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].

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]

Endocrine		Genetic		Rheumatic and auto-immune	
Endocrine	Acromegaly	**Genetic**	Cystic fibrosis	**Rheumatic and auto-immune**	Ankylosing spondylitis
	Addison's disease		Ehlers-Danlos syndrome		Graves' disease
	Adrenal insufficiency		Glycogen storage diseases		Lupus
	Cushing's syndrome		Gaucher's disease		Psoriasis
	Diabetes mellitus		Homocystinuria		Rheumatoid arthritis
	Hyperparathyroidism		Hypophosphatasia	**Others**	Alzheimer's disease
	Thyrotoxicosis		Idiopathic hypercalciuria		Amyloidosis
Digestive and gastrointestinal	Alcoholism		Marfan syndrome		Cancer (breast, prostate, etc.)
	Celiac disease		Menkes syndrome		Chronic metabolic acidosis
	Crohn's disease		Osteogenesis imperfecta		Congestive heart failure
	Gastrectomy		Porphyria		COPD, asthma, chronic bronchitis
	Gastrointestinal bypass		Primary hemochromatosis		Depression
	Malabsorption syndrome		Riley-Day syndrome		End stage / chronic kidney disease
	Primary biliary cirrhosis	**Hypogonadal states**	Androgen insensitivity		Epilepsy
	Primary sclerosing cholangitis		Anorexia nervosa		Idiopathic scoliosis
	Secondary hemochromatosis		Female athlete triad		Immobilization
	Ulcerative colitis		Hyperprolactinemia		Multiple sclerosis
	Weight loss surgery		Klinefelter's syndrome		Muscular dystrophy
Hematologic	Hemophilia		Panhypopituitarism		Organ transplants
	Leukemias		Premature menopause		Osteonecrosis / avascular necrosis
	Lymphomas		Turner's syndrome		Parkinson's disease
	Multiple myeloma	**Chronic viral**	HBV / HCV fibrosis or cirrhosis		Sarcoidosis
	Sickle cell disease		HIV / AIDS		Smoking
	Systemic mastocytosis		Poliomyelitis		Spinal cord injuries
	Thalassemia				Stroke

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 (Prelone), dexamethasone (Decadron, Hexadrol), or cortisone (Cortone) [22-25]. Glucocorticoids are used to the 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, nafarelin, 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).
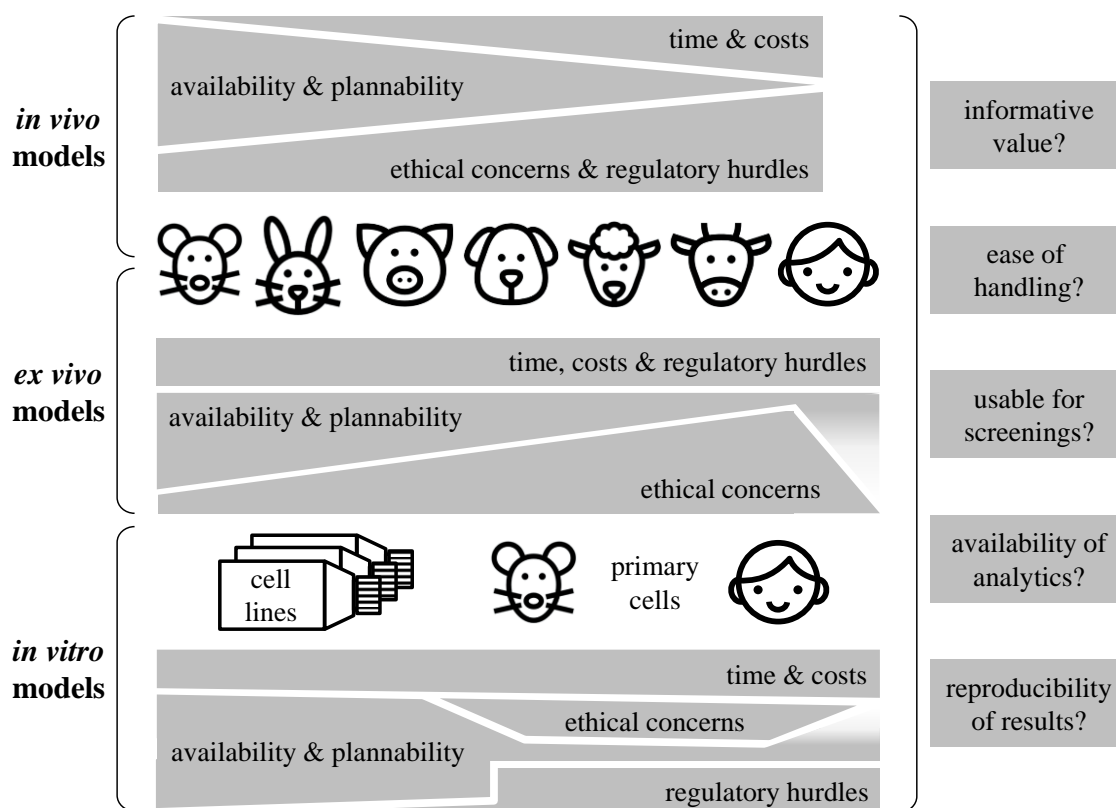

