1 Cell attachment to the well-plate

To determine the amount of DNA as a measure of the number of cells per well after 3 weeks of culture, DNA content was measured by Qubit dsDNA HS assay kit (Life Technologies, Q32851). Cell-seeded wells (n=3 per group) were rinsed with PBS and incubated in 500 µL of cell lysis solution containing 0.2% (v/v) Triton-x-100 (Merck, 1.08603.1000) in 5 mM $MgCl_2$ (Sigma-Aldrich, M2393) for 30 minutes. Next, the content of the well was transferred into an Eppendorf tube and incubated with 500 µL digestion buffer (500 mM phosphate buffer, 5 mM L-cystein (Sigma-Aldrich, C1276), 5 mM EDTA (Sigma-Aldrich, 1.08421.1000)) containing 140 µg/ml papain (Sigma-Aldrich, P-5306) at 60°C for 16 hours in a water bath shaker (300 rpm). Next, samples were centrifuged at 3000g for 10 minutes and the DNA concentration was measured. Briefly, 10 µL of samples and standards were mixed thoroughly with DNA buffer containing DNA reagent (1:200) and the DNA concentration was measured with Qubit 2.0 Fluorometer.

4.2.4.1.2 Immunohistochemistry

After 3 weeks of culture, cell-seeded wells were rinsed with PBS and fixed with 10% neutral buffered formalin for 30 minutes at 4°C. Then, the wells were rinsed 3 times with PBS and covered with 100 µL 0.5% (v/v) Triton-X 100 (Merck, 1.08603.1000) in PBS for 5 minutes to permeabilize cells. Then, the cells were rinsed with PBS and incubated with 5% (v/v) normal donkey serum and 1% (w/v) BSA (Roche, 10.7350.86001) in PBS for 1 hour at room temperature to block the non-specific antibody binding. Wells were then incubated for 16 hours at 4°C with the primary antibody (Table 4.5). The wells were washed with PBS 3 times and incubated for 1 hour with secondary antibody solution (Table 4.5). This was followed by 3 times rinsing of the wells with PBS, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI (Sigma-Aldrich, D9542), diluted to 0.1 µg/ml in PBS) for 15 minutes

Wells were rinsed with PBS 3 times and then covered with PBS. The expression of proteins was visualized with a Leica TCS SP5X microscope and images were processed with ImageJ (version 1.53f51). Figures were chosen to be representative images per group for all the samples assessed.

Table 4.5 Overview of antibodies used for immunohistochemistry.

Primary antibody	**Source**	**Cat. No**	**Label**	**Species**	**Dilution**
RUNX2	Abcam	Ab23981	-	Rabbit	1:500
Osteopontin	Thermo Fisher	14-9096-82	-	Mouse	1:200
DMP-1	Sigma-Aldrich	HPA037465	-	Rabbit	1:200
Collagen type 1	Santa Crus	SC-59773	-	Mouse	1:200
Secondary antibody	**Source**	**Cat. No**	**Label**	**Species**	**Dilution**
Anti-rabbit IgG (H+L)	Jackson Immumo Research	711-605-152	A647	Donkey	1:200
Anti-mouse IgG (H+L)	Jackson Immumo Research	715-545-150	A488	Donkey	1:200

4.2.4.1.3 Alizarin Red staining

After 3 weeks of culture, cell-seeded wells were rinsed with PBS and fixed with 10% neutral buffered formalin for 30 minutes at 4°C. Then, the wells were rinsed 3 times with PBS and covered with 500 µL 2% Alizarin Red solution (Sigma-Aldrich, 05600) diluted in UPW for 15 minutes. Next, the wells were rinsed with UPW and air dried until imaged with a Zeiss Axio Observer Z1. Figures show representative images per group of all the sample assessed.

4.2.4.1.4 Measurement of deposited calcium in the well-plate

After 3 weeks of culture, cell-seeded wells (n=3 per group) were rinsed with PBS and incubated in 500 µL of 5% trichloroacetic acid (TCA, Sigma-Aldrich, T6399) in UPW for 30 minutes. Next, the content of the well was transferred into an Eppendorf tube and incubated for 48 hours at room temperature. Then, the solids were separated by centrifugation (3000g, 10 min). Five µL of the supernatant were mixed with 95 µL of calcium working solution (Stanbio Calcium (CPC) LiquiColor® Test, Stanbio Laboratories) and incubated at room temperature for at least 1 minute. In this assay, the calcium ion concentration is measured by the chromogenic complex formed between calcium ions and o-cresolphthalein. Absorbance at 550 nm was measured and calcium concentration was calculated by comparison to standards of known calcium chloride concentrations.

4.2.4.2 Cells seeded on 3D scaffolds (3D set-up)

Scaffolds were pre-wetted with medium containing FBS or serum substitute which was supplemented with 5 µg/ml vitronectin for 1 hour at 37 °C. Next, the media were removed, and scaffolds were seeded with $1*10^6$ cells each in 20 µL FBS containing medium or serum substitute medium supplemented with 5 µg/ml vitronectin and incubated in an incubator (37°C, 5% CO_2) for 16 hours. The next day, cell-

seeded scaffolds (n=4 per group) were individually placed in wells of a 48-well-plate. Each well was filled with 1 mL of either **FBS containing medium** or **Serum substitute medium**, both supplemented with osteogenic differentiation factors (50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, 10 mM β-glycerophosphate). The medium was refreshed 3 days a week for 3 weeks.

4.2.4.2.1 Live/dead assay

To indicate the amount of living and dead cells at the end of the culture, a live/dead assay was performed. This assay is a two-color fluorescence cell viability assay that is based on the simultaneous determination of living and dead cells. To perform the assay at the end of the culture, scaffolds were rinsed with PBS and incubated in 500 µL of 2 µM Calcein AM (Sigma-Aldrich, 17783) and 4 µM propidium iodide (Invitrogen, P3566) solution prepared in PBS at 37°C, in dark. After 30 minutes, the staining solution was aspirated, and the scaffolds rinsed with PBS. The scaffolds were then imaged with a TCS SP5X confocal microscope (Leica). The number of live and dead cells was counted using ImageJ (version 1.53f51). From each group, 5 images were used, the channels were separated, and the threshold of each channel was adjusted to 20. Then the images were converted to mask and the analyze tool was used to calculate the percentage of cells from each channel.

4.2.4.2.2 Cell attachment to the scaffolds and cell number

To determine the amount of DNA as a measure of the number of cells per scaffold, assuming all cells of the same type have the same amount of DNA, after 24 hours and 3 weeks of culture, DNA content was measured by Qubit dsDNA HS assay kit (Life Technologies, Q32851). Scaffolds (n=3 per group) were washed with PBS and each disintegrated in 600 µL of digestion buffer (500 mM phosphate buffer, 5 mM L-cystein, 5 mM EDTA) containing 140 µg/ml papain using steel balls and a Mini-BeadBeater™ (Biospec, USA). Samples were incubated at 60°C for 16 hours in a water bath shaker (300 rpm). Next, the DNA concentration of the sample was measured as explained in the section 4.2.4.1.1.

4.2.4.2.3 Immunohistochemistry and histology

Sample preparation

After 3 weeks of culture, scaffolds were cut in half, washed with PBS and fixed with 10% neutral buffered formalin overnight at 4°C. Then, the scaffolds were dehydrated in graded ethanol solutions and embedded in paraffin, cut into 10 µm thick sections and mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific, The Netherlands). The sections were dewaxed with xylene (VWR, 1330.20.7) (2 times each time for 5 minutes) and rehydrated to water through ethanol solutions (3 times in 100% ethanol each time for 2 minutes, 1 time in 96% ethanol for 2 minutes, 1 time in 70% ethanol for 2 minutes, and finally 2 minutes in UPW). Then, the sections were ready for immunohistochemical and histological staining and Raman spectroscopy.

Immunohistochemistry

Sections were incubated in primary and secondary antibodies as explained in section 4.2.4.1.2. After staining the nuclei with DAPI for 15 minutes, sections were rinsed with PBS 3 times and then mounted

on microscope glass slides with Mowiol (Sigma-Aldrich, 81381). The expression of proteins was visualized with a Leica TCS SP5X microscope and images were processed with ImageJ (version 1.53f51). Figures were chosen to be representative images per group for all the samples assessed.

Alizarin Red staining

To identify mineral deposition, sections were stained with 2% Alizarin Red solution diluted in UPW for 15 minutes. Then, the sections were washed with UPW and dehydrated with acetone (Boom, 76050006) and acetone-xylene (1:1) solution each for 30 seconds. Next, the sections were cleared in xylene for 5 minutes and mounted in Entellan (Sigma-Aldrich, 1.07960.0500). The samples were imaged with a Zeiss Axio Observer Z1.

Picro-Sirius Red staining

To determine collagen production, sections were stained with 0.1% Picro-Sirius Red (Direct red 80, Sigma-Aldrich, CI35872) for 1 hour. Then, the sections were rinsed in 0.1% acidified water and 0.5% acidified water (Acetic acid, Merck, 1.00056.2500) each for 1 minutes. Next, the sections were dehydrated in 70% ethanol for 1 minute, 95% ethanol for 1 minute, and 3 times in 100% ethanol each for 1 minute, and 2 times in xylene for 5 minutes each. Finally, the sections were mounted in Entellan. The samples were imaged with a Zeiss Axio Observer Z1.

Hematoxylin and Eosin (H&E)

To localize the cells within the scaffolds, sections were stained with Mayer's hematoxylin solution (Sigma-Aldrich, MHS16) for 10 minutes. Then, the sections were rinsed in 0.1% acidified water for 1 minute following 5 minutes washing in slow running tap water. Next, the samples were stained in aqueous eosin Y solution (Sigma, HT110-2-16) for 3 minutes followed by washing in slow running tap water for 1 minute. The sections were dehydrated through 30 seconds in 70% ethanol, 30 seconds in 96% ethanol, 3 times in 100% ethanol each for 30 seconds, and 2 times in xylene for 3 minutes. Finally, the sections were mounted in Entellan. The samples were imaged with a Zeiss Axio Observer Z1.

4.2.4.2.4 Measurement of deposited calcium on cell-seeded scaffolds

After 3 weeks of culture, scaffolds (n=6 per group) were washed with PBS, and each was disintegrated in 500 µl of 5% trichloroacetic acid (TCA) in UPW using steel balls and a Mini-BeadBeater™ (Biospec, USA). After 48 hours of incubation at room temperature, the solids were separated by centrifugation (3000g, 10 min). The calcium concentration was measured as explained in section 4.2.4.1.4.

4.2.4.2.5 Raman spectroscopy

Raman measurements were performed using Alpha 300R confocal Raman microscope (WiTec, Germany) on samples that were prepared as described in section 2-4-2-3. Raman imaging of surface areas of 30*30 μm^2 with a resolution of 1 µm per spectrum of each sample was conducted using a 532 nm excitation laser with a laser power of 10 mW, incubation time of 2s per spectrum, and 50x (NA 0.55) objective. Focus was acquired within the area of the sample using topography correction with manual learning of a 5x5 surface. One plot was cropped due to wrong focus into an area of 30 by 20 mm. Grating was set at 1200 mm^{-1}. All acquired spectra were processed using WiTec Project FIVE

5.1.8.64 software (Witec, Ulm). Before analysis, cosmic rays were removed from data with filter size 2 and dynamic factors 6 and background was subtracted with shape size 500. Then, automated True Component Analysis (TCA) was performed to map the location of mineral, collagen, and non-mineralized scaffold. The components identified with TCA were then used to graph the spectrum of minerals and collagen. Mineral and collagen peaks were also found within the samples using a filter on the maximum at peaks 961 +/-20, and 1666 +/- 30.

4.2.4.3 Cells cultured under dynamic condition (Dynamic culture)

The cells were seeded on 3D scaffolds as explained in section 4.2.4.2. The day after cell incubation with vitronectin solution, cell-seeded scaffolds (n=4 per group) were transferred to spinner flask bioreactors [24]. Each bioreactor contained a magnetic stir bar and was placed on a magnetic stirrer (RTv5, IKA, Germany) at 300 rpm in an incubator (37 °C, 5% CO_2) [24]. Each bioreactor was filled with 5 mL either **FBS containing medium** or **Serum substitute medium**, both supplemented with osteogenic differentiation factors (50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, 10 mM β-glycerophosphate). The medium was refreshed 3 days a week for 3 weeks. After 3 weeks of culture, DNA content of scaffolds and deposited calcium were measured and immunohistochemistry, histology, and Raman spectroscopy were done on the samples the same as explained in section 4.2.4.2.

4.2.5 Statistics

GraphPad Prism 9.0.2 (GraphPad Software, La Jolla, CA, USA) was used to perform statistical analysis and to prepare the graphs. To test for differences in DNA and calcium content in 2D set-up (Figure 4.1A and D, respectively), DNA content in 3D set-ups after 24 hours and 3 weeks (Figure 4.2A and F, respectively), percentage of live and dead cells (Figure 4.2I), calcium content in 3D set-up (Figure 4.4E), DNA content under dynamic condition (Figure 4.5B), and calcium content under dynamic condition (Figure 4.7E), an unpaired Mann−Whitney test was performed. Data of DNA content under dynamic condition after 24 hours (Figure 4.5A) was tested for differences with the non-parametric Kruskal-Wallis with Dunn's post hoc tests. Differences between groups were considered statistically significant at a level of $p<0.05$.

4.3 Results

4.3.1 Serum substitute medium maintained hBMSCs attachment and supported osteoblastic differentiation in a 2D set-up

Cell attachment to the substrate

For tissue engineering purposes, anchorage dependent cells should be able to attach to a substrate. FBS has adhesion-promoting properties due to presence of proteins such as fibronectin and vitronectin [26], [27]. The serum substitute medium should enable cells to maintain their attachment to the substrate. For the serum substitute medium, vitronectin was chosen as the component to promote cell attachment. Without the vitronectin coating, cells did not attach to the wells (data not

shown). The wells of a well-plate were coated with vitronectin, and the cells were seeded in the wells. The maintenance of cell attachment to the surface of well-plates was investigated through measurement of the DNA content of cells attached to the wells. The DNA content of the cells cultured in serum substitute medium showed that the cells maintained their attachment to the surface of the well-plate for 3 weeks (Figure 4.1A).

hBMSCs differentiation towards osteoblasts/osteocytes

After 3 weeks of culturing hBMSCs in either FBS containing medium or serum substitute medium supplemented with osteogenic differentiation factors, the expression of runt-related transcription factor-2 (RUNX-2) and collagen type 1 as early osteoblast specific markers, osteopontin as a late osteoblast specific protein, and dentin matrix protein-1 (DMP-1) as early osteocyte specific marker was investigated [53], [54]. The cells expressed RUNX-2 and osteopontin after 3 weeks of culture in FBS containing medium (Figure 4.1B) which confirmed the differentiation of hBMSCs towards osteoblasts. As in FBS containing medium, cells expressed RUNX-2 and osteopontin when cultured in serum substitute medium (Figure 4.1C). The expression of collagen type 1 by cells cultured in FBS containing medium was limited, while cells cultured in serum substitute medium significantly expressed collagen type 1 (Figure 4.1B and C). Interestingly, the cells cultured in serum substitute medium formed nodules where collagen type 1 and DMP-1 were highly expressed, while DMP-1 expression was not detected in FBS containing medium cultures.

Extracellular matrix deposition

After 3 weeks of culture, the calcium content of wells was measured. Calcium was deposited in both FBS containing medium and serum substitute medium. The calcium content of the cells cultured in FBS containing medium was always higher compared to serum substitute medium, even though statistical analysis did not show any significant differences (Figure 4.1D). The histological staining of the wells showed that calcium was deposited homogenously over the well in FBS containing medium group, while in serum substitute medium, large mineral nodules were formed on few spots over the well (Figure 4.1E and F). It might be due to not homogenously distributed cells over the well-plate in serum substitute medium, and it might be possible that more ECM could form if the cells have been confluent. Nevertheless, formulated serum substitute medium preserved the attachment of hBMSCs to the surface of the well-plate, stimulated hBMSCs differentiation towards osteoblasts/early osteocytes, and supported formation of collagen and mineral nodules.

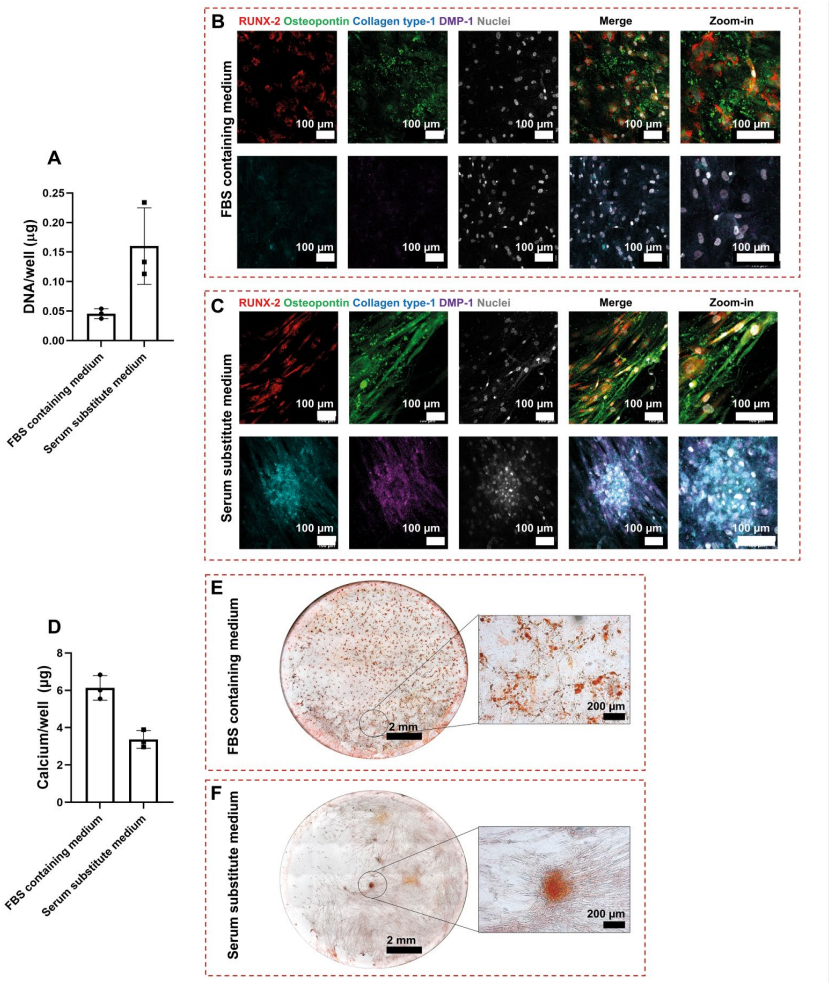

Figure 4.1 The serum substitute medium supported maintenance of cell attachment to the surface of the well-plate (A). hBMSCs were differentiated towards osteoblasts in FBS containing medium as shown by expression of RUNX-2, collagen type 1, and osteopontin (B). DMP-1 as early osteocyte marker was not expressed by cells cultured in FBS containing medium (B). The serum substitute medium stimulated osteoblast and early osteocyte differentiation of hBMSCs after 3 weeks (C). Calcium was deposited in both FBS containing medium and serum substitute medium. The calcium content in FBS containing medium was higher than in serum substitute medium, even though statistical analysis did not show any significant differences (D). Calcium phosphate deposits were homogenously distributed all over the plate in FBS containing medium (E) while only few mineral nodules were formed in serum substitute medium (F). Mann−Whitney tests did not show any significant differences between FBS containing medium and serum substitute medium in figures A and D. Merge stands for the overlay of first 3 channels.

4.3.2 Serum substitute medium supported hBMSCs attachment and osteoblastic differentiation in a 3D set-up

Cell attachment to the substrate

Upscaling to larger 3D BTE constructs requires the use of biomaterial scaffolds that provide a 3D environment to the cells. Serum substitute medium should also be able to promote the attachment of anchorage dependent cells to the 3D scaffolds. In analogy to the 2D study, vitronectin was used as a component to support the attachment of cells to the 3D silk fibroin scaffolds (Figure SI 4.1). The attachment of cells to the silk fibroin scaffolds incubated overnight in either FBS containing medium, or serum substitute medium was investigated through measuring the amount of DNA of cells attached to the scaffolds. The DNA content of cells attached to the scaffolds when cultured in serum substitute medium containing 5 µg/ml vitronectin showed no significant differences compared to cultured cells in FBS containing medium (Figure 4.2A). The Hematoxylin and Eosin (H&E) staining showed the location of seeded cells within the scaffold. In both media, the cells were distributed over the entire scaffold and located within the pores, spanning the void space (Figure 4.2B-E). These results indicated that the serum substitute medium is able to support cell attachment to the silk fibroin scaffolds. The serum substitute medium also supported the maintenance of cell attachment to the silk fibroin scaffolds in serum substitute medium (Figure 4.2F) which were shown to be present between the pores of the scaffolds (Figure 4.2G and H). The live/dead assay indicated that on average more than 80% of cells were alive in both FBS containing medium and serum substitute medium while on average less than 20% of cells were dead in both groups after 3 weeks of culture (Figure 4.2I, J, and K). However, it should be mentioned that there is a possibility that dead cells have been washed away during staining process and the number of the living cells might be overestimated.

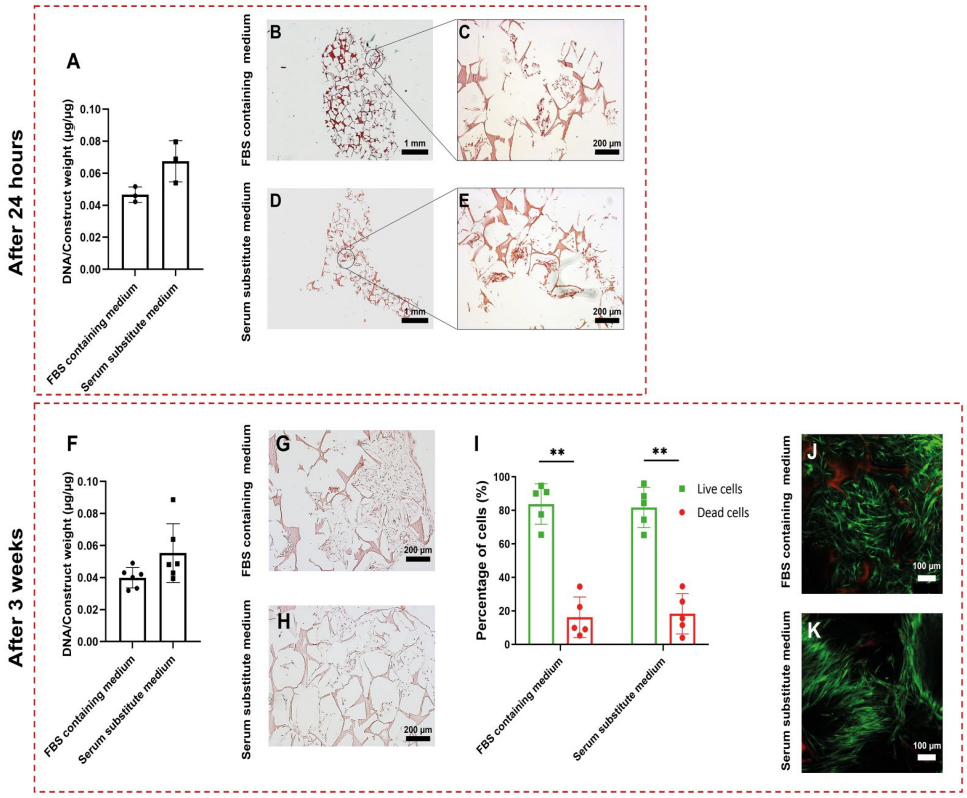

*Figure 4.2 The serum substitute medium supported cells to attach to silk fibroin scaffold: (A) The DNA content of scaffolds after 24 hours of incubation of cells in FBS containing medium or serum substitute medium containing 5 µg/ml vitronectin showed an equal DNA content in both media. H&E staining showed that the cells were distributed all over the scaffold volume and located between the pores of the scaffold in both FBS containing medium (B-C) and serum substitute medium (D-E). The DNA content of attached cells to the scaffolds was equal in FBS containing medium and serum substitute medium after 3 weeks of culture (F). H&E staining showed that the cells were located between the pores and attached to the silk fibroin scaffolds in in both media equally (G and H). The cells survived in both media equally after 3 weeks of culture (I, J and K). **p<0.01. Mann-Whitney tests did not show any significant differences in figures A and F. Significant differences were detected in figure I using Mann-Whitney tests between either FBS containing medium or serum substitute medium groups.*

hBMSCs differentiation towards osteoblasts/osteocytes

Cells cultured in the serum substitute medium expressed RUNX-2 and osteopontin similarly as in FBS containing medium (figure 4.3A and B). The extracellular matrix protein collagen type 1 was expressed in both medium types. DMP-1 as an early osteocyte marker was also expressed within the nuclei and cells in FBS containing medium and serum substitute medium.

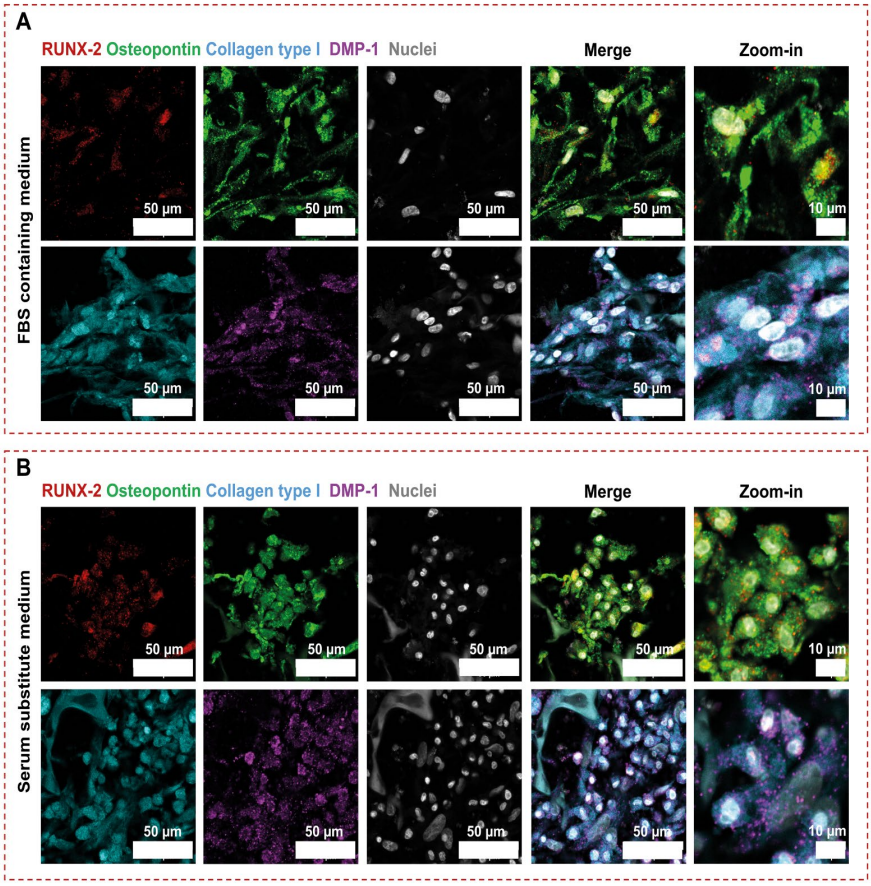

Figure 4.3 Osteoblast markers expressed by cells cultivated in either medium type. Cells expressed both the early osteoblast differentiation marker RUNX-2 and osteoblast marker osteopontin when cultured in FBS containing medium (A) or in serum substitute medium (B). Collagen, a protein expressed by osteoblasts, was detected in both cultures. DMP-1, an osteocyte-specific protein, was also expressed by cells cultured in FBS containing medium and serum substitute medium. Merge stands for the overlay of first 3 channels.

Extracellular matrix deposition

In BTE studies, the medium needs to be able to stimulate cells to express collagen and deposit mineral (mainly carbonated hydroxyapatite), as mineralized collagen forms the basis of bone tissue. Picro-Sirius Red staining of samples confirmed the production of collagen by cells cultured in serum substitute medium (Figure 4.4C) similar as in FBS containing medium (Figure 4.4A). The produced collagen in serum substitute medium was located in the voids of the pores of the scaffolds as in FBS containing medium. Calcium deposits were detected on cell-seeded scaffolds cultured in FBS containing medium (Figure 4.4E). Alizarin Red staining showed that these calcium deposits were

located on the silk fibroin scaffolds (Figure 4.4B, yellow asterisk) as well as in the ECM produced by cells close to the scaffolds (Figure 4.4B, black arrow). However, cells cultured in serum substitute medium did not deposit calcium phosphate on the scaffolds or within the produced ECM (Figure 4.4D). Raman spectroscopy was performed on the cell-seeded scaffolds cultured in FBS containing medium and serum substitute medium. The presence of peaks at approximately 962 cm^{-1}, 1299 cm^{-1}, and 1441 cm^{-1} representative for phosphate, amide III, and methylated side chains, respectively [55], in FBS containing medium group confirmed the deposited mineralized collagen in this group (Figure 4.4F). The phosphate and amide peaks were missing in the Raman spectrum of serum substitute medium group (Figure 4.4F).

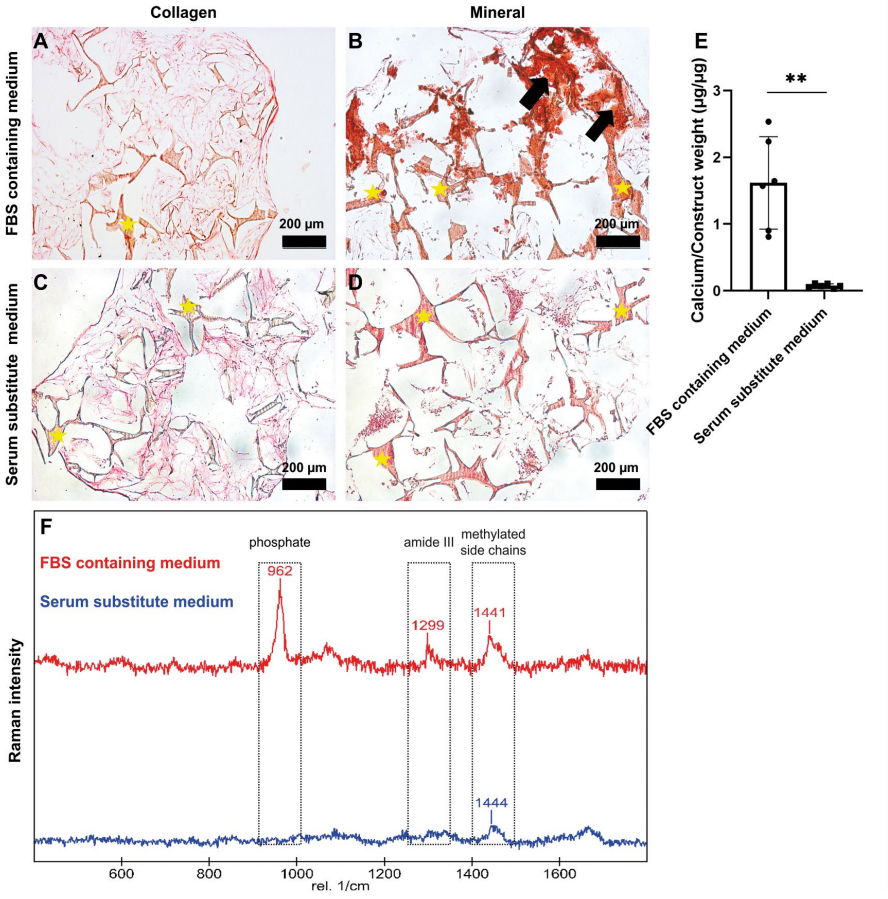

*Figure 4.4 Cells cultured in FBS containing medium produced collagen and showed mineral deposition on scaffolds/ECM (A and B). The cells cultured in the serum substitute medium produced collagen as shown by Picro-Sirius Red staining, but no mineral was detected on the scaffolds/ECM (C and D). The calcium assay confirmed the alizarin red staining on the minerals (E). The Raman spectroscopy showed no mineralized collagen formation in serum substitute medium (F). **p<0.01. Significant differences were detected in figure E using Mann-Whitney tests between FBS containing medium and serum substitute medium groups.*

4.3.2 Serum substitute medium supported hBMSCs attachment and osteoblastic differentiation in dynamic conditions

Cell attachment to the substrate

In order to make sure that cells can form a tissue and investigate the cellular behavior in dynamic conditions, cells need to maintain their attachment to the substrate when they are exposed to mechanical loading. Due to the adhesion-promoting properties of FBS, cells can maintain their attachment to the scaffolds when cultured in FBS containing medium. Serum substitute medium also needs to be able to preserve the attachment of cells to the scaffolds when subjected to mechanical loading such as shear stress. To investigate whether shear stress could result in detachment of cells from the scaffolds in the serum substitute medium, cell-seeded scaffolds were kept in either static or dynamic condition for 24 hours after cell seeding. The DNA content of cell-seeded scaffolds did not change after 24 hours of applied shear stress (Figure 4.5A). The DNA content of cell-seeded scaffolds was also measured after 3 weeks of culture in either FBS containing medium or serum substitute medium under dynamic condition. The results indicated that the shear stress applied in the dynamic condition did not lift off the cells in serum substitute medium, however the DNA content of cell-seeded scaffolds cultured in serum substitute medium was significantly higher than DNA content of cell-seeded scaffolds cultured in FBS containing medium (Figure 4.5B). H&E staining confirmed the presence of the cells in the scaffolds (Figure 4.5C and D). Moreover, it showed that the cells were well distributed in the scaffolds in both FBS containing medium (Figure 4.5C) and in serum substitute medium (Figure 4.5D).

