Feasibility of Cell Lines for In Vitro Co-Cultures Models for Bone Metabolism

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Abstract

Today, over 70 diseases and health conditions are known that negatively affect the bone quality directly or indirectly by their medical treatment, establishing the term metabolic bone disease. Already every third hospitalized patient in Europe suffers from musculoskeletal injuries or diseases. Facing an ageing society and a more and more sedentary lifestyle the number of chronic diseases and consequently metabolic bone diseases are expected to continuously increase. In order to investigate the various disease constellations and/or develop new treatment strategies suitable models representing bone metabolism are required. Many in vivo, ex vivo and in vitro models have been described, which have their advantages and limits. We here summarize the advantages and challenges of frequently used models to investigate bone metabolism, focusing on in vitro co-cultures of bone forming osteoblasts and osteoclasts. Comparing own data with published models, we further elaborate the feasibility of commonly used cells lines for such in vitro co-culture models, in order to provide an easy, constantly available, and up-scalable model system for screening alterations in bone metabolism.

Keywords: Osteoblast/Osteocyte; Osteoclast; Cell Lines; Co-culture; Bone Metabolism.

1. Introduction

Alterations in bone metabolism often affect bone stability. Osteoporosis is by far the most common metabolic bone disease worldwide. The international osteoporosis foundation estimates that approx. every third woman and every fifth man are affected. The world health organization (WHO) defined osteoporosis as “a reduction in bone mineral density (BMD) of 2.5 standard deviations or more below that of the mean peak BMD of young adults when measured by dual-energy x-ray absorptiometry” [1]. The actual bone loss does not cause any symptoms (silent disease), therefore, many people affected only get to know when having a so called fragility fracture [1]. Currently in Europe approx. 3.5 million fragility fractures occur every year. The most common are in the spine, the hip, and the distal forearm, with a yearly economic burden estimated to be at least 37 billion € [2].

There are a number of different ways in which osteoporosis can develop. Typically, an increased bone resorption and/or decreased bone formation results in loss of bone mineral density (BMD), affecting most of the skeleton. But osteoporosis can also occur locally, as a result of reduced muscle forces on the bone, for example during paralysis. Based on the cause of the disease, there exist a variety of different types of osteoporosis [2-4].

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1.1. Primary Osteoporosis

Primary osteoporosis is the most commonly described type of osteoporosis. It results from loss of BMD and alterations in bone structure that occur as people age. Thus, it is often referred to as age-related osteoporosis. Although primary osteoporosis develops in both sexes, it is two to three times more common in women than men [5]. While men typically show slow but continuous bone loss (up to 25% of cortical and trabecular bone) during aging, women show an additional phase of rapid bone loss at the beginning of the menopause that lasts for about 4 to 8 years. During this initial rapid phase of bone loss women lose up to 10% of cortical bone and up to 30% of trabecular bone. Thus, the term post-menopausal osteoporosis is also used [6]. Thus, primary osteoporosis is mainly caused by estrogen deficiency [7], with the consequence that bone resorption is increased and bone formation is decreased. This also applies for men. However, their deficiency in sex hormones is mainly due to an increase in sex hormone binding globulin, which inactivates testosterone and estrogen [6, 7]. In the late continuous phase of bone loss calcium balance might play a crucial role. As a result of impaired calcium absorption by the intestine and/or impaired calcium conservation by the kidneys, serum calcium levels are reduced. To compensate for the lowered calcium levels parathyroid hormone (PTH) levels are increased, causing thinning of the bones by the release of calcium [8]. To compensate for the thinning of the cortical bone shell, the outside diameter of weight bearing bones might increase with age [9].

Very rarely children or young adults develop a primary osteoporosis, which is then called idiopathic primary osteoporosis. Although, the exact causes of the disease are often unknown mainly bone growth is impaired in this juvenile form of primary osteoporosis [10].

1.2. Secondary Osteoporosis

Nowadays, facing an ageing society and a sedentary lifestyle the number of chronic diseases continuously increases. As a consequence of these chronic diseases, other health condition and their medication, osteoporosis affects more and more people of all ages. In fact by now there are over 70 diseases and health conditions known that contribute to the development of osteoporosis [11-13]. These include endocrine disorders (e.g. Diabetes mellitus, hyperparathyroidism, or Cushing’s syndrome), digestive and gastrointestinal disorders (e.g. Crohn’s disease, malabsorption syndrome, or liver diseases), hematologic disorders (e.g. leukemia, haemophilia, or lymphomas), genetic disorders (e.g. osteogenesis imperfecta, homocystinuria, or cystic fibrosis), hypogonadal states (e.g. with anorexia nervosa, Klinefelter’s or Turner’s syndrome), chronic viral infections (e.g. HBV/HCV fibrosis or cirrhosis), rheumatic and auto-immune diseases (e.g. rheumatoid arthritis, lupus, or psoriasis), and others (for summary see Table 1). Affected patients are said to have “secondary” osteoporosis.

Table 1. Diseases that cause or contribute to the development of secondary osteoporosis [11-13]