Figure 1. Factors Affecting the Choice of Models Suitable for Screening Changes in Bone Metabolism. Diverse *in vivo*, *ex vivo* and in *vitro* models to investigate bone cell functions exist. The different models vary strongly in their planning and handling, which in turn affects the timing and costs. 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.

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 inbreed 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 ¾ 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.
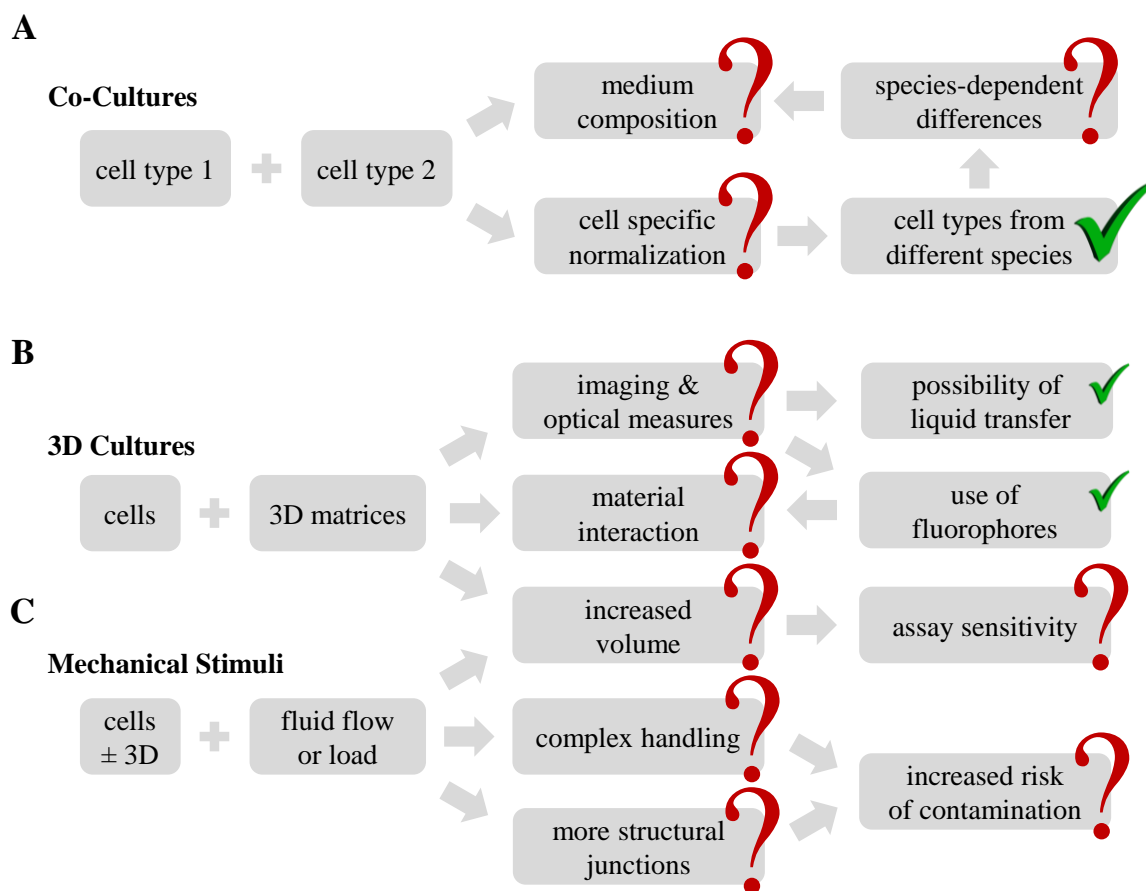