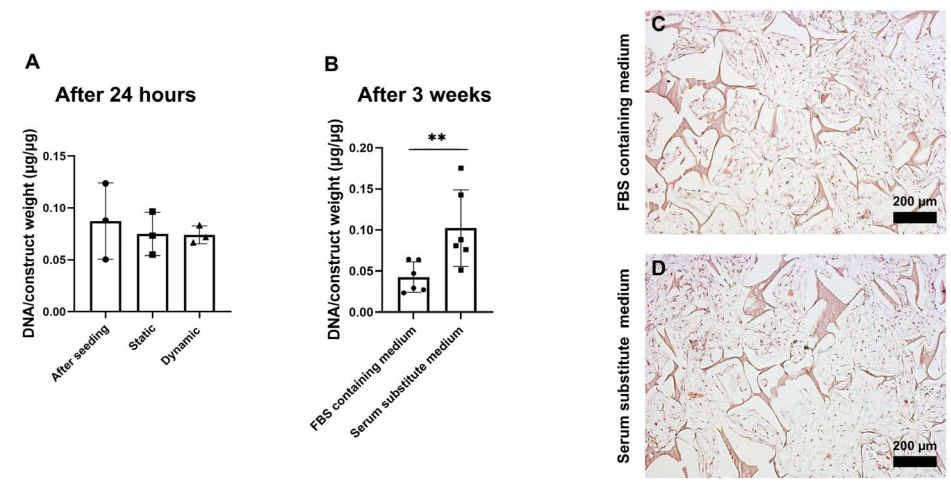

*Figure 4.5 Applied shear stress did not result in cell removal from the scaffolds in serum substitute medium after 24 hours (A). DNA content of scaffolds cultured in FBS containing medium and serum substitute medium for 3 weeks indicated that the cells maintained their attachment to the scaffolds under the dynamic condition (**p<0.01) (B). The H&E staining showed that the cells were distributed between the pores of the scaffold in FBS containing*

*medium (C) and in serum substitute medium (D). **p<0.01. Significant differences were detected in figure B using Mann-Whitney tests between FBS containing medium and serum substitute medium groups*

hBMSCs differentiation towards osteoblasts/osteocytes

hBMSCs were cultured under continuous shear stress in both media supplemented with osteogenic differentiation factors for 3 weeks. Cells cultured in FBS containing medium expressed RUNX-2, osteopontin, collagen type 1, and DMP-1 after 3 weeks of culture which indicated the differentiation of hBMSCs towards osteoblasts and early osteocytes (Figure 4.6A). hBMSCs also differentiated towards osteoblasts in serum substitute medium as shown by expression of RUNX-2 and osteopontin (Figure 4.6B). Expression of DMP-1 by cells embedded within the collagenous matrix indicated early osteocyte differentiation after 3 weeks of culture in serum substitute medium under mechanical loading (Figure 4.6B).

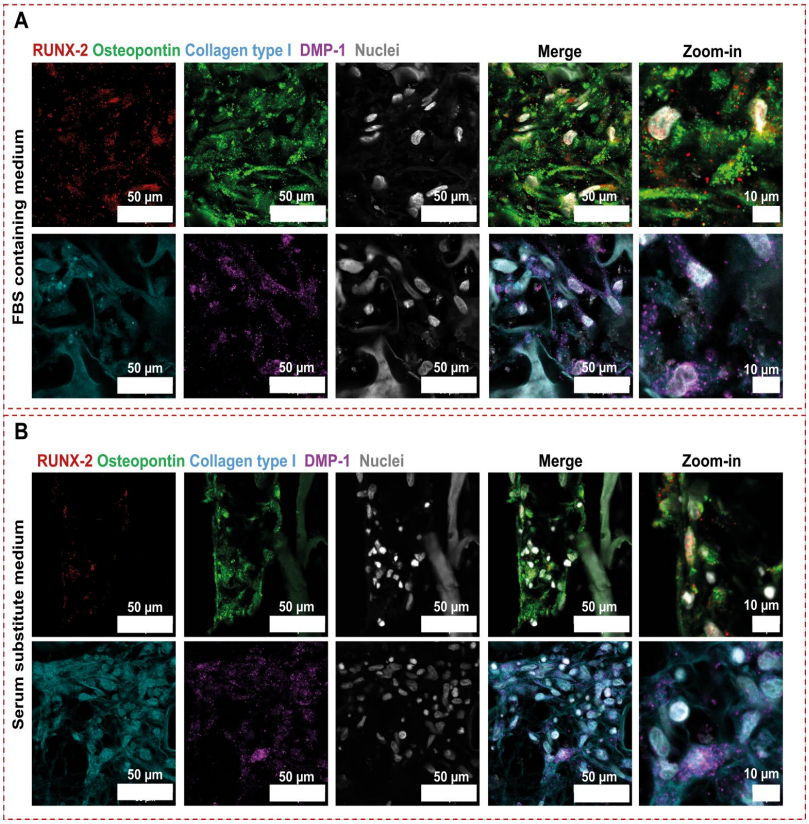

Figure 4.6 Osteoblast/osteocyte markers expressed by cells cultivated in either medium type under continuous shear stress. Cells expressed both the early differentiation marker RUNX-2 and osteoblast marker osteopontin when cultured in FBS containing medium (A) or in serum substitute medium (B). Collagen type 1, a protein expressed by osteoblasts, was detected in abundance in both cultures. DMP-1, an osteocyte-specific protein was detected in both cultures. Merge stands for the overlay of first 3 channels.

Extracellular matrix deposition

After 3 weeks of culturing hBMSCs under dynamic conditions in either medium, collagen was expressed as confirmed by histological images (Figure 4.7A and C). The collagen was laid down between the pores of the scaffolds in both media conditions. The expressed collagen by cells cultured in serum substitute medium seemed denser between the scaffold pores compared to FBS containing medium. Under dynamic conditions, the amount of deposited calcium in serum substitute medium was equal to the FBS containing medium group (Figure 4.7E). The deposited calcium on the scaffolds and/or ECM was enhanced in the serum substitute medium group under dynamic condition compared to 3D culture in static condition where no mineral was formed (Figure 4.4E and 4.7E). Alizarin red staining indicated that in the FBS containing medium group, minerals were deposited on/within the silk fibroin scaffold (yellow asterisk) and ECM (black arrow), while, in the serum substitute medium, the minerals were found between the pores of the scaffolds (Figure 4.7B and D) where collagen was also deposited (Figure 4.7C). Raman spectroscopy was performed on the cell-seeded scaffolds cultured in FBS containing medium and serum substitute medium under dynamic conditions. The presence of the peaks of phosphate (approximately 960 cm^{-1}), amide III (1240-1320 cm^{-1}), methylene side chains (CH_2 at 1450 cm^{-1}), and amide I (1616-1720 cm^{-1}) demonstrated the formation of mineralized collagenous matrix in serum substitute medium (Figure 4.7F).

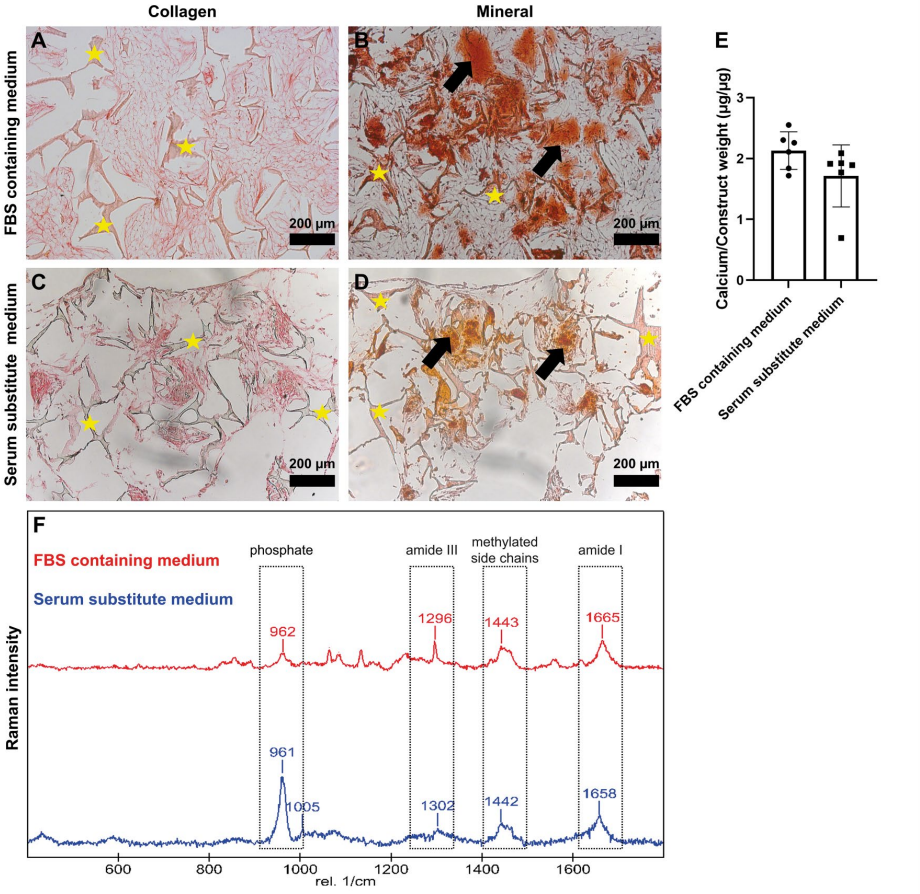

Figure 4.7 Cells cultured in FBS containing medium and serum substitute medium produced collagen and showed mineral deposition on scaffolds/ECM as detected by Picro-Sirius Red staining and Alizarin Red staining, respectively (A-D) under dynamic condition. Equal amount of calcium was detected in both FBS containing medium and serum substitute medium (E). The Raman spectroscopy showed mineralized collagen formation in both groups (F). Mann-Whitney tests did not show any significant differences in figure E.

4.4 Discussion

FBS is still routinely used as a cell culture supplements in many research lines despite its known disadvantages. The undefined, complex, and inconsistent composition of FBS has been shown to greatly affect the experimental outcomes and to contribute towards low reproducibility of data [2], [56]. Thus, FBS needs to be replaced, ideally by a chemically defined medium. Due to the differences in cells' requirements in growth, the defined media need to be optimized for each specific cell type and application. In the present study, we developed a serum substitute medium in a step-by-step approach for BTE studies. The potential of this medium in hBMSCs attachment to the substrate, their differentiation towards osteoblasts/osteocytes, and production of a mineralized collagenous matrix in 2D and 3D substrates and under the application of mechanical loading was evaluated.

In the present study, the developed serum substitute medium stimulated hBMSCs to differentiate into osteoblasts as shown by expression of RUNX-2 and osteopontin when the cells where cultured in 2D well-plates and on 3D silk fibroin scaffolds with or without the application of mechanical loading. RUNX-2 is a transcription factor which is expressed at early stages of osteoblast differentiation and regulates the expression of several specific genes related to osteoblasts [57]. The ECM protein osteopontin is expressed by mature osteoblasts and it is believed that it induces mineralization of collagenous fibrils through crystal growth inhabitation in ECM [58], [59]. The components of serum substitute medium also supported cells to differentiate further into early osteocytes shown by expression of DMP-1. FBS has been shown to be a cocktail of growth factors including b-FGF and BMPs which greatly influence cell growth and differentiation [56], [60]. b-FGF and BMP-2 were chosen as two specific growth factors to stimulate osteoblast differentiation of hBMSCs. b-FGF is a potent mitogen that enhances MSCs proliferation and maintains their differentiation potential [61]. BMP-2 is a member of the transforming growth factor-β superfamily that is well-known for its potential in osteoblast differentiation of MSCs and bone formation *in vitro* and *in vivo* [62]. Without any growth factors, hBMSCs could not differentiate towards osteoblasts (SI 4.2C) and the addition of b-FGF to the serum substitute medium seemed to be essential to induce osteogenic differentiation of hBMSCs (SI 4.2D). The presence of BMP-2 alone in serum substitute medium negatively influenced the number of cells (SI 4.2E), while the addition of BMP-2 to the serum substitute containing b-FGF did not show any significant influence on osteogenic differentiation of hBMSCs (SI 4.2F). Previous studies on the influence of combination of b-FGF and BMP-2 on osteoblast differentiation of MSCs and bone formation showed that b-FGF had a proliferative role during early stages of differentiation while BMP-2 promoted osteogenic differentiation in the later stages [37], [63], [64]. It seemed like, in serum substitute medium, b-FGF was needed to induce cell proliferation which further differentiate towards osteoblast in the presence of osteogenic differentiation supplements (dexamethasone, ascorbic acid, and β-glycerophosphate) but the addition of BMP-2 was not needed.

Collagen is the most prominent constituent of the bone organic matrix which is synthesized by osteoblasts. Glycine and proline are among the amino acids that form the collagen structure and their insufficient availability could result in lack of collagen synthesis [34], [65]. Influence of proline on collagen synthesis by fibroblasts as the main collagen synthesized cells has been shown before. The exogenous proline upregulated collagen expression by fibroblasts in the absence of glutamine [34].

Another study indicated the collagen synthesis, mainly collagen type 2, by chondrocytes has improved in the presence of glycine [33]. Basal medium (DMEM) already contains several amino acids such as glycine, however, the addition of 1% v/v NEAA containing 10 mM glycine and 10 mM proline to the serum substitute medium was able to improve ECM production by hBMSCs during osteogenic differentiation (SI 4.3).

The secreted collagen by osteoblasts gets mineralized during bone formation. To mineralize bone, osteoblasts express membrane-bound alkaline phosphatase (ALP) or secreted matrix vesicles containing alkaline phosphatase (ALP) which cleaves phosphate sources (i.e., pyrophosphate). The combination of free phosphate with calcium results in calcium phosphate deposition within ECM [66]. FBS also contains ALP which could affect the mineralization of collagenous matrix [67], [68]. Thus, the deposition of minerals in FBS containing medium could be influenced by both cellular ALP activity and FBS ALP activity. While in serum substitute medium, hBMSCs needed to differentiate into osteoblasts which express ALP. These osteoblasts cleave β-glycerophosphate which resulted in increased free phosphate in the medium and deposition of calcium phosphate only in a few spots.

Interestingly, mineral deposition did not take place in cells in 3D set-ups cultured in serum substitute medium unlike in 2D culture. The differences in matrix mineralization in 2D and 3D cultures could be due to less cell-cell contact in 3D cultures compared to 2D cultures. The 3D porous structure of silk fibroin scaffolds might influence the cell-cell interaction which resulted in lack of mineral nodules formation. Previous studies have shown the direct influence of cell density with ECM formation/mineralization during osteoblast differentiation [69], [70]. An increase in cell density might enhance the cell-cell interactions and increase the deposition of collagenous matrix. Thus, it would be good to investigate the influence of a higher cell density on matrix mineralization. To increase calcium phosphate deposition, serum substitute medium was supplemented with either vitamin D (10 nM) or high concentration of rhBMP-2 (1000 ng/ml). Vitamin D showed no effect on calcium phosphate deposition despite what was expected [71], [72]. The high/non-physiological dose of rhBMP-2 is able to push cells to deposit calcium phosphate, while at the same time, it inhibited the collagenous matrix production (SI 4.4). Moreover, stimulating cells with high doses of growth factors might potentially disguise the influence of other soluble factors on cells as the combination of growth factors could have synergistic effects on cells [37], [73], [74].

Bone is continuously subjected to different mechanical forces such as shear stress, hydrostatic pressure, and mechanical stretch and tension due to body movement [75]. Early *in vivo* studies have shown that mechanical loading stimulates the formation of woven bone [76]–[80]. Woven bone forms during skeletal development or fracture healing where a rapid pace of matrix deposition is needed [77]. Woven bone formation is triggered under a variety of loading conditions where pre-osteoblasts lay down randomly oriented collagen that becomes highly mineralized [80], [81]. In the current study, shear stress has been applied to hBMSCs seeded on silk fibroin scaffolds. The application of shear stress increased mineral formation in FBS containing medium and induced mineralization in the serum substitute medium compared to static condition. The serum substitute medium without the presence of components such as ALP which could influence mineralization process during *in vitro* bone formation indicated the crucial role of mechanical loading in deposition of bone-like tissue *in vitro*.

Bone tissue engineering principles could be applied to develop *in vitro* functional engineered tissue for subsequent implantation into bone defects [82], [83]. Such constructs will need to be developed in a defined medium with components that are approved for clinical use. To the best of our knowledge, GlutaMax, NEAA, and ITS were manufactured at facilities registered with the US Food and Drug Administration (FDA) as a medical device manufacturer. Human serum albumin use is approved by the FDA. Anti-Anti is composed of penicillin, streptomycin, and amphotericin B which are all approved by the FDA. The use of b-FGF has not yet been approved by the FDA, however, products containing b-FGF such as Fiblast®, a topical spray has been marketed for skin ulcers in Japan [84], [85]. CDlipid contains many chemically defined components some of which such as cholesterol are approved by the FDA to be used. Since the use of some components of CDlipid are not (yet) approved by the FDA to be used in clinics such as linolenic acid or palmitoleic acid, the use of CDlipid might need to be reconsidered in case the *in vitro* tissue is developed for clinical use.

The *in vitro* functional engineered tissue models could be used to investigate human bone physiology, pathology, and drug discovery and screening while addressing the principle of replacement, reduction, and refinement of animal experiments (3Rs). To replace animal experiments with *in vitro* models, high standards of reproducibility and reliability need to be established and maintained in the field of tissue engineering [86]. The use of FBS in cell culture media results in low experimental reproducibility and is against the 3R principles, thus, the development of serum substitute media has become a major goal in the last few decades [87], [88]. In the current study, the development of serum substitute medium for bone tissue engineering contributes to reduce the harvesting of FBS from bovine fetuses which is in agreement with 3R principles. Besides, standardization and improvement of cell culture protocols are feasible with the serum substitute medium to achieve more reproducible and reliable *in vitro* bone models. The same method of serum substitute medium development could also be useful for development of other types of *in vitro* models without the use of FBS.

The current study obviously has its limitations. For example, in the present study, FBS has been used as the gold standard/control meaning that the outcomes of the developed serum substitute medium were compared to the FBS containing medium. This comparison raises the question of how to validate the results if the outcomes of the control group could be unreliable due to the variable nature of FBS. It should also be noted that in the current study, the focus was on development of a serum substitute medium for osteogenic differentiation of hBMSCs and the isolation and expansion of hBMSCs were still done in FBS containing medium. Exposure of cells to FBS during isolation and expansion steps might affect the cell phenotype and their differentiation capacity. A recent study showed that BMSCs lost their chondrogenic potential after expansion in FBS containing medium [89]. Thus, the serum substitute medium for hBMSCs isolation and expansion also needs to be optimized to have fully FBS-free studies. This study was limited to two donors of hBMSCs while it is known that different donors can react differently [90], [91]. Thus, the serum substitute medium needs to be tested for more donors in the future studies. As every component within the serum substitute medium is known, in future studies, different components can be added to this medium to systematically investigate their influence on cells in *in vitro* bone formation, allowing to extract influential parameters in a more precise way.

4.5 Conclusion

In this study we developed a serum substitute medium able to replace FBS in BTE studies. The developed serum replacement medium supported hBMSCs attachment, differentiation, and extracellular matrix production both in 2D and 3D. The most prominent difference was that for mineralization in 3D, mechanical stimulation was essential and representative of mineralization process during woven bone formation. The serum substitute medium has the potential to eliminate the use of FBS in the creation of *in vitro* bone models using BTE approaches and provides an opportunity to systematically study the influence of soluble factors on the bone formation in a less variable environment and without being overshadowed by unknown factors within FBS. Further studies should be performed with hBMSCs from different donors to reveal the suitability of the developed serum substitute medium for more donors.

Acknowledgement

This work has been financially supported by the Dutch Ministry of Education, Culture and Science (Gravitation Program 024.003.013). We would like to thank Dewy van der Valk for her help in Raman spectroscopy measurement and analysis.

Supplementary information

Vitronectin was used as a component to promote the attachment of cells to the 3D silk fibroin scaffolds. Cells were incubated in either FBS containing medium or serum substitute medium with or without 5 µg/ml vitronectin. The attachment of cells to the silk fibroin scaffolds incubated overnight in either FBS containing medium or serum substitute medium was investigated through measuring the amount of DNA of cells attached to the scaffolds. The presence of 5 µg/ml vitronectin in serum substitute medium promoted the attachment of cells to the substrate (Figure SI 4.1A). After 3 weeks of culture, H&E staining showed the distribution of cells between the pores of scaffolds in FBS containing medium (Figure SI 4.1B) and serum substitute medium containing 5 µg/ml vitronectin (Figure SI 4.1D). The cells incubated in serum substitute medium without vitronectin distributed sporadically between scaffold pores (Figure SI 4.1C).

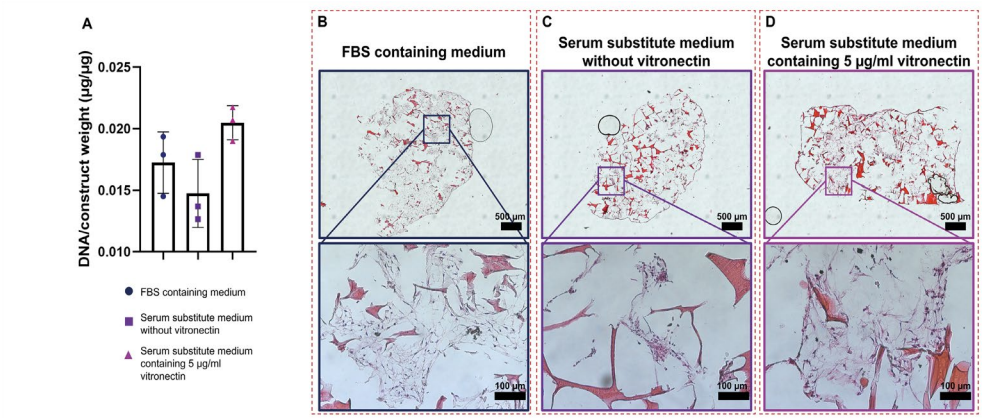

Figure SI 4.1 The DNA content of scaffolds after 24 hours of incubation showed that the serum substitute medium containing 5 µg/ml vitronectin increased attachment of cells to silk fibroin scaffold (A). H&E staining showed that after 3 weeks of culturing cells in FBS containing medium and serum substitute medium containing 5 µg/ml vitronectin distributed all over the scaffolds (B and D). While in serum substitute medium without vitronectin, cells sporadically spread between scaffold pores (C). Kruskal-Wallis with Dunn's post hoc tests did not show any significant differences in figure A.

Without any growth factors, hBMSCs could not differentiate towards osteoblasts (SI 4.2C) and the addition of b-FGF to the serum substitute medium seemed to be essential to induce osteogenic differentiation of hBMSCs (SI 4.2D). The presence of BMP-2 alone in serum substitute medium negatively influenced the number of cells (SI 4.2A and E), while the addition of BMP-2 to the serum substitute containing b-FGF did not show any significant influence on osteogenic differentiation of hBMSCs (SI 4.2F).

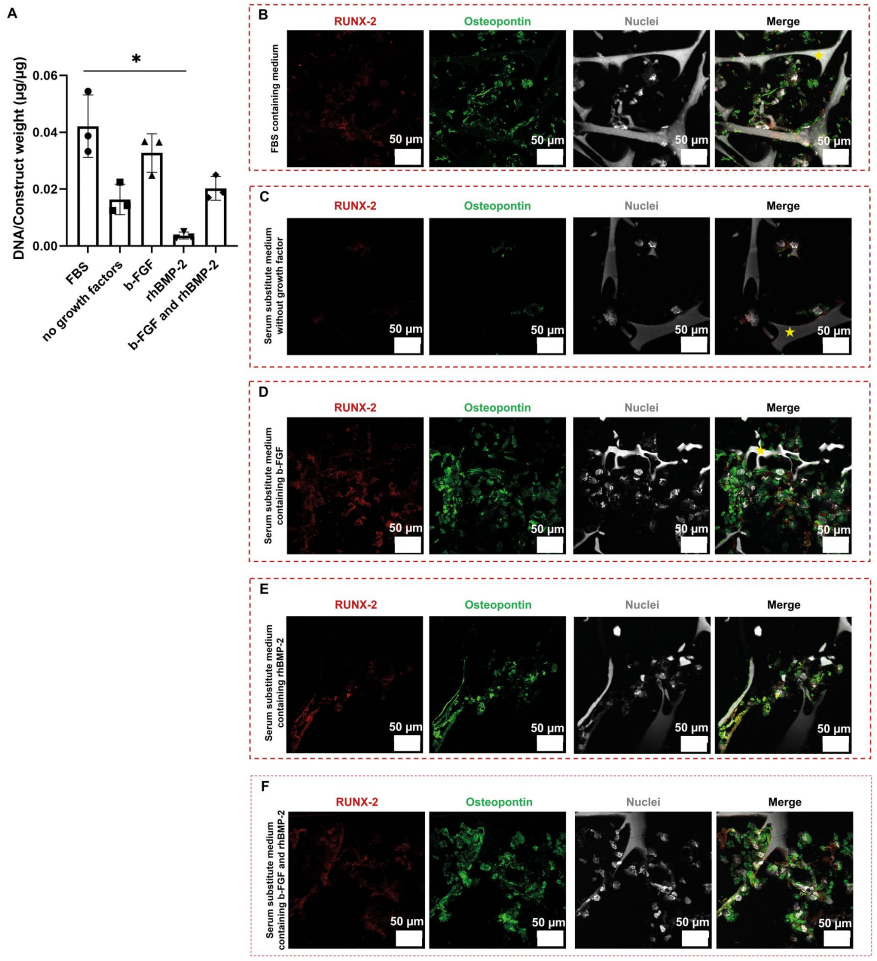

*Figure SI 4.2 hBMSCs could differentiate towards osteoblasts as shown by the expression of RUNX-2 and osteopontin in FSB containing medium (B). Lack of growth factors did not induce osteoblast differentiation in hBMSCs (C). The presence of b-FGF was needed for osteogenic differentiation in serum substitute medium (D). The addition of BMP-2 alone to serum substitute medium negatively affected the number of cells (A), while in combination of b-FGF did not show any significant changes in osteogenic differentiation of hBMSC. Yellow asterisks are the scaffold parts. *p<0.05. Kruskal-Wallis with Dunn's post hoc tests detected significant differences in figure A between FBS and rhBMP-2 groups. Merge stands for the overlay of first 3 channels.*

Collagen is the most prominent constituent of the bone organic matrix which is synthesized by osteoblasts. Glycine and proline are among the amino acids that form the collagen structure, and their insufficient availability could result in lack of collagen synthesis. Influence of proline on collagen synthesis by fibroblasts as the main collagen synthesized cells has been shown before. In this study, exogenous proline upregulated collagen expression by fibroblasts in the absence of glutamine. Another study indicated the collagen synthesis, mainly collagen type 2, by chondrocytes has improved in the presence of glycine. Basal medium (DMEM) already contains several amino acids such as glycine, however, the addition of 1% v/v NEAA containing 10 mM glycine and 10 mM proline to the serum substitute medium was able to improve ECM production by hBMSCs during osteogenic differentiation (SI 4.3).

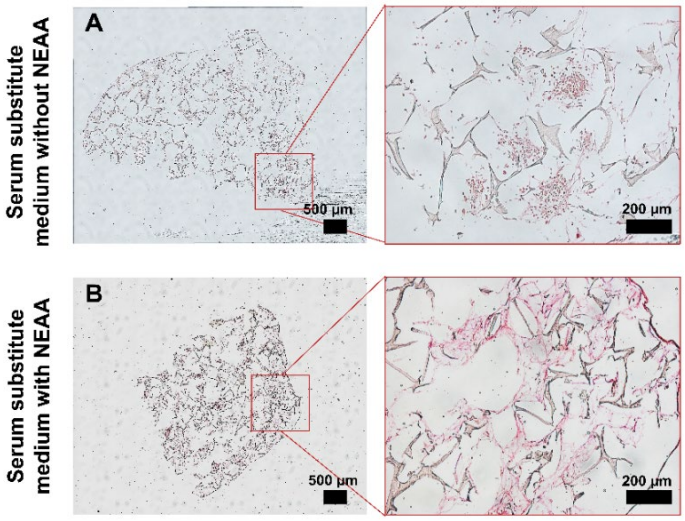

Figure SI 4.3 Exogenous NEAA containing glycine and proline enhanced collagen formation in the serum substitute medium shown by Picro-Sirius Red staining.

To increase calcium phosphate deposition, serum substitute medium was supplemented with either vitamin D (10 nM) or high concentration of rhBMP-2 (1000 ng/ml). Vitamin D showed no effect on calcium phosphate deposition despite what was expected. The high/non-physiological dose of rhBMP-2 is able to push cells to deposit calcium phosphate, while at the same time it inhibited the collagenous matrix production (SI 4.4).

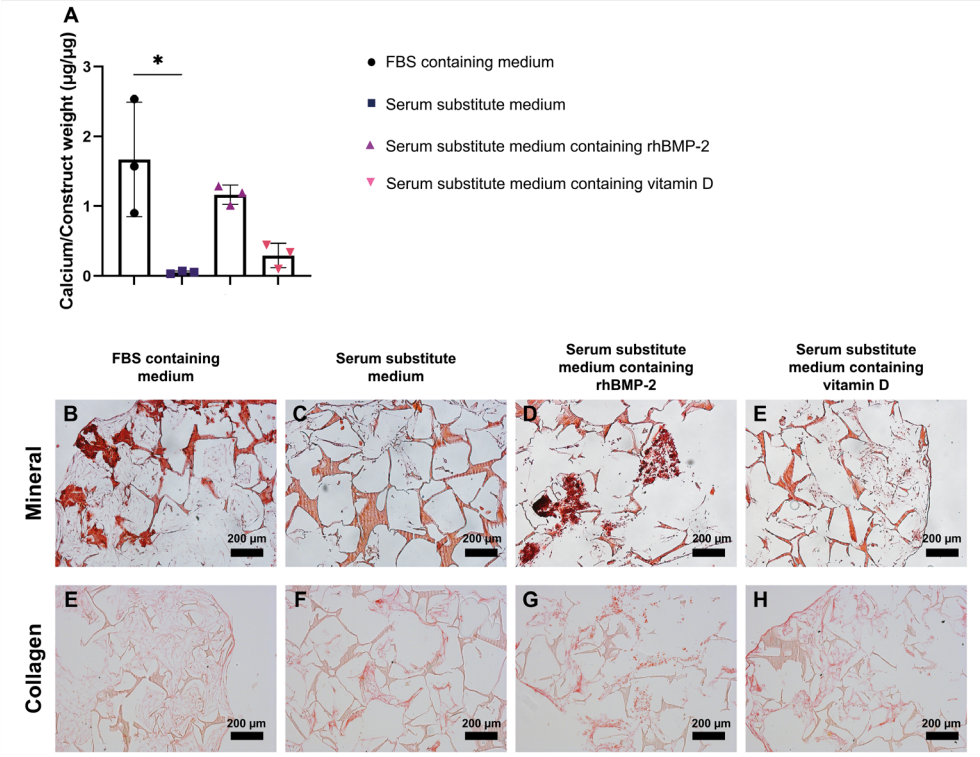

*Figure SI 4.4 Addition of vitamin D to serum substitute medium did not have any influence of collagen production and mineral deposition (A, E, H). Addition of high dosage of rhBMP-2 to serum substitute medium increased mineral deposition (A and D) while negatively influenced collagen production (G). *p<0.05. Kruskal-Wallis with Dunn's post hoc tests detected significant differences in figure A between FBS containing medium and Serum substitute medium groups.*

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Chapter 5

Towards development of serum substitute medium to induce osteoclast differentiation of human peripheral blood derived monocytes

This chapter is based on:

Sana Ansari, Keita Ito, Sandra Hofmann, Towards development of serum substitute medium to induce osteoclast differentiation of human peripheral blood derived monocytes, Submitted.

Abstract

Fetal bovine serum (FBS) is a widely used supplement in cell culture media despite its known drawbacks, including ethical, safety, and scientific issues. To overcome the drawbacks of using FBS in cell culture, a defined serum substitute medium needs to be developed. The development of such a medium depends on the cell type, which makes it impossible to use one universal serum substitute medium for all cells. Osteoclasts are large, multinucleated cells originated from the hematopoietic stem cell lineage that play an important role in regulating bone mass and quality. To date, no defined serum substitute medium formulations have been reported for osteoclast differentiation of monocytes derived from peripheral blood mononuclear cells (PBMCs). Here, we have attempted to develop such a serum substitute medium for the osteoclastogenesis process in a stepwise approach. Essential components were added to the medium while monocytes were cultured in 96-well plates and in Osteo-Assay well plates to analyze the formation of tartrate resistant acid phosphatase (TRAP) expressing multinucleated osteoclasts with distinct actin ring and to analyze the resorption activity of mature osteoclasts for 21 days, respectively. The serum substitute medium was aimed at supporting differentiation of monocytes into multinucleated osteoclasts and the resorption of mineralized matrix as a measure of functionality. All points were achieved after 21 days of culture in the developed serum substitute medium. This serum substitute medium could potentially replace FBS in osteoclastogenesis studies eliminating its debated use. Moreover, the well-defined serum substitute environment simplifies the study of factors released by the cells that were so far overwhelmed by the complexity of FBS.