<table>
<thead>
<tr>
<th>Endocrine</th>
<th>Cystic fibrosis</th>
<th>Rheumatic and auto-immune</th>
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<tr>
<td>Acromegaly</td>
<td>Ehlers-Danlos syndrome</td>
<td>Ankylosing spondylitis</td>
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<td>Addison’s disease</td>
<td>Glycogen storage diseases</td>
<td>Graves’ disease</td>
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<td>Adrenal insufficiency</td>
<td>Gaucher’s disease</td>
<td>Lupus</td>
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<td>Cushing’s syndrome</td>
<td>Homocystinuria</td>
<td>Psoriasis</td>
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<td>Diabetes mellitus</td>
<td>Hypophosphatasia</td>
<td>Rheumatoid arthritis</td>
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<td>Hyperparathyroidism</td>
<td>Idiopathic hypercalciuria</td>
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<td>Thyrotoxicosis</td>
<td>Marfan syndrome</td>
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<td>Primary biliary cirrhosis</td>
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<td>Primary sclerosing cholangitis</td>
<td>Osteogenesis imperfecta</td>
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<td>Secondary hemochromatosis</td>
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<td>Ulcerative colitis</td>
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<td>Weight loss surgery</td>
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<td>Genetic</td>
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<td>Others</td>
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<td>Hemophilia</td>
<td>Anorexia nervosa</td>
<td>Alzheimer’s disease</td>
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<td>Leukemias</td>
<td>Female athlete triad</td>
<td>Amyloidosis</td>
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<td>Lymphomas</td>
<td>Hyperprolactinemia</td>
<td>Cancer (breast, prostate, etc.)</td>
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<td>Multiple myeloma</td>
<td>Klinefelter’s syndrome</td>
<td>Chronic metabolic acidosis</td>
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<td>Sickle cell disease</td>
<td>Panhypopituitarism</td>
<td>Congestive heart failure</td>
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<td>Systemic mastocytosis</td>
<td>Premature menopause</td>
<td>COPD, asthma, chronic bronchitis</td>
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<td>Thalassemia</td>
<td>Turner’s syndrome</td>
<td>Depression</td>
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<td>Hematologic</td>
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<td>End stage / chronic kidney disease</td>
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<td>Chronic viral</td>
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<td>HBV / HCV fibrosis or cirrhosis</td>
<td>Idiopathic scoliosis</td>
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<td>HIV / AIDS</td>
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<td>Poliomyelitis</td>
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<td>Stroke</td>
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These patients typically lose more BMD than a normal individual of the same age, gender and race. Their bone loss is fostered by various factors. These include secondary effects of the diseases themselves, e.g. calcium and phosphorus malabsorption in the intestines, insufficient calcium conservation in the kidneys, impaired availability of vitamin D, reduced production of sex and growth hormones, excessive production of glucocorticoids or PTH, constant production of inflammatory cytokines (chronic inflammation), or impaired mobility (missing mechanical stimulation) and balance, but also side effects of the required medication [14-16]. For example in a representative German level 1 trauma center 80.5% of all patients suffer from one or more chronic diseases requiring medication (Ø 4.3 drugs per patient): approx. 13% are diabetics [17, 18], 22% are at risk for malnutrition [19], 15% drink alcohol on a daily base, and 42% are smokers [20]. These patients are above-average in developing post-surgical complications, e.g.; delayed wound or fracture healing, which in turn results in significantly prolonged hospital stays [21], and often aggravates the underlying disease.

1.3. Effects of Drugs on Bone Metabolism

The most commonly known drugs associated with secondary osteoporosis are glucocorticoids, e.g. prednisone (Deltasone, Orasone, etc.), prednisolone (Preloene), dexamethasone (Decadron, Hexadrol), or cortisone (Cortone) [22-25]. Glucocorticoids are used to treat a wide variety of inflammatory and autoimmune diseases, including the above mentioned asthma, COPD, rheumatoid arthritis, and Crohn’s disease. Glucocorticoids strongly affect osteoblast function but may also stimulate osteoclast function [23, 26-28]. This imbalance in bone metabolism induces a rapid loss of BMD early in the course of treatment. Even low dose therapies (equivalent to 2.5–7.5 mg prednisone per day) are associated with a low BMD and an increased fracture risk [29, 30]. Thus, long-term glucocorticoid treatment is reported to lead to fractures in up to 50% of the patients [23, 31].

Continuous intake of anti-coagulants, commonly used as anti-thrombotic agents, including the classical heparins, vitamin K antagonists (e.g. warfarin, acenocoumarol, phenprocoumon, and fluindione), and novel oral anti-coagulants (e.g. rivaroxaban, edoxaban, apixaban, and dabigatran) are associated with an increased prevalence of osteoporosis [32]. Based on their pharmacological mode of action these drugs may both decrease bone formation by inhibiting osteoblasts but also increase bone resorption by stimulating osteoclasts [33]. Interestingly, despite having positive effects on BMD, there is no evidence so far, that daily intake of acetylsalicylicacid, commonly known as aspirin, does reduce fracture risk [34].

On the contrary, frequent consumption of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and diclofenac, ibuprofen, naproxen, indomethacin, celecoxib, oxaprozin, and others, as used during chronic pain management is even associated with a high risk for delayed fracture healing and fracture non-unions [35-37]. Methotrexates, e.g. Trexall or Rheumatrex, represent the most effective and widely used drugs for the treatment of rheumatoid arthritis and other inflammatory types of arthritis [38]. They are also used at higher doses to treat malignancies [39]. Methotrexates, being folate antagonists, may lower BMD by interfering with the bone remodeling [39].

Gonadotropin-releasing hormone (GnRH) agonists are used amongst others for the treatment of hormone-sensitive cancers (e.g. prostate or breast cancer) or endometriosis, in fertility medicine, or as a part of transgender hormone therapy. GnRH agonists, including buserelin, gonadorelin, goserelin, histrelin, leuprorelin, and triptorelin, reduce both estrogen and testosterone levels, which may cause significant bone loss and fragility fractures [40-42].

In hypothyroidism, lack of thyroid stimulating hormone (TSH) and consequently thyroxin can lead to decreased BMD [43]. However, treatment with exogenous thyroxin is also associated with a high prevalence of additional bone loss in these patients [44], possibly caused by an additional stimulation of bone resorption [45, 46].

Immunosuppressive drugs, e.g. sirolimus, tacrolimus or cyclosporine A, commonly used with glucocorticoids to prevent rejection of a transplanted organ, are associated with a particularly severe form of osteoporosis when administered at higher doses [47, 48]. Especially, cyclosporine A and tacrolimus treatment are reported to induce osteoclast activity resulting in a high bone turnover [49]. Antiviral agents, e.g.; ribavirin, may favor thinning of bones both by inhibiting bone formation by osteoblasts and inducing bone resorption by osteoclasts [50-52].

Osteopenia and osteoporosis are also reported with several frequently prescribed anticonvulsants, including diphenylhydantoin, phenobarbital, sodium valproate, and carbamazepine [11, 53]. Besides the possible direct effects on bone cells, many of the anticonvulsants are proposed to disturb biological activation of vitamin D in the liver [54-56]. The resulting reduced vitamin D levels cause a lowering of BMD (rickets and osteomalacia). While increased vitamin D uptake might improve BMD in these patients, while high intakes of vitamin A may cause the opposite [57-59].