Figure 2. Challenges of Complex Culture Systems. Overview on challenges for (A) co-cultures, (B) 3D cultures, and (C) mechanically stimulated cultures is given. Combining different aspects, the complexity of the model system increases and new challenges arise. These may limit the available methodology 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 cells 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 SJSA-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 D_3), 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* (runt-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).
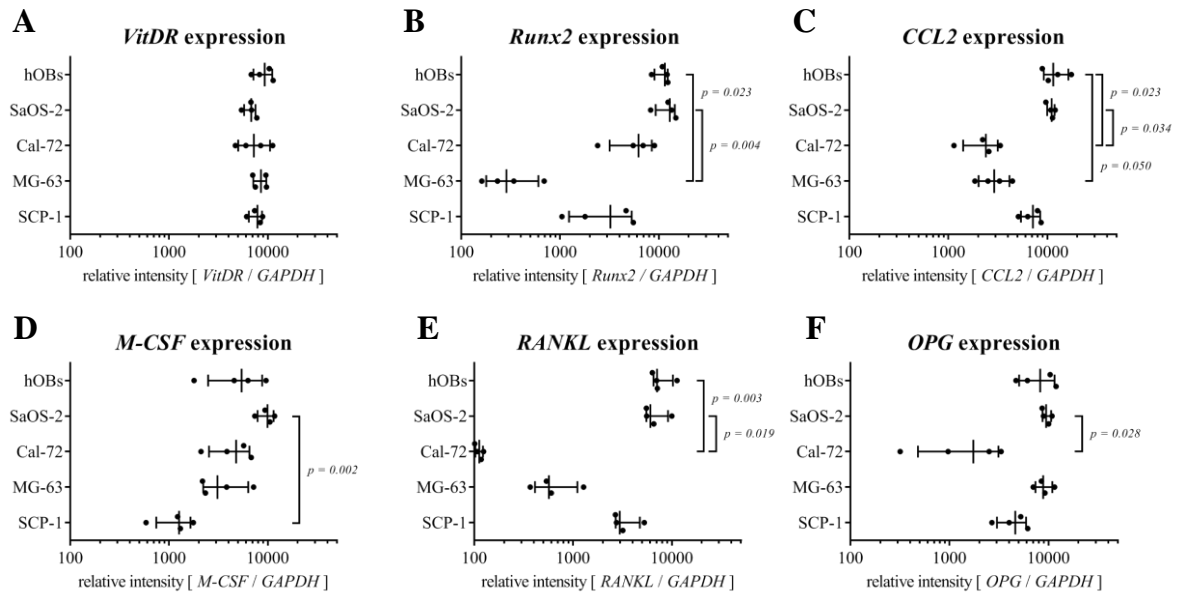
Figure 3. Comparison of the Cell Lines MG-63, Cal-72, and SaOS-2, with Primary Human Osteoblasts and the Immortalized Human MSC Cell Line SCP-1. Primary human osteoblasts (hOBs), SaOS-2, Cal-72, MG-63, and SCP-1 cells were osteogenically differentiated for 72 h. Gene expression levels of (A) *VitDR* = vitamin D receptor, (B) *Runx2* = runt-related transcription factor 2, (C) *CCL2* = C-C motif chemokine ligand 2, (D) *M-CSF* = macrophage colony-stimulating factor, (E) *RANKL* = receptor activator of nuclear factor kappa-B ligand, and (F) *OPG* = osteoprotegerin 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). Kruskal-Wallis test followed by Dunn's multiple comparison tests was used to compare different groups.