5.1 Introduction

Fetal bovine serum (FBS) is a cell culture supplement containing hormones, growth factors, attachment factors, protease inhibitors, vitamins, and proteins that support cell survival, cell adhesion, and cell growth (Figure 5.1) [1]. FBS is easily accessible, inexpensive to produce, and effective to culture most types of human and animal cells, which makes it a widely and commonly used supplement in *in vitro* cell/tissue culture experiments [2]. However, the use of FBS in the culture medium bears several drawbacks: 1) ethical aspects regarding the collection of blood from bovine fetuses, 2) biosafety aspects as FBS might contain endotoxins or viral contaminants, 3) shortage in global supply which might not meet the global demand of FBS in the future, and 4) scientific aspects as FBS is a mixture of components with qualitative, quantitative, geographical, and seasonal batch-to-batch variations. The last issue could be the reason for irreproducible and unexpected experimental outcomes within and between research groups [2], [3]. To overcome the disadvantages of using FBS in culture, defined serum substitute media with known components should be developed and replace FBS in culture [4].

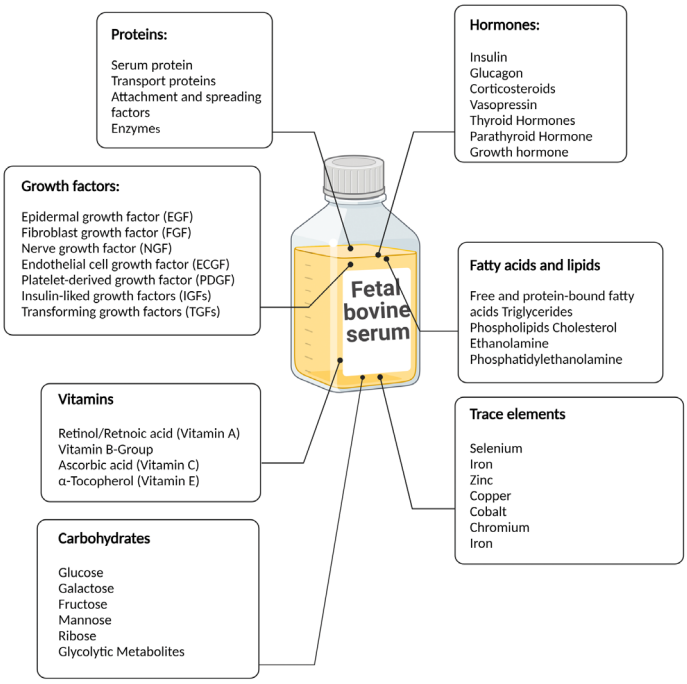

Figure 5.1 Components of FBS adapter from [1], [5]*. Created with BioRender.com.*

Using serum substitute medium in *in vitro* studies holds many advantages such as: 1) avoid suffering for cows and their fetuses, 2) reduced variability in cell culture composition to obtain reliable/reproducible results, 3) support cell homeostasis in the physiological state of a specific tissue, and 4) recreate the non-physiological state of a specific tissue to study possible treatments [6]–[9]. Development of a defined serum substitute medium for cell/tissue cultures has been the focus of research for decades [4]. The identification of factors essential for cells out of many unknown and complex components of FBS is the main challenge to develop a specialized serum substitute medium which is a timely and costly process. Recently, it has been found that FBS can be replaced in the medium of some cell types, such as mesenchymal stromal cells (MSCs), with addition of certain components to maintain cell survival, growth, and differentiation potential [10], [11]. The development of these defined media depends on many factors including cell type, cell sources, applications, and species. Thus, it has been proposed that development of a universal serum substitute medium is too challenging and that the focus should be on developing a suitable one for each cell type and/or application first [4].

Osteoclasts are large, multinucleated cells originating from the hematopoietic stem cell lineage and playing a vital role in the regulation of bone mass and quality [12]. During balanced bone remodeling, osteoclast progenitor cells are recruited to the site of remodeling from the bloodstream or bone marrow and differentiate into osteoclasts through the major influence of two cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) which, *in vivo*, are produced by osteoblasts, osteocytes, and stromal cells [12]. These factors bind to colony-stimulating factor-1 receptor (c-fms) and receptor activator of nuclear factor kappa-B (RANK), respectively, which are the receptors on the surface of osteoclast progenitor cells. These progenitor cells fuse together to form mature multinucleated osteoclasts. Activated osteoclasts secrete hydrogen ions and proteolytic enzymes which degrade the inorganic and organic components of bone [13]–[15].

In vitro studies on osteoclast differentiation, activity, and their interaction with other cell types such as osteoblasts make use of cell culture medium containing FBS [16]–[19]. Studies on osteoclast differentiation in a serum substitute medium are still in their infancy probably due to the limited availability of osteoclasts, their complex differentiation process, high donor variability, and short lifespan which make studying osteoclasts challenging in general [20]–[22]. It has been reported that FBS can be replaced with specific components which could support osteoclastic differentiation in a murine monocytic cell line, RAW 264.7, but not in human monocytes derived from peripheral blood mononuclear cells (PBMCs) [23]. Thus, there is still a need to develop a serum substitute medium for osteoclast differentiation of human PBMCs derived monocytes in a defined serum substitute medium.

The serum substitute medium was developed in a stepwise approach by adding factors to stimulate monocyte fusion and osteoclast differentiation in a basal medium containing essential components for cell survival, differentiation, and activation (Table 5.1). After 3 weeks of culture, the differentiation of monocytes into osteoclasts, their activity and expression of tartrate resistant acid phosphatase (TRAP), formation of multinucleated cells with rearrangement of actin cytoskeleton, and their functionality measured as resorption potential were studied in the newly developed serum substitute medium and compared with the results of the FBS containing medium (Figure 5.2). Our study shows

the potential of the newly developed osteoclast specific serum substitute medium for differentiation of human PBMCs derived monocytes into mature multinucleated osteoclasts. This specialized serum substitute medium provides the opportunity to investigate the influence of different soluble factors on osteoclast formation without the complex effect of serum.

Table 5.1 Serum substitute medium components.

Component	**Impact of the components on cells**	**Reference**
Vitronectin	Enhances attachment of cells to the substrate	[24], [25]
Human Serum Albumin (HSA)	Acts as antioxidant to prevent oxidative damages to cells through binding to metal ions including copper or iron	[26]
	Binds to various compounds including fatty acids, amino acids, hormones, vitamins, and metal ions (such as Zinc), and transport them to the cells	
Antibiotic/antimycotic	Controls the growth of bacterial and fungal contamination	[27]
Insulin/Transferrin/Selenium	Insulin: enhances glucose and amino acid uptake by cells, intercellular transport, and synthesis of proteins	[28]–[30]
	Transferrin: transports iron to cells which is a co-factor for enzymes involved in DNA synthesis and prevent extracellular oxidation	
	Selenium: protects cells from oxidative damages by reducing the production of free radical	
Glutamine	An essential amino acid for the synthesis of proteins and nucleic acids	[4], [31]
	Acts as a secondary energy source for metabolism	
Chemically defined lipids	Serves as structural constitutes of cell membrane, energy stores, and signaling molecules	[4], [32]
Sodium Pyruvate	A moderate excess of sodium pyruvate enhances osteoclast differentiation as this process requires significant increase in energy consumption	[33]
Vitamin D	Increasing the expression of osteoclast adhesion molecule $\alpha_v\beta_3$ and RANK	[34], [35]
Low-density lipoprotein (LDL)	Induces cell-cell fusion and controls osteoclast formation and survival	[36], [37]

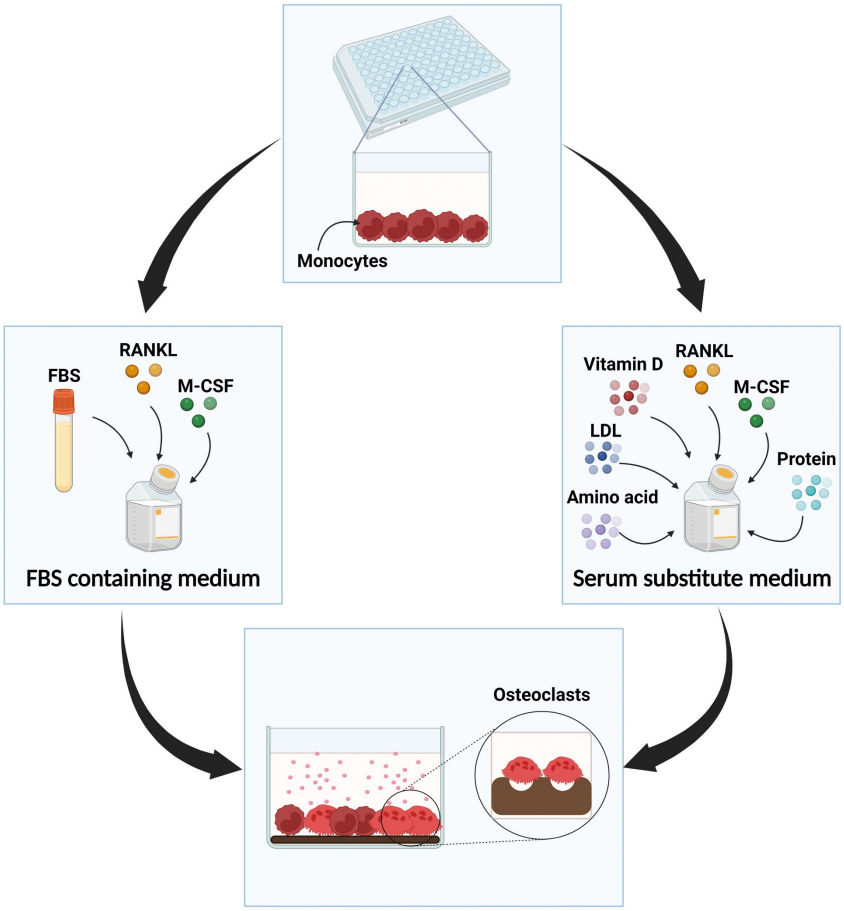

Figure 5.2 Monocytes were cultured in either FBS containing medium or serum substitute medium in which they are needed to differentiate into TRAP-positive multinucleated osteoclasts with resorption capability. Image was created with BioRender.com.

5.2 Materials and methods

5.2.1 Materials

α-Minimum Essential Medium (α-MEM, 41061) and Dulbecco's Modified Eagle Medium (DMEM, 22320) were purchased from Thermo Fisher Scientific (The Netherlands) and FBS (SFBS, Lot. No. 51113) was purchased from Bovogen (Australia). Antibiotic/antimycotic (Anti-Anti, 15240062), Insulin-Transferrin-Selenium (ITS-G, 41400045), sodium pyruvate (11360), human serum albumin (HSA, A1653), human low-density lipoprotein (LDL, LP2), and 1α,25-Dihydroxyvitamin D_3 (vitamin D, D1530) were obtained from Sigma-Aldrich (The Netherlands). Non-essential amino acids (NEAA, 11140050), GlutaMAX (35050061), and chemically defined lipid concentrate (CDlipid, 11905031) were from Life Technologies (The Netherlands). Basic fibroblast growth factor (b-FGF, 100-18B), recombinant human vitronectin (140-09), macrophage colony stimulating factor (M-CSF, 300-25), and receptor activator nuclear factor kappa-B ligand (RANKL, 310-01) were purchased from Peprotech (UK). Recombinant human bone morphogenetic protein-2 (rhBMP-2, 7510200) was purchased from Medtronic Sofamor Danek (USA). Unless noted otherwise, all other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich (The Netherlands).

5.2.2 Serum substitute medium development

In the first step of development of serum substitute medium for osteoclast cultures, we decided to use the medium that we previously developed for osteoblast cultures [38]. We aimed at investigating whether the same medium supplemented with osteoclast differentiation supplements (M-CSF and RANKL) would be sufficient for initiating osteoclastogenesis of human PBMC derived monocytes. In the next step, as the osteoblast-specific serum substitute medium supplemented with M-CSF and RANKL did not seem to be sufficient to initiate osteoclastogenesis, we aimed at developing a novel serum substitute medium specific for osteoclast cultures. To induce the formation of multinucleated mature osteoclasts, several components such as vitamin D and LDL were added to the primary serum substitute medium. Figure 5.3 illustrates the stepwise approach of components added.

5.2.2.1 Step 1- osteoblast serum substitute medium

Initially serum substitute medium consisted of DMEM, 1% w/v HSA, 1% v/v Anti-Anti, 1% v/v ITS-G, 1% v/v GlutaMax, 1% v/v NEAA, 0.1% v/v CDlipid, 10 ng/ml b-FGF, and 100 ng/ml rhBMP-2 and supplemented with osteogenic differentiation factors: 50 µg/ml ascorbic-acid-2-phosphate (Sigma-Aldrich, A8960), 100 nM dexamethasone (Sigma-Aldrich, D4902), 10 mM β-glycerophosphate (Sigma-Aldrich, G9422), as previously described for osteoblasts [38]. To this, osteoclast differentiation factors were added: 50 ng/ml M-CSF for the first 48 hours, and 50 ng/ml M-CSF and 50 ng/ml RANKL for the rest of the experiment [39].

5.2.2.2 Step2- primary serum substitute medium

To develop a serum substitute medium specifically for osteoclast cultures, we selected α-Minimum Essential Medium (α-MEM, Cat. No. 41061) as it has been shown before to best support osteoclast formation from monocytes derived from PBMCs when supplemented with 10% FBS [39], [40]. α-MEM

contains a wide variety of amino acids, vitamins, inorganic salts, glucose, and sodium pyruvate that each have specific functions essential for cell function (Table 5.2).

Table 5.2 The components of α-MEM and their function on cells.

Components	Impact of the components on cells	References
Amino acids	Basic building blocks of proteins and essential for cell proliferation and viability	[41]
Vitamins	Essential for cell growth and proliferation, act as co-factor for enzymes	[4], [42]
Inorganic salts	Maintain the osmotic balance and regulate membrane potential which is necessary to transport ions through cell membrane	[43], [44]
Glucose	Main source of energy for cell metabolism	[27]
Sodium Pyruvate	By-product of glucose consumption in the glycolytic pathway and functions as a source of energy	[45]

To induce osteoclast differentiation, the primary serum substitute medium was developed by adding 1% w/v HSA, 1% v/v Anti-Anti, 1% ITG-S, 1% v/v GlutaMAX, 0.1% v/v CDlipid, 1% v/v sodium pyruvate to α-MEM. Again, this medium was supplemented with 50 ng/ml M-CSF for the first 48 hours, and 50 ng/ml M-CSF and 50 ng/ml RANKL for the rest of the experiment.

5.2.2.3 Step 3- primary serum substitute medium + vitamin D

To enhance the osteoclast differentiation of monocytes, the primary serum substitute medium (5.2.2.2) was additionally supplemented with 20 nM vitamin D. It has been shown that vitamin D can support the formation of multinucleated osteoclasts [34], [46]. This medium was also supplemented with 50 ng/ml M-CSF for the first 48 hours, and 50 ng/ml M-CSF and 50 ng/mL RANKL for the rest of the experiment.

5.2.2.4 Step 4- primary serum substitute medium + vitamin D + LDL

Since it seemed that cell-cell fusion was still insufficient with medium 5.2.2.3, 20 µg/ml LDL was additionally added. LDL has been shown to be essential for cell-cell fusion [36], [37]. Again, this medium was supplemented with 50 ng/ml M-CSF for the first 48 hours, and 50 ng/ml M-CSF and 50 ng/ml RANKL for the rest of the experiment.

5.2.2.5 Control medium

As controls, either DMEM (for step 1) or α-MEM (for steps 2-4) were supplemented with 10% FBS, 1% Anti-Anti, and osteoclast differentiation factors: 50 ng/ml M-CSF for the first 48 hours, and 50 ng/ml M-CSF and 50 ng/ml RANKL for the rest of the experiment.

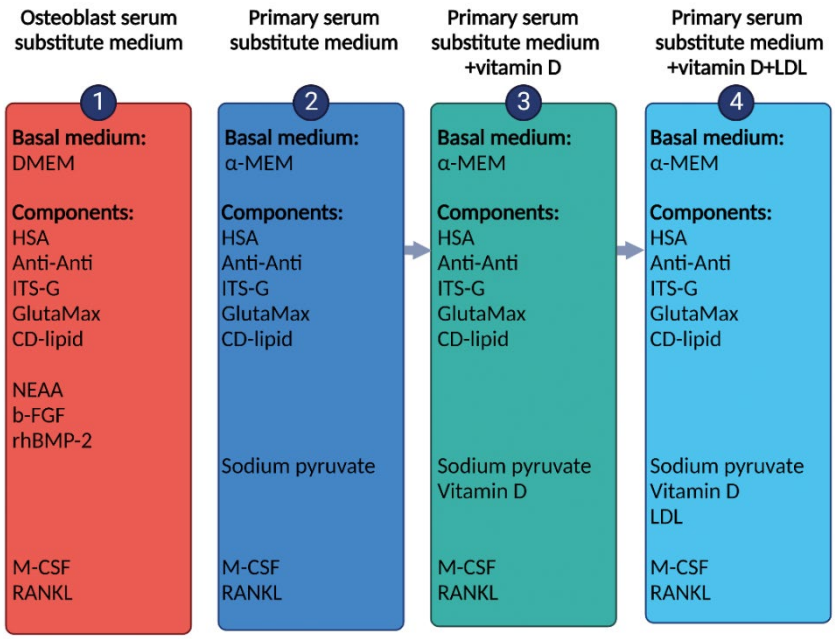

Figure 5.3 The steps of development serum substitute medium for osteoclast cultures. Image was created with BioRender.com.

5.2.3 Monocyte isolation, seeding, and cultivation

Monocyte isolation

Human peripheral blood buffy coats from 2 healthy donors were collected under institutional guidelines with informed consent per declaration of Helsinki (Sanquin, Eindhoven, The Netherlands). Fifty mL of buffy coats were diluted with 0.6% (w/v) sodium citrate in PBS (citrate-PBS) up to a final volume of 200 mL and layered per 25 mL on top of 10 mL Lymphoprep™ (Stem Cell Technologies, Koln, Germany). The samples were centrifuged at 800g with the lowest break for 20 minutes. PBMCs were collected, resuspended in citrate-PBS, washed 4 times in citrate-PBS supplemented with 0.01% bovine serum albumin (BSA, Roche, 10.7350.86001). PBMCs were frozen at 50*10^6 cells/ml in freezing medium containing α-MEM, 20% human platelet lysate (hPL, PE20612, PL BioScience, Aachen, Germany), and 10% dimethyl sulfoxide (DMSO, VWR, 1.02952.1000. Radnor, PA, USA) and stored in liquid nitrogen until further use. To isolate monocytes, PBMCs were thawed in α-MEM containing 10% hPL and 1% Anti-Anti, centrifuged at 300g for 10 minutes, and resuspended in isolation buffer (0.5% w/v BSA in 2 mM EDTA-PBS). Monocytes were isolated with magnetic activated cell separation (MACS) using the Pan Monocyte Isolation Kit (130-096-537, Miltenyi Biotec, Leiden, The Netherlands) and LS columns (Miltenyi Biotec, 130-042-401) according to the manufacturer's instructions.

Monocyte seeding and cultivation

The wells of a 96-well plate were coated with 5 µg/ml vitronectin diluted in PBS. Briefly, 100 µL of vitronectin solution was added to each well and incubated in an incubator (37°C, 5% CO_2) for 2 hours and then at 4°C for 16 hours. The following day, the vitronectin solution was aspirated, the wells were rinsed 3 times with PBS, and the well-plate was pre-warmed in an incubator before seeding the cells. Monocytes were seeded in the well-plate at a density of $9*10^4$ cells per well (n=3-5). Monocytes were cultured in either FBS containing medium or the respective Serum substitute medium (section 5.2.2) containing 50 ng/ml M-CSF for the first 48 hours. After 48 hours, the medium was replaced with FBS containing medium or the respective Serum substitute medium containing 50 ng/ml M-CSF and 50 ng/ml RANKL to induce osteoclast differentiation. The medium was replaced 3 times per week for 3 weeks.

For Step 4, to study the resorption activity of differentiated osteoclast in FBS containing medium and serum substitute medium, monocytes of each donor were seeded in a vitronectin coated 96 Osteo-Assay well-plate (CLS3988, Corning, Amsterdam, The Netherlands) at a density of $9*10^4$ cells per well (n=3-5). Monocytes were cultured in either FBS containing medium or Serum substitute medium containing 50 ng/ml M-CSF for the first 48 hours. After 48 hours, the medium was replaced with FBS containing medium or Serum substitute medium containing 50 ng/ml M-CSF and 50 ng/ml RANKL to induce osteoclast differentiation. The medium was replaced 3 times per week for 3 weeks.

5.2.4 Immunohistochemistry

To visualize cell nuclei, the actin cytoskeleton, and the expression of TRAP, immunohistochemistry was performed. Cell-seeded wells were rinsed with PBS and fixed with 10% neutral buffered formalin for 30 minutes at 4°C. Then, the wells were rinsed 3 times with PBS and covered with 100 µL 0.5% (w/v) Triton x-100 (Merck, 1.08603.1000) in PBS for 5 minutes to permeabilize cells. Then, the wells were rinsed with PBS and incubated with 5% (v/v) normal goat serum and 1% (w/v) BSA in PBS for 1 hour at room temperature to block non-specific antibody binding. Wells were then incubated for 16 hours at 4°C with primary antibody solution containing 5% (v/v) normal goat serum, 1% (w/v) BSA in PBS, and anti-TRAP antibody (Abcam, ab185716, 1:200). The following day, the wells were washed with PBS 3 times and incubated for 1 hour with secondary antibody solution containing 5% (v/v) normal goat serum, 1% (w/v) BSA in PBS, and anti-mouse (Molecular probes, A21240, 1:200). This was followed by 3 times rinsing of the wells with PBS and incubation of cells with 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI (Sigma-Aldrich, D9542) and 50 pmol Atto 488-conjugated Phalloidin (Sigma-Aldrich, 49409, diluted in PBS) for 30 minutes at room temperature to stain nuclei and actin cytoskeleton, respectively. Wells were rinsed with PBS 3 times and then covered with PBS. The expression of proteins was visualized with a Leica TCS SP5X microscope and images were processed with ImageJ (version 1.53f51). Figures were chosen to be representative images per group for all the samples assessed.

5.2.5 Measurement of number of nuclei per osteoclasts and size of osteoclasts

TRAP-positive cells with 3 or more nuclei were considered to be osteoclasts. To quantify the number of nuclei per osteoclasts and size of osteoclasts, 6-10 immunohistochemistry images of cells grown in

step 4 and control medium were analyzed using ImageJ (version 1.53f51). The number of nuclei per TRAP-positive cells were manually counted. The longest diameter of osteoclasts was measured using the length measurement function of ImageJ after calibrating it using the scale bar of each image.

5.2.6 Tartrate resistant acid phosphatase (TRAP) assay

The release of TRAP to the culture medium as a measure for osteoclast differentiation was measured in the cell supernatant. Ten µL supernatant or p-nitrophenol standard was incubated in 90 µL p-nitrophenyl phosphate buffer (1 mg/ml p-nitrophenyl phosphate disodium hexahydrate (Sigma-Aldrich, 71768), 0.1 M sodium acetate, 0.1% Triton x-100, and 30 µL/mL tartrate solution (Sigma-Aldrich, 3873) in PBS) in a 96-well-plate for 90 minutes at 37 °C. The reaction was stopped by adding 100 µL 0.3 M NaOH. Absorbance was measured using a plate reader at 405 nm and TRAP activity was calculated by comparison to standards of known p-nitrophenol concentration.

5.2.7 Resorption assay

The resorption activity of osteoclasts was measured after 21 days of culture in FBS containing medium and complete serum substitute medium. First, cells seeded on Osteo-Assay wells were removed by incubating in 5% bleach in ultra-pure water (UPW) for 5 minutes, following rinsing the wells twice with UPW. To visualize the non-resorbed surface, the remaining calcium phosphate within the wells were stained with Von Kossa as described previously [40], [47]. Briefly, wells were incubated in 5% (w/v) silver nitrate (Sigma-Aldrich, 209139) in UPW for 30 minutes in dark, following rinsing with UPW, and incubation in 5% (w/v) sodium carbonate (Sigma-Aldrich, S7795) in 10% neutral buffered formalin for 4 minutes. The solution was completely aspirated, and the plates were dried at 50°C for 1 hour. The wells were captured with bright field microscope (Zeiss Axio Observer Z1) using tile scanning function. The tile scans were stitched with Zen Blue software (version 3.1, Zeiss). The segmentation and resorption quantification were done as previously described [40]. Briefly, first image contrast was increased using ImageJ. Then, a clipping mask was created in Illustrator (Adobe Inc., San Jose, Ca, USA) in order to remove the edges of the wells. Segmentation was done using MATLAB (version 2019b, The MathWorks Inc., Natrick, MA, USA), using Otsu's method for binarization with global thresholding. The total number of pixels within the well and the number of resorbed pixels were determined to quantify the percentage of resorbed area.

5.2.8 Statistics

GraphPad Prism 9.0.2 (GraphPad Software, La Jolla, CA, USA) was used to perform statistical analysis and to prepare the graphs. Data used for statistical analysis was tested for normality using the Shapiro-Wilk normality test. The TRAP assay data (Figure 5.4C, 5.5D, and 5.6E) were normally distributed. These data were compared using Repeated Measures Two-Way Analysis of Variances (ANOVA) followed by Tukey's post hoc tests with adjusted p-value for multiple comparisons. Geisser-Greenhouse correction was used to account for unequal variances. The data is presented as mean and standard deviation. Number of nuclei per osteoclasts (Figure 5.6C), size of osteoclasts (Figure 5.6D), and resorption data (Figure 5.7C) were not normally distributed and were tested with Mann-Whitney test and are presented as median and interquartile range. Differences were considered statistically significant at a level of $p<0.05$.

5.3 Results

5.3.1 Serum substitute medium for osteoblast cultures containing M-CSF and RANKL did not support osteoclast formation

In the first step to develop a serum substitute medium for osteoclast cultures, we used a previously developed serum substitute medium for osteoblast cultures [38] supplemented with osteoclast differentiation factors: M-CSF and RANKL. This was done to investigate whether only adding the osteoclast differentiation factors to an already functional serum substitute medium would be sufficient for osteoclast cultures. The results indicated that monocytes did not differentiate into multinucleated osteoclasts in both FBS containing medium (Figure 5.4A) and osteoblast serum substitute medium (Figure 5.4B). In both medium types, cells seemed to remain as mononucleated monocytes despite the presence of M-CSF and RANKL. Interestingly, TRAP release into cell culture supernatant increased significantly over time in FBS containing medium (Figure 5.4C), while in serum substitute medium, only a slight increase could be detected.

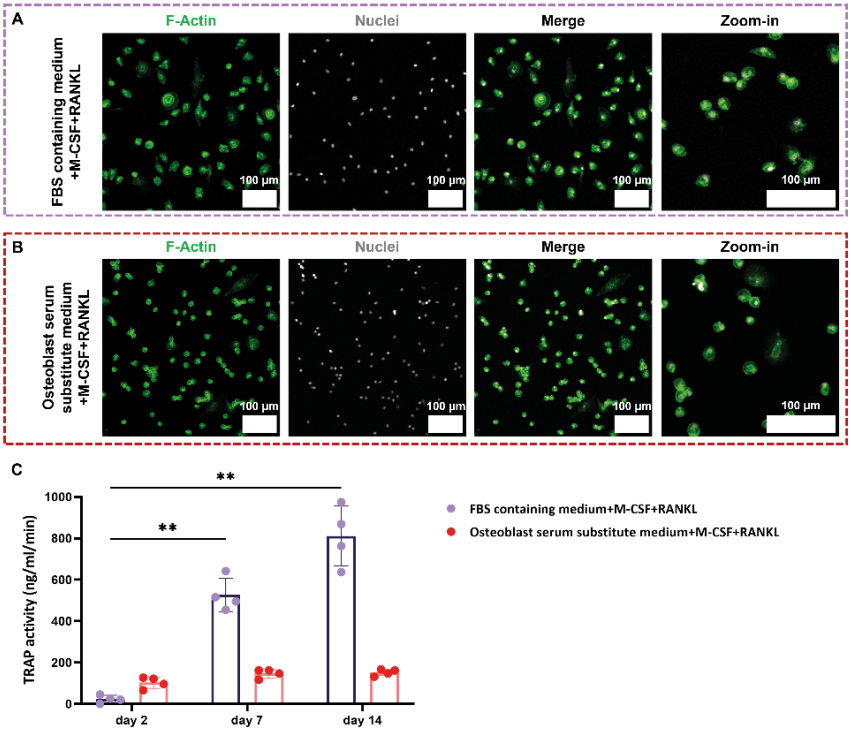

*Figure 5.4 In step 1 of development serum substitute medium for osteoclast cultures, the previously developed serum substitute medium for osteoblast cultures supplemented with M-CSF and RANKL was used. Monocytes did not differentiate into multinucleated osteoclasts in FBS containing medium (A) and osteoblast serum substitute medium (B) after 14 days of culture. TRAP release into the medium increased significantly over time in FBS containing medium (**p<0.01- detected by Repeated Measures Two-Way ANOVA followed by Tukey's post hoc tests). Only a slight increase in TRAP release was detected in osteoblast specific serum substitute medium (C). Merge stands for the overlay of first 2 channels.*

5.3.2 Development of serum substitute medium for osteoclastogenesis

Monocytes were cultures in the "primary serum substitute medium" (Figure 5.2-Step 2) supplemented with 50 ng/ml M-CSF for the first 48 hours and 50 ng/ml M-CSF and 50 ng/ml RANKL for the next 21 days. As a control, monocytes were also cultured in FBS containing medium supplemented with the same amount of M-CSF and RANKL. Multinucleated osteoclasts were formed in FBS containing medium (Figure 5.5A), while in the primary serum substitute medium monocytes remained mononucleated and did not differentiate into multinucleated osteoclasts (Figure 5.5B). TRAP release into the cell culture supernatant increased significantly over time in the FBS containing medium (Figure 5.5D). The primary serum substitute medium did not induce any TRAP activity by cells (Figure 5.5D).

In the next step of developing a serum substitute, it was chosen to additionally supplement the primary serum substitute medium with vitamin D to support the formation of multinucleated osteoclasts [34], [46]. The addition of 20 nM vitamin D to the primary serum substitute medium supported some fusion of cells into double-nucleated cells (Figure 5.5C). But more often, mononucleated monocytes remained in each other's vicinity and only in a few spots, cells did fuse together (Figure 5.5C, arrows). However, the addition of vitamin D was able to increase TRAP activity to an equal level as the FBS containing control group (Figure 5.5D). After 21 days of culture, no significant differences were detected in TRAP activity of cells cultured in FBS containing medium and primary serum substitute medium containing vitamin D.

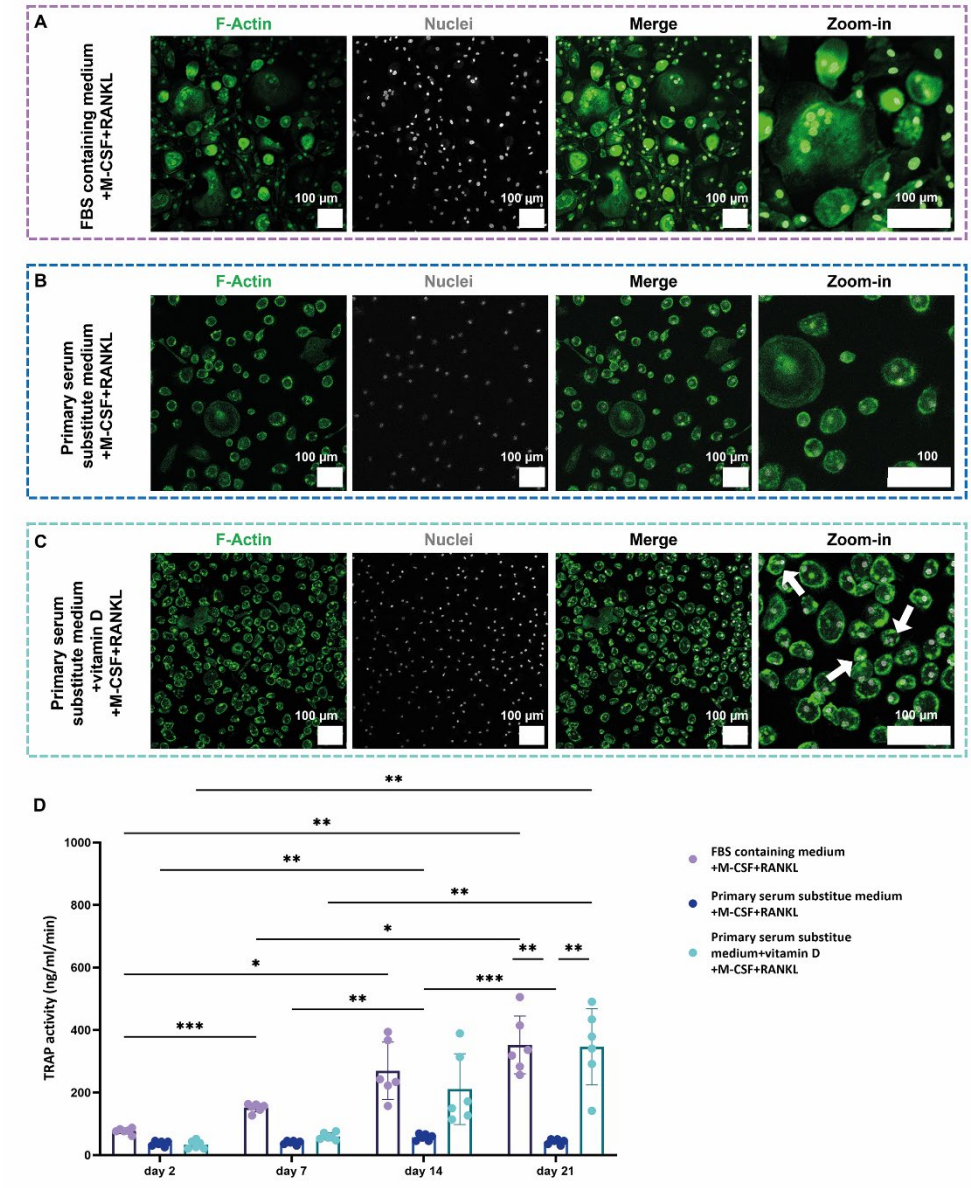

*Figure 5.5 Vitamin D in serum substitute medium enhanced the formation of osteoclasts in culture. Monocytes differentiated into multinucleated osteoclasts in FBS containing medium (A). Primary serum substitute medium did not induce osteoclast formation (B). Addition of vitamin D induce formation of few double-nucleated cells (C). TRAP activity of cells cultured in FBS containing medium increased significantly over time. The primary serum substitute medium did not affect the TRAP release by cells. Addition of vitamin D to primary serum substitute medium significantly enhanced TRAP activity of cells (D). *p<0.05, **p<0.01, ***p<0.001. Significant differences were detected by Repeated Measures Two-Way ANOVA followed by Tukey's post hoc tests. Merge stands for the overlay of first 2 channels.*

5.3.3 Promote cell-cell fusion in serum substitute medium

Osteoclasts are characterized as large (10-300 µm in diameter), multinucleated cells with at least 3 nuclei per cell and a typical actin ring [54], [55]. In the next step, we tried to further promote the cell-cell fusion. This was done by adding low-density lipoprotein (LDL) to the primary serum substitute medium containing vitamin D. The addition of LDL led to the formation of large, multinucleated cells with a clear actin ring (Figure 5.6B), similar to FBS containing medium (Figure 5.6A). The formed multinucleated cells contained more nuclei compared to double-nucleated cells formed in the absence of LDL (Figure 5.6B-arrows vs. Figure 5.5C-arrows). This serum substitute medium induced the formation of larger osteoclasts with less nuclei compared to FBS containing medium in which smaller osteoclasts were formed with more nuclei per osteoclast (Figure 5.6C and 5.6D). In both FBS containing medium and serum substitute medium, round mononucleated cells were also present which suggested that not all cells differentiated into osteoclasts. TRAP was expressed both in monocytes and osteoclasts cultured in both FBS containing medium and serum substitute medium.