Furthermore, many drugs acting on the central nervous system, e.g. anti-depressants, anxiolytics, lithium, neuroleptics, and sedatives, may alter both bone stability and postural balance. Anti-depressants, for example, show a dose-dependent increase in fracture risk. For a yet unknown reason, the fracture risk seems to be higher with selective
inhibitors of serotonin re-uptake than with tricyclic anti-depressants [60, 61]. Benzodiazepines, as part of the anxiolytics and sedatives, have been associated with an increased fracture risk, probably due to an increased risk of falls [62]. In addition, neuroleptics have been associated with decreased BMD. Lithium on the contrary is associated with an increase in BMD, which might be linked to its stimulatory effects on the Wnt signaling pathway [63].

Even patients receiving long term parenteral nutrition develop osteoporosis. The exact cause for lowered BMD in these patients is unknown, but is thought to be multifactorial – including significant gastrointestinal failure and malabsorption, components of the nutritional solution itself and additional medication, as well as limited physical mobilization [64].

1.4. Need to Investigate Effect of Drugs on Bone Metabolism

These examples show that there are many drugs that negatively affect bone metabolism, resulting in a decrease in BMD in many of these individuals. As the actual bone loss does not cause any symptoms, preventive therapies are often not considered, which in turn increases the risk for fractures and disturbed fracture healing in affected patients [1]. Therefore, facing new regulations on the re-evaluation and licensing of drugs and medical devices, it should be mandatory to examine the effect of drugs, especially drugs for sustainable medication, on the bone health. However, to do so a suitable screening platform is required. In the human body, bones of the skeleton constantly adapt to the stress exposed. New bone structures are formed by cells of the osteogenic lineage, e.g. mesenchymal stem cells (MSCs), osteoblasts, lining cells, and osteocytes. Damaged or poor quality bone matrix is resorbed by osteoclasts, derived from the hematopoietic lineage [65]. Therefore, in order to display healthy bone function, the interplay between bone forming and resorbing cells is essential. The direct interplay between the cells is often mediated via paracrine and systemic mediators, but also perfusion, availability of nutrients, or mechanical load play a critical role [65]. Therefore, many factors have to be considered when searching for a suitable screening model (for overview see Figure 1).

![Figure 1. Factors Affecting the Choice of Models Suitable for Screening Changes in Bone Metabolism. Diverse in vivo, ex vivo and in vitro models to investigate bone cell functions exist. The different models vary strongly in their planning and handling, which in turn affects the timing and costs. Furthermore, the use of animals or animal tissue may give ethical concerns. All these factors have to be considered when searching for a suitable screening model.](image-url)

2. Models to Investigate Bone Metabolism

2.1. In Vivo Models

So far, the interplay between the different cells can only be displayed in vivo in animal models. Although, the natural blood supply, cell-cell- and cell-matrix-interaction are given in vivo, there exist several limitations. Partly huge
species-dependent differences in bone metabolism are present [66]. This may be partly explained by the altered posture between animals (tetrapod motion) and humans (upright walk), which may significantly affect the mechanical strain the bones experience [67]. It is indisputable that animals are not exposed to the same environmental influences as patients due to strictly controlled housing conditions. This is of special importance when investigating disease conditions. Furthermore, the use of inbred rodent-strains cannot display the great inter-individual differences observed in humans, without using a vast amount of animals, which raises great ethical concerns.

2.2. Ex Vivo Bone Cultures

Culture models closest to the in vivo situation are so called ex vivo organ cultures. Depending on the research interest different ex vivo bone cultures exist. Most described ex vivo bone cultures are done with bones derived from animals. However, using explant cultures proposed to reduce the overall number of experimental animals and reduce possible harm to the animals during in vivo experiments [68, 69].

Long bone cultures are supposed to be the best suitable model to investigate linear bone growth and hypertrophic ossification [70-78]. Therefore, almost ⅔ of ex vivo bone growth studies are performed using ex vivo long bone cultures [79]. The first described model used proximal phalanges, metacarpal, and metatarsal bones dissected from the paws of young rats – these bones were kept several days in culture, during which the bones grew and mineralized [76, 77]. The method was then adapted for long bones of mice [70, 78, 80-82] or chicken [71]. Preserving the surrounding soft tissue, as done in so called limb bud cultures, promised even better representation of the in vivo situation [72-75].

The second most portion (approx. 16%) of ex vivo bone growth studies, represent murine or rat femur head and calvarial cultures [79, 83-87]. However, these cultures are also used to investigate bone and cartilage metabolism or bone defect healing. When co-cultured with cancer cells this model was used to investigate bone metastases [88-92]. Similarly, a co-culture of this model with immune cells was used to investigate inflammatory bone diseases [93]. It has been critically discussed, whether this type of ex vivo bone cultures can significantly reduce the amount of experimental animals used, when per animal only two conditions can be tested [68, 69].

Better efficiency in reducing the number of experimental animals is expected, when the explanted bones are sliced, e.g. in rat femur slice cultures [94], or rat mandible slice cultures. Slice cultures are not frequently used to investigate ex vivo bone growth [79], but to investigate stem cell behavior and bone repair [95-98]. It was even possible to adapt this model to the human system by culturing slices of immature molars from young adults; however endochondral bone formation is not represented in this model [99, 100].

Trabecular core cultures utilize bones of larger animals and even humans [101-109]. A direct comparison of ovine, bovine, and human trabecular core cultures emphasized the species-specific differences [108]. These cultures are primarily used to investigate bone metabolism. Due to their larger size even mechanical load can be applied [102, 108, 110]. Trabecular core cultures are also used to investigate biocompatibility of materials [103], or cancer-dependent effects on bone [90]. The possibility to generate these ex vivo cultures from human bone slices or biopsies, even allows investigating molecular mechanisms in metabolic bone diseases [111, 112].

However, the availability of the required native material is limited, such that these models cannot be easily used for large scale substance screenings. This raises the need for a permanently available and up-scalable in vitro model, adequately representing human bone metabolism.