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 D$_3$ [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.
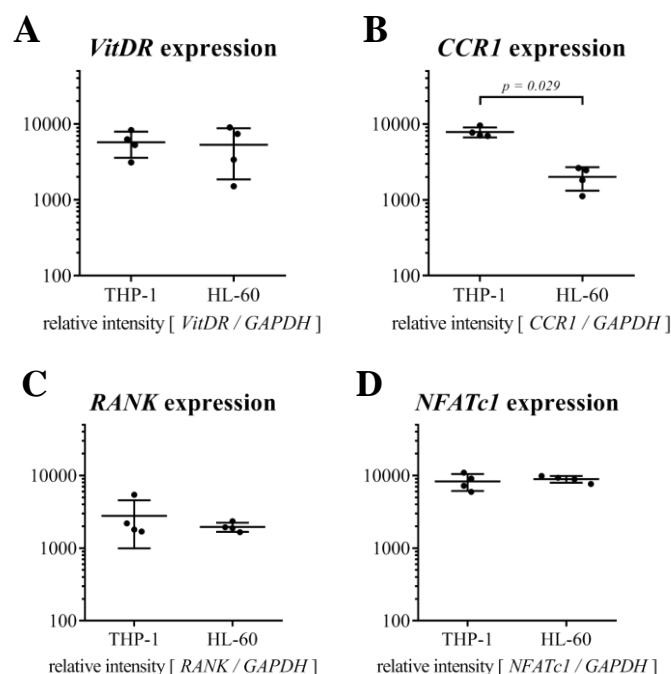