As expected, in FBS containing medium, TRAP release into the medium increased significantly over time. Similarly, TRAP activity of cells cultured in the serum substitute medium containing vitamin D and LDL increased over time. The variation in TRAP activity was larger compared to the FBS containing medium (Figure 5.6E). The release of TRAP to the serum substitute medium was in general slightly higher than in FBS containing medium, however only on day 14, the TRAP activity of cells in serum substitute medium was significantly higher than in FBS containing medium. Higher TRAP release in serum substitute medium after 14 days compared to FBS containing medium could suggest that the serum substitute medium stimulated cells to express and release TRAP to the medium faster than in FBS containing medium.

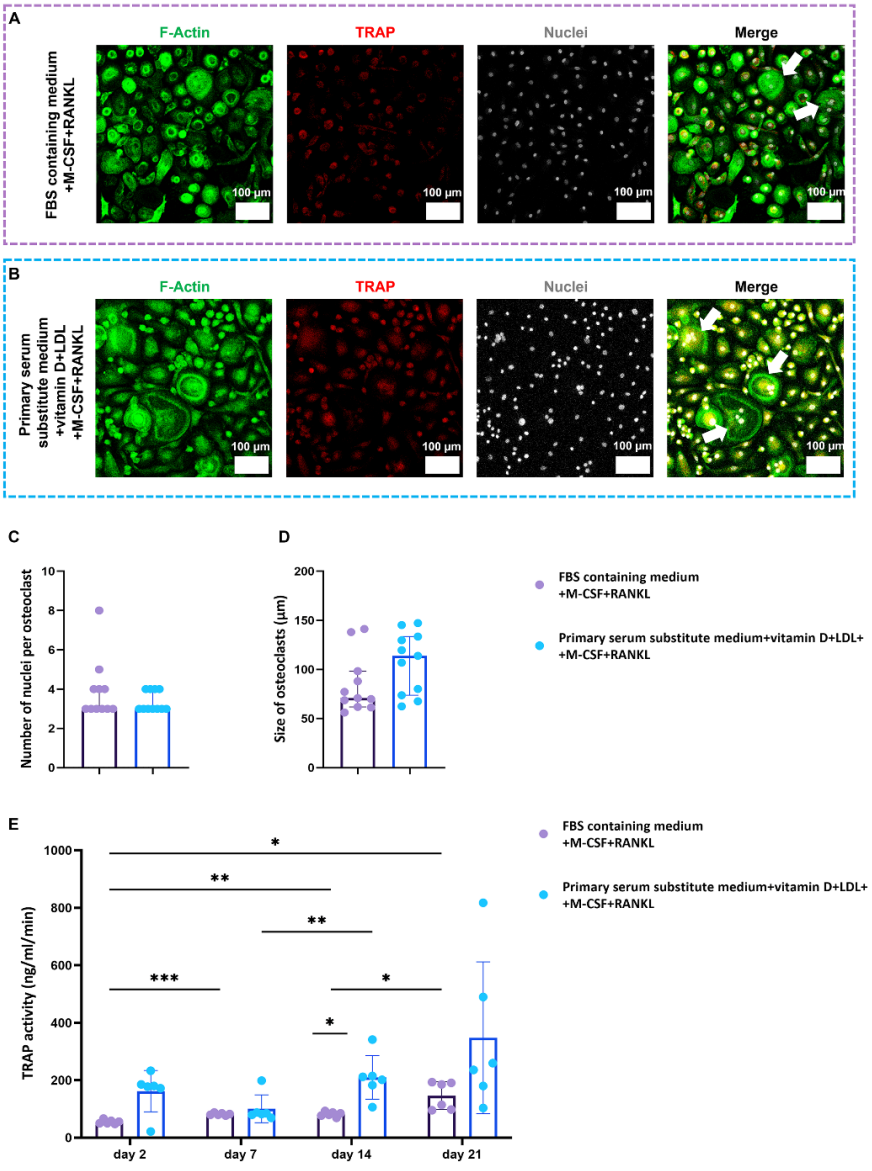

*Figure 5.6 The serum substitute medium induced osteoclast differentiation. Monocytes differentiated into multinucleated osteoclasts in FBS containing medium (A). Primary substitute medium containing vitamin D and LDL supported the formation of multinucleated osteoclasts after 21 days of culture (B). The serum substitute medium induced the formation of larger osteoclasts with less nuclei compared to FBS containing medium in which smaller osteoclasts were formed with more nuclei per osteoclast (C and D). TRAP activity of cells cultured in serum substitute medium increased over time in both groups (E). *p<0.05, **p<0.01, ***p<0.001. Significant differences in figure E were detected by Repeated Measures Two-Way ANOVA followed by Tukey's post hoc tests. Merge stands for the overlay of first 3 channels.*

5.3.4 Serum substitute medium containing vitamin D and LDL supported osteoclast activity

The functionality of osteoclasts *in vitro* in FBS containing medium and serum substitute medium containing vitamin D and LDL was analyzed by the assessing their capacity to resorb calcium phosphate surfaces (Osteo-Assay plates). Osteoclasts formed in both FBS containing medium (Figure 5.7A) and serum substitute medium (Figure 5.7B) were able to resorb parts of the Osteo-Assay plates. The resorbed and un-resorbed area are shown in white and black, respectively. Quantification of the resorbed area of the Osteo-Assay plates by differentiated osteoclasts in FBS containing medium revealed a variation from 0.04% to 11% (Figure 5.7C). This variation could be attributed to a donor-dependent response of osteoclasts to FBS, which has been described earlier [40]. Osteoclasts formed in serum substitute medium were not able to resorb the whole wells (Figure 5.7B) and the quantification of the resorbed area of Osteo-Assay plates by differentiated osteoclasts in complete serum substitute medium showed to be 2.86 +/- 1.28% (Figure 5.7C).

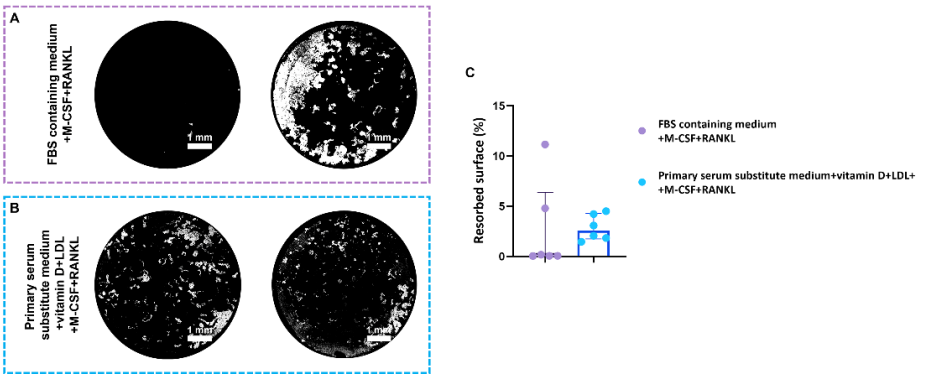

Figure 5.7 Osteoclasts formed in serum substitute medium containing vitamin D and LDL resorbed the calcium phosphate surface. The Osteo-Assay surface was resorbed in both FBS containing medium (A) and serum substitute medium (B). Quantification of resorbed area showed differences in resorption potential of differentiated osteoclasts in FBS containing medium and serum substitute medium (C). Mann-Whitney tests did not detect any significant differences in figure C.

5.4 Discussion

FBS is a commonly used growth supplement in most cell/tissue culture media despite its known disadvantages in scientific, biosafety, and ethical aspects [2]. To overcome the disadvantages of using FBS in cell culture media, defined media replacing FBS need to be developed. Due to the differences in cell's requirements in growth and differentiation, the defined media might need to be optimized for each specific cell type. In the present study, we developed a serum substitute medium in a step-by-step approach for osteoclastogenesis of human PBMCs derived monocytes. By doing so, we could also elucidate the effect that the added supplements have during osteoclastogenesis. The suitability of our newly developed serum substitute medium was determined based on its capability to support

the differentiation of monocytes into resorbing TRAP-positive multinucleated osteoclasts with a clear actin ring.

Osteoclasts are large multinucleated cells formed by fusion of mononuclear monocytes capable of resorbing bone. Each osteoclast contains 3 or more nuclei and the cell size varies in diameter between 10 to 300 µm [54]. Despite many attempts to develop a serum substitute medium for different cell types [3]–[5], a defined serum substitute medium for osteoclast differentiation from human monocytes has not been reported yet. This might be attributed to the challenges of studying osteoclasts in general due to their limited availability, complex differentiation process, donor variation, and short lifespan [20]–[22]. In an early study, there have been attempts to define a serum substitute medium to differentiate human monocytes and the murine monocytic cell line, RAW 264.7 into osteoclasts [23]. This defined serum substitute medium contained albumin, transferrin, insulin, β-mercaptoethanol, LDL, ascorbic acid, epidermal growth factor (EGF), platelet derived growth factor-BB (PDGF-BB), dexamethasone, and glutamine and has been shown to support the formation of TRAP releasing multinucleated osteoclasts from RAW 264.7. However, the same medium did not support osteoclast formation of human PBMCs derived monocytes [23]. Thus, to be able to study human osteoclast differentiation in a more defined biochemical environment, there is still a need to develop a serum substitute medium for osteoclastogeneis of human monocytes.

We used a stepwise approach for serum substitute development. In the first step, a previously developed serum substitute medium for osteoblast cultures which was able to support osteogenic differentiation of MSCs and ECM deposition after 21 days of culture has been used [38]. This step was done to investigate whether the same medium supplemented with osteoclast differentiation factors (M-CSF and RANKL), which are expressed by mature osteoblasts [56], would be sufficient for osteoclast differentiation of monocytes. The potential of using the same serum substitute medium was in view of using it for studies mimicking the bone remodeling process [39]. In such bone remodeling models, bone forming osteoblasts and bone resorbing osteoclasts act closely together in a co-culture. One of the challenges in creating such models has been the choice of medium composition [39], [57]. A suitable medium for co-culture experiments needs to support the growth and function of both cell types. Thus, it would have been ideal if the same serum substitute medium developed for osteoblast cultures could also support osteoclast formation. However, here we have shown that culturing monocytes in osteoblast serum substitute medium supplemented with osteoclast differentiation factors was not able to induce osteoclast differentiation. The inability of FBS containing medium to support differentiation of monocytes into osteoclasts could be due to the use of DMEM as basal medium. Osteoblast serum substitute medium did not induce osteoclast differentiation probably due to not only the use of DMEM as basal medium but also the presence of factors that inhibit the osteoclast formation such as dexamethasone. DMEM contains phenol red (pH indicator). Previous studies have shown that phenol red can mimic the action of estrogen and inhibit osteoclast formation and activity [48], [49], [50], [51]. Moreover, the presence of dexamethasone in high concentrations (100 nM) in osteoblast serum substitute medium has been shown to inhibit the formation of TRAP-positive multinucleated osteoclasts [52], [53]. These results indicated that serum substitute medium containing factors that support osteoblast cultures could interferes with monocyte

differentiation into osteoclasts even when potent osteoclast differentiation factors are present. This observation suggested that for monocyte differentiation into osteoclasts, different formulation of serum substitute medium would be needed. In future studies, a serum substitute medium should be developed that supports both cell types in a co-culture. It might be that interaction between the cells could limit the need to add exogenous factors. For instance, the expression of M-CSF, RANKL, and osteoprotegerin (OPG) by mature osteoblasts could support osteoclast formation in a co-culture [40], [58].

In the next step, we focused on developing a specialized serum substitute medium for osteoclast differentiation from human PBMC derived monocytes. We selected α-MEM (without phenol red) as a basal medium as it has been shown before to support osteoclast formation when supplemented with 10% FBS [39], [40]. Phenol red is a pH indicator in cell culture media, but studies have shown that it mimics the action of estrogen and its effect should be taken into account when studying estrogen-responsive cells such as osteoclasts [48], [49]. Estrogen itself has been shown to inhibit osteoclast formation and activity [50], [51]. Thus, the use of phenol red-free α-MEM was a logical choice for osteoclast studies.

The basal medium needed to be supplemented with a number of components that supported the formation of osteoclasts *in vitro* [4]. The complex process of fusion of monocytes during osteoclast differentiation made the process challenging. One of the components that appeared to be essential for osteoclast formation was 1α,25-dihydroxyvitamin D_3 (vitamin D). It has been shown previously that culture of osteoclast progenitors in the presence of vitamin D led to an increased expression of integrin $\alpha_v\beta_3$ on the cell surface [59]. This integrin is known to be expressed on the membrane of osteoclasts and their progenitors and to promote osteoclast progenitors' attachment to the bone matrix through binding to vitronectin receptors and initiate osteoclast differentiation [25], [35], [59]. Besides, vitamin D was shown to enhance the formation of multinucleated TRAP-positive osteoclasts through increasing RANK expression in osteoclast progenitor cells [34], [60]. RANK is the receptor to which RANKL binds, which is needed for osteoclast differentiation and activation [61]. In our study, adding vitamin D only slightly promoted the formation of multinucleated cells. This effect was probably through inducing the expression of the integrin $\alpha_v\beta_3$ on monocyte surface and promoting the attachment and spreading of cells to the vitronectin coated surface. While most cells remained mononuclear, they seemed to migrate closer to each other, only missing a cue to fuse together. Vitamin D also increased TRAP activity even though most of the cells still seemed to be mononuclear monocytes. The TRAP release could be from mononuclear TRAP-positive cells which might be cells on the pathway to a multinucleated phenotype [62].

Cholesterol is one of the major components of biological membranes which affects their structure and function [63]. Cholesterol is insoluble in water, and it can be imported to the cells through LDL. LDL is a complex particle with a hydrophobic core of cholesterol and triglycerides which is surrounded by a hydrophilic membrane consisting of phospholipids, free cholesterol, and apolipoproteins [64]. LDL can enter cells via LDL receptors and upon its degradation, cholesterol can be released into the cytoplasm [64]. The regulation of cholesterol by LDL plays an important role in osteoclasts. It has been shown that depletion of LDL from serum impaired cellular membrane fusion events and osteoclast formation

in vitro, while osteoclast formation was restored by adding LDL to the LDL-depleted serum [36], [37], [63]. In our study, when LDL was added as a supplement, multinucleated TRAP-positive cells were formed. Furthermore, TRAP activity of cells and their resorption capability indicated the formation of active osteoclasts in the complete serum substitute medium, with values comparable to FBS supplemented medium which contains not only LDL but also other types of lipoproteins such as high-density lipoprotein (HDL) [65], [66].

A major limitation of the current study is that FBS, an ill-defined cell culture supplement, has been used as the gold standard/control of the experiments. This means that the outcomes of the serum substitute medium have been compared to the outcomes collected from FBS containing medium which we stated ourselves could be unreliable due to the nature of the FBS and can be different in case of using different FBS batches/brands.

It should also be noted that the components of serum substitute medium did not induce osteoclastic differentiation in some of the investigated donors. The serum substitute medium might need further optimization. Various other molecules have been reported to have an influence on the differentiation of monocytes into multinucleated osteoclasts which were not investigated in the current study, for example vascular endothelial growth factor (VEGF) [62], [67], [68], platelet-derived growth factor-BB (PDGF-BB) [69], [70], low concentrations (less than 10 nM) of dexamethasone [71], or epidermal growth factor (EGF) [72]. All these factors have been shown to promote osteoclast differentiation and survival. The benefit of adding any of these factors to our serum substitute medium should be investigated in future studies. Our new serum substitute medium for osteoclast cultures might help shed light on their precise functions on osteoclastogenesis of human monocytes without the overshadowing effect of the complex FBS composition.

5.5 Conclusion

In this study, we have developed a serum substitute medium with potentials to promote the osteoclastogenesis of human PBMC derived monocytes. The developed serum substitute medium was able to support the formation of resorbing TRAP-positive multinucleated osteoclasts from human PBMCs derived monocytes, however it is still less optimal than medium containing FBS and needs to be more optimized in future studies. The serum substitute medium has the potential to eliminate the use of FBS when studying osteoclast differentiation and activity and provides an opportunity to study the effect of many other soluble factors on the bone resorption process without being dominated by unknown components of FBS.

Acknowledgement

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Chapter 6

Matrix vesicles: Role in bone mineralization and potential use as therapeutics

This chapter is based on:

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Abstract

Bone is a complex organ maintained by three main cell types: osteoblasts, osteoclasts, and osteocytes. During bone formation, osteoblasts deposit a mineralized organic matrix. Evidence shows that bone cells release extracellular vesicles (EVs): nano-sized bilayer vesicles, which are involved in intercellular communication by delivering their cargoes through protein–ligand interactions or fusion to the plasma membrane of the recipient cell. Osteoblasts shed a subset of EVs known as matrix vesicles (MtVs), which contain phosphatases, calcium, and inorganic phosphate. These vesicles are believed to have a major role in matrix mineralization, and they feature bone-targeting and osteo-inductive properties. Understanding their contribution in bone formation and mineralization could help to target bone pathologies or bone regeneration using novel approaches such as stimulating MtV secretion *in vivo*, or the administration of *in vitro* or biomimetically produced MtVs. This review attempts to discuss the role of MtVs in biomineralization and their potential application for bone pathologies and bone regeneration.

6.1 Introduction

Bone is a multifunctional organ that is maintained through lifelong remodeling by the bone-forming osteoblasts, bone-resorbing osteoclasts, and the bone-regulating osteocytes. These cells reside on/in a composite matrix, comprising mainly the organic collagen type 1 and the mineral hydroxyapatite which are highly organized at multiple hierarchical levels [1]. This matrix and its organization give bones their remarkable mechanical properties and support their function as a storage for calcium and phosphate ions [2].

Osteoblasts are responsible for the creation of the mineralized organic matrix. They produce collagen type 1, which is considered to be the template for mineral nucleation and mineral crystal growth [3]–[5]. After mineral precursors have entered the collagen gap region, hydroxyapatite crystals grow outside the dimensions of the fibril, forming an interconnected continuous cross-fibrillar pattern [6]. This mineralization is believed to be regulated by osteoblasts via indirect and direct mechanisms [7]. The indirect mechanism involves the synthesis of negatively charged non-collagenous proteins which are believed to be associated with the collagen gap region where they may direct mineral precursors into the collagen fibril [8], [9]. The direct mechanism is thought to be associated with extracellular vesicles (EVs) produced by osteoblasts [7], [10].

Osteoblast-derived EVs, similar to other EVs produced by different cell types, are phospholipid-enclosed nanoparticles containing a variety of lipids, proteins and nucleic acids. There is increasing evidence that osteoblast-derived EVs have a multitude of functions, including the promotion of osteogenic differentiation, inducing osteoclast formation, and mineralization of the organic matrix [7], [10]–[17]. In general, vesicles involved in matrix mineralization are referred to as matrix vesicles (MtVs); they can bind to the collagen matrix and are equipped with mineralization-specific components such as phosphatases, calcium, and inorganic phosphate [10], [18], [19]. These calcium and phosphate ions can remain amorphous or can crystalize inside the MtVs into hydroxyapatite. Upon crystallization, they can break through the vesicle's membrane to form a mineral nodule [20].

Besides MtVs' essential role in mineralization of the organic bone matrix, they also have shown innate osteo-inductive properties [17]. In addition, chondrocyte- and osteoblast-derived MtVs are involved in endochondral ossification; a process which is part of natural bone regeneration [21]. This indicates their therapeutic potential for bone pathologies and fracture healing. Accordingly, osteoblast-derived EVs, from which MtVs are likely a significant part, were already proposed as a promising therapeutic for osteoporosis and fracture healing [17], [22]–[24]. In this review article, the roles of EVs—and more specifically, MtVs—in bone and the mineralization of the organic matrix are summarized. Current methods for the isolation and characterization of MtVs are discussed. An overview of the current therapeutic applications of MtVs for bone disorders, and possible future applications of (biomimetic) MtVs, are presented.

6.2 EV Subtypes, Biogenesis, and Their Biological Potentials

The term "extracellular vesicles" was chosen in September 2011 as a generic term by the International Society for Extracellular Vesicles (ISEV), a group of scientists with collective long-term expertise in the field of EV biology [25]. EVs stand for particles naturally released or secreted from prokaryotic and

eukaryotic cells that are delimited by a lipid bilayer [25]–[27]. The first use of an "extracellular vesicle" in the title of a scientific publication was in 1971, when Aaronson et al. showed that the eukaryotic alga *Ochromonas danica* could produce a large variety of small and large intra- and extracellular membrane-bound vesicles. These vesicles were recovered in the centrifugates of the cell-free *Ochromonas danica* after ultracentrifugation [28]. In 1967, Peter Wolf had found that fresh plasma freed of intact platelets through ultracentrifugation contained particulate material which he called platelet-dust. This platelet-dust was the first scientific appearance of platelet-derived EVs [29].

EVs are found in most biological fluids; for example, cell culture supernatant, blood, urine, saliva, amniotic fluid, milk, synovial, and seminal fluids [30]–[32]. Furthermore, EVs can be extracted from tissues such as brain, tumor, bone, and cartilage [21], [33]–[36]. In general, EVs are heterogenous in their size and morphology, and they can contain a variety of organic and inorganic cargoes such as lipids, proteins, genetic materials (DNA/RNA), and minerals derived from their parent cells [20], [25], [26], [37].

6.2.1 Biogenesis of EVs

Consensus has not yet emerged on specific markers of EV subtypes; therefore, assigning an EV to a particular biogenesis pathway remains a challenge. Nevertheless, based on the current knowledge in the field, EVs can be classified into three subtypes: (i) plasma membrane-derived ectosomes (shedding microparticles/microvesicles); (ii) endosome-originated exosomes; and (iii) apoptotic bodies (Figure 6.1) [32], [37].

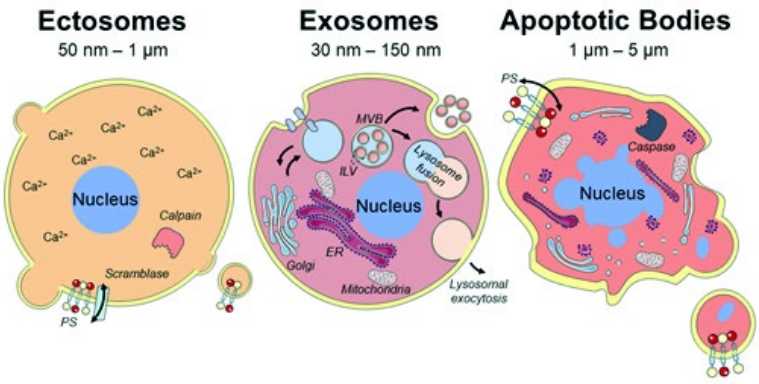

Figure 6.1 Subtypes of extracellular vesicles (EVs) based on their possible biogenesis pathways. EVs can appear as ectosomes that bleb from the cell membrane, as exosomes that are formed inside the cell after endocytosis, or as apoptotic bodies that derive from cells undergoing apoptosis. MVB, multi vesicular body; ILV, intra luminal vesicle; ER, endoplasmic reticulum; PS, phosphatidylserine. The figure was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (http://smart.servier.com/, accessed on 20 January 2021).

6.2.1.1 Plasma Membrane-Derived Ectosomes

Ectosomes are produced via the outward budding of plasma membranes [32], [38]. These vesicles are often generically referred to as microvesicles/microparticles, although they are extremely heterogeneous in size, ranging from exosome-like EVs of 50 nm to microvesicles as large as 1 μm [37]–[39]. An increased influx of calcium ions in the cell, which may be triggered after a plasma membrane injury, seems to initiate the biogenesis of ectosomes and generate heterogeneity in their sizes and cargoes [40], [41]. The increase in cytosolic calcium concentration can activate the proteins scramblase and calpain [42]. The activation of scramblase leads to a loss of membrane phospholipid asymmetry and exposure of the negatively charged phospholipid phosphatidylserine (PS) [43]. The activation of calpain leads to the calcium-dependent degradation of various proteins, allowing the outward budding of ectosomes from the plasma membrane [38], [43], [44]. Additionally, an increase in cytosolic calcium due to plasma membrane injury may trigger the endosomal sorting complex required for transport (ESCRT) machinery, which in turn results in ectosomes being released during excision of the injured membrane [40], [45]–[47].

6.2.1.2 Endosome-Originated Exosomes

The biogenesis of exosomes begins with the inward budding of small parts of the plasma membrane containing several membrane protein components and the formation of early endosomes. Upon inward budding of the plasma membrane, the extracellular proteins/molecules are encapsulated into small vesicles. These vesicles fuse with early endosomes which serve as the focal point of the endocytic pathway. At this stage, there is also continual fusion of transport vesicles containing newly synthesized molecules from the trans-Golgi network. The early endosomes then become mature and transform into late endosomes [48], [49]. Molecules are also sorted into smaller vesicles that bud from the perimeter membrane into the endosome lumen, forming intraluminal vesicles (ILVs). This leads to the multivesicular appearance of late endosomes known as multivesicular endosomes or multivesicular bodies (MVBs) [50], [51]. When molecules are destined for degradation inside the cell, MVBs fuse with lysosomal membranes and release ILVs into lysosomes for degradation. In some cases, for instance when there is an increased influx of calcium ions into the cytosol due to plasmalemmal damage and tissue remodeling, lysosomes can also move towards the plasma membrane, fuse, and release their intraluminal vesicle cargoes [52]. When molecules are destined for recycling outside the cell, instead of fusing with lysosomes, MVBs can also fuse with the associated plasma membrane. ILVs are then released extracellularly as exosomes, small EVs with a diameter of around 30–150 nm, which carry the cargoes of the MVBs [31], [53]. What defines the choice for degradation or recycling of the cargoes of MVBs is unknown and is likely related with the multiple machineries proposed in the biogenesis of ILVs for MVBs [32], [54]. The ESCRT is the most extensively described pathway of MVB biogenesis, responsible for the sorting of ubiquitinated proteins into ILVs [51], [53]. Several ESCRT-associated proteins such as PDCD6IP, tumor susceptibility gene 101, and heat shock protein 70 have been used to identify exosomes [31], [53], [55]–[60]. Interestingly, sorting of exosomal cargoes into MVBs can also occur in an ESCRT-independent manner, which seems to be driven by the presence of certain lipids, such as lysobisphosphatidic acid and ceramides, within the endosomal membrane [53], [61]. These lipids might organize into specialized endosomal regions, bend inward, and ultimately

form vesicles enriched in tetraspanins such as CD9, CD63, CD81, and CD82 [61]. Whether each pathway acts in different MVBs, or if they simultaneously act on the same MVB, is not known.

6.2.1.3 Apoptotic Bodies (ApoBDs)

Unlike other types of EVs such as exosomes and ectosomes, ApoBDs with sizes between 1 and 5 µm are generated only via budding of the plasma membrane of cells undergoing apoptosis which include caspase activation and DNA fragmentation [62]–[64]. Like ectosomes, ApoBDs expose PS on the outer leaflet of their membrane and retain cell type-specific markers [62], [64]. It is thought that ApoBDs are released during the late stages of cell death, whereas ectosomes are released during the early stages of apoptosis [42], [62], [65]. Some ApoBDs, thus, contain DNA and nuclear proteins such as histones, which can be useful markers to distinguish ApoBDs from other EV subtypes [62], [64].

6.2.2 Biological Potentials of EVs

Although initially considered as inert cellular debris, EVs are now recognized as being important mediators in intercellular communication and many biological processes [31], [32], [66]. It has been shown that the concentration, composition, and cellular origin of EVs in body fluids differ between healthy subjects and patients suffering from diseases such as cancer, and cardiovascular and inflammatory diseases [31], [67]–[72]. Therefore, there is a growing scientific and medical interest in EVs as valuable biomarkers in diagnostics and as therapeutics.

6.2.2.1 Use of EVs in Diagnosis

EVs are present in various body fluids at relatively high numbers—for example, blood plasma contains more than 10,000 EVs per milliliter—therefore, their enumeration in body fluids offers quantitative advantages [73]. Furthermore, obtaining body fluids to isolate EVs for diagnostic purposes is less invasive and relatively low-cost compared to obtaining tissue biopsy. Thus, many clinicians and biotechnology companies are attracted to research and develop EVs as a “liquid” biopsy [74]. One of commercialized EV-based diagnostics is ExosomeDx™. This is a non-invasive urine test for prostate cancer initially developed by Exosome Diagnostic, and now commercialized by Bio-Techne [75].

6.2.2.2 Use of EVs for Therapy

EVs are naturally derived from cells and carry various molecules and lipids which are targeted at other specific cell types. This indicates that EVs are exploitable for therapeutic purposes to target specific cells or tissue components [76]–[79]. Similar to nanoparticles, EVs (particularly exosomes and ectosomes) are in the nanometer range; they can be loaded with drugs or other inorganic particles [79]–[83]. The added value of EVs as drug nanocarriers is that EVs are not foreign to the host immune system because they are naturally derived. Therefore, EVs would overcome the issues of toxicity as well, which can occur when synthetic biomaterial nanoparticles are used to deliver drugs to target cells [84]. Recently, the use of EVs as therapeutic nanocarriers in the clinical settings have been reviewed [85]–[90]. One example of a successful EV-based therapeutic is Bexsero®, a meningitis serogroup B vaccine, which was approved in 2013 by the European Medicines Agency and the U.S. Food and Drug Agency. This vaccine contains EVs, referred to as outer membrane vesicles (OMVs), derived from Gram-negative bacteria *Neisseria meningitidis*. This vaccine is used for the prevention

of meningococcal disease caused by *Neisseria meningitidis* group B bacteria in individuals from two months old through to 25 years of age [79], [91].

6.2.3 EVs Derived from Bone Cells

The skeletal system functions and maintains itself based on communication between cells of various origins. Osteoblasts and osteoclasts, responsible for bone formation and resorption, respectively, are of importance for bone homeostasis. Thus far, the communication between osteoblasts and osteoclasts has been suggested to occur at the protein level via a direct contact (i.e., membrane-bound ligands and gap junctions), secreted cytokines, and deposited growth factors in the bone matrix [12], [92]. However, it has also been reported that osteoblasts and osteoclasts can communicate via an indirect contact through EVs. Such communication regulating bone remodeling can, for example, happen through the interactions with ligands that are present on the EV's surface or by transferring EV cargoes exchanging EV enclosed genetic information, such as microRNAs (miRNAs) [22], [92]–[96].

Cappariello et al. found that osteoblasts pre-treated with parathyroid hormone (PTH) generated EVs carrying the receptor activator of nuclear factor kappa-B ligand (RANKL) and demonstrated that these EVs supported the survival of osteoclasts *in vitro* [22]. *In vivo*, intraperitoneal injection of EVs from wild-type osteoblasts into RANKL−/− mice lacking tartrate-resistant acid phosphatase (TRAP) expression increased the presence of TRAP-positive cells in trabecular bone, which is indicative of neo-osteoclastogenesis [22], [97]. Mature osteoblasts also release EVs with specific characteristics involved in matrix mineralization. These EVs are anchored to protein components of the surrounding extracellular matrix and are known as MtVs [20], [98]. Their contribution to bone mineralization is discussed in Section 6.3.