2.3. In Vitro Models

In the past years, several attempts have been done to establish model systems, which can adequately represent bone metabolism. To display bone metabolism both bone formation by osteogenic cells and bone degradation by osteoclastic cells have to be addressed. This can only be done in some sort of co-culture. The described co-cultures of bone forming and resorbing cells differ not only in the type of cells used, but also in their individual setup. By means of using culture inserts or transfer of media, some models do not allow direct cell-cell-interaction. As these types of co-culture are most comparable to the classical 2D mono-cultures, cell specific effects can easily be described. Comparing these most basic co-cultures with a direct co-culture facilitating cell-cell-contact revealed that the cells do not only interact via paracrine factors, but also directly via cell-cell-interactions [113], which are proposed to be essential to activate osteoclast function [114]. Furthermore, there is evidence that presence of bony matrix is required to properly regulate osteoclast formation [115] and regulates bone cell functions [116, 117]. However, only few co-cultures provide cell-matrix-interactions [118-122] and even less consider the natural 3D organization of the cells within the matrix [120, 122-124]. In vivo bones constantly adapt to the mechanical forces applied. Therefore, few models tried to include mechanical stimulation of the bone cells [123, 125, 126].

With each factor considered, the in vitro models should better represent the in vivo situation. Simultaneously, the complexity of the respective model system also increases which effectively limits the analytical methods available. Similar to the ex vivo models, the most complex in vitro models rely solely on histological and immuno-histological
stainings, when it comes to their characterization. Factors limiting the analytical methods include the direct co-culture itself, where cell specific effects are sometimes hard to observe, as conventional methods for normalization do not distinguish between different cell types. This is a negligible factor, when the cells in co-culture originate from different species. In this case species-specific expression analysis can be performed [127]. However, due to the species-dependent diversity, secreted factors regulating the function of the co-culture may not be as efficient as when combining cells of the same origin of species.

Another factor to be considered during analysis is the presence of 3D matrices. Typically, by applying the cells on a 3D carrier, the culture or reaction volumes have to be increased. An increase in liquid volume dilutes factors secreted in the culture medium and thus effectively lowers the sensitivity of established analytical methods. In a static 3D co-culture on a porous carrier this increase in volume might be compensated by the increase in surface area available for the cells to attach and grow. But not only alterations in liquid volume but also the carrier itself may represent a challenge. When being non-transparent, the carrier limits microscopic analyses, a factor that might be partly circumvented by using fluorophores [128]. However, penetration depth and possible autofluorescence of the carrier material remain limiting factors. In this case, classical histological and immune-histological analyses of sections may be applied.

Furthermore, assay substrates and reaction product not only require time for perfusion but may also react with the carrier material. This effect may be reduced by actively or passively (mechanical stimuli) perfusing the 3D model. However, further application of mechanical stimuli or perfusion may additionally increase the liquid volume. Without a comparable increase in the carrier volume compensation in cell numbers cannot be expected. This might be a criterion for exclusion when considering primary cells, where only a limited number of donors and amount of donor tissue is available. Furthermore, the more complex technical settings do not only complicate the handling of the system but also with an increased number of structural junctions may increase the risk for contaminations.

By carefully choosing and adapting existing assays some of the described limitations may be avoided, but, it is obvious that advances on the engineering side, e.g. scaffold preparation and reactor settings are more rapidly advancing than the adaption of the analytical methods. But this is an important requirement to fully use the potential of the developed model systems. For overview see Figure 2.

Figure 2. Challenges of Complex Culture Systems. Overview on challenges for (A) co-cultures, (B) 3D cultures, and (C) mechanically stimulated cultures is given. Combining different aspects, the complexity of the model system increases and new challenges arise. These may limit the available methodology for characterization of the model system.
3. In Vitro Co-Culture Models for Bone Metabolism using Cell Lines

Previous attempts to establish co-cultures based on primary human bone cells proved to be strongly donor-dependent and time-consuming [120, 121, 129, 130]. Primary human osteoblasts and/or osteocytes have to be expanded for several weeks up to months in order to obtain a sufficient number of cells. By that time the donors will no longer be available for a blood donation to isolate monocytes as precursors for osteoclasts. Thus, a compatible donor for the isolation of monocytes has to be found. Using osteoprogenitor cells, e.g. mesenchymal stem cells (MSCs) derived-from bone marrow or fat tissue may shorten the expansion time, at the risk of an elongated period of differentiation [113, 131, 132], which lasts up to 6 weeks depending on the protocol used [118-124, 129, 133, 134].

To quickly obtain larger amounts of cells, some models utilize human cell lines for their co-cultures [119, 134]. Fortunately, there is a great variety of human osteogenic cell lines established, often derived from osteosarcomas, so that inter-species differences have not to be worried about. These cell lines strongly differ, not only regarding their proliferation, migration, or invasion capacity [135, 136], but also regarding their osteogenic features [137-140]. Therefore, these may not be equally useful for a co-culture model to investigate bone metabolism. Finding a suitable candidate for the intended co-culture of bone forming and bone resorbing cells may give stable and reproducible results, however, one should bear in mind that using a single cell line cannot represent the inter-individual differences observed in humans.

3.1. Choice of Suitable Osteogenic Cell Lines

When investigating osteogenic differentiation in vitro, the commercially available murine muscle myoblast cell line C2C12 (ATCC® CRL-1772) and calvarial pre-osteoblast cell line MC3T3-E1 (ATCC® CRL-2593 till 2596) are frequently used. More differentiated osteocyte features are found in the murine MLO-Y4 cell line [141]. However, when it comes to co-culture approaches for investigating bone metabolism, these cell lines were not often reported. C2C12 cells were shown to induce osteoclast features in the murine Raw264.7 macrophage cell line in an indirect co-culture approach [142]. More is reported on the more osteoblast like MC3T3-E1 cells, which were reported to stimulate resorption activity of primary rat osteoclasts [114], mouse bone marrow cells [143, 144], or murine Raw264.7 cells [145, 146]. Similarly, the more mature MLO-Y4 cells have been shown to stimulate osteoclast differentiation of mouse bone marrow cells [147-150] and Raw264.7 cells in both direct and indirect co-culture approaches [151-155]. Although some of these models can even show influence on bone metabolism [144, 146], similar to most in vivo and ex vivo models these in vitro models cannot obviate species-specific differences to the human situation. Therefore, bone co-culture models using human cell lines are preferred.