Figure 4. Activated THP-1 and HL-60 Cells Express Receptors and Transcription Factors Involved in Osteoclastogenensis. THP-1 and HL-60 cells were treated with 200 nM PMA for 24 h. Gene expression levels of (A) *VitDR* = vitamin D receptor, (B) *CCR1* = C-C chemokine receptor type 1, (C) *RANK* = receptor 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 monocytic 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 MEMα 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).
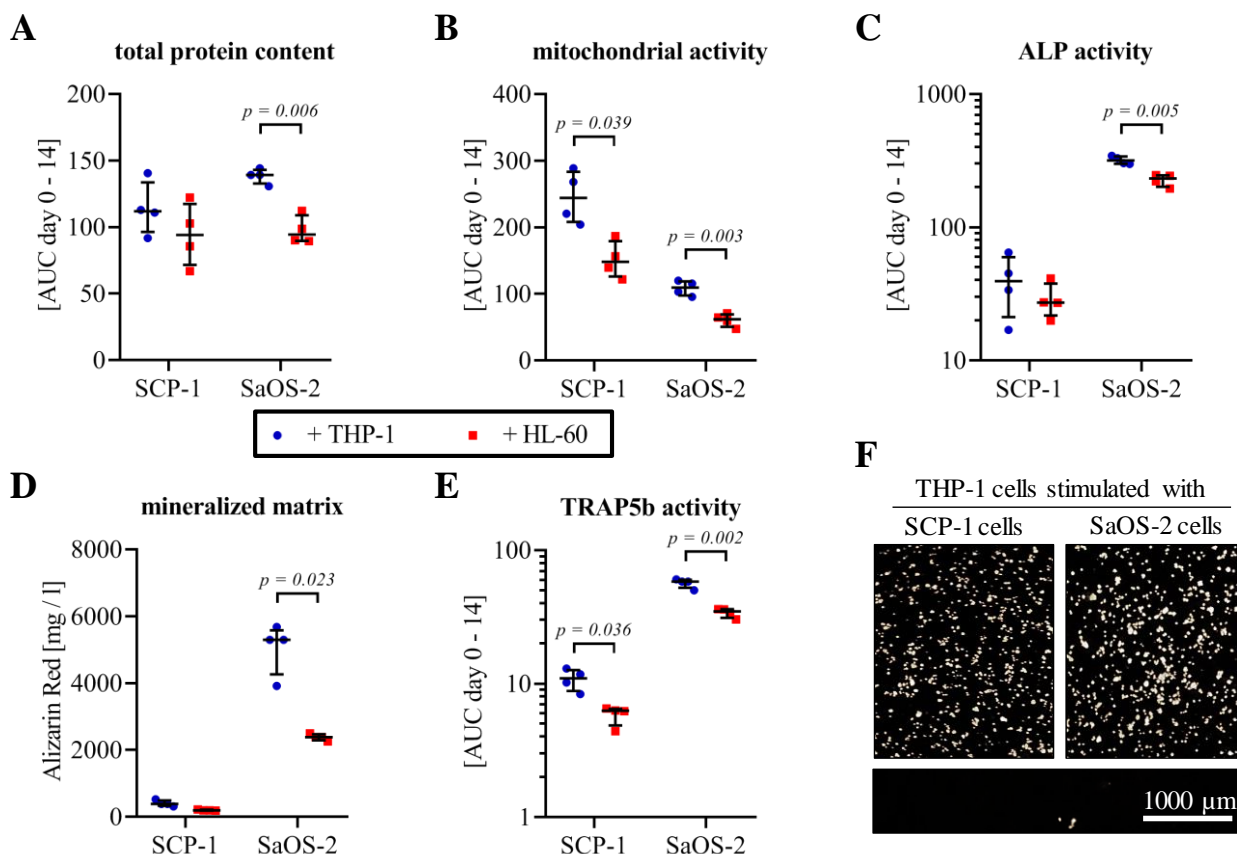
Figure 5. Osteogenic and Myeloid Cell Lines in Direct Co-Culture. THP-1 cells and HL-60 cells were activated with 200 nM PMA. After 24 h medium was removed and osteogenic SCP-1 or SaOS-2 cells were added and osteogenically differentiated for 14 days. (A) Total protein content was determined by Sulforhodamine 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].
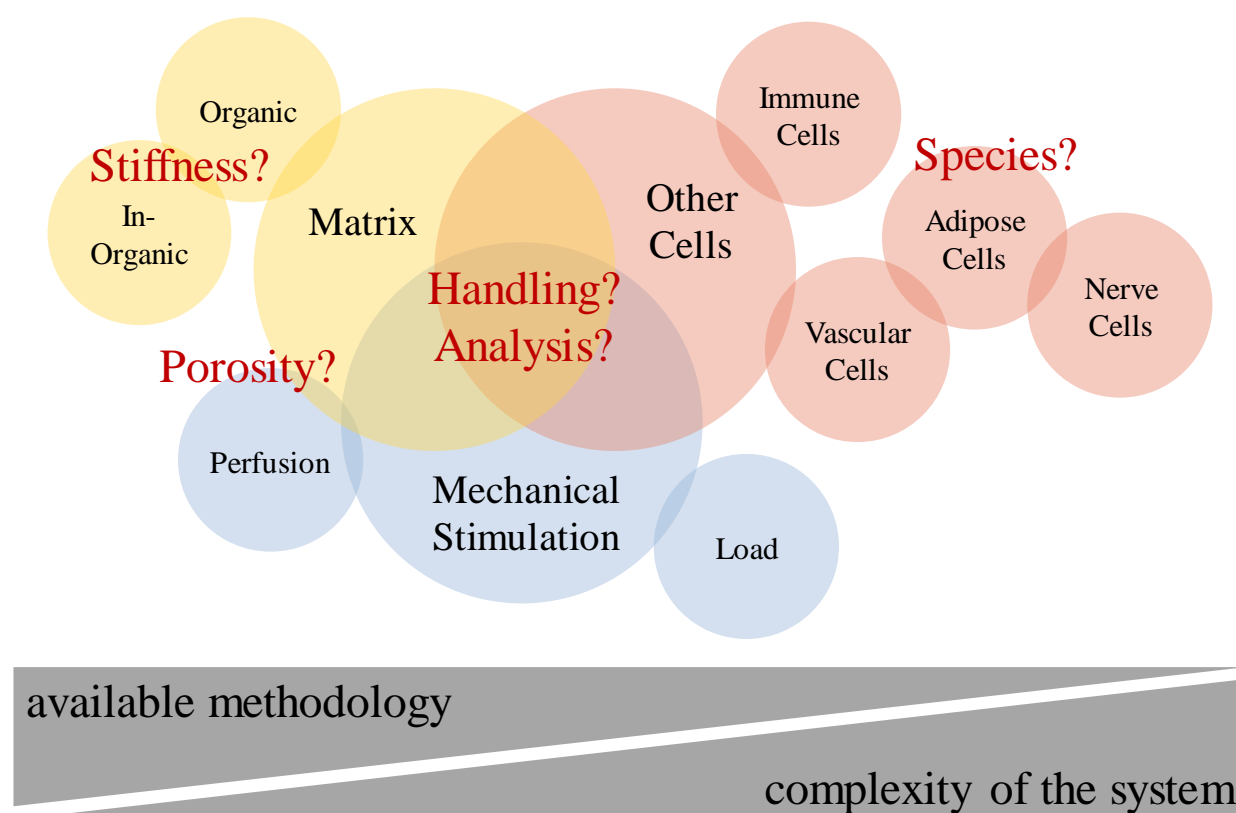