Osteoclasts and their precursors have also been described to generate EVs with a diameter between 25 and 120 nm, similar to the size of exosomes [96]. Their membranes are enriched with epithelial cell adhesion molecule, CD63, and RANK. Interestingly, RANK-rich osteoclast-derived EVs act as inhibitors of osteoclastogenesis through competitively decreasing the RANK–RANKL interaction with, e.g., osteoblasts [96], [99]. During bone remodeling, osteoclasts undergo apoptosis at the end of the bone resorption phase, and produce large amounts of ApoBDs [100]. These vesicles promote osteogenesis via RANKL reverse signaling. These studies show that the maintenance of bone homeostasis relies heavily on cellular communication between osteoclasts and osteoblasts through the RANKL interactions; EVs are likely to play a role in this process.

It also has been shown that communication between osteoclasts and osteoblasts is mediated by the transfer of miRNAs which are contained within EVs. Through direct incubation of osteoclast-derived exosomes containing miR-214 with osteoblasts *in vitro*, osteoblast activity was inhibited [95], [101]. In the case of osteoblasts, 43 miRNAs were found to be highly abundant in mineralized exosomes of the MC3T3-E1 pre-osteoblast cell line [93]. Those osteogenic miRNAs were able to promote bone marrow stromal cell (ST2) differentiation into osteoblasts [93]. Osteoblasts released EVs carrying miR-125b and miR-503 with anti-osteoclastogenic activity [102], [103]. Interestingly, it has been shown that EVs carrying miR-125b could prevent bone loss in a mouse model of post-menopausal

osteoporosis [102]. Thus, EVs carrying miR-125b could be able to inhibit bone resorption, emphasizing the role of EVs in bone remodeling.

Osteocytes, the most abundant cellular component of mature bone, are terminally differentiated osteoblasts, which reside deep within the bone matrix [104]. The osteocytes orchestrate the actions of both osteoblasts and osteoclasts through relaying external mechanical signals, to trigger the deposition or resorption of bone possibly via the expression of osteoprotegerin (OPG) and RANKL [105]. It has been shown that osteocyte mechanosensitivity is encoded through unique intracellular calcium dynamics [41]. Upon fluid flow, osteocytes showed a transient increase in intracellular calcium ions and these cells released a substantial amount of EVs containing bone regulatory proteins such as sclerostin, RANKL, and OPG into the culture medium [41]. Osteocytes also released EVs containing miR-218, which inhibited sclerostin and influenced the differentiation of osteoblasts [106]. Interestingly, the miR-218 contained in these EVs can be suppressed by myostatin secreted by muscles [106]. These results indicate possible bone–muscle communications.

Summary of the bone cell populations producing EVs including their cargoes and functions can be found in Table 6.1.

Table 6.1 Cargo and function of EVs derived from bone cells.

Cell source of EVs	Cargo of EVs	Function of EVs
Osteoblast	1. RANKL [22], [97]; 2. miR-1192, miR-680 and miR-302a [93]; 3. miR-125b and miR-503 [102], [103].	1. Supports survival of osteoclasts *in vitro* and neo-osteoclastogenesis *in vivo*; 2. Promotes osteogenic differentiation, as manifested by the up-regulated expression of osteogenic marker genes RUNX-2 and ALP, as well as enhanced matrix mineralization; 3. Have anti-osteoclastogenic activity.
Osteoclast	1. RANK, EpCAM, CD63 [96], [99]; 2. miR-214 [95], [101].	1. Maintain bone homeostasis through the RANK–RANKL interaction; 2. Inhibits osteoblast activity *in vitro*.
Osteocyte	1. LAMP1, sclerostin, RANKL, and OPG [41]; 2. miR-218 [106].	1. Attenuate bone formation *in vivo*; 2. Inhibits sclerostin and influences the differentiation of osteoblasts.

RANKL, receptor activator of nuclear factor kappa-B ligand; miR, microRNA; RUNX-2, runt-related transcription factor 2; ALP, alkaline phosphatase; RANK, receptor activator of nuclear factor kappa-B; EpCAM, epithelial cell adhesion molecule; LAMP1, lysosomal-associated membrane protein 1; OPG, osteoprotegerin.

6.3 MtVs and Their Contribution to Bone Mineralization

MtVs are a subgroup of EVs. They were first detected in cartilage and bone through electron microscopy methods [33], [34], [107]. They range in size from 10–400 nm and are considered to derive from mineralization-mediating cells such as chondrocytes, osteoblasts, and odontoblasts [21], [35]. Similar to EVs, their biogenesis can most likely be through multiple distinct pathways. Therefore, unlike other types of EVs (e.g., exosomes or ectosomes), MtVs cannot simply be classified based ontheir size or biogenesis mechanism. Instead, MtVs are normally referred to by their function and location in the extracellular matrix of mineralized tissues.

Most MtVs are electron-dense and equipped with mineralization supportive proteins [10], [18], [108]–[110]. It is believed that these MtVs accumulate calcium and inorganic phosphate and, upon supersaturation, precipitate into needle-like hydroxyapatite [$Ca^{10}(PO^4)^6(OH)$] crystals whose growth eventually disrupts the MtVs' membrane [35], [109]. Under transmission electron microscopy (TEM), these crystals feature spicules or a ribbon-like structural profile, approximately 25 nm wide, 10 nm high, and 50 nm long [20]. Subsequently, their growth can continue in the extravesicular space to form more stable crystals, or to propagate on the collagen fibrils [18], [35], [111]. Some MtVs are electron-lucent and are referred to as empty MtVs [112], [113]. Whether these MtVs will still gradually accumulate calcium and phosphate over time or whether they remain electron-lucent is unknown.

6.3.1 MtV Biogenesis and Characteristics

Currently, MtVs are often only described as microvesicles or ectosomes which are formed by budding from the plasma membrane of their parent cell [21], [35]. However, once MtVs are released from their parent cell, assigning them to a distinct biogenesis pathway is extraordinarily difficult unless the vesicle is caught in the act of being released; for example, by live imaging techniques [25]. Multiple MtV biogenesis mechanisms have been reported. Probably, these different biogenesis mechanisms also will result in some specific characteristics of the MtVs. However, most studies only focus on the biogenesis or the characterization of MtVs, making it difficult to couple specific MtV characteristics to a specific biogenesis pathway. Here, we describe the characteristics, as reported in the literature, for the different biogenesis pathways of MtVs (Figure 6.2 and Table 6.2).

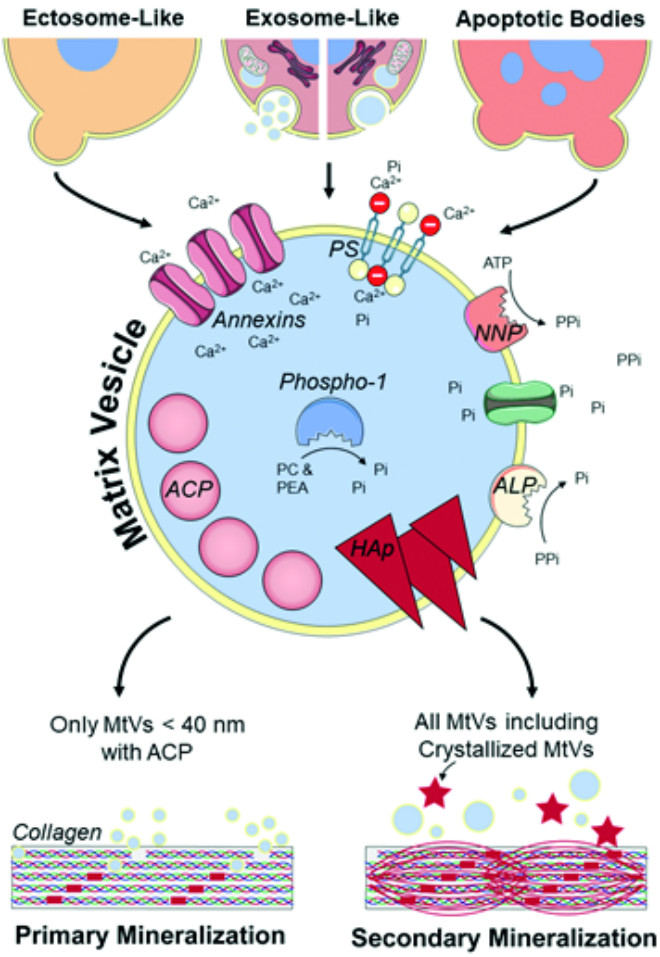

Figure 6.2 Possible MtV biogenesis pathways and collagen mineralization mechanisms. MtV, matrix vesicle; ALP, alkaline phosphatase; NNP, nucleotide pyrophosphatase; PS, phosphatidylserine; ACP, amorphous calcium phosphate; HAp, hydroxyapatite. The figure was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License. (http://smart.servier.com/, accessed on 20 January 2021).

6.3.1.1 Ectosome-Like MtVs

There is morphological evidence indicating that some MtVs are formed by budding and then pinching off from the plasma membrane of cells mediating mineralization [21], [35]. To be able to accumulate calcium and inorganic phosphate, the membranes of these MtVs are enriched with mineralization-related proteins (Figure 6.2). These include PS-binding annexin proteins and phosphatases [18], [114]–[117]. Alkaline phosphatase (ALP) on the MtV's membrane, which is also present on the membrane of osteoblasts, dephosphorylates pyrophosphate [18]. Phosphate transporters can subsequently transfer free phosphate into the MtVs [18]. Nucleotide pyrophosphatase-1 (NNP-1) can also be found on the plasma membrane of MtVs and their parent cells. This enzyme frees pyrophosphate from adenosine triphosphate (ATP), indirectly providing a new phosphate source for the MtV [118]. However, pyrophosphate also functions as an inhibitor of mineralization [119]. Therefore, ALP and NNP-1 are believed to control the pyrophosphate/inorganic phosphate ratio that is needed for physiological matrix mineralization [18]. In addition to these phosphatases on the MtV's membrane, Phospho1 inside the vesicle might be involved in MtV mineralization by producing phosphate from phosphocholine and phosphoethanolamine [118], [120]. Besides these phosphatases, the high level of PS on the plasma membrane of MtVs indicates that PS is an important contributor to the mineralization process as well; their potential function is described in Section 6.3.2. [42]. Annexin proteins (annexin A2, A5, and A6) are also present at substantial concentrations on the MtV membrane [114], [121], [122]. Annexin A5, which can strongly bind to PS, increases the permeability of MtVs for calcium [114], [121]. However, the function of annexins seems to not be critical for mineralization; bone development was not impaired in annexin A5 and A6 knockout mice [123], [124].

6.3.1.2 Exosome-Like MtVs

More recently, evidence was found for the formation of MtVs intracellularly through an exosome-like mechanism [125]–[127]. This process was first described by Boonrungsiman et al., who found that there is transport of calcium and phosphate ions from mitochondria to intracellular vesicles that support mineralization [7]. These mitochondria are likely provided with calcium and phosphate from the endoplasmic reticulum [127]. The amorphous calcium phosphate-containing mitochondria can fuse with lysosomes, becoming autolysosomes, where they subsequently undergo mitophagy [125], [127]. Within the lysosomal compartment, membrane components of the mitochondria are degraded, and amorphous calcium phosphate is freed in the autolysosome which could be released via exocytosis in the extracellular matrix as an MtV [125]. Due to the acidic environment within lysosomes, amorphous calcium phosphate crystallization is prevented [126]. Thus, lysosomes seem to fulfil the role of intracellular transporters of amorphous calcium phosphate-containing MtVs to the plasma membrane [126]. At early stages of osteoblastic differentiation, vacuolization of mitochondria was observed, probably leading to the formation of MVBs, and thus smaller and multiple MtVs from one mitochondrion [126], [127]. Whether these exosome-like MtVs could be identified upon release from their parent cell and how exactly they differ from the ectosome-like MtVs described earlier needs more detailed investigation.

6.3.1.3 ApoBDs Involved in Mineralization

Mineralization often also occurs with apoptosis. For example, in growth plates where terminally differentiated chondrocytes undergo apoptosis, this process results in the formation of ApoBDs [121]. This is also thought to be an important initiator of vascular calcification [128]. These ApoBDs are also able to accumulate calcium and phosphate, but they contain only a few calcium channel-forming annexins [121]. Unlike ectosome-like MtVs, ApoBDs do not need annexins to induce mineralization, because they do not accumulate calcium inside the vesicle [129]. ApoBDs are rich of externalized PS, which could function as mineral nucleation sites because they can stabilize calcium and phosphate [130]. Thus, ApoBDs seem to accumulate calcium and phosphate on their membrane.

ApoBDs also seem to influence mineralization indirectly, by enhancing ALP and inhibiting NNP-1 exposure on the membranes of endplate chondrocytes [131]. This results in more available inorganic phosphate, which promotes mineralization [131]. Taken together, because MtVs exhibit multiple characteristics that are involved in calcium and phosphate accumulation, all MtVs may contribute to matrix mineralization regardless of their biogenesis pathway. Our findings on the different types of MtVs and their physical, biological, and functional properties as described in the previous sections are summarized in Table 6.2.

Table 6.2 Different types of MtVs and their physical, biological, and functional properties

Biogenesis	Physical properties	Biological properties	Functional properties
Ectosome-like	~50 nm–1 μm	-Rich in PS [114] -Rich in Annexins [115], [116] -Exhibit membrane proteins and phosphatases from their parent cells (e.g., ALP, NNP-1) [18] -Accumulate calcium and phosphate internally upon release from their parent cell [18] -Calcium and phosphate can crystalize into HAp which can grow and disrupt the vesicle's membrane to form a nodule [20]	Most likely secondary or extrafibrillar collagen mineralization
Exosome-like	~30 nm–150 nm	-Receive calcium and phosphate intracellularly from the ER via mitochondria [7], [125], [127] -Likely transported to the matrix by lysosomes that provide an acidic environment [126], [127] -Acidity prevents crystallization of ACP [126]	Primary or intrafibrillar and secondary or extrafibrillar collagen mineralization [127]
Apoptotic bodies	~1 μm–5 μm	-Rich in PS [129] -Accumulate calcium and phosphate externally [130]	Vascular calcification [128] and Endochondral ossification [128], [129], [131]

MtV, matrix vesicle; ALP, alkaline phosphatase; NNP, nucleotide pyrophosphatase; PS, phosphatidylserine; ACP, amorphous calcium phosphate; HAp, hydroxyapatite.

6.3.2 Potential Collagen Mineralization Mechanisms

MtVs can bind to the collagen matrix through, e.g., annexins and ALP [19]. How MtVs subsequently interact with the collagen matrix and how this leads to intra- or extrafibrillar mineralization, is still largely unknown. Most likely, primary intrafibrillar mineralization of newly formed collagen is regulated by MtVs smaller than 40 nm containing amorphous calcium phosphate, which could enter the collagen gap region (~40 nm) (Figure 6.2) [7]. These vesicles were found to be secreted by mid-to-late stage differentiated osteoblasts [17]. In the later stage of osteogenic differentiation, needle-like crystals were found, probably matured MtVs from which the membrane was disrupted by the crystals [17]. These membrane-free crystals were less able to induce mineralization than the vesicles with amorphous calcium phosphate [17]. This indicates that the vesicle's membrane has an important role in inducing primary or intrafibrillar mineralization (i.e., in the collagen gap region), and that these crystallized MtVs probably only support secondary or extrafibrillar collagen mineralization (Figure 6.2). In this regard, ectosome-like MtVs and mineralizing ApoBDs are rich with externalized PS, creating a more negatively charged vesicle membrane [16], [42]. This PS on the vesicle's membrane can stabilize calcium and phosphate [130]. Interestingly, negatively charged non-collagenous proteins are believed to form these complexes as well, and their importance for mineralization is indisputable [9]. This might suggest that externalized PS could help, as non-collagenous proteins do, to guide MtVs to the collagen gap-region [9]. Most likely, MtVs larger than 40 nm also contribute to secondary collagen mineralization, although their exact role has yet to be elucidated. Thus, MtVs can vary in (membrane) composition, calcium phosphate crystallinity, and size, depending on the biogenesis of the vesicle and the differentiation stage of their parent cell. Probably, their function to induce primary intrafibrillar or secondary extrafibrillar mineralization is defined by these characteristics.

6.4 Isolation and Characterization of MtVs

To understand the role of MtVs during the mineralization process, attempts have been made to isolate these vesicles, separate them from other EVs, and characterize them based on their physical and biological properties and their functionality. The approaches to isolate and characterize MtVs are discussed in this section.

6.4.1 Isolation of MtVs

For the isolation of MtVs, centrifugation-based methods are commonly used. Around two-thirds of the reviewed papers which included MtV isolation in their methodology used differential centrifugation (DC) combined with a final ultracentrifugation (UC) (>10,000g) step [17], [102], [107], [132]–[134]. Even though DC is most often used to isolate MtVs, the exact execution differs in the number of steps, centrifugation speed, and centrifugation time used. Other common strategies for isolating MtVs include DC/UC in combination with ultrafiltration [23], [135]–[139]. Size-exclusion chromatography, fluid flow fractionation, polymer precipitation, immunoaffinity isolation, and microfluidic-based technologies are often used to isolate and/or concentrate EVs, although none of these techniques has been reported to be used for the isolation of MtVs [140]. The size and some

biological characteristics of MtVs overlap with those of other EVs; therefore, the isolation techniques used for EVs may also be suited for isolating MtVs.

Hutcheson et al. hypothesized that time-dependent UC may enrich mineral-containing vesicles more than other vesicular populations, because of their greater physical density [141]. They found electron-dense vesicular structures to be associated with hydroxyapatite crystals with high ALP activity and mineralization potential in the pellet after only 10 minutes of UC of the conditioned media of calcifying coronary artery smooth muscle cells. Significantly, greater amounts of protein were pelleted after 10 minutes UC for the calcifying samples compared to the control samples. If the amount of protein per vesicle in both samples is approximately constant, they estimated that vesicles in the calcifying samples were 35% more dense than those in control samples. Shortening the time of UC decreased the contamination of other vesicle populations, but the number of calcifying vesicles was not increased. Hutcheson et al. suggested performing collagenase digestion of the cell culture prior to UC to increase the yield of MtVs [141].

Collagenase breaks down collagen fibers and is believed to release the MtVs that are embedded in the matrix [142], [143]. Therefore, to isolate MtVs from tissue, a digestion step with collagenase should be performed prior to MtV isolation [107], [132], [133]. MtVs can also be isolated from cell culture samples [17], [102], [144]. Some of the literature reported that a collagenase digestion step was added to release MtVs or mineralizing-EVs from the cell culture matrix [102], [134], [145]–[147]. To distinguish MtVs isolated from the cell culture matrix with the use of collagenase from MtVs isolated from the cell culture supernatant, the vesicles are sometimes referred to as collagenase-released matrix vesicles and medium matrix vesicles, respectively [35], [148]. The effect of collagenase digestion on EV characteristics still needs thorough investigation. There are indications that collagenase treatment prior to EV isolation results in an isolated EV population with higher mineralization potential, as indicated by more apatite-like mineral, higher ALP activity, and higher cholesterol/lipid content than isolation without collagenase treatment [148], [149]. Thus, collagenase treatment does not only improve the harvest of total EVs, but also yields MtVs with better mineralization potential.

6.4.2 Characterization of MtVs

In 2018, the ISEV introduced guidelines for EV experimental research [25], [26]. They recommend that for the preparation of EVs: (i) quantitative measures of the source are given; (ii) the number of EVs is estimated; (iii) the sample is tested for the presence of specific markers associated with EVs and EV subtypes; and (iv) the sample is tested for the presence of non-vesicular, co-isolated components. In this section, we discuss the characterization of MtVs based on these recommendations. Methods of characterization of MtVs are based on physical properties, biological properties, and functionality. There is no single perfect characterization method of MtVs; therefore, a combination of different methods is necessary.

6.4.2.1 Physical Properties

To increase the reproducibility of EV experiments and to allow for comparisons between studies, the source of the EVs should always be described in detail. For tissue derived MtVs, the tissue type, its

origin, condition, and preferably also the size and volume, are reported. When MtVs are collected from cell culture, a more elaborate source description is required. This includes the cell source, the number of secreting cells, cell passage, the types of culture medium, and the time between the last medium refreshing and EV isolation. MtVs can be obtained both from the culture supernatant or from the matrix with the use of collagenase; therefore, the isolation technique used needs to be described in detail in the methods section.

Besides the source of MtVs, their physical properties such as number, size, and morphology need to be reported. Nanoparticle tracking analysis (NTA) [41], [134], [136], [137], [143], [150], dynamic light scattering (DLS) [23], [135], and tunable resistive pulse sensing (TRPS) [139], [151] can be used to determine particle size and bulk concentration. MtVs, like other subtypes of EVs, are heterogenous in size, and some non-vesicular contaminants such as protein aggregates may be co-isolated and interfere with measurement; therefore, these techniques are not optimal for characterizing a single MtV and measuring the concentration of MtVs.

Singular vesicle size, topology, and morphology have been studied using atomic force microscopy (AFM) [135]. AFM has also been used in combination with antibody-coated surfaces to capture specific subsets of EVs and simultaneously characterize the single vesicle size and topology/morphology. This approach has been shown for the characterization of specific subsets of EVs derived from platelets [39]. It also has been demonstrated that AFM operated in peak force quantitative nanomechanical property mapping (AFM-PFQNM) was able to measure the nanomechanical and morphological properties of individual MtVs under both mineralizing (with addition of calcium ions) and non-mineralizing fluid conditions [152]. TEM is a common method to characterize the physical properties of MtVs, including the size and the crystals present inside MtVs [17], [23], [134], [144], [149]. When the characterization of certain surface proteins on MtVs is needed, immunogold labeling of MtVs prior to TEM can also be performed. This method was used, for example, by New et al., to visualize the release of CD68-positive MtVs from macrophages [153].

6.4.2.2 Biological Properties

The concentration of the total MtV can be estimated by measuring the total protein content of the sample. This is often assessed by a bicinchoninic acid (BCA) protein assay [93], [128], [135]. Similar to other subtypes of EVs, MtVs carry transmembrane proteins such as CD9, CD63, and CD81 [25], [26], [136], [139]. The presence of these proteins can be demonstrated with Western blotting and flow cytometry [23], [132], [148]. Besides general membrane proteins, other markers are used to characterize MtVs. The MtV membrane contains a high concentration of PS, which can be detected with flow cytometry by binding to annexin A5 conjugated to a fluorescent probe [138]. A high concentration of PS does, however, not mean that the isolated vesicles are indeed MtVs, because ectosomes and ApoBDs are generally rich in PS [154]. MtVs can also be characterized using markers which are present on their source cells. Nahar et al. found that MtVs from chondrocytes contain several annexins (A1, A2, A4, A5, A6, A7 and A11), BMPs (1–7), VEGF, osteopontin, osteocalcin, osteonectin, and bone sialoprotein [132]. Even though most markers will not be MtV-specific, combining several markers helps to better characterize MtVs. ISEV also recommends checking isolated EV samples for the presence of non-vesicular components which could be co-isolated [25]. There have

been several markers used to exclude non-vesicular components in MtV isolates such as GM130, which can normally be found at the Golgi membrane, β-tubulin as a cytosolic marker, and histone 1 as a nuclear marker [17], [155].

6.4.2.3 Functional Properties

MtVs can be identified based on their distinct functionality such as their ALP activity, and calcium and phosphate levels. The ALP activity from a sample of isolated vesicles can be measured with a p-nitrophenyl phosphate substrate [148]. The calcium and phosphate levels within the vesicles can also be determined. Calcium levels within MtVs can be measured by a colorimetric analysis, whereas phosphate levels can be measured by a modified version of the method defined by Ames using ascorbic acid [121], [129], [156], [157]. A unique property of MtVs is their ability to form crystals and to mineralize collagen. An *in vitro* mineralization assay to study the calcification potential of MtVs can be performed by adding MtVs to a collagen type 1 coated culture dish containing a calcification medium comprising 15% fetal bovine serum (FBS) and 10 mM β-glycerophosphate [158], [159]. However, it should be considered that FBS may contain ALP as well that might interfere with the mineralization assay results. Y. Kunitomi et al. embedded MtVs in a collagen hydrogel and incubated this gel in medium containing only 10 mM β-glycerophosphate [144]. The MtVs mineralized the collagen in the gel, but mineralization was more successful after fragmentation of the MtVs [144]. After a set incubation time, the amount of deposited calcium could be measured colorimetrically by the *O*-cresolpthalein complexone method [147]. Another slightly different method was used by Chaudhary et al., where isolated MtVs were incubated with calcium chloride and ATP as a phosphodiester substrate in a calcifying solution for 5.5 hours [134]. The assay demonstrated that MtVs accumulate calcium ions from the extravesicular environment in a dose-dependent manner. Thus, these functional assays are useful.

6.5 Therapeutic Potential of Osteoblast-Derived EVs and MtVs

EVs are considered complex biological mediators of tissue development and regeneration that may feature innate therapeutic potential for diseases/regeneration [10]. At present, research into the therapeutic application of EVs, and particularly MtVs, to treat bone diseases is in its infancy. This might be due to the complexity of MtV components and technical difficulties in the isolation and characterization of MtVs [17]. Only few bone-related clinical trials are registered, as revealed by a search in the United States National Library of Medicine [160]. Two clinical studies on EVs are documented, one on bone inflammation and one on osteoarthritis [161], [162]. Unfortunately, clinical trials on MtVs were not found. Nevertheless, interest is rapidly growing, with several publications outlining the potential therapeutic utility of EVs for the regeneration of a wide range of tissues, including bone [10].

Strikingly, therapeutic bone-related studies generally mention EVs, but not particularly MtVs. In fact, EVs comprise MtVs, and therefore these studies presumably included MtVs without characterizing them. Wei et al. specifically investigated the proportion of MtVs in EVs released by mineralizing MC3T3-E1 osteoblast precursors at different stages of differentiation using TEM. Their study showed

that MtVs accounted for a considerable proportion of EVs, and MtVs derived at different stages showed varying sizes and crystallinities [17].

6.5.1 Cellular Source of MtVs for Therapeutics

Various cell types can secrete MtVs with the potential to induce mineralization. Osteoblasts, chondrocytes, and odontoblasts are involved in biomineralization, and vascular smooth muscle cells (VSMCs) and macrophages could stimulate pathological calcification [163], [164]. MtVs secreted by these cell types show similar characteristics, such as the PS membrane containing annexin A2, annexin A5, and phosphatases, and the formation of hydroxyapatite inside the vesicles [146], [153], [165]. However, many other components/cargoes in MtVs likely affect their targeting potential.

Many studies have used mesenchymal stromal cell (MSC)-derived EVs [139], [166]. However, the advantages and disadvantages of a cellular source of EVs for certain therapeutic applications has not been investigated thoroughly [17], [167], [168]. It is probable that the cellular source of EV is essential for targeting certain tissue. For example, EVs derived from mineralizing osteoblasts were shown to possess innate bone-targeting potential [17]. These EVs were successfully labeled with fluorescent PKH67; when injected into the tail vein of mice, the fluorescence intensities of EVs in the femurs were strongly visible after two hours [17]. Thus, the use of osteoblast-specific EVs might be a more effective choice, because it has shown to better target bone tissue.

6.5.2 Potential Targets of MtVs for Bone Mineralization Disorders

Most studies that report osteoblast-derived EVs focus on osteoporosis or fracture healing [22]–[24]. For example, intravenous injection of EVs derived from osteoblasts in an osteoporotic mouse model significantly lowered bone loss as measured with micro-computed tomography [17]. In addition, the same study showed the osteo-inductive potential of MtVs visible by the induced expression of the osteogenic-related genes runt-related transcription factor 2 (RUNX-2), collagen type 1, and ALP in mesenchymal stromal cells cultured under growth conditions [17]. This osteo-inductivity makes MtVs an interesting target for bone regeneration applications.

Next to fracture healing and osteoporosis, mineralization of bone's organic matrix can be impaired in different ways causing various diseases; for example, hypophosphatasia, where low ALP activity leads to low levels of inorganic phosphate. Hypocalcemia or hypophosphatemia, on the other hand, are a result of low blood levels of calcium or phosphate, respectively [169]. Impaired mineralization can ultimately lead to the development of rickets (in children) or osteomalacia (in adults), i.e., the softening of bone, potentially causing skeletal deformities and severely influencing the quality of life of affected patients [169], [170]. Currently, treatments for these disorders include the supplementation of vitamin D and/or calcium (hypocalcemia), administration of the phosphate-regulating FGF23 antibody burosumab (hypophosphatemia), and enzyme replacement therapy (hypophosphatasia). Additional supplementation with MtVs to current treatments might be beneficial to achieve enhanced mineralization and thus to counteract the softening of the bones.

6.5.3 Potential Risk of the Application of MtVs

The mineralizing potential of MtVs also has a major drawback, when it occurs at unwanted locations in the body. This unwanted mineralization is featured in pathological conditions such as vascular calcification, often seen in diabetic, hypertensive, and/or chronic kidney disease patients [21], [164], [171], [172]. For example, the mechanism of initiation and progression of vascular calcification has been shown to be similar to physiological bone formation [164], [173]. A high extracellular phosphate concentration upregulates the inorganic phosphate transporter 1 (Pit1), raising intracellular levels of inorganic phosphate. This results in the activation of RUNX2, enhancing the osteogenic transition of VSMCs [163], [164], [174]. These osteogenic VSMCs produce MtVs that cause microcalcifications inside the vessel wall that play a pathological role in the onset and progression of vascular disease [164]. The similarity of the mechanisms of soft tissue calcification and bone mineralization indicate the need for extra caution when considering MtVs for therapeutic purposes in mineralization-related bone diseases.

6.6 Prospect for Therapeutic Applications of MtVs

To use MtVs for therapeutic applications, we propose three approaches (Figure 6.3): (i) stimulate MtV secretion *in vivo*, particularly at the location where the mineralization is needed; (ii) stimulate MtV secretion *in vitro* by stimulating MtV-producing cells; and (iii) engineer biomimetic MtVs. For the second and third approaches, MtVs and biomimetic MtVs need to be delivered to the region of interest either by localized injection or biomaterial implantation. These approaches will be discussed further in the next sections.

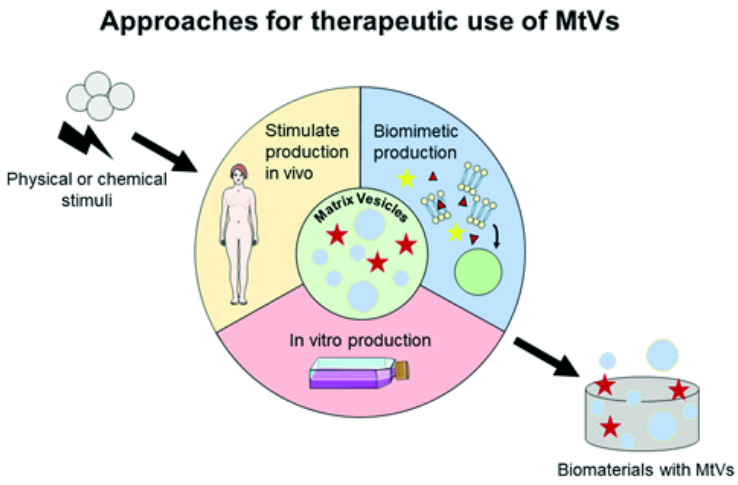

Figure 6.3 Possible approaches to use MtVs for therapeutics. MtV production can be stimulated in vivo by applying physical or chemical stimuli on MtV-secreting cells. Biomimetic or in vitro produced MtVs could be used in combination with biomaterials, for example. The figure was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (http://smart.servier.com/, accessed on 20 January 2021).

6.6.1 Stimulation of MtV Secretion *In Vitro* and *In Vivo*

To maximize the potential of MtVs for desired mineralization, it is important to identify factors which can influence the characteristics and/or the composition of MtVs [175], [176]. These factors could be used to guide MtV-producing cells to produce targeted MtVs *in vitro* and *in vivo* for therapeutics.

6.6.1.1 Chemical Stimuli for MtV Secretion

Calcium and phosphate, as the main components of bone's inorganic matrix, can trigger MtV secretion by MtV-producing cells. Treatment of osteoblasts with phosphate resulted in the secretion of MtV expressing high levels of ALP, Phospho1 and annexin A5, and containing hydroxyapatite crystals [134]. VSMCs and macrophages can also be stimulated with phosphate to induce MtV secretion which contained high levels of annexin A2, annexin A5, ALP and hydroxyapatite nucleation inside the vesicles [146], [153], [158]. Elevated calcium concentration, both extracellularly and intracellularly, can cause the secretion of MtVs and enhance mineralization as well [165], [177]. Exposure of VSMCs to calcium ions led to the increased production of MtVs, which were accumulated by annexin A6 on the outer surface of MtVs, upregulation of PS, and formation of crystalline hydroxyapatite associated with both the outer and inner membrane of MtVs [178]. In addition, the concentration of intracellular calcium ions could be increased by ionomycin [127]. This facilitated the formation of calcium phosphate granules in the endoplasmic reticulum, and the transport to the mitochondria which resulted in the formation of MtVs intracellularly.

The combination of calcium and phosphate has also been introduced to osteoblasts as a trigger for MtV secretion. For instance, synthesized calcium phosphate powders, comprising tricalcium phosphate and hydroxyapatite, induced the formation of spherical nodules containing calcium phosphate, indicating the formation of MtVs [179]. However, the formed MtVs were not fully characterized. Another study also demonstrated that the incubation of osteoblasts with hydroxyapatite nanoparticles resulted in the secretion of MtVs, as shown by TEM images [180].