Investigating osteogenic differentiation in vitro human osteosarcoma cell lines are often used as alternatives to primary human MSCs, osteoblasts or osteocytes. Based on the publication frequency, most commonly used human osteosarcoma cell lines are the commercially available cell lines MG-63 (ATCC® CRL-1427; male), SaOS-2 (ATCC® HTB-85, female), Cal-72 (DSMZ® ACC-439; male), U2OS (ATCC® HTB-96, female), HOS (ATCC® CRL-1543 till 1547, female), and OSA also known as SJA-1 (ATCC® CRL-2098, male). As these cell lines are already established in many laboratories, they represent ideal candidates for osteogenic cells in a co-culture model. HOS and OSA cells are considered as highly tumorigenic cells with strong proliferation, migration, and invasion [135]. A similarly strong proliferation, migration, and invasion capacity show U2OS cells [135]. From the remaining three cell lines Cal-72 cells are reported to proliferate strongest [135], which is in line with our own observations. Considering that in the intended co-culture osteoclastic cells may not proliferate, overgrowing osteogenic cells may be a disadvantage. Therefore, when considering the more strongly proliferative MG-63 cells for the intended co-culture, fewer cells might be needed as when using Cal-72 cells or SaOS-2 cells.

Proliferation is usually suppressed when these cells are osteogenically differentiated. However, the osteogenic differentiation medium frequently contains dexamethasone, a glucocorticoid with well-known immune-suppressive action. By altering the immune response of the myeloid cells, dexamethasone may interfere with osteoclast formation in the intended co-cultures [156, 157]. Dexamethasone in the differentiation medium may be replaced by cholecalciferol (vitamin D₃), which acts via the vitamin D receptor (VitDR) both on osteogenic and osteoclastic cells [131, 158]. Comparing VitDR expression levels in MG-63, Cal-72, and SaOS-2 cells with VitDR expression levels in primary human osteoblasts and an immortalized MSC cell line (SCP-1 [159]) revealed no significant difference between the different cell types (Figure 3A). This is different when it comes to the osteogenic key transcription factor Runx2 (run-related transcription factor 2). There is evidence that in osteosarcoma cells expression of Runx2 correlates with the differentiation status of the cells [137]. This is supported by our observations, where expression of Runx2 was highest in the most mature SaOS-2 cells, followed by Cal-72 cells and MG-63 cells. Expression levels of Runx2 in SaOS-2 cells were comparable to primary human osteoblasts, while expression levels of Runx2 in MG-63 cells were even below that of the SCP-1 cells (Figure 3B).
High levels of the C-C motif chemokine ligand 2 (CCL-2), a marker for cellular stress, are associated with increased osteoclast activation [160]. Therefore, we determined the expression levels of CCL-2 in these cells. Primary human osteoblasts and SaOS-2 cells expressed comparably high levels of CCL-2, followed by SCP-1 cells, MG-63 cells, and Cal-72 cells (Figure 3C). However, the main initiators driving osteoclastogenesis are thought to be M-CSF (macrophage colony-stimulating factor) and RANKL (receptor activator of nuclear factor kappa-B ligand) [161], which are common supplements in osteoclast differentiation medium. Usually these factors are produced by osteoblasts in order to induce osteoclast formation [162]. To adequately display this known interplay between bone forming osteoblasts and bone resorbing osteoclasts the osteogenic cell line of choice should best represent primary human osteoblasts. When comparing MG-63, Cal-72, and SaOS-2 cells, expression of M-CSF strongly varies [139], and may be even absent depending on the subset of cells in culture [138]. Comparing M-CSF levels in these cells with M-CSF levels in primary human osteoblasts and SCP-1 cells showed that expression of M-CSF may be dependent on the differentiation status of the cells (Figure 3D). Expression of RANKL showed similarities to the expression of CCL2 (Figure 3E). Highest levels were observed in primary human osteoblasts and SaOS-2 cells, followed by SCP-1 cells and MG-63 cells. Low expression levels of M-CSF and RANKL may explain why co-culture attempts using MG-63 cells additionally supplement the medium with recombinant M-CSF and RANKL [163, 164], which is not reported for co-culture approaches of SaOS-2 cells with murine RAW 264.7 macrophages [165-167], mouse or human peripheral blood mononuclear cells [115, 168-170], or human clonal osteoclast precursor cells FLG 29.1 [171, 172].

Supplementation of the medium with exogenous factors may suppress the interaction of the two cell types, and thus possibly camouflage effects on bone metabolism. Therefore, Cal-72 cells, which barely express RANKL, may not suitable for the intended co-culture. A factor that should not be neglected in this regard is osteoprotegerin (OPG), which acts as a decoy receptor, capturing RANKL and thus inhibiting osteoclast formation [173]. Expression of OPG in SaOS-2 cells and MG-63 cells was most comparable to primary human osteoblasts and lowest in Cal-72 cells (Figure 3F).

### 3.2. Choice of Suitable Osteoclast Precursor Cells

Same holds for precursors of osteoclasts. While there are several publications that generate osteoclasts from the murine macrophage cell lines RAW264.7 [165, 174-182] and J774 [183-187], little is known about the osteoclast differentiation potential of human myeloid cell lines. There are few reports describing osteoclast features in differentiated human myeloid THP-1 cells [188], U937 [183, 189], HL-60 cells [190, 191], UG-3 [192], ML-2, or Mono Mac 6. Despite likely species-specific differences, the use of murine macrophage cell lines in a co-culture for bone metabolism also bares advantages. When combined with an osteogenic cell line originating from another species,
e.g., human, cell specific expression analysis is much easier as when combining two cell types of the same species where in direct co-culture approaches analytical methods face limits [154, 181, 182].

Furthermore, as macrophage cell lines RAW264.7 and J774 cells are adherent cells by nature. On the contrary, human mononuclear cell lines, e.g., THP-1, U937, or HL-60, are suspension cells that require activation to attach and differentiate into osteoclasts. This is frequently done by exposure to PMA (phorbol 12-myristate 13-acetate) [193, 194], which in turn may affect function of other cells in the co-culture and thus prevent simultaneous cell seeding. Furthermore, the most common human myeloid cell lines THP-1, U937 and HL-60 are not subject to any special regulatory issues, as the RAW264.7 cell line that can only be handled under safety level S2 in many countries.

However, when considering human myeloid cell lines for a direct co-culture representing bone metabolism the cultivation procedure has to be carefully optimized. Critical will be to find the right density of myeloid cells to start the co-culture. During seeding the density of the myeloid cells has to be high to allow fusion of the cells to pre-osteoclast [195]. Then the right dose and duration of the PMA treatment for activation of the cells [188] has to be chosen such that osteogenic cells are not affected. To prevent exposure to PMA, seeding of osteogenic cells may be done directly after activation and attachment of the myeloid cells. Added osteogenic cells then have to provide a sufficient amount of CCL-2, M-CSF and RANKL to induce cell fusion and osteoclastogenesis [160, 161]. Their effect may be even enhanced in the presence of IL-4 and/or vitamin D₃ [192], which is of advantage when replacing dexamethasone by cholecalciferol in the co-culture medium as suggested before [131, 158]. To efficiently respond to these stimuli, the osteoclast precursor cells have to express the relevant receptors, e.g., VitDR, CCR1 (C-C chemokine receptor type 1), or RANK (receptor activator of nuclear factor kappa-B), and associated transcription factors, e.g., NFATc1 (nuclear factor of activated T-cells 1) [161, 196].

Comparing PMA-activated THP-1 and HL-60 cells, there was no significant difference in expression of VitDR (Figure 4A). Although, CCR1 was strongly expressed in both cell lines, its expression was significantly lower in activated HL-60 cells than in activated THP-1 cells (Figure 4B). This is of importance, as inhibition of CCR1 is associated with impaired osteoclastogenesis [197]. CCR1 is thought to promote recruitment and motility of osteoclasts, in a RANKL dependent manner [198]. Therefore, reduced levels of CCR1 may indirectly suppress RANKL effect, although no significant difference in expression of its direct receptor RANK was observed between activated THP-1 and HL-60 cells (Figure 4C). Successful activation of the described receptors shall then promote osteoclastogenesis by activating the transcription factor NFATc1 [161, 196]. Basal expression levels of NFATc1 were comparable between activated THP-1 and HL-60 cells (Figure 4D), such that no clear preference for any of the two cell lines could be made.

![Image](image-url)

**Figure 4.** Activated THP-1 and HL-60 Cells Express Receptors and Transcription Factors Involved in Osteoclastogenesis. THP-1 and HL-60 cells were treated with 200 nM PMA for 24 h. Gene expression levels of (A) VitDR = vitamin D receptor, (B) CCR1 = C-C chemokine receptor type 1, (C) RANK = receptor activator of nuclear factor kappa-B, and (D) NFATc1 = nuclear factor of activated T-cells 1 were determined by semi-quantitative RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as house-keeping gene. Experiments were repeated 4 times (N=4) in duplicates (n=2). Comparison of groups was performed by Kruskal-Wallis test followed by Dunn’s multiple comparison tests.
3.3. Compatibility of Human Osteogenic and Myeloid Cell Lines for Direct Co-Culture

Considering the above expression profile (Figure 3), from the three osteosarcoma cell lines SaOS-2 cells resemble best primary human osteoblasts. MG-63 cells showed lower expression of the key osteogenic transcription factor Runx2, and the osteoclast inducers CCL2 and RANKL. Taking into account the reduced expression of these key regulatory factors, MG-63 cells might be less suitable for the intended direct co-culture than SaOS-2 cells. In a direct comparison, co-culture of MG-63 cells with CD14+ monocytes showed less sensitively sex hormone-dependent changes in bone resorption than a co-culture of SaOS-2 cells with CD14+ monocytes [199, 200].

In other studies using MG-63 cells in osteoblast-osteoclast co-cultures the medium had to be additionally supplemented with recombinant M-CSF and RANKL to induce osteoclast like features in mononuclear cells [163, 164]. When this is not provided, THP-1 cells remain macrophage like such that the model was used as inflammatory bone model [134], further favoring the use of SaOS-2 cells. Supplementation of medium was not required to induce osteoclast features in murine RAW 264.7 cells by SaOS-2 cells [165]. Cal-72 cells basically disqualified due to the lack in RANKL expression. Notwithstanding, it has to be addressed that many osteosarcoma cell lines highly express hypoxia-inducible factor 1-alpha (HIF-1α) due to their cancerous origin [201, 202]. HIF-1α is a well-known inducer of vascular endothelial growth factor (VEGF), which has been reported to be a substitute for M-CSF in RANKL-induced osteoclastogenesis [164, 203]. Therefore, immortalized SCP-1 cells [159] are also of potential interest, due to their non-cancerous origin. SCP-1 cells proliferate strongly, and expressed VitDR, Runx2, CCL2, RANKL and OPG in levels comparable to the SaOS-2 cells (Figure 3). Solely, M-CSF expression was lower in SCP-1 cells than in SaOS-2 cells.

Therefore, compatibility of cell lines was tested with SCP-1 cells and SaOS-2 cells as representatives of osteogenic cells and THP-1 cells and HL-60 cells as representative of myeloid osteoclast precursors. SaOS-2 cells, THP-1 cells, and HL-60 cells all used RPMI 1640 as basis medium, such that no unwanted effect due to the medium was expected. SCP-1 cells were grown in MEMu Medium, which induced cell death in the myeloid cell lines. Vice versa RPMI 1640 medium was not suitable to grow and differentiate SCP-1 cells. As a 1:1 mixture of both media sustained the highest number of viable cells in direct co-culture (data not shown) this mixture was used for the experiments.