The most biologically active vitamin D, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), which acts through binding to the vitamin D receptor, promotes osteoblast differentiation of MSCs and primary osteoblasts *in vitro* and *in vivo* [181], [182]–[185]. *In vitro* treatment of osteoblasts during the pre-mineralization phase with 1α,25-$(OH)_2D_3$ stimulates and accelerates matrix mineralization through increasing the number of secreted ALP-positive MtVs [186], [187]. Other components capable of increasing MtV secretion are glycosaminoglycans (GAGs), which are key organic components of the extracellular matrix and play an essential role in the development of bone tissue. It has been shown that GAGs including hyaluronic acid (HA) and its synthetically sulfated derivatives could promote the osteogenic differentiation of MSCs and result in the secretion of MtVs [188]. Furthermore, treatment of osteoblasts with sulfated HA derivative had great influence on the proteome of MtVs [110]. The affected proteins such as thrombospondin-1 and -2, fibrillin-1, latent transforming growth factor β-binding protein 2, and fibronectin-1 can regulate vesicle–extracellular matrix interactions and MtV activity, leading to extracellular matrix formation and mineralization [110], [189]. Besides the mentioned components, other factors have shown potential to enhance mineralization *in vitro* and *in*

vivo [190]; thus, they can potentially stimulate MtV-producing cells to secrete MtVs. The secretion of MtVs under the influence of such chemical stimuli should be investigated further.

6.6.1.2 Physical Stimuli for MtV Secretion

The secretion of MtVs under physical stimuli has not been thoroughly investigated, however stimuli such as mechanical forces, electrical and electromagnetic stimulation, ultrasound, shock wave and low-level laser therapy have been shown to promote bone regeneration *in vivo* and *in vitro* [191]–[193]. These physical stimuli led to increased osteogenesis and bone formation *in vivo*. For instance, pulsed electromagnetic field stimulation treatment reduced lumber vertebral osteoporosis by increasing bone formation and reducing bone resorption [194]. Whether these methods did induce the release of MtVs *in vivo* under physical stimulation has not been investigated. *In vitro* studies demonstrated that the application of these physical stimuli not only induced osteogenic differentiation, but also enhanced mineralization. This could be associated to the release of MtVs by the cells in culture. A recent *in vitro* study showed that static magnetic fields enhanced the osteogenic differentiation of MSCs and increased mineral formation through the release of MtVs into the extracellular matrix [195]–[197].

Overall, the MtV-producing cells can be chemically and physically stimulated, and induced MtV secretion *in vitro.* Secreted MtVs can be isolated and delivered to the site of interest via localized or systemic applications, for example, via injection. For both local and systemic delivery, a major challenge is to attain a suitable concentration of vesicles at the target site [144], [198]. The use of biomaterials with integrated MtVs could help in delivering the vesicles to the targeted region in an appropriate concentration and allows for a controlled release of vesicles in this region. The *in vivo* application of chemical and physical stimuli has showed increased mineralization; thus, the release of MtVs can potentially be enhanced in the applied area. Further studies should investigate the *in vivo* secretion of MtVs under such stimuli.

6.6.2 Engineering Biomimetic MtVs

Apart from using cell-derived MtVs, one could also synthetically engineer biomimetic MtVs for therapeutic applications. These engineered MtVs give the possibility to fine-tune the cargoes and increase the specificity of MtVs to a certain region for targeted biomineralization. In the past few years, several attempts have also been made to create MtVs to better understand MtV-mediated mineralization. For example, the incorporation of ion carriers to transfer phosphate and calcium ions into triblock copolymer vesicles has shown that it is possible to form minerals inside such vesicles [199]. These primary vesicles are still rather simple models, but the concept of mimicking MtVs artificially seems a promising strategy.

6.6.2.1 Proteoliposomes Mimicking MtVs

Proteoliposomes are lipid membranes with incorporated proteins that have been used as a tool for lipid–protein interactions studies, biotechnological applications, and the modeling of biological membranes [200]. They have been used to mimic MtVs to unravel the enzymatic activity of these

vesicles and their contribution to the biomineralization process [201]. Various different combinations of enzymes and proteins associated to MtVs, such as ALP, NPP1 and annexin A5, were inserted in the lipid membranes [122], [202]–[205]. It was found that the lipid composition could influence the formation of minerals and the activity of the enzymes or proteins [206], [207]. For instance, the addition of cholesterol to the lipid membrane of the vesicles had an influence on the incorporated ALP [208]. A higher concentration of cholesterol in the membrane resulted in the formation of a more rigid monolayer which hampered ALP incorporation. Nevertheless, the enzymatic activity of incorporated ALP was enhanced in membranes with higher concentrations of cholesterol [208], [209]. Moreover, a recent study has shown that the presence of cholesterol and sphingomyelin in the membrane of ALP-harboring proteoliposomes promoted amorphous calcium phosphate precipitation *in vitro* [210].

6.6.2.2 Polymeric Vesicles Mimicking MtVs

Lately, other strategies have been developed to mimic MtVs. In a recent study, high concentrations of serine were used to stabilize amorphous calcium phosphate (S-ACP), which was then mixed with polyethylene glycol (PEG) to form PEG-S-ACP nanoparticles [11]. These nanoparticles were added to polysorbate to form micelles carrying these nanoparticles to establish a model of MtVs carrying amorphous calcium phosphate *in vitro*. It has been shown that changes in pH and surface tension of these models caused two forms of minerals, including crystalline mineral nodules and amorphous calcium phosphate particles. When introducing these models to collagen fibers, the crystalline needle-shaped apatites deposited on the surface of the collagen fibrils led to extrafibrillar mineralization, while amorphous calcium phosphate particles released from MtV-mimicking micelles showed infiltration of particles into the fibrils and were deposited intrafibrillarly [11]. In another study, ultrasmall black phosphorus nanosheets were encapsulated in poly (lactic-co-glycolic acid) nanoparticles to develop biomimetic MtVs [211]. The surfaces of the nanoparticles were modified with osteoblast-targeting aptamers, which are single-stranded oligonucleotides that use distinct structures to specifically bind to target cells [212]. The aptamers direct the MtVs to bind to osteoblasts, whereas the black phosphorus enhanced the local concentration of inorganic phosphate which facilitated the mineralization process. These biomimetic MtVs have shown great potential in facilitating *in vivo* biomineralization and promoting new bone formation [211].

More research on engineered biomimetic MtVs could address several issues regarding the mineralization of organic bone matrix. Besides targeting mineralization, these biomimetic MtVs could serve as efficient tools for various applications, including studying enzymatic defects associated with mineralization-related bone diseases and the screening of small molecules capable of modulating enzymatic activity of MtVs for potential therapeutic applications. The therapeutic potential application of MtVs as described in the previous sections are summarized in Table 6.3.

Table 6.3 Approaches for therapeutic uses of MtVs.

Possible approaches to use MtVs	**Methods of MtV secretion or production**	**Methods for delivery of MtVs**
Stimulate MtV secretion *in vivo*	- Physical stimulation [194]	- Locally applied on the region of interest
Stimulate MtV secretion *in vitro*	- Physical stimulation [197] - Chemical stimulation [134,186,188]	- Localized injection - Biomaterial implantation
Engineer biomimetic MtV	- Proteoliposomes [201] - Polymeric vesicles [11], [211]	- Localized injection - Biomaterial implantation

6.7 Conclusion

MtVs are a subset of EVs that are secreted by, e.g., osteoblasts, chondrocytes, and odontoblasts, to directly induce mineralization of the organic matrix. Multiple biogenesis pathways are described for MtVs, and consequently, they are very heterogenous. Regardless of their biogenesis and heterogenicity, MtVs are all located in the extracellular matrix and all contribute to biomineralization through multiple mechanisms. In addition to their role in biomineralization, MtVs also feature innate bone-targeting potential and osteo-inductive properties. These features of MtVs imply their therapeutic potential in bone regeneration or for treating bone pathologies such as osteoporosis. The therapeutical use of MtVs is still in its infancy; therefore, further characterization of MtVs, their cargoes, and their biomineralization potentials are important. Finally, exploiting *in vivo* and *in vitro* approaches to generate MtVs including to design biomimetic MtVs will accelerate the research on MtVs for therapeutic purposes.

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Chapter 7

Matrix vesicles isolation from a three-dimensional *in vitro* bone model

This chapter is based on:

Sana Ansari, Lotte van Dasler, Yuana Yuana, Miguel Castilho, Keita Ito, Sandra Hofmann. Matrix vesicles isolation from a three-dimensional *in vitro* bone model, Submitted.

Abstract

Extracellular vesicles (EV) are nano-sized bilayer vesicles that are involved in biological functions and secreted by a wide variety of cells. Osteoblasts, the bone forming cells, can release a subset of EVs known as matrix vesicles (MtVs) which are believed to be involved in matrix mineralization and feature bone forming properties. We have previously developed a functional three-dimensional (3D) self-organizing co-culture of osteoblasts and osteocytes that represents the woven bone formation using human bone marrow MSCs (hBMSCs). The formation of highly mineralized matrix in this 3D *in vitro* woven bone-like construct could be partly associated to the release of MtVs by osteoblasts. This woven bone-like structure could potentially be used as a platform to isolate MtVs, which is the aim of the current study. In this study, secreted EVs to the condition medium during osteogenic differentiation of hBMSCs in the 3D *in vitro* woven bone-like constructs were isolated and characterized. hBMSCs were cultured in spinner flask bioreactors which induced wall shear stress on cells and directed the cells to differentiate into osteoblasts and osteocytes. The EVs secreted into the culture medium were isolated and characterized based on their morphological, biological, and functional properties. The characteristics of a part of isolated EVs shared similarities with MtVs. These vesicles were electron-dense and electron-lucent, showed alkaline phosphatase (ALP) activity, increased the amount of released free phosphate into the culture medium, and increased the amount of deposited phosphate within the ECM. The results indicate that our complex 3D environment mimicking bone development could stimulate MtV-producing cells to secrete targeted MtVs *in vitro*. These MtVs potentially could be used as a biological factor for bone regeneration and fracture healing through, for instance, integration with biomaterials to target bone formation locally.

7.1 Introduction

Bone is a rigid connective tissue that provides structural support for the body, allows movement, protects internal organs, and serves as storage for calcium and growth factor. Despite its passive appearance, bone is a highly dynamic tissue that continuously undergoes a physiological process called bone remodeling [1]. This process maintains bone strength and mineral homeostasis through close interaction between bone-forming osteoblasts, bone-resorbing osteoclasts, and bone-regulating osteocytes [1]. Osteoblasts are bone forming cells derived from mesenchymal stromal cells (MSCs). Under appropriate mechanical and/or chemical stimuli, MSCs differentiate towards osteoblasts [2]. Osteoblasts are responsible for the formation of the bone mineralized tissue which consists of mainly collagen type 1 and carbonated hydroxyapatite deposited within/outside of these collagen fibrils, forming a continuous inter-connected cross-fibrillar pattern [3]. This composite matrix is highly organized at multiple hierarchical levels giving bone its mechanical properties [4].

Coordinating the regulatory processes between bone specific cells during bone remodeling is thought to happen partly through extracellular vesicles (EVs) produced by each cell type [5]. EVs have complex membrane structures and can be secreted by almost all cell types, including osteoblasts. EVs are phospholipid-enclosed nanoparticles containing lipids (to protect the bioactive substances within EVs), proteins (to give them targeting abilities), and nucleic acids (to transmit information to other cells) [5]. Osteoblast-derived EVs have been shown to be involved in multiple processes such as osteogenic differentiation, osteoclast formation, and inorganic matrix deposition [6]–[9]. A subset of osteoblast-derived EVs is known as matrix vesicles (MtVs). These vesicles range in size from 50-1000 nm and can bind to the collagenous matrix [10]. The membrane of these vesicles is enriched with phosphatidylserine (PS)-binding annexin proteins such as annexin A5 and phosphatases such as alkaline phosphatase (ALP). These membrane proteins facilitate entry of calcium and phosphate ions into MtVs. These ions then form amorphous or crystalized minerals inside the MtVs which can rupture the membrane and form mineral nodules in the extracellular matrix (ECM) [10], [11]. MtVs have shown to target bone *in vivo* and induce bone formation *in vitro* and *in vivo* [12]–[15]. For instance, EVs derived from the osteoblast cell line MC3T3-E3 added to bone marrow derived mesenchymal stromal cells (BMSCs) accelerated the osteogenic differentiation of BMSCs and mineral deposition [12], [13].

We have previously developed a functional 3D self-organizing co-culture of osteoblasts and osteocytes that represents the woven bone formation using human bone marrow MSCs (hBMSCs) [16]. Woven bone forms during skeletal development where rapid pace of matrix mineralization is needed [17]. In this process, pre-osteoblasts lay down randomly oriented collagen that becomes highly mineralized [18]. The formation of highly mineralized matrix in this 3D *in vitro* woven bone construct could be partly associated to the release of MtVs by osteoblasts. In our current study, we used this setup and isolated the osteoblast-derived EVs or MtVs released by cells in the cell culture supernatant. We hypothesized that those EVs would show similar characteristics as MtVs and promote collagenous matrix mineralization *in vitro.*

To create the woven bone constructs, hBMSCs were seeded on silk fibroin scaffolds. These cell-seeded constructs were placed inside a spinner flask bioreactor which induce continuous wall shear stress on

cells for 4 weeks (Figure 7.1A). After concentrating cell culture supernatant, EVs were isolated using size-exclusion chromatography (Figure 7.1B) and characterized based on their morphological, biological (Figure 7.1C), and functional properties (Figure 7.1D). The culture medium for hBMSCs differentiation contained fetal bovine serum (FBS). To account for EVs that were already present in FBS, EVs from FBS containing medium were also isolated and characterized using the same approach. To study the potential of isolated EVs on osteogenic differentiation, the EVs from cell culture supernatant (Cell-EVs) were added to hBMSCs cultured in the previously developed serum substitute medium (SSM) for 3 weeks [19]. The influence of MtVs on osteoblast differentiation of hBMSCs and deposition of mineralized matrix were studied.

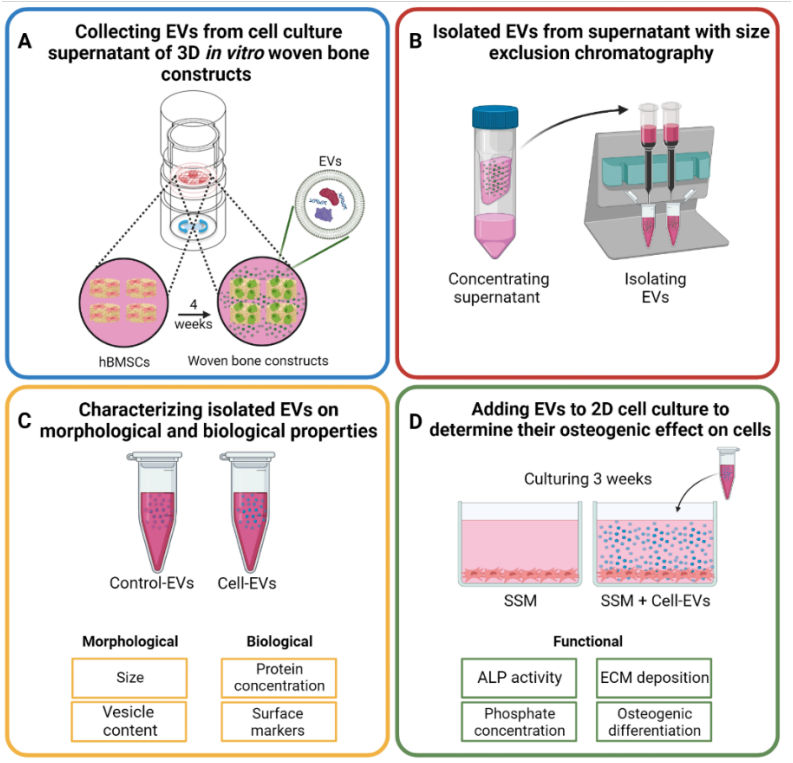

Figure 7.1 Isolation and characterization of MtVs from cell culture supernatant of osteogenic differentiated hBMSCs during in vitro woven bone formation. hBMSCs were cultured under continuous shear stress for 4 weeks which could release EVs to the medium (A). Cell culture supernatant was first concentrated using a spin-column and then EVs were isolated from the collected cell culture supernatant (Cell-EVs) and control group (Control-EVs) using size-exclusion chromatography (B). Isolated EVs were characterized based on their morphological and biological properties (C). hBMSCs were cultured in serum substitute medium (SSM) containing Cell-EVs for 3 weeks to study the influence of EVs on the osteogenic differentiation process (D). Created with BioRender.com.

7.2 Materials and methods

7.2.1 Materials

Dulbecco's modified eagle medium (DMEM high glucose, 41966 and low glucose, 22320), non-essential amino acids (NEAA, 11140050), antibiotic/antimycotic (Anti-Anti, 15240062), and trypsin-EDTA (0.5%, 2530054), Insulin-Transferrin-Selenium (ITS-G, 41400045), GlutaMAX (35050061), chemically defined lipid concentrate (CDlipid, 11905031) were from Life Technologies (The Netherlands). For cell expansion, FBS (F7524) from Sigma-Aldrich (The Netherlands) and for osteogenic differentiation, FBS (SFBS) from Bovogen (Australia) were used. Basic fibroblast growth factor (b-FGF, 100-18B) was purchased from Peprotech (UK). Recombinant human bone morphogenetic protein-2 (rhBMP-2, 7510200) was purchased from Medtronic Sofamor Danek (USA). Human serum albumin (HSA, A1653) was purchased from Sigma-Aldrich (The Netherlands). Unless noted otherwise, all other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich (The Netherlands).

7.2.2 Silk fibroin scaffold fabrication

Silk fibroin scaffolds were prepared by cutting and cleaning 3.2 grams of Bombyx mori L. silkworm cocoons (Tajima Shoki., Ltd. Japan). The cocoons were then degummed by boiling in 1.5 L ultra-pure water (UPW) containing 0.02 M Na_2CO_3 (Sigma-Aldrich, S7795) for 1 hour, whereafter it was rinsed with 10 L cold UPW to extract sericin. Dried purified silk fibroin was dissolved in 9 M lithium bromide (LiBr, Acros organics, 199870025) solution in UPW at 55°C for 1 hour and dialyzed against UPW for 36 hours using SnakeSkin Dialysis tubing (molecular weight cut-off (MWCO): 3.5 kDa, Thermo Fisher Scientific, The Netherlands). The silk fibroin solution was frozen at -80°C for at least 2 hours and lyophilized (Freezone 2.5, Labconco, USA) for 4 days. Lyophilized silk fibroin (1.7 grams) was then dissolved in 10 mL 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, Fluorochem, 003409) at room temperature for 5 hours resulting in a 17% (w/v) solution. One mL of silk-HFIP solution was added to a Teflon container containing 2.5 grams NaCl with a granule size between 250-300 µm. After 3 hours, HFIP was allowed to evaporate for 4 days in a fume hood. Silk fibroin-NaCl blocks were immersed in 90% (v/v) methanol (Merck, The Netherlands) in UPW for 30 minutes to induce the protein conformational transition to β-sheets and let dry overnight [20]. Scaffolds were cut into disks of 3 mm height with an Accutom-5 (Struer, Type 04946133, Ser. No. 4945193), followed by immersion in UPW for 2 days to extract NaCl. Disc-shaped scaffolds were made with a 5 mm diameter biopsy punch (KAI medical, Japan) and autoclaved in phosphate buffered saline (PBS, Sigma-Aldrich, P4417) at 121°C for 20 minutes.

7.2.3 Medium collection and EV isolation

7.2.3.1 Cell expansion and seeding

The hBMSCs were isolated from human bone marrow (Lonza, USA) and characterized as previously described [21]. Passage 4 hBMSCs were expanded (2500 cell/cm^2) in expansion medium (DMEM high glucose containing 10% FBS Sigma, 1% Anti-Anti, 1% NEAA, and 1 ng/ml b-FGF) for 9 days, the medium was replaced 3 times per week. At day 9, cells were 80% confluent and trypsinized using trypsin-EDTA

and dynamically seeded on scaffolds as previously described [22]. Briefly, each scaffold (n=8) was incubated with a cell suspension (1*10^6 cells/4 mL control medium, i.e., DMEM, 10% FBS Bovogen, and 1% Anti-Anti) in 50 mL tubes placed on an orbital shaker at 150 rpm for 6 hours in an incubator at 37°C. Then, cell-seeded scaffolds were transferred to spinner flask bioreactors (n=4 per bioreactor). Each bioreactor contained a magnetic stir bar and was placed on a magnetic stirrer (RTv5, IKA, Germany) at 300 rpm in an incubator (37°C, 5% CO_2) [23]. Each bioreactor was filled with 5 mL control medium containing osteogenic differentiation factors (50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, and 10 mM β-glycerophosphate, β-GP). The medium was refreshed 3 days a week for 4 weeks.

7.2.3.2 Extracellular vesicle (EV) isolation

Cell culture supernatant (5 mL from each bioreactor) was collected at each medium change and centrifuged at 200g for 10 minutes. The supernatant was transferred to a new tube and centrifuged again at 2000g for 10 minutes to remove dead cells and debris. The supernatant was collected in a new tube, frozen in liquid nitrogen, and stored at -80°C. This process was done after each medium change for 4 weeks of osteogenic differentiation of hBMSCs in spinner flask bioreactors. To isolate EVs, the stored medium aliquots were thawed at 37°C and combined. Fifteen mL of the combined medium was concentrated using Amicon Filter Units (MWCO=100 kDa, ACS510024, Merck Millipore) centrifuged at 4000g for 15 minutes. EV isolation was done using qEV1/70 nm column (IC1-70, Izon Science, France). As mentioned in the supplier's user manual, these columns are suitable to isolate vesicles in the range of 70-1000 nm. The columns were prepared before use as described in the user manual. Briefly, the columns were rinsed with 11.5 mL 0.5M filtered NaOH which was followed by 2 times rinsing with 11.5 mL filtered UPW. Then, the columns were rinsed with 11.5 mL 20% ethanol and 2 times with 11.5 mL sterile PBS. The concentrated medium (around 1 mL) was overlaid on the column. The sample was allowed to run completely into the loading frit, the layer separating loading reservoir from the gel part of the column. Once the sample has passed into the frit, 3 mL filtered PBS was added to the column. Four mL of the flowthrough PBS was discarded as there should be no particles in this volume. We added 2.8 mL of filtered PBS again and collected 2.8 mL of this flowthrough directly. This collected fraction should contain the EVs. The collected EVs were concentrated again using Amicon Filter Units resulting in up to 500 µL of EV solution. These EVs were referred as Cell-EVs in this study. The culture medium for hBMSCs differentiation contained FBS. To account for EVs that were already present in FBS, the same procedure was done on 15 mL of control medium (DMEM, 10% FBS Bovogen, and 1% Anti-Anti). EVs isolated from control medium were referred as Control-EVs. The EVs were characterized and used in the cell culture experiment as follows.

7.2.4 Characterization of EVs

7.2.4.1 Bicinchoninic acid (BCA) assay

Protein content of EVs was determined using Micro BCA protein assay kit (23235, Thermo Fisher Scientific, The Netherlands) according to the manufacturer's instructions. Briefly, 150 µL of EV solution was mixed with 150 µL of Micro BCA working Reagent in a 96-well plate. The plate was covered with a sealing tape and incubated at 37°C for 2 hours. Absorbance was measured at 562 nm on a plate

reader and protein concentration was calculated by comparison to standards of known bovine serum albumin (BSA) concentrations.

7.2.4.2 Alkaline phosphatase (ALP) assay

The membrane of MtVs is equipped with mineralization-specific components such as ALP. To determine whether the isolated EVs from the cell culture supernatant contained active ALP, the ALP activity of EVs was measured as follow. EV solution (240 µL) was mixed with 240 µL of 0.2% (v/v) Triton X-100 and 5 mM $MgCl_2$ solution and incubated for 30 minutes at room temperature. In a 96-well-plate, 80 µL of the samples was mixed with 20 µL of 0.75 M 2-amino-2-methyl-1-propanol buffer and 100 µL of 10 mM p-nitrophenylphosphate solution and incubated until colour developed, before 0.2 M NaOH was added to stop the conversion of p-nitrophenylphosphate to p-nitrophenol. Absorbance was measured in at 405 nm on a plate reader and ALP activity was calculated by comparison to standards of known p-nitrophenol concentration.

7.2.4.3 Measure the amount of free phosphate in medium with/without the presence of EVs

To determine the potential of EVs on increasing the concentration of phosphate in the medium, the serum substitute medium was supplemented with EVs and 10 mM β-GP. β-GP is a phosphate source in osteogenic differentiation cultures that can be cleaved by ALP (expressed by osteoblasts during differentiation or present in FBS [24]) and released free phosphate to the medium. Since the membrane of MtVs are equipped with ALP, it was hypothesized that isolated EVs can increase the amount of the free phosphate in the medium containing β-GP. To measure the amount of free phosphate in the medium, serum substitute medium with and without EV supplementation (n=3 per group) was incubated with β-GP at 37°C for 48 hours. The phosphate concentration was then measured according to the manufacturer's instructions (Malachite Green Phosphate Assay Kit, Sigma-Aldrich, The Netherlands). Briefly, 80 µL of 1:200 (v/v) diluted samples in UPW were mixed with 20 µL of working reagent and incubated for 30 minutes at room temperature. In this assay, a green complex is formed between molybdate and free phosphate. Absorbance was measured in at 620 nm on a plate reader and phosphate concentration was calculated by comparison to a phosphate standard provided in the kit.

7.2.4.4 Nanoparticle tracking analysis (NTA)

NTA was used to determine the size and concentration of EVs. This method is based on the Brownian motion of individual particles in solution. The movement of the particles is tracked based on light scattering. A Nanosight NS300 instrument (Malvern Panalytical) equipped with a scientific complementary metal-oxide-semiconductor (sCMOS) camera was used. The camera was mounted on an optical microscope, allowing visualization of the light scattered by the injected particles that were present in the focus of an 80 µm beam generated by a single mode laser diode with a blue laser (488 nm). During the measurement, temperature was kept at 24°C. The samples were diluted 10 times in filtered PBS and 1 mL of the samples was injected into the Nanosight chamber. One mL filtered PBS was used as a control. NTA 3.2 software was used to analyze the data. Five videos of 60 seconds were captured per sample at camera level 10 and screen gain of 5. During the data analysis the screen gain was set to 5 with the detection threshold of 10.

7.2.4.5 Flow cytometry

Flow cytometry was used to detect and measure the proportion of annexin A5 and CD-9, as typical EV surface markers. Briefly, 20 µL of EV solution was first mixed with 20 µL of flow cytometry buffer, i.e., 1% BSA dissolved in PBS containing 1% binding buffer (521103674, Miltenyl Biotec). The binding buffer contains calcium which is needed for the binding of annexin A5 to phosphatidylserine (PS). Next, either 1 µL of annexin A5 conjugated with FITC (BioLegend, 640906) and/or 1 µL of CD-9 antibodies conjugated with APC (BioLegend, 312107) was added to the samples. The samples were then incubated at 4°C for 30 minutes in the dark. Another 260 µL flow cytometry buffer was added to each sample prior to the measurement done by FACSymphony A3 (660937, BD Biosciences). The triggering threshold was set on forward scatter at 200. All samples were analyzed at low speed (35 µL/second) for 2 minutes. The obtained results were analyzed with FlowJo (v10.8.1, BD Biosciences).

7.2.4.6 Cryo-Transmission electron microscopy (Cryo-TEM)

Vitrified thin films for Cryo-TEM analysis were prepared using an automated vitrification robot (FEI Vitrobot Mark IV) by plunge vitrification in liquid ethane. Before vitrification, a 200-mesh copper grid covered with a Quantifoil R 2/2 holey carbon film (Quantifoil Micro Tools GmbH) was surface plasma treated for 40 seconds using a Cressington 208 carbon coater. Cryo-TEM imaging was carried out on the cryoTITAN (Thermo Fisher, previously FEI), equipped with a field emission gun (FEG), a post-column Gatan imaging filter (model 2002) and a post-GIF 2k × 2k Gatan CCD camera (model 794). The microscope was operated at 300 kV acceleration voltage in bright-field TEM mode with zero-loss energy filtering at a nominal magnification of 6.500×; or at 24.000× magnification both with a 1s image acquisition time.

7.2.5 Addition of EVs to osteogenically differentiating hBMSCs

To determine the influence of EVs on osteogenic differentiation, hBMSCs were seeded on well-plates and cultured in the previously developed serum substitute medium (SSM) [19]. The use of serum substitute medium was to avoid the influence of EVs present in FBS [25].

7.2.5.1 Cell expansion and seeding

Passage 4 hBMSCs were expanded (2500 cell/cm^2) in expansion medium (DMEM high glucose containing 10% FBS Sigma, 1% Anti-Anti, 1% NEAA, and 1 ng/ml b-FGF) for 9 days, the medium was replaced 3 times per week. At day 9, cells were 80% confluent, trypsinized and seeded on vitronectin coated 48-well-plate (Greiner bio-one, CELLSTAR, 677-180). The wells of a 48-well-plate were coated with 5 µg/ml vitronectin (Peprotech, 140-09) diluted in PBS to increase cell attachment to the surface of well plate in the serum substitute medium [19]. In short, 100 µL of vitronectin solution was added to each well and incubated in an incubator (37°C, 5% CO_2) for 2 hours and then at 4°C for 16 hours. The following day, the vitronectin solution was aspirated, the wells were rinsed 3 times with PBS, and the well-plate was pre-warmed in an incubator before seeding cells into the wells. We seeded 2500 cells per well resulting in 2500 cells/cm^2 and cultured them in serum substitute medium (Table 7.1), additionally supplemented with osteogenic differentiation factors (50 µg/ml ascorbic-acid-2-phosphate, 100 nM dexamethasone, 10 mM β- glycerophosphate). The medium was refreshed 3 days

a week during the first week. After 1 week of culture, a group of cells was continued to be cultured without EVs in serum substitute medium, while the medium of the other group was supplemented with EVs at a concentration of 800 ng/mL (the maximum EV concentration isolated each time). The medium was refreshed 3 days a week for 2 more weeks.

Table 7.1 Concentrations of serum substitute medium components [19].

Component	Concentration
HSA	1% w/v
Anti-Anti	1% v/v
ITS-G	1% v/v
GlutaMax	1% v/v
NEAA	1% v/v
CD-lipid	0.1% v/v
b-FGF	10 ng/mL
rhBMP-2	100 ng/mL

7.2.5.2 ALP assay on cells

ALP is an enzyme expressed by osteoblasts and present of the cell surface. To measure the ALP activity of osteoblasts, cell-seeded wells (n=6 per group) were rinsed thoroughly with PBS several times and incubated in 250 µL of 0.2% (v/v) Triton X-100 and 5 mM $MgCl_2$ solution for 30 minutes at room temperature to disturb the cell membrane. Next, the content of the well was transferred into an Eppendorf tube and centrifuged at 3000g for 10 minutes. In a 96-well plate, 80 µL of the supernatant was mixed with 20 µL of 0.75 M 2-amino-2-methyl-1-propanol buffer and 100 µL 10 mM p-nitrophenylphosphate solution and incubated until colour developed, before 0.2 M NaOH was added to stop the conversion of p-nitrophenylphosphate to p-nitrophenol. Absorbance was measured in at 405 nm on a plate reader and ALP activity was calculated by comparison to standards of known p-nitrophenol concentration.

7.2.5.3 DNA assay

After performing ALP assay, the remaining solution was used to measure the amount of DNA per well (n=6 per group) as a measure for the number of cells. Briefly, 90 µL of the remaining solution after ALP assay was mixed with 90 µL of digestion buffer (500 mM phosphate buffer, 5 mM L-cystein (Sigma-Aldrich, C1276), 5 mM EDTA (Sigma-Aldrich, 1.08421.1000)) containing 140 µg/ml papain (Sigma-Aldrich, P-5306) at 60°C for 16 hours in a water bath shaker (300 rpm). Next, samples were centrifuged at 3000g for 10 minutes and the DNA concentration was measured by Qubit dsDNA HS assay kit (Life Technologies, Q32851). Briefly, 10 µL of samples and standards were mixed thoroughly with DNA buffer containing DNA reagent (1:200) and the DNA concentration was measured with Qubit 2.0 Fluorometer.

7.2.5.4 Immunohistochemistry

Cell-seeded wells were rinsed with PBS and fixed with 10% neutral buffered formalin for 30 minutes at 4°C. To stain and detect the mineral deposition within the matrix, OsteoSense 680 EX (28422525, PerkinElmer) was used. OsteoSense 680 EX has a high affinity to hydroxyapatite both *in vitro* and *in vivo*. The dye was diluted in PBS (1:200), added to cell-seeded wells, and incubated at 37°C overnight in dark. The next day, the wells were rinsed with PBS twice and covered with 100 µL 0.5% (v/v) Triton-X 100 (Merck, 1.08603.1000) in PBS for 5 minutes to permeabilize cells. Then, the cells were rinsed with PBS and incubated with 5% (v/v) normal donkey serum and 1% (w/v) BSA (Roche, 10.7350.86001) in PBS for 1 hour at room temperature to block the non-specific antibody binding. Wells were then incubated for 16 hours at 4°C with the primary antibody (Table 7.2). The wells were washed with PBS 3 times and incubated for 1 hour with the secondary antibody solution (Table 7.2). This was followed by 3 times rinsing of the wells with PBS. Then, nuclei, actin cytoskeleton, and collagen were stained with 4',6-diamidino-2-phenylindole (DAPI (Sigma-Aldrich, D9542), diluted to 0.1 µg/ml in PBS), 50 pmol Phalloidin-TRITC, and 1 µM CNA35-OG [26], respectively, for 30 minutes. Wells were rinsed with PBS 3 times and then covered with PBS. The expression of proteins was visualized with Zeiss Axio Observer Z/Apotome microscope and images were processed with ImageJ (version 1.53f51). Figures were chosen to be representative images per group for all the samples assessed.