In order to define adequate cell-cell-ratio several factors had to be considered. As described above, myeloid cells had to be plated at a high cell density to facilitate cell fusion and osteoclast formation [195]. However, to resemble the cell-cell-ratio observed in bone, where up to 95% of the cells are osteoblasts [117], the final number of osteoclasts should not be too high. It can be assumed that THP-1 and HL-60 cells stop proliferation upon activation with PMA [204]. However, the osteogenic cell lines remain proliferating even during maturation. In the co-cultures of myeloid cells with SaOS-2 cells a cell-cell-ratio of 2:1 resulted in measurable osteoblast and osteoclast markers. Considering that SCP-1 cells proliferate faster than SaOS-2 cells, in these co-cultures a cell-cell-ratio of 8:1 (THP-1:SCP-1) was sufficient.

Conspicuously, direct co-cultures with HL-60 cells had significantly less total protein and mitochondrial activity than direct co-cultures with THP-1 cells (Figure 5A&B), suggesting that with THP-1 cells osteogenic cells survived better. In line with other reports, SaOS-2 cells had very low mitochondrial activity but strongly produced matrix [205].

Alkaline phosphatase (ALP) activity was significantly higher in co-cultures with SaOS-2 cells, which have a very high basal ALP activity [205], than in co-cultures with SCP-1 cells, which normally show an increase in ALP activity with differentiation [206] (Figure 5C). Consequently, after 14 days of culture formation of mineralized matrix was also higher in co-cultures with SaOS-2 cells than in co-cultures with SCP-1 cells (Figure 5D). Despite the differences observed due to the osteogenic cells, ALP activity and formation of mineralized matrix were significantly lower in co-cultures with HL-60 cells than in co-cultures with THP-1 cells. TRAP5b activity was determined as common osteoclast marker. It was only detectable after few days in culture. In general, TRAP5b activity increased faster in co-cultures with SaOS-2 cells, but remained longer in co-cultures with SCP-1 cells. Over the entire 14 days, TRAP5b activity was higher in cultures with SaOS-2 cells or THP-1 cells than in co-cultures with SCP-1 cells or HL-60 cells (Figure 5E). As TRAP5b activity represents only an indirect marker for osteoclastogenesis, resorption activity of differentiated THP-1 cells was determined using an artificially generated mineralized matrix [207]. Both stimulation by SCP-1 cells and SaOS-2 cells resulted in formation of resorption pits by THP-1 cells after 14 days of culture (Figure 5F).
Figure 5. Osteogenic and Myeloid Cell Lines in Direct Co-Culture. THP-1 cells and HL-60 cells were activated with 200 nM PMA. After 24 h medium was removed and osteogenic SCP-1 or SaOS-2 cells were added and osteogenically differentiated for 14 days. (A) Total protein content was determined by Sulforhodamine B (SRB) staining. (B) Mitochondrial activity was assessed by resazurin conversion assay. (C) Alkaline phosphatase (ALP) activity and (E) tartrate-resistant acidic phosphatase (TRAP5b) were determined photometrically. (D) Formation of mineralized matrix by the direct co-culture was quantified by Alizarin Red staining. (F) To visualize resorption of mineralized matrix by osteoclastic cells, THP-1 cells were differentiated on chemically generated mineralized matrix as described [207]. Resorption pits were visualized by von Kossa staining. Experiments were repeated 4 times (N=4) in triplicates (n=3). AUC = area under the curve, summarizing the data over 14 days of differentiation. Comparison of groups was performed by Kruskal-Wallis test followed by Dunn’s multiple comparison tests.

3.4. Technical Capabilities to Improve and Stabilize the Co-Culture

Our data suggest that THP-1 cells are better suitable as osteoclast precursors than HL-60 cells in a direct co-culture approach. Regarding osteogenic cells both SaOS-2 cells and SCP-1 cells were able to induce osteoclastic features in THP-1 cells. However, SCP-1 cells seem to require more time than SaOS-2 cells to obtain a stable co-culture, but have the advantage that the co-culture can be kept longer (> 2 weeks) in culture. During this elongated culture period osteogenic differentiation can be observed in SCP-1 cells, which is basally advanced in SaOS-2 cells. These are factors that may be decisive depending on the planned investigation.

These data are supported by reports successfully showing osteoclast features in murine RAW 264.7 macrophages [165-167], human FLG 29.1 osteoclast precursors [171, 172] or peripheral blood mononuclear cells [115, 168-170], when co-cultured with different osteogenic cells. However, there are several factors that are not addressed in these simple 2D direct co-cultures. Summarizing these studies, a direct co-culture is superior to an indirect co-culture [171], where culture supernatants are transferred such that only a one-directional transfer of secreted factors is given. Similarly, THP-1 cells gained more osteoclastic features, when directly co-cultured with differentiated human MSCs than in indirect co-culture [208-210].

The artificially generated mineralized coating of the cell culture dish (Figure 5F) was able to nicely and reproducibly visualize the resorption capacity of the co-culture system [207]. It is self-explanatory, that these 2D cultures cannot sufficiently display the bone environment, as the organic (mostly collagen) and inorganic (mostly hydroxyapatite) matrix characteristic for bone is missing [112]. Addition of 3D bony matrix not only provides a framework for a more natural 3D organization of the cells but also regulates bone cell functions via cell-matrix-interactions [116, 117]. Furthermore, there is evidence that some kind of bone matrix is required to observe the full
interplay between the two cell types, as membrane- or matrix-associated forms of M-CSF and RANKL are reported to be essential for osteoclast formation [115]. So far only a few co-culture models consider cell-matrix-interactions in 2D [118-122] and in 3D [120, 122-124]. These examples clearly show that 3D cultures are more beneficial for the differentiation of the osteogenic cells when compared to 2D cultures, especially, when a stiff and porous carrier was used [211]. When the carrier is too soft, monocytic cells may cave into the matrix, which effectively prevents their fusion early in osteoclastogenesis. Similarly soft carriers are thought to induce expression of stem cell markers, e.g. Sox2, in MSCs, which proved to inhibit osteogenic differentiation [212-215] in favor of adipogenic differentiation [216]. Therefore, carrier stiffness over 60 kPa is proposed to favor osteogenic differentiation of MSCs [217, 218]. Yet, the scaffolds should not be too stiff in order to pass on mechanical stimuli to the cells [219], as it is observed in vivo where bones constantly adapt to the mechanical forces applied. Thus, few models tried to include mechanical stimulation of the bone cells [123, 125, 126].

Recently, there is growing evidence that osteogenic and osteoclastic cells are not only influenced by the surrounding matrix [116, 117] and mechanical stimuli [220], but also strongly by immune cells [221, 222], adipose cells [223-225], nerve cells [226, 227], or endothelial cells [228] (for overview see Figure 6). However, considering the cell-cell- and cell-matrix-interactions, as well as the 3D conformation and mechanical stimuli and including these or other cells within the model systems would rapidly increase the complexity of the system, such that analytical limits are quickly reached [229].

Figure 6. Overview of Factors that may Affect Bone Metabolism in a Model System. Novel model systems try to address as many of these factors as possible. However, with increasing complexity of the model system the number of available methods for the characterization of the model decreases.

4. Summary and Conclusion

Undoubtedly, in vitro co-cultures of bone forming and bone resorbing cells represent a great chance for investigating bone metabolism. We here show that commercially available human cell lines can be used to generate such a basic co-culture model, which is of special interest when the model system should be used for screening purposes. Regarding time, a direct co-culture of SaOS-2 cells and THP-1 cells faster developed osteoblast and osteoclast markers than the direct co-culture of SCP-1 cells and THP-1 cells. However, the later was stable for a longer time frame and could display features of osteogenic differentiation. Considering these factors, the two co-cultures represent a great base for further advanced co-cultures, possibly considering cell-matrix-interactions in a 3D environment or even mechanical stimulation. However, to fully address the potential of the model system analytical methods have to be adapted and optimized.
5. Materials and Methods

5.1. Search Criteria

On the 12th of April 2020, a search was performed with PubMed and Web of Science, limited to manuscripts in English or German language. The search strategy is summarized in Table 2.

Table 2. Search Strategy

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5.2. Cell Culture

Primary human osteoblasts from spongy bone tissue (ethical vote: 539/2016BO2) were isolated by collagenase digestion as described before [222, 230, 231]. Cells were expanded in DMEM medium supplemented with 5% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM L-ascorbate-2-phosphate, 50 μM β-glycerol phosphate.

Immortalized bone marrow-derived mesenchymal stem cell line SCP-1 was kindly provided by Prof. Matthias Schieker [23]. SCP-1 cells were cultured in MEMα Medium supplemented with 5% FCS. The osteogenic cell lines MG-63, Cal-72, and SaOS-2 (obtained from the DSMZ) were all expanded in RPMI 1640 medium, supplemented with 5% FCS [222]. The myeloid cell lines THP-1 and HL-60 (DSMZ) were used as osteoclastic precursor cells. Cells were expanded as suspension culture in RPMI 1640 Medium, supplemented with 5% FCS [222].

Cells were kept at 37°C and 5% CO₂ in a humidified atmosphere. Medium was changed twice a week. Cells were sub-cultured when a confluence of 80-90% or cell density of 1* 10⁶ cells/ml were reached in order to prevent spontaneous differentiation. Experiments with primary osteoblasts were performed in passage 3 or 4 and experiments with cell lines were performed within the first 15 passages. For differentiation the respective media were supplemented with 2% FCS, 200 μM L-ascorbic acid 2-phosphate, 5 mM β-glycerol phosphate, 25 mM HEPES, 1.5 mM CaCl₂, and 5 μM cholecalciferol.

5.3. Conventional RT-PCR

Total mRNA was isolated using the Trifast reagent, according to the manufacturer’s protocol. Quantification of mRNA was done photometrically and integrity of mRNA was checked by gel electrophoresis. cDNA was synthesized from 2 µg mRNA using the First-Strand cDNA Synthesis Kit (ThermoFisherScientific, Karlsruhe, Germany) according to the manufacturer’s protocol. For semi-quantitative RT-PCR a standardized amount of template cDNA was tested for the expression level of each target gene (primer sequences and PCR conditions are listed in Table 3) using the Red HS Taq Master Mix (Biozym, Hessisch Oldendorf, Germany).

Table 3. Information of PCR Conditions

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5.4. Viability and Proliferation

Mitochondrial activity (resazurin conversion assay) of the cells was determined as described before [230, 231]. Briefly, cells were incubated with reaction substrate (0.025% resazurin in medium) for up to 2 h. Every 30 min the fluorescence was determined. Resazurin conversion was calculated for each cell line within their linear range. Total protein content was determined by SRB staining of ethanol fixed cells as described [230].

5.5. Functional Assays

As early osteoblast marker ALP activity was measured, as described [231]. Briefly, cells were incubated with reaction buffer (pH = 10) containing 0.2% p-nitrophenyl-phosphate for up to 1 h. A kinetic was measured and p-nitrophenol (pNP) formation rate was calculated for each cell line within their linear range. As osteoclast marker TRAP5b activity was measured, as described [232]. One volume culture supernatant was mixed with 3 volumes of the reaction buffer (pH = 5.5) containing 0.2% p-nitrophenyl-phosphate. After 6 h of incubation at 37°C, reaction was stopped with NaOH. From the resulting absorption pNP formation rate was calculated.

5.6. Mineralized Matrix

Mineralized matrix was visualized by von Kossa staining and quantified by Alizarin red staining as described before [231, 233]. Mineralized matrix for resorption assays was generated as described in Mari et al. [207].

5.7. Statistics

Results are presented as floating bars (mean ± 95% confidence interval). Each experiment was performed 4 times (N = 4) in duplicates or more (n ≥ 2). Statistical analyses were performed using the GraphPad Prism Software (GraphPad, El Camino Real, USA). Different groups were compared using the Kruskal-Wallis H-test followed by Dunn’s multiple comparison tests. A p-value below 0.05 was considered statistically significant.

6. Acknowledgements

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8. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

9. Author Contributions


10. Ethical Approval

The manuscript does not contain experiments on animals and humans; hence ethical permission not required.

11. References