Table 7.2 Overview of antibodies used for immunohistochemistry.

Primary antibody	**Source**	**Cat. No**	**Label**	**Species**	**Dilution**
RUNX2	Abcam	Ab23981	-	Rabbit	1:500
Osteopontin	Thermo Fisher	14-9096-82	-	Mouse	1:200
Secondary antibody	**Source**	**Cat. No**	**Label**	**Species**	**Dilution**
Anti-rabbit IgG (H+L)	Jackson Immumo Research	711-605-152	A647	Donkey	1:200
Anti-mouse IgG (H+L)	Jackson Immumo Research	715-545-150	A488	Donkey	1:200

7.2.5.5 Measure the amount of deposited phosphate within ECM

hBMSCs were cultured in serum substitute medium containing the osteogenic differentiation factors with or without the presence of EVs for 3 weeks. At the end of the culture, the deposited phosphate on the cell-seeded well plates was measured (n=4 per group). Cell-seeded wells were rinsed with PBS and incubated in 500 µL of 5% trichloroacetic acid (TCA, Sigma-Aldrich T6399) in UPW for 30 minutes. Next, the content of the each well was transferred into an Eppendorf tube and incubated for 48 hours at room temperature. Then the solids were separated by centrifuging at 3000g for 10 minutes. Eighty

µL of 1:200 (v/v) diluted samples in UPW were mixed with 20 µL of working reagent and incubated for 30 minutes at room temperature. Absorbance was measured in at 620 nm on a plate reader and phosphate concentration was calculated by comparison to a phosphate standard provided in the kit.

7.2.6 Statistics

GraphPad Prism 9.0.2 (GraphPad Software, La Jolla, CA, USA) was used to perform statistical analysis and to prepare the graphs. As tested with Shapiro-Wilk test, the data were normally distributed. To test for differences, an independent t-test for particle size, mode, and concentration (Figure 7.2C, D, E), amount of protein (Figure 7.3A), ALP activity of EVs and the amount of free phosphate in the medium (Figure 7.6), amount of DNA and ALP activity of cells (Figure 7.7), and deposited phosphate (Figure 7.9C) was performed. The data were presented as mean +/- standard deviation and differences were considered statistically significant at a level of $p<0.05$.

7.3 Results

EVs were isolated from culture medium of hBMSCs differentiating towards osteoblasts/osteocytes in a previously developed 3D *in vitro* woven bone model (Cell-EVs) [16]. As a control, EVs were isolated from control medium (containing 10% FBS) without having had cell contact (Control-EVs). EVs were characterized based on their morphological, biological, and functional properties.

7.3.1 Morphological properties of EVs

NTA showed the size distribution of EVs isolated from hBMSCs during osteogenic differentiation (Cell-EVs) and control medium (Control-EVs) (Figure 7.2). The particle size of Cell-EVs was shown to be significantly larger and more heterogenous compared to the ones of Control-EVs with smaller and more homogenous particles (Figure 7.2A and B). The main peak of the Control-EVs seemed to be present in the Cell-EVs graph, but Cell-EVs graph contains more peaks that could be attributed to the EVs secreted by cells to the medium (Figure 7.2A and B). The measurement revealed that the mean particle size and mode (most frequent population of particle sizes) of Control-EVs were 168.9 +/- 2.4 nm and 172.6 +/- 5.0 nm, respectively, and the Cell-EVs had a mean particle size of 373.0 +/- 25.3 nm and mode of 289.9 +/- 52.4 nm (Figure 7.2C and D). The particle concentration measured by NTA was $5.19*10^7$ +/- 6.34 $*10^6$ particles/mL in Cell-EVs which was significantly higher than Control-EVs with particle concentration of $1.72*10^7$ +/- $5.52*10^6$ particles/mL (Figure 7.2E). Cryo-TEM showed the presence of Cell-EVs with heterogeneity diameters which correlates with the NTA result of Cell-EVs (Figure 7.2F and G). No particles could be visualized in Control-EVs with cryo-TEM which may be due to their low concentration. The Cell-EVs were electron-dense (Figure 7.2F) and electron-lucent (Figure 7.2G) as shown by TEM images, which could suggest mineral accumulation or protein aggregation within some EVs.

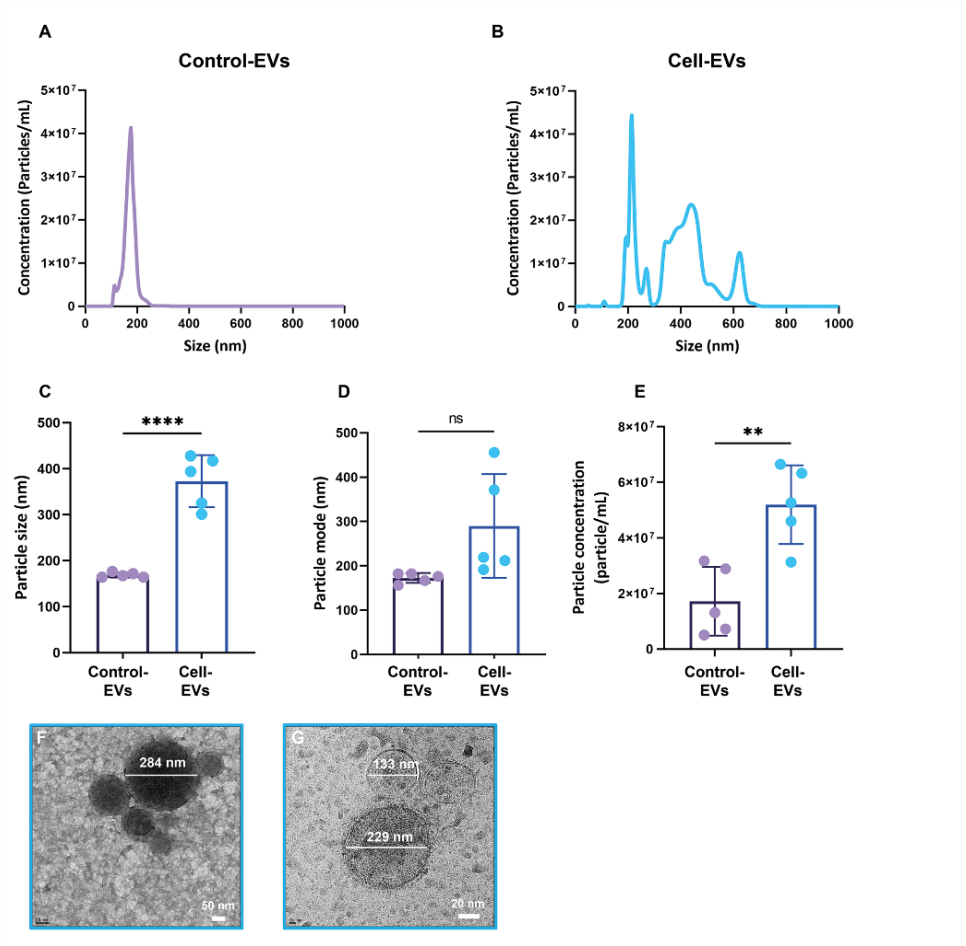

*Figure 7.2 Distribution of Control-EVs (A) and Cell-EV (B) showed larger mean particle size and mode in Cell-EVs compared to Control-EVs. The particle size and mode of Cell-EVs were significantly larger than of Control-EVs (C and D). The measured particle concentration by NTA in Cell-EVs group was also significantly higher than in Control-EVs (E). Cryo-TEM images confirmed the existence of a heterogeneous population of Cell-EVs in their diameter and electron dense regions (F and G). **p<0.01, ***p<0.001. Significant differences in figures C and E were detected by independent t-test.*

7.3.2 Biological properties of EVs

The total amount of protein within Cell-EVs and Control-EVs was measured using a Micro BCA assay. The amount of protein in Cell-EVs isolated from 15 mL of cell culture supernatant was 1150.39 +/- 48.79 ng which was significantly higher than the amount of protein in Control-EVs isolated from 15 mL control medium which was 189.45 +/- 41.15 ng (Figure 7.3A). EVs were further characterized using flow cytometry. CD-9 and annexin A5, two EV surface markers were used. CD-9 is one of the general markers present on the surface of EVs [27]. Flow cytometry analysis demonstrated that Control-EVs

did not show any CD-9 positive events while 1.11% of isolated Cell-EVs were CD-9 positive (Figure 7.4A and 7.5A). Annexin A5 is one of the annexin proteins that are present at substantial concentrations on the MtV membrane [28]. Flow cytometry analysis revealed that 1.58% of Control-EVs and 53.67% of Cell-EVs were annexin A5 positive (Figure 7.4B and 7.5B). This indicates the presence of MtV in the Cell-EV fraction. Double staining of the Control-EVs and Cell-EVs revealed that none of the Control-EVs were both CD-9 and annexin A5 positive while a small percentage (0.13%) of isolated Cell-EVs showed to be both CD-9 and annexin A5 positive (Figure 7.4C and 7.5C).

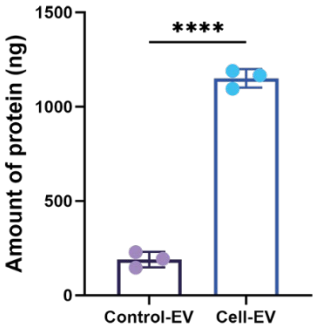

*Figure 7.3 Micro BCA assay showed a significantly higher amount of protein in Cell-EV compared to Control-EV (A). ****p<0.0001. Significant differences were detected by independent t-test.*

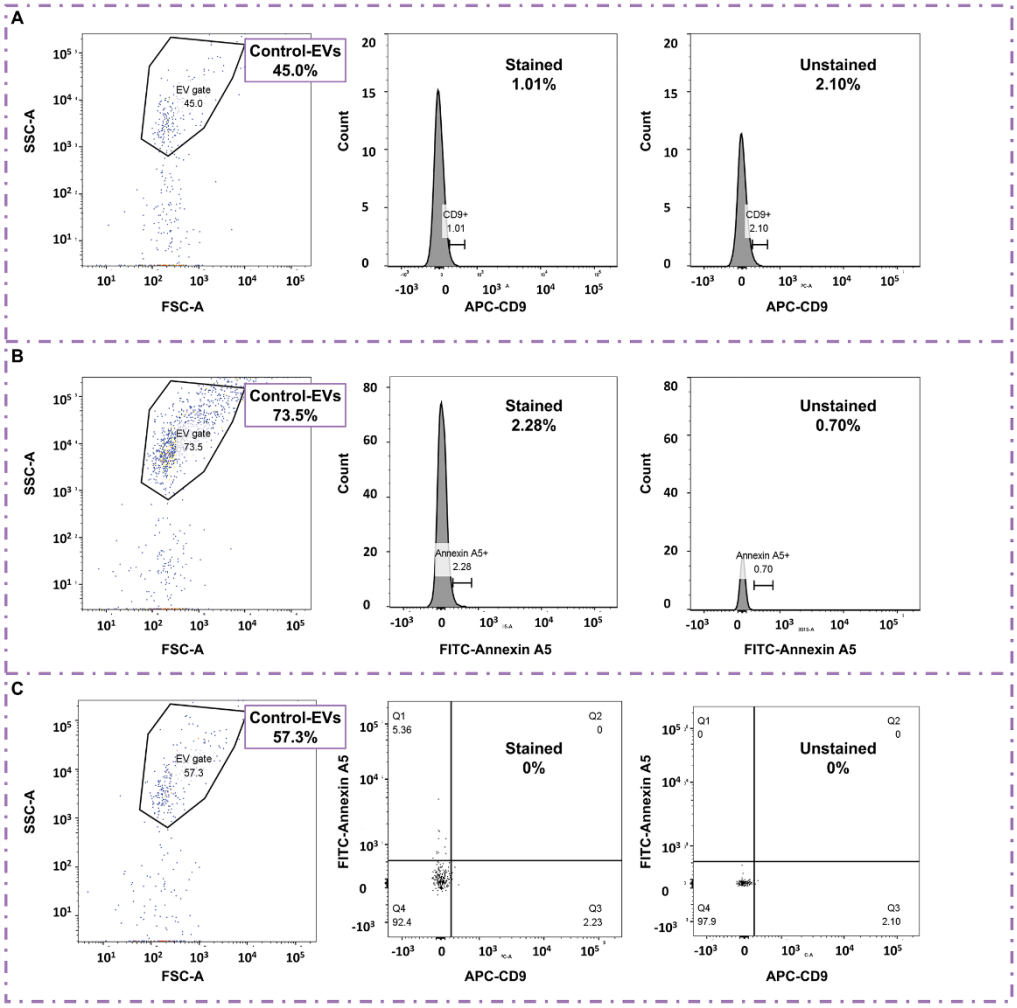

Figure 7.4 Flow cytometry analysis demonstrated that Control-EVs did not show any CD-9 positive events (A). These Control-EVs were 1.58% annexin A5 positive (B). Double staining of Control-EVs did not also show any positive events (C).

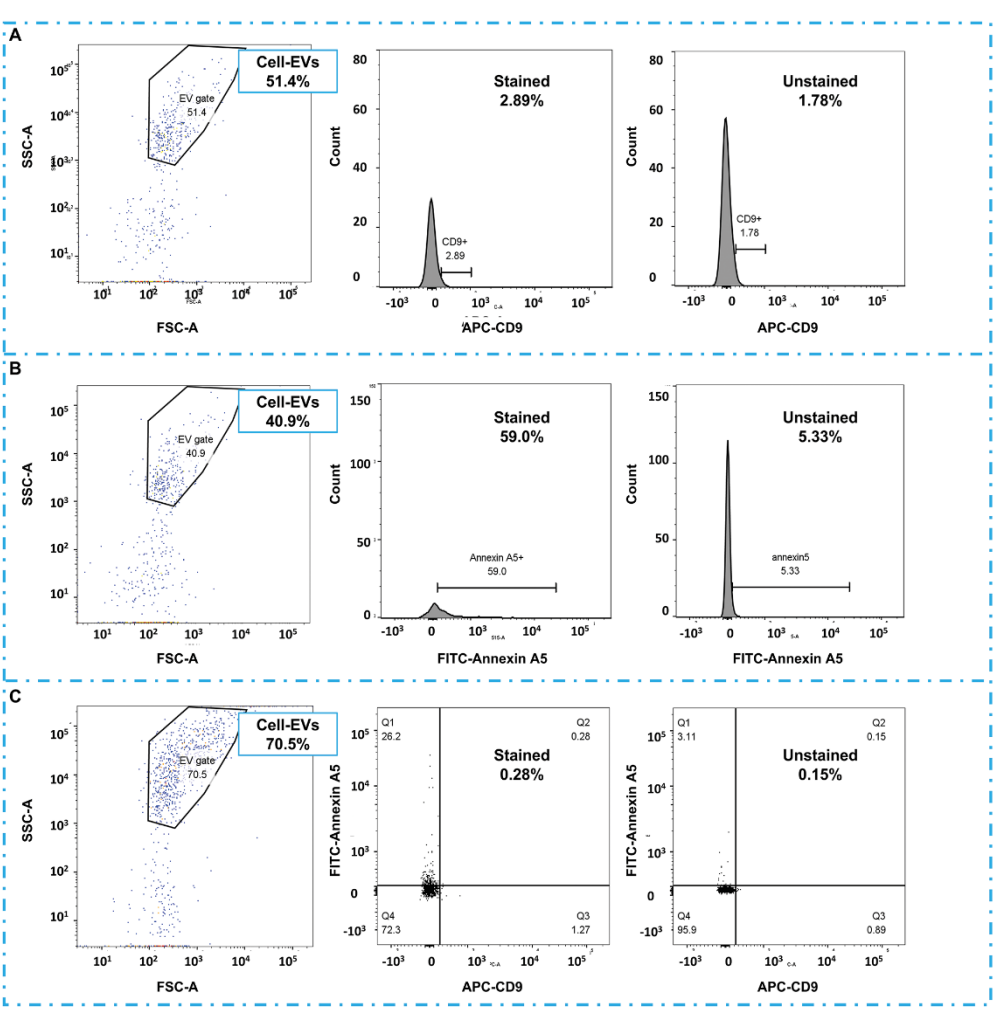

Figure 7.5 Flow cytometry analysis demonstrated that 1.11% of Cell-EVs were CD-9 positive (A). These Cell-EVs were 53.67% annexin A5 positive (B). Double staining of Control-EVs showed 0.13% positive events (C).

7.3.3 Functionality of EVs

7.3.3.1 ALP activity of Cell-EVs and its influence on the release of free phosphate from a phosphate source

ALP, an enzyme present on the MtV membrane and responsible for dephosphorylation of pyrophosphates, was measured [29]. The ALP activity of Cell-EVs was significantly higher than the ALP activity of the same amount of Control-EVs (Figure 7.6A), knowing that there was lower concentration of particles in Control-EVs group. These results suggested that if Cell-EVs were incubated with a phosphate source such as β-GP, the EVs would be able to release free phosphate to the culture medium - an effect that is usually mediated by active osteoblasts. To test this, serum substitute medium containing 10 mM β-GP was incubated without or with 800 ng/mL Cell-EVs. The results indicated that the amount of free phosphate in the medium supplemented with Cell-EVs was significantly higher compared to when no Cell-EVs were added to the medium (Figure 7.6B). This effect confirmed the presence of active ALP on the surface of Cell-EVs.

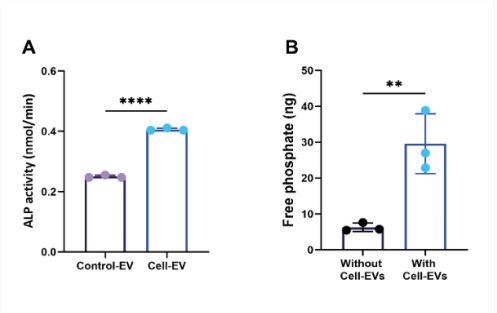

*Figure 7.6 Cell-EVs showed significantly higher ALP activity compared to Control-EVs (A). The amount of free phosphate increased significantly in the medium supplemented with Cell-EVs and 10 mM β-GP compared to when no Cell-EVs were added to the medium containing 10 mM β-GP (B). ** p<0.01), ****p<0.0001. Significant differences were detected by independent t-test.*

7.3.3.2 Influence of Cell-EVs on osteogenic differentiation of hBMSCs

Cell-EVs increased the amount of DNA and ALP activity of cells

The influence of Cell-EVs on osteogenic differentiation of hBMSCs was studied by culturing cells in serum substitute medium supplemented with osteogenic differentiation factors (dexamethasone, ascorbic acid, and β-GP) and Cell-EVs. As a control, hBMSCs were cultured in serum substitute medium without the presence of Cell-EVs, but with osteogenic differentiation factors. The amount of DNA as a measure of number of cells increased significantly in the presence of Cell-EVs (Figure 7.7A). The addition of Cell-EVs with their own ALP activity to the cells also increased the ALP activity of the cells significantly compared to when no EVs were added to the cells (Figure 7.7B). Next, the measured ALP activity was normalized to the amount of DNA which also showed a significant increase in the ALP activity of cells in the presence of Cell-EVs compared to the control group (without Cell-EVs) (Figure 7.7C).

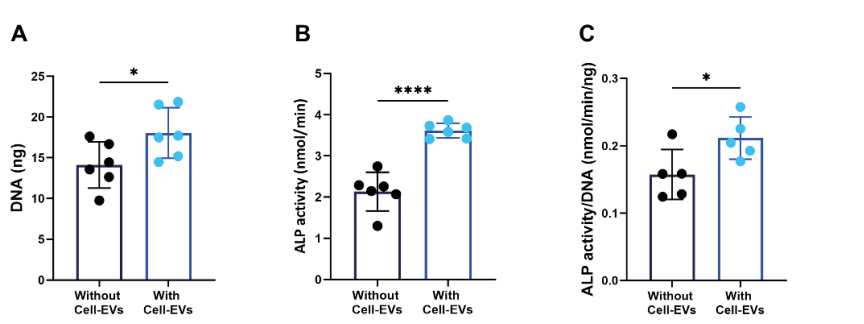

*Figure 7.7 The amount of DNA (A), the ALP activity of cells with the presence of Cell-EVs increased significantly compared to when no Cell-EVs were added (B) ALP activity of cells was normalized to the DNA of cells and showed significant influence of Cell-EVs on ALP activity of cells during osteoblast differentiation (C). *p<0.05, ****p<0.0001). Significant differences were detected by independent t-test.*

Cell-EVs did not have an effect on the osteogenic differentiation of hBMSCs

hBMSCs were cultured in osteogenic differentiation medium supplemented with or without Cell-EVs. After 21 days of culture, osteoblast specific markers such as RUNX-2 and osteopontin were expressed in both groups (Figure 7.8A and B). The results revealed that adding Cell-EVs to the osteogenic culture medium did not affect the expression of such ECM proteins and did not hinder osteoblastic differentiation of hBMSCs.

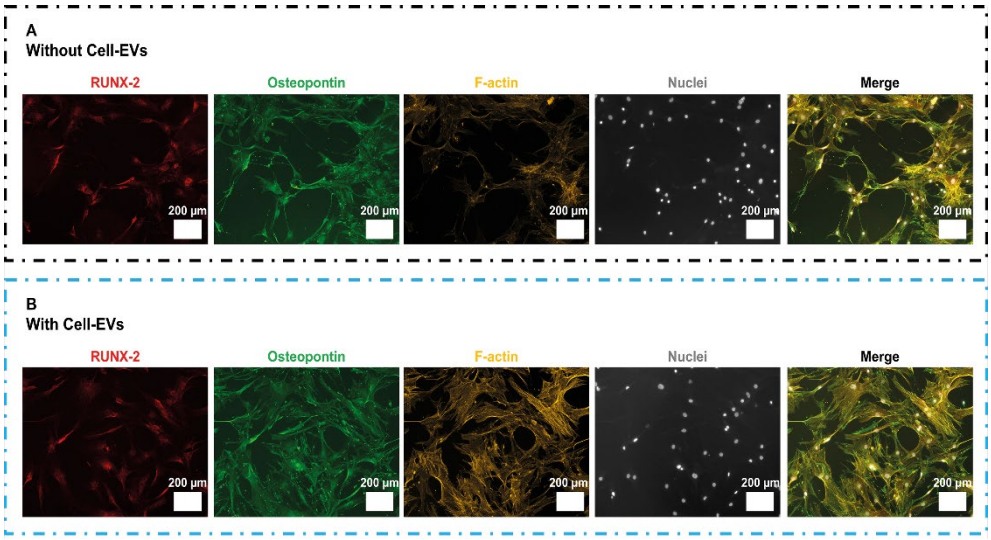

Figure 7.8 After 21 days of culturing hBMSCs in the absence (A) and presence (B) of Cell-EVs, no differences could be detected in the expression of RUNX-2 and osteopontin, and F-actin. Merge stands for the overlay of the first 4 channels.

Cell-EVs did not have an effect on collagen production but increased mineral deposition

Even though adding Cell-EVs to the cells cultured in osteogenic differentiation medium did not have any influence on osteoblast differentiation of hBMSCs, there seemed to be an influence on the production of ECM, particularly ECM mineralization, when Cell-EVs were added to the cells. Collagen type 1 as the main ECM protein of bone was laid down both in the presence and absence of Cell-EVs (Figure 7.9). The main effect of adding Cell-EVs to the medium was in mineral deposition. In the cell culture medium without Cell-EVs, only small mineral nodules were formed (Figure 7.9A), while there were distinctly more, and larger mineral spots present in the group where Cell-EVs were added (Figure 7.9B). The measurement of the phosphate in the ECM also showed significantly more deposited phosphate after 21 days in the presence of Cell-EVs compared to when no EVs were added to the medium (Figure 7.9C).

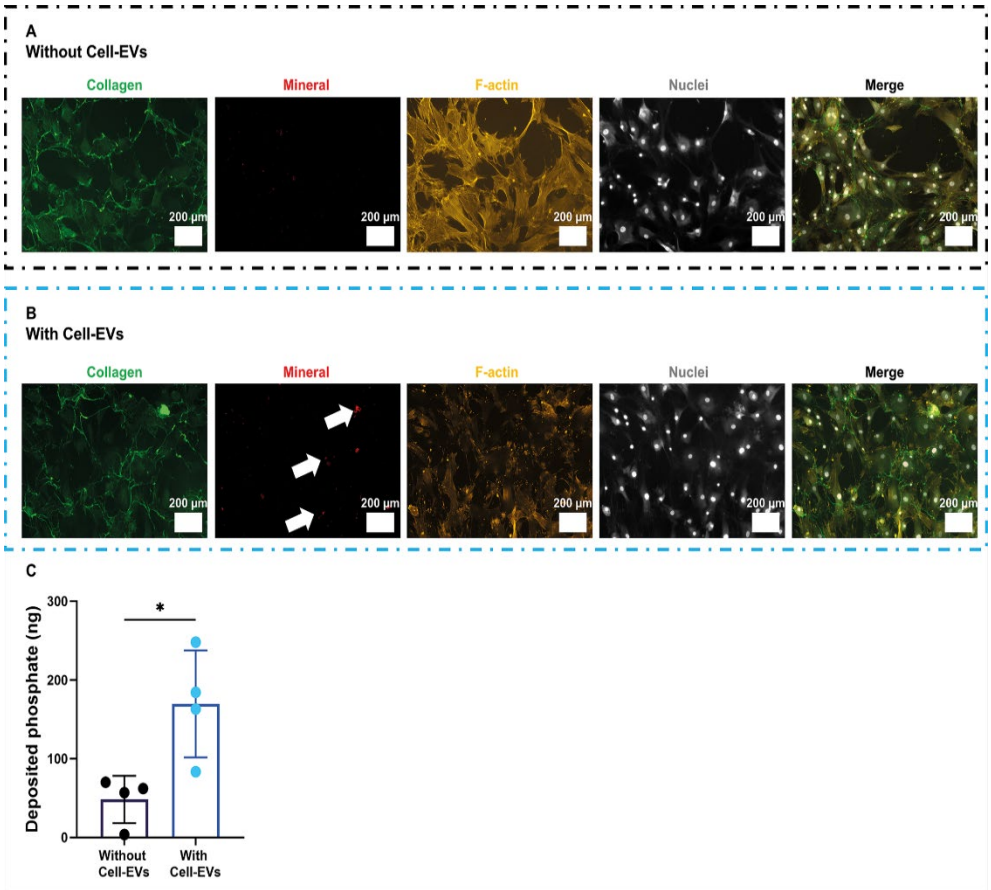

*Figure 7.9 Collagen type 1 was expressed regardless of the presence of Cell-EVs in the culture (A and B). The presence of Cell-EVs in the culture increased the mineral deposition after 21 days of culture (A and B) and phosphate assay (C). * p<0.05. Significant differences were detected by independent t-test. Merge stands for the overlay of the first 4 channels.*

7.4 Discussion

EVs released by osteoblasts have shown remarkable potential in osteogenesis, osteoclastogenensis, and mineralization of the organic matrix [5], [7], [8], [30]. A subset of osteoblast-derived EVs is known as matrix vesicles (MtVs) which are involved in matrix mineralization and are equipped with mineralization-specific components such as phosphatases, calcium, and inorganic phosphate. These MtVs also showed to accelerate the osteogenic differentiation of MSCs *in vitro,* have bone-targeting potential, and induce bone formation *in vivo* [12], [13]. We have previously developed a functional 3D self-organizing co-culture of osteoblasts and osteocytes that represents the woven bone formation using human bone marrow MSCs (hBMSCs) [16]. The formation of highly mineralized matrix in this 3D *in vitro* woven bone construct could be due to the release of MtVs by osteoblasts. Woven bone forms during skeletal development where rapid pace of matrix mineralization is needed [17]. In this process, pre-osteoblasts lay down randomly oriented collagen that becomes highly mineralized [18]. During this rapid pace mineralization process, MtVs might be highly secreted and induce the matrix mineralization. In our study, we first create the 3D *in vitro* woven bone-like constructs to further isolate the osteoblast-derived EVs or MtVs released by cells in the cell culture supernatant. The *in vitro* woven bone-like construct was created through the differentiation of hBMSCs seeded on silk fibroin scaffolds and placed inside spinner flask bioreactors which induce wall shear stress to cells [16], [23]. hBMSCs were differentiated into a functional 3D co-culture of osteoblasts and osteocytes and formed a mineralized matrix mimicking native woven bone [16]. In the current study, we isolated the osteoblast-derived EVs or MtVs released by cells cultured in this setup and it was hypothesized that EVs released into the culture medium show similar characteristics as MtVs, such as rich in annexin A5, exhibit membrane phosphatases such as ALP, and induce mineral deposition. EVs released by hBMSCs during osteoblast differentiation were isolated and characterized based on their morphological, biological, and functional properties. The latter was done through culturing hBMSCs in the presence and absence of EVs.

The released EVs were isolated from the cell supernatant during 4 weeks of culture. To isolate EVs, size-exclusion chromatography with columns specified for isolating EVs in the size range of 70-1000 nm has been used. This method, unlike the commonly used ultracentrifugation method, is faster, maximizes the purity of EVs, and prevents EV degradation, aggregation, and fusion due to intense gravitational force [31]–[35]. The mean particle size of isolated EVs from the cell culture supernatant was 373.0 +/- 25.3 nm which was at the same size range of ectosome-like MtVs [11]. Ectosome-like MtVs are formed by budding and pinching off from the membrane of mineralization-mediating cells. The membrane of such MtVs is enriched with PS-binding annexins such as annexin A5 and ALP facilitates the accumulation of calcium and phosphate ions within MtVs [36], [37]. We confirmed the presence of EVs by showing the presence of the transmembrane protein CD-9, one of the EV marker proteins [27]. We also confirmed the presence of annexin A5 on the membrane of a part (not all) of Cell-EVs. The isolated EVs also contained active ALP which was able to release free phosphate from the phosphate source. These results indicated that at least a part of the isolated Cell-EVs shared similar characteristics as ectosome-like MtVs isolated from native bone or MtV-producing cells *in vitro* [38], [39].

Cell-EVs were isolated from cell culture supernatant containing FBS. It is widely known that FBS itself contains EVs which might be co-isolated with the EVs of interest and contaminate the population of Cell-EVs [25]. To avoid EV contamination, cells have been typically cultured in EV-depleted medium or serum-free medium. However, this approach could cause cellular stress, changes in cellular phenotype, and significantly affected the growth and viability of cells [25], [40]–[42]. In the present study, EVs were isolated from control medium (containing FBS and not in contact with cells) to investigate if Control-EVs could have been co-isolated with Cell-EVs. Although, isolated Control-EVs showed significantly less protein content and ALP activity compared to Cell-EVs, this fraction still expressed annexin A5. These results indicated that control medium contained a small population of MtVs which formed a subpopulation of the Cell-EVs, but still there were substantially more EVs present in Cell-EVs. To avoid the Control-EVs co-isolation with Cell-EVs, specialized serum substitute medium for osteogenic differentiation cultures could be used to develop an *in vitro* woven bone and isolate the secreted MtVs [19]. Using a defined medium would guarantee that only EVs expressed by cells are being isolated.

The functional properties of Cell-EVs were investigated through culturing hBMSCs in serum substitute medium supplemented with osteogenic differentiation factors and with or without Cell-EVs. Use of serum substitute medium was to prevent the influence of FBS and its EVs on cells. We previously showed that different types of FBS contain various levels of active ALP that might be due to the different levels of MtVs present in FBS [24]. These different FBS types significantly affected the matrix mineralization which was correlated with the level of active ALP in each FBS brand [24]. Our results indicated that RUNX-2 and osteopontin were expressed during osteogenic differentiation of hBMSCs regardless of the presence of Cell-EVs and these EVs did not hinder the osteogenic differentiation potential of hBMSCs, but the presence of Cell-EVs significantly increased the cellular ALP activity after 3 weeks of culture. Increasing the cellular ALP activity in the presence of Cell-EVs could be attributed to the slightly positive influence of Cell-EVs on osteogenic differentiation. Lack of differences in expression of osteoblast-specific factors in the presence and absence of Cell-EVs could be due to either the presence of potent osteogenic differentiation factors which could overshadow the effect of Cell-EVs or low concentration of Cell-EVs added to osteogenic differentiation medium. A previous study has also demonstrated that osteoblast-derived EVs did not induce any significant changes in osteoblastic differentiation of hBMSCs when added to medium supplemented with osteogenic differentiation factors [13]. However, to draw a stronger conclusion on the influence of Cell-EVs on osteogenic differentiation, quantified approaches such as quantitative real-time polymerase chain reaction (qPCR) needs to be performed. Furthermore, to determine the influence of Cell-EVs only on osteogenic differentiation of hBMSCs, addition of EVs to the medium without the presence of osteogenic differentiation factors could be studied in the future. However, the presence of β-GP, as a phosphate source in the medium, might be required. In most studies, the concentration of added EVs to the osteogenic culture medium was reported to be between 2.5 to 10 μg/mL [12], [13], [43]. The highest Cell-EV concentration that we could isolate from the cell culture medium collected during 4 weeks of experiment using size-exclusion chromatography was 800 ng/mL. Apart from soluble EVs and MtVs, MtVs can also bind to collagen matrix, for example through annexin proteins and ALP. The use of collagenase before isolation of osteoblast-derived EVs from osseous tissue was reported as an

approach to release MtVs from the matrix [44]. This approach resulted in a population of MtVs with a higher ALP activity and mineral formation potential compared to the non-collagenase treated osseous tissue [45]. A potential way to increase the concentration of isolated Cell-EVs could be breaking down the collagen matrix using collagenase [46], [47]. In this case, the effect of collagenase on the characteristics of Cell-EVs should be investigated as well.

Matrix mineralization is an important step in *in vitro* bone tissue formation. MtVs are known to play an essential role in mineralization of the organic bone matrix. These vesicles can bind to the collagen matrix. The membrane proteins of MtVs facilitate entry of calcium and phosphate ions into MtVs. These ions then form amorphous or crystalized minerals inside the MtVs which can rupture the membrane and form mineral nodules in the ECM [10], [11]. Adding Cell-EVs to hBMSCs showed an increase in deposited hydroxyapatite, as detected by OsteoSense 680 EX with a high affinity to hydroxyapatite in both *in vitro* and *in vivo*, compared to when hBMSCs were continued to be cultured in serum substitute medium in the absence of Cell-EVs. In the presence of Cell-EVs, the deposited phosphate within the matrix increased significantly compared to when no EVs were added to the culture. The mineral formation could be directly due to the contribution of ALP activity of Cell-EVs on increasing the amount of free phosphate in the medium or indirectly through the increase in cellular ALP activity in the presence of Cell-EVs. Previous studies also demonstrated the influence of MtVs on increasing the cellular ALP activity and matrix mineralization [12], [13], [43].

Previous studies have shown that EVs isolated from different stages of osteogenic differentiation of MSCs showed different characteristics [48]–[50]. A recent study revealed that EVs isolated on days 21, 28, and 35 of osteogenic differentiation of hBMSCs have different potentials in hBMSCs proliferation, osteoblast differentiation, and ECM mineralization [48]. It has been shown that EVs isolated from earlier time points exhibited a high proliferation stimulus; while EVs isolated from later time points resulted in more osteogenic differentiation and mineral deposition [48]. In our study, EVs were isolated from the mixture of osteogenic medium collected during 28 days of culture. For future studies, to increase the purity of MtVs, either EVs secreted by hBSMCs during later stages of osteogenic differentiation using the same setup could be isolated or another step of isolation could be added to the current isolation method. For instance, after isolating EVs using size-exclusion chromatography, the affinity chromatography method could be used to isolate EVs with specific surface markers, for example, ALP [51]–[53].

Osteoblast derived EVs and more specifically MtVs are increasingly being considered as a promising therapeutic factor for bone regeneration and fracture healing [7], [12], [15]. Considering advances in the development of *in vitro* bone models using bone tissue engineering approaches [54], more physiologically relevant models could be used to guide MtV-producing cells to produce targeted MtVs *in vitro*. Later, these secreted MtVs could be integrated in biomaterials and delivered at a controlled release rate to the site of interest to regulate bone regeneration locally. The future studies should focus on development of such MtV-integrated biomaterials for therapeutic applications for bone tissue.

7.5 Conclusion

We have shown that our previously developed 3D human *in vitro* woven bone models could result in secretion of MtVs from osteogenic differentiated hBSMCs. Secreted EVs during osteogenic differentiation of hBSMCs were isolated using size-exclusion chromatography and characterized based on their morphological, biological, and functional properties. A part of these EVs shared the similarities as MtVs in size, CD-9 and annexin A5 surface markers, ALP activity, increase the concentration of free phosphate in the medium as well as deposited phosphate on ECM. These isolated MtVs could potentially be used as a biological factor for bone regeneration and fracture healing.

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Chapter 8

General discussion

Preface

Fetal bovine serum (FBS) is a widely used supplement in cell culture media that provides cells with vital factors, including growth factors, hormones, and vitamins, essential for cell function, survival, growth, and division. However, the use of FBS in *in vitro* cell culture is controversial. FBS is a variable and undefined medium supplement with unknown and complex composition, which can change between FBS batches. This undefined complex composition has been shown to affect experimental outcomes and FBS containing medium needs to be avoided. In tissue engineering studies, FBS could be avoided by formulating a defined and more controlled medium supplementation. The aim of this thesis was to develop defined serum substitute media for bone tissue engineering studies. Such medium formulations should not only diminish the influence of unknown factors of FBS but should also enable the investigation of the effect of different soluble factors or cell-derived extracellular vesicles (EVs) on cell behavior and *in vitro* bone tissue formation/resorption. This chapter gives an overview of the main findings of the current thesis, the remaining challenges, and the future perspective.

8.1 Main findings

Bone tissue engineering has emerged to facilitate the regeneration of large bone defects through 1) *in situ* bone regeneration using scaffolds with biocompatible, osteoinductive, and osteoconductive properties or 2) development of *in vitro* engineered functional tissues making use of scaffolds, progenitor cells, biochemical and/or biomechanical stimuli for subsequent implantation into bone defects. The advances in the development of such engineered tissues could also be applied to create three-dimensional (3D) *in vitro* human bone models for studying human bone physiology, pathology, or for preclinical drug testing and creating personalized tissue models. In case of using such models to create personalized *in vitro* bone models, the cell heterogeneity among patients needs to be considered. In **Chapter 2**, we described the possibility of development of personalized *in vitro* bone models using patient-specific cells and the potential sources to isolate both osteoblast and osteoclast progenitor cells. In this chapter, we discussed the advantages and disadvantages of bone marrow and peripheral blood as two main sources to isolate both progenitor cells. We concluded that peripheral blood can be considered to be a suitable source for osteoblast and osteoclast progenitor cells, being less invasive for patients.

The next part of the thesis focused on the biochemical environment of bone tissue engineering studies. It has been shown in the previous studies that FBS can affect the amount of deposited calcium phosphate during the osteogenic differentiation process *in vitro* [1], [2]. **Chapter 3** focused on measuring the enzymatic activity of alkaline phosphatase (ALP) in FBS. We demonstrated that FBS from different suppliers showed different levels of ALP activity. The ALP activity intrinsic to FBS contributed to osteoblast differentiation of human bone marrow mesenchymal stromal cells (hBMSCs) and matrix mineralization in osteogenic differentiation cultures. The results indicated that in the media with low inherent ALP activity, the cellular ALP activity was higher compared to the media with higher inherent ALP activity. Moreover, in the medium with high ALP activity, the amount of deposited calcium phosphate was higher compared to medium with low ALP activity, in the presence or absence of cells. This study highlighted the need to replace FBS with a defined and more controlled serum substitute medium.

To overcome the disadvantages of using FBS in culture, namely, ethical, safety, and scientific issues, in **Chapter 4** and **Chapter 5**, we described the development of serum substitute media. The focus of **Chapter 4** was on the development of a serum substitute medium in a stepwise approach for osteogenic differentiation cultures. Essential components were added to the basal medium while hBMSCs, osteoblast progenitor cells, were cultured in 2D and 3D substrates for three weeks. In both conditions, the final composition of the serum substitute medium was able to support the attachment of cells to the substrate, cell survival, differentiation of hBMSCs towards osteoblasts and the deposition of collagenous matrix. The use of this serum substitute medium was evaluated when culturing cells under mechanical stimuli in the form of shear stress. The results of the study showed that the application of shear stress seemed to be essential to induce the mineralization of the deposited matrix. These observations were in agreement with early *in vivo* studies which showed that mechanical loading stimulates the formation of woven bone.

In **Chapter 5**, a specialized serum substitute medium for osteoclast differentiation cultures was developed. With the same stepwise approach, essential components were added to the basal medium. Monocytes, osteoclast progenitor cells, were seeded in 2D well-plates and on Osteo-Assay plates and cultured in the serum substitute medium. The formation of tartrate-resistant acid phosphatase (TRAP) expressing multinucleated osteoclast with distinct actin ring and the resorption activity of mature osteoclasts after three weeks of culture were analysed. The results indicated that the developed serum substitute medium was able to support cell attachment, monocyte differentiation towards TRAP expressing multinucleated osteoclasts, and the resorption of mineralized matrix. However, this medium is still less optimal than medium containing FBS and needs to be more optimized.

These two specialized serum substitute media could pave the way in eliminating the use of controversial FBS and providing a better-defined biochemical environment for cells in bone tissue engineering studies.

Extracellular vesicles (EVs) are nano-sized bilayer vesicles secreted by all cell types and involved in cell-cell communication. A subset of EVs produced by bone-forming osteoblasts is known as matrix vesicles (MtVs). **Chapter 6** described the different types of EVs released by bone-specific cells with a focus on MtVs. This chapter discussed the role of MtVs in biomineralization and their potential application for bone-related diseases and bone regeneration. Here, we proposed various novel approaches to target bone-related diseases and bone regeneration, such as stimulating MtV secretion *in vivo*, stimulating MtV secretion *in vitro* by stimulating MtV-producing cells and engineering biomimetic MtVs. MtVs derived from the second and third approach, cellular MtVs and engineered MtVs, have the advantage that they could be delivered to the region of interest either by localized injection or by implantation of MtV-loaded biomaterials.

Considering the role of MtVs in bone mineralization, in **Chapter 7**, we aimed at isolating the secreted EVs during osteogenic differentiation of hBMSCs from the human 3D *in vitro* woven bone model previously developed by our group [3]. The EVs secreted into the cell culture medium during the development of *in vitro* woven bone were isolated using size-exclusion chromatography columns and characterized based on their morphological and biological properties. The secreted EVs showed similar characteristics as MtVs. Later, isolated MtVs were used as a biological component in the developed serum substitute medium in **Chapter 4**. Culture of hBSMCs in serum substitute medium containing MtVs resulted in an increase of deposited mineralized matrix compared to when no MtVs were added to the medium. The results of this study highlighted that MtVs can be secreted during the development of *in vitro* woven bone. These MtVs potentially could be used as a biological component for bone regeneration and fracture healing through, for instance, integration with biomaterials to target bone formation locally.

8.2 Remaining challenges and future perspective

8.2.1 Alternatives to FBS: Complexity vs simplicity

FBS is a complex mixture of different components, such as growth factors, vitamins, trace elements, hormones, etc., crucial for the growth and maintenance of cells [4]. Although this complex composition provides nutrients for almost all cell types, it could mask the effects of exogenous or endogenous factors on cells. For instance, it has been shown that FBS blocked the effect of transforming growth factor β-3 on chondrocyte maturation [5]. Another study confirmed that adding FBS damped the intracellular calcium signaling in chondrocytes which could ultimately affect various physiological processes such as gene expression [6]. Furthermore, the presence of growth factors such as basic fibroblast growth factor, epidermal growth factors, insulin-like growth factors, and hormones such as parathyroid hormone and estrogen in FBS could affect osteoblasts and osteoclasts growth and differentiation in culture [7], [8]. The presence of such factors in FBS could potentially limit the study of the influence of these factors on bone-specific cells, as they are already present in FBS in unknown concentrations. Moreover, FBS contains significant quantities of EVs that confound quantitative and qualitative analyses of the EVs secreted by cells *in vitro* [9]. The EVs of FBS could also interfere the intercellular communication through cellular EVs [10]. The complex and unknown composition of FBS, along with its batch-to-batch variation, ethical, and safety issues, urges the need to find alternatives to FBS.

Over the past few decades, many sources have been introduced to substitute FBS in culture. Human platelet lysate (hPL) is among them, which could address ethical and safety issues of FBS [11], [12]. Like FBS, hPL contains many factors that promote cell attachment, growth, and proliferation *in vitro* [11], [13]. However, the complex composition and concentration of growth factors present in hPL are not known and can vary depending on the preparation procedure [11], [13]. Therefore, using hPL in culture could affect the reproducibility of the experimental outcomes and limit the study of the soluble factors on cells. A chemically defined serum-free medium represents an ideal formulation for consistency across all cell culture experiments and labs [14]. Investigations into cell function have resulted in the identification of many components which have been helpful in the development of chemically defined media [4]. There are many chemically defined media commercially available for cell cultures. These commercially available media are not without disadvantages. They are mostly cell-specific and are not capable of replacing FBS to culture all kinds of cell types, thus requiring further optimization for the cell of interest [13]. Moreover, the exact formulation of these media is not known and are often a corporate secret [11]. The unknown composition of these commercially available media makes them as well unfavorable to study the effect of soluble factors on cellular behavior and function.

Moving from the complex and unknown biochemical environment of FBS and its current alternatives towards a simple and defined serum substitute medium with known concentrations of growth factors and components in which cells can retain their growth and function could address the reproducibility, ethical, and safety issues. The serum substitute media developed in **Chapter 4** and **Chapter 5** for osteoblast and osteoclast cultures, respectively, contains the minimum number of components that were required for cell survival, differentiation, and function. This simplicity of the media avoids the

unwanted and uncontrolled experimental outcomes resulting from a complex biochemical environment of FBS. Future studies could make use of the developed media to investigate the influence of other soluble chemical or biological factors on cellular behavior and the interactions between cells and materials without being overshadowed by FBS.

8.2.2 Validation of the outcomes from serum substitute medium

A challenge in the development of serum substitute medium is the validation of the newly formulated medium. The outcomes of the serum substitute medium have been compared mainly to the current standard: medium containing FBS. This raises the question of how to validate the results obtained from serum substitute medium if the outcomes of the FBS containing medium are unreliable or inconsistent due to the variable nature of FBS. The influence of FBS and its composition on bone tissue engineering has been addressed before. Bone tissue engineering studies have demonstrated that the highly variable chemical composition of FBS between different brands/batches acts as a strong biochemical cue that can affect the deposition of the mineralized matrix during *in vitro* osteoblast differentiation [1], [2]. Interestingly, a previous study has demonstrated that different FBS types could affect the deposition of mineralized matrix under the application of mechanical loading [2]. We showed that these variations in mineralized matrix deposition could be due to different levels of ALP activity of FBS. The FBS containing medium with higher ALP activity resulted in more mineral deposition compared to the medium with lower ALP activity of FBS, with or without the presence of cells. Since FBS can affect the experimental outcomes, when developing a serum substitute medium, choosing a different FBS brand/batch as a control group could change our expectations of the experimental outcomes and it might cause the craving to constantly change the serum substitute medium composition to reach the outcomes of experiments using medium containing FBS. Therefore, comparing the experimental outcomes obtained in a serum substitute medium with the results of an experiment performed in one type of FBS might be misleading. The main objective of developing a serum substitute medium could be retaining the desired cellular functions and validating the experimental outcomes with prior knowledge obtained through *in vivo* observations. For instance, mesenchymal stromal cells (MSCs) condense and directly differentiate into osteoblasts during bone formation through intramembranous ossification [15]. The osteoblast differentiation is initiated by the activation of runt-related transcription factor-2 (RUNX-2). Then, differentiated osteoblasts secrete unmineralized collagenous matrix, which further gets mineralized [16], [17]. Bone resorption occurs through the recruitment of monocytes to the resorption site and further their differentiation towards bone-resorbing multinucleated osteoclasts under the influence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [18]. These factors, *in vivo*, are produced by osteoblasts, osteocytes, and stromal cells. Activated osteoclasts secrete hydrogen ions and proteolytic enzymes which degrade the inorganic and organic components of bone [19], [20]. These cellular behavior and functions could be retained in the serum substitute media that were developed for osteoblast and osteoclast cultures. However, more detailed investigations might be needed to validate the serum substitute media further. For instance, the signaling networks that control the differentiation process could be studied when cells are cultured in the serum substitute media. These signaling pathways have been identified through *in vivo* molecular and genetic studies,

and their activation is necessary for osteoblast differentiation, deposition of mineralized extracellular matrix, and the fusion of monocytes and their differentiation towards multinucleated bone-resorbing osteoclasts [21]–[25].

8.2.3 Development of serum substitute medium for co-culture systems

In this thesis, two types of specialized serum substitute media have been developed: one for osteoblast and one for osteoclast cultures. However, many research lines in bone tissue engineering require a co-culture of two or more cell types, for instance, to understand the cellular interactions within bone remodeling processes. Bone remodeling is tightly regulated by a crosstalk between osteoblasts, osteocytes, and osteoclasts. Osteoblasts, osteocytes, and osteogenic progenitor cells secrete M-CSF, RANKL, and osteoprotegerin (OPG) that regulate survival and proliferation of mononuclear monocytes and their differentiation into bone-resorbing multinucleated osteoclasts [26]. Osteoclasts also release soluble factors such as sphingosine 1 phosphate (S1P) and collagen triple helix repeated containing 1 (CTHRC1) and complement component C (C3) that regulate osteoblast differentiation and matrix deposition [27]. A serum substitute medium for a culture system with both osteoblast and osteoclast progenitor cells needs to be composed of all the components that can support their growth, differentiation towards respective lineage, and function. The interaction between osteoblasts and osteoclasts through their released soluble factors could change the composition of the serum substitute media developed for each cell type and limit the addition of exogenous stimulations. For instance, an early study reported the development of a serum substitute medium for the co-culture of osteoblasts derived from human trabecular bone and monocytes derived from human peripheral blood [28]. This medium supported the phenotypic maturation of osteoblasts and induced the highest ratio expression of RANKL/OPG by osteoblasts, and when co-cultured with monocytes, TRAP-positive multinucleated osteoclasts were formed without the need for exogenous osteoclast stimulators in the serum substitute medium [28]. This medium could be a good starting point to develop a serum substitute medium for the co-culture of osteoblasts and osteoclasts. However, only mature osteoblasts were used in this study and the potential of the medium to support osteoblast differentiation of MSCs was not studied. Future studies could investigate the development of a serum substitute medium that can support both osteoblast and osteoclast progenitor cells differentiation towards their respective lineage. Such a defined medium could control the cellular interaction between osteoblasts and osteoclasts without the concerns of using FBS.

Osteoblasts and osteoclasts are not the only cells involved in bone remodeling. Osteocytes are terminally differentiated osteoblasts that become embedded within the bone matrix during bone formation [29]. Osteocytes are star-shaped cells with multiple cytoplasmic processes that contact other bone cell types. The osteocyte network senses the mechanical loads and releases paracrine factors that regulate bone remodeling [26], [30]. Studying the influence of osteocytes on the bone remodeling process *in vitro* in a biochemically controlled environment would require a serum substitute medium that supports osteoblasts, osteoclasts, and osteocytes. Although osteocytes are differentiated from osteoblasts, their culture medium requirement might not be exactly similar to

osteoblast cultures. For instance, dexamethasone as a factor that initiates osteoblast differentiation of MSCs does not seem to be needed for osteocyte cultures [31]–[34].

Blood vessels in the skeletal system not only transport gases, nutrients, metabolic wastes, or cells but also provide signaling molecules involved in bone regeneration and remodeling. Direct contact of capillaries with bone tissue makes endothelial cells communicate with osteoblasts and osteoclasts through cell-cell connection or release of proteins and EVs [35]. Several studies have shown that endothelial cells secrete signaling molecules such as vascular endothelial growth factor, RANKL, and OPG, each can influence bone formation and resorption during the bone remodeling process [36]–[39]. Developing a serum substitute medium to support endothelial and bone-specific cells in a controlled environment could facilitate investigating the interaction between these cell types without being masked by the complex and undefined composition of FBS.

8.2.4 Methods to develop complex serum substitute media

In this thesis, the serum substitute medium was developed using the classical and commonly used method of a step-by-step approach. The media pyramid, a modular approach for the development of serum substitute media, has been introduced by Jan van der Valk [4]. In this method, general essential supplements for cellular growth, which were defined by several previous studies, have been added to the basal medium when excluding FBS from the culture. The bottom of the pyramid contains the basal medium supplemented with insulin-transferrin-selenium (ITS). Then, proteins, amino acids, and lipids which have significant roles in cellular activities, need to be added to the medium. Moving to the tip of the pyramid in media formulation development, specific soluble and growth factors need to be added to the medium. This approach and several others similar to this one, such as Ham's, Sato's, top-down, and bottom-up approaches, which consist of trial and error of various components with different concentrations, have been applied in different studies [40]–[43]. Even though these approaches could be efficient and sufficient for developing specialized serum substitute medium for one specific cell type, for more complex cultures with multiple cell types involved, more advanced approaches are needed. Different components and growth factors required for each cell type in a culture medium might act synergistically. In this regard, a statistical experimental strategy called a design of experiments (DoE) could be a way to investigate specific interactions between growth factors under screening [43]. DoE is a tool for investigating a system's mathematical relationships between input and output variables. The DoE is mainly composed of screening designs and response surface designs [44], [45]. The screening design aims at identifying the most crucial components from many potential components. Once these components are identified, a response surface design can be used to estimate the optimized concentrations and interactions of each component [45]. These approaches could be a powerful tool for the development of a serum substitute medium when many different factors with various effects on multiple types of cells in co-cultures need to be considered. DoE approaches also have their own disadvantages, such as resulting in a high number of time- and/or cost-consuming experiments in complex systems. Moreover, these approaches are based on user-defined choices of the components and their concentrations obtained through literature or experience, which could potentially affect the whole composition of the medium [46]. Nevertheless,

for future studies, the statistical approaches would be recommended over the stepwise approaches to develop a complex serum substitute medium for multiple cell types in future studies.

8.2.5 Reduce the bias in studies with randomization and blinding

Randomization and blinding are considered to be important tools in determining the effectiveness of a new treatment, preventing any subjective biases, and maximizing the validity of study results [47]. Randomization is a method of allocating subjects/samples to treatment groups such that every subject/sample has an equal chance of receiving any one of the treatments [47], [48]. The randomization techniques ensures that subjects/samples are randomly assigned to different treatment groups which prevent the systematic arrangement of treatments and avoid predictability of the outcomes [48]. Blinding is used in combination with randomization to eliminate intentional or unintentional bias, increase the objectivity of results, and ensure the credibility of study conclusions by making the researcher unaware of treatment groups [48]. The randomization and blinding techniques are common practices in conducting human clinical and animal preclinical studies [49], [50]. To reduce potential sources of bias such as selection bias or detection bias in *in vitro* studies, applying the same techniques when planning a study might need to be considered. In cell culture experiments, randomization has been done to some extent. For instance, cell suspension is pipetted into different wells of a well-plate or on different scaffolds meaning that cells are randomized to the wells or scaffolds [51]. Furthermore, the wells or scaffolds containing cells can be randomized to the treatments. To reduce the bias in collecting the results, at the end of the experiment, blinding techniques can be implemented to make the researcher or investigator unaware of the treatment groups, for instance, coding the cell-seeded scaffolds that have been cultured in either dynamic or static conditions by someone other than the main researcher. This procedure would be able to reduce the detection bias by preventing looking for the desired outcomes. For future studies, it would be suggested, if possible, consider applying such bias-reducing measures when planning a study.

8.3 General conclusion

Taken together, this thesis highlighted that use of FBS in *in vitro* cell/tissue cultures is best avoided due to its scientific, ethical, and safety issues. FBS is composed of unknown and undefined components that affect the experimental outcomes. ALP was identified as one of the components of FBS. Its variability from one batch/brand to the other affects mineralized matrix deposition and osteogenic differentiation of MSCs. The unreliable and irreproducible experimental outcomes obtained due to the complex and unknown composition of FBS urge the development of a defined serum substitute medium. This thesis focused on developing two types of serum substitute media for osteoblast and osteoclast cultures. The defined and known composition of these serum substitute media contributes to revealing the cell-cell interactions within a biological process and the influence of chemical or biological components on bone-specific cell behavior and function *in vitro* without the influence of complex composition of FBS. The developed serum substitute media in this thesis pave the way in eliminating the use of FBS in bone tissue engineering disciplines and could be a guide to develop such defined media for other tissue engineering applications.

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Summary

Tissue engineering is a discipline that integrates biology with engineering to repair, restore, or regenerate living tissue using biomaterials, cells, and biochemical factors alone or in combination. Bone tissue engineering has emerged to facilitate bone regeneration of large bone defects resulting from trauma, metabolic diseases, infection, or tumor removal. To induce bone regeneration in situ, specific scaffolds have been designed and developed that could be decorated with bioactive factors to induce bone formation. In addition to the cell-free approach to support bone regeneration, bone tissue engineering principles have been applied to develop functional engineered tissue *in vitro* for subsequent implantation into the bone defect. In this approach, cells are seeded onto a three-dimensional (3D) scaffold with or without bioactive molecules to form an *in vitro* tissue. Advances in the development of such engineered tissues can also be used to create 3D *in vitro* human bone models. These tissue models facilitate the study of complex physiological and pathological processes and the evaluation of new therapeutic approaches. In addition to using sophisticated scaffolds (to mimic the 3D environment of the tissue), cell types, bioreactors (to apply mechanical loads needed for tissue growth), and biochemical factors (to control cellular differentiation and activity) need to be considered in bone tissue engineering studies.

In this thesis, chapter 1 provided a general overview of bone tissue engineering and its potential in the development of *in vitro* bone models, fetal bovine serum (FBS) and its use in *in vitro* studies, and the need to replace FBS with a defined serum substitute medium. The selection of cells in bone tissue engineering studies as a tool to develop human *in vitro* bone models is crucial. The cell heterogeneity among patients and cell donor variability need to be considered in the development of such *in vitro* models. In chapter 2, we reviewed the cell sources for the development of personalized human *in vitro* bone models. In this review, the importance of using osteoblast and osteoclast progenitor cells as the most promising cell types for the development of *in vitro* bone models was discussed. Furthermore, osteoblast and osteoclast progenitor cells obtained from bone marrow and peripheral blood of individuals, the main two cell sources to obtain bone-specific progenitor cells, were compared in terms of the ease of cell isolation, proliferation capacity, and differentiation potential of progenitor cells. Besides donor heterogeneity, cell culture composition plays an important role in bone tissue engineering and the development of *in vitro* tissue models. FBS is a widely used cell culture supplement that provides cells with a broad spectrum of macromolecules, proteins, lipids, trace elements, vitamins, and attachment factors essential for cell survival and growth. However, many disadvantages have been attributed to the use of FBS in culture, such as ethical, safety, and scientific issues. The undefined, complex, and inconsistent composition of FBS has been shown to greatly affect the experimental outcomes. In bone tissue engineering, the deposited mineralized matrix is one of the main outcomes but using different brands/batches of FBS can result in great variations in the deposition of minerals within the matrix. In chapter 3, we showed that alkaline phosphatase (ALP), an enzyme produced by osteoblasts during bone formation, is present in FBS and contributes to changes in phosphate concentration of the medium, mineral deposition within the produced extracellular matrix (ECM) *in vitro*, and osteogenic differentiation. The result of this study highlighted the importance to develop specialized serum substitute media for tissue engineering studies. In

chapters 4 and 5, we aimed at developing such specialized serum substitute medium for bone-specific cell types, bone-forming osteoblasts and bone-resorbing osteoclasts. These media could support cell survival, growth, differentiation of osteoblast and osteoclast progenitor cells to their respective lineage, and cell function. Such medium formulation not only diminishes the influence of unknown factors of FBS but also enables to study the effect of different soluble factors, chemical or biological, on cell behavior and *in vitro* bone tissue. One of the biological factors that influences bone tissue formation is extracellular vesicle (EV). Osteoblasts shed a subset of EVs known as matrix vesicles (MtVs), which contain phosphatases, calcium, and inorganic phosphate. These MtVs have a major role in matrix mineralization and feature innate bone-targeting and bone formation properties. In chapter 6, we reviewed the EVs secreted by bone-specific cells, with a focus on MtVs, their biogenesis, characteristics, and contribution to bone mineralization. Finally, the potential therapeutic application of MtVs to treat bone-related diseases or fractures was discussed. In Chapter 7, we aimed at isolating the released EVs during osteogenic differentiation of hBMSCs in the human 3D *in vitro* woven bone models previously developed by our group. The characteristics of a part of isolated EVs shared similarities with MtVs. These vesicles showed alkaline phosphatase (ALP) activity, increased the amount of released free phosphate into the culture medium, and increased the amount of deposited phosphate within the ECM. These results indicated that a complex 3D environment mimicking bone development is favorable to stimulate MtV-producing cells to produce targeted MtVs *in vitro*. In chapter 8, the main findings of this thesis are presented, as well as potential future directions.

To conclude, in this thesis, we reported the development of serum substitute media for bone tissue engineering applications. The development of such media enables the replacement of FBS in bone tissue engineering studies and provides the opportunity to study the influence of soluble factors on bone formation/resorption in a less variable environment and without being overshadowed by unknown factors within FBS.

Curriculum vitae

Sana Ansari was born on July 23rd, 1992, in Tehran, Iran. After finishing her pre-university education in 2010 at Khavarmanesh high school in Tehran, Iran, she started the Bachelor of Science program in Materials Science and Engineering at Amirkabir University of Technology in Tehran. In 2015, she started the Master of Science program in Biomedical Engineering at Amirkabir University of Technology. In her master's end project, she worked at the Pasteur Institute of Iran and focused on the development of drug-loaded nanofibrous scaffolds and studied the drug release from the scaffolds and its influence on cartilage-specific cells (chondrocytes). The release of drugs from the nanofibrous scaffolds and its influence on cells were also studied under the application of hydrostatic pressure. In July 2018, Sana moved to the Netherlands to start her Ph.D. project in the Orthopaedic Biomechanics (OPB) group at the Biomedical Engineering department of Eindhoven University of Technology. In her research, she worked under the supervision of dr. Sandra Hofmann and prof.dr. Keita Ito on the development of serum substitute medium for bone tissue engineering applications. The results of her Ph.D. research are presented in this thesis.

Scientific output

Related to this thesis:

Sana Ansari, Keita Ito, Sandra Hofmann. Cell sources for human in vitro bone models. *Current Osteoporosis Reports*, 19, 88-100 (2021). Doi: 10.1007/s11914-020-00648-6

Sana Ansari†, Bregje W. M. de Wildt†, Michelle A. M. Vis, Carolina E. de Korte, Keita Ito, Sandra Hofmann, Yuana Yuana. Matrix vesicles: Role in bone mineralization and potential use as therapeutics, *Pharmaceuticals*. 14(4), 289, (2021). Doi: 10.3390/ph14040289

†Equal contribution

Sana Ansari, Keita Ito, Sandra Hofmann. Alkaline phosphatase activity of serum affects osteogenic differentiation cultures. *ACS Omega*, 15, 12724-12733 (2022). Doi: 10.1021/acsomega.1c07225

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Sana Ansari, Keita Ito, Sandra Hofmann. Towards development of serum substitute medium to induce osteoclast differentiation of human peripheral blood derived monocytes, Submitted. Preprint available at BioRxiv. Doi: 10.1101/2023.01.18.524526

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Other publications:

Bregje W.M. de Wildt, **Sana Ansari**, Nico A.J.M. Sommerdijk, Keita Ito, Anat Akiva, Sandra Hofmann. From bone regeneration to three-dimensional *in vitro* models: tissue engineering of organized bone extracellular matrix. *Current Opinion in Biomedical Engineering*, 10, 107-115 (2019). Doi: 10.1016/j.cobme.2019.05.005

Anat Akiva, Johanna Melke, **Sana Ansari**, Nalan Liv, Robin van der Meijden, Merijn van Erp, Feihu Zhao, Merula Stout, Wouter H. Nijhuis, Cilia de Heus, Claudia Muñiz Ortera, Job Fermie, Judith Klumperman, Keita Ito, Nico A.J.M. Sommerdijk, Sandra Hofmann. An organoid for woven bone. *Advanced Functional Materials.* 31(17), 2010524 (2021). Doi: 10.1002/adfm.202010524

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Conference contributions:

Sana Ansari, Anat Akiva, Johanna Melke, Claudia Muniz-Ortera, Sandra Hofmann, Nico A.J.M. Sommerdijk. Evaluation and characterization of bone tissue formation, CHAINS 2018, *poster presentation*.

Sana Ansari, Claudia Muniz-Ortera, Anat Akiva, Johanna Melke, Kieta Ito, Sandra Hofmann, Nico A.J.M. Sommerdijk. Correlation of Non-collagenous proteins and collagen mineralization in a 3D tissue culture, MDR annual meeting 2018, *poster presentation.*

Sana Ansari, Johanna Melke, Claudia Muniz Ortera, Keita Ito, Nico A.J.M. Sommerdijk, Anat Akiva, Sandra Hofmann. Correlation of collagen mineralization and non-collagenous proteins expression during osteoblast/osteocyte differentiation process, Dutch biophysics 2019, *poster presentation.*

Sana Ansari, Johanna Melke, Keita Ito, Sandra Hofmann. Effect of glucose concentration on osteogenic differentiation *in vitro* in a defined serum substitute medium, NBTE 2019, *oral presentation.*

Sana Ansari, Keita Ito, Sandra Hofmann. Serum alkaline phosphatase activity impacts the osteogenic differentiation process, NBTE 2020, *poster presentation.*

Sana Ansari, Keita Ito, Sandra Hofmann. Modulating glucose concentration in a fully defined medium for successful *in vitro* osteogenic differentiation. TERMIS-EU 2020, *poster presentation.*

Sana Ansari, Keita Ito, Sandra Hofmann. Development of serum substitute medium for 3D human *in vitro* bone models, NBTE 2021, *poster presentation.*

Sana Ansari, Keita Ito, Sandra Hofmann. Influence of serum alkaline phosphatase activity on osteogenic differentiation, TERMIS-World, 2021, *poster presentation.*

Sana Ansari, Keita Ito, Sandra Hofmann. Development of a serum substitute medium for bone tissue engineering, MDR annual meeting 2022, *oral presentation.*

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