Figure 6. Overview of Factors that may Affect Bone Metabolism in a Model System. Novel model systems try to address as many of these factors as possible. However, with increasing complexity of the model system the number of available methods for the characterization of the model decreases.

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

	Search criteria	all	review articles
#1	"osteoblast" AND "osteoclast" AND "co-culture"	125	2
#2	"osteocyte" AND "osteoclast" AND "co-culture"	13	0
#3	"MSC" AND "osteoclast" AND "co-culture"	1	0
#4	"mesenchymal stem cell" AND "osteoclast" AND "co-culture"	6	1
#5	"bone cell" AND "co-culture"	21	2
#6	"bone metabolism" AND "*in vitro*" AND "co-culture"	15	0
#7	#1 OR #2 OR #3 OR #4 OR #5	166	4

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% CO_2 in a humidified atmosphere. Medium was changed twice a week. Cells were sub-cultured when a confluence of 80-90% or cell density of $1* 10^6$ 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 $CaCl_2$, 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

Gene name	Forward primer	Reverse primer	Gene bank accession number	Annealing temp. (°C)	# of cycles	Product length (bp)
CCL2	CCTTCATTCCCCAAGGGCTC	GGTTTGCTTGTCCAGGTGGT	002982.3	60	30	236
CCR1	AGTCCCTTGGAACCAGAGAGAAG	GGACCACCAGGATGTTTCCA	001295.2	60	35	197
M-CSF	AAGTTTGCCTGGGTCCTCTC	CCACTCCCAATCATGTGGCT	000757.5	60	35	289
NFATc1	TGCAAGCCGAATTCTCTGGT	CTTTACGGCGACGTCGTTTC	172390.2	60	35	228
OPG	CCGGAAACAGTGAATCAACTC	AGGTTAGCATGTCCAATGTG	002546.3	60	30	313
RANK	TTGTGATACAGGCAAGGCCC	TGCACACTGTGTCCTTGTTG	003839.3	55	35	182
RANKL	TCCCAAGTTCTCATACCCTGA	CATCCAGGAAATACATAACACTCC	033012.3	56	30	245
RUNX2	CGGCAAAATGAGCGACGTG	CACCGAGCACAGGAAGTTG	004348	60	30	268
VitDR	GACATCGGCATGATGAAGGAG	GCGTCCAGCAGTARGGCAA	000376	54	30	158
GAPDH	GTCAGTGGTGGACCTGACCT	AGGGGTCTACATGGCAACTG	002046.4	56	25	420

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

7. Funding

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

Conceptualization: S.E. and A.K.N.; literature research and review, S.E., R.A.-W., S.Z., and K.C.N.; methodology: S.E. and B.B.; investigations: S.E., C.L., R.A.-W., V.H., B.B., and W.W.; formal analysis: S.E. and B.B.; validation: C.L., R.A.-W. and A.K.N.; original draft preparation: S.E.; review and editing: all authors; visualization: S.E.; supervision: S.E. and AKN.; funding acquisition: S.E. and AKN.

10. Ethical Approval

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

11. References

[1] Sambrook P, Cooper C (2006) Osteoporosis. Lancet 367: 2010-2018. doi:10.1016/S0140-6736(06)68891-0.

[2] Hernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, McCloskey EV, Jonsson B, Kanis JA (2013) Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). Arch Osteoporos 8: 136. doi:10.1007/s11657-013-0136-1.

[3] Stathopoulos IS, Ballas EG, Lampropoulou-Adamidou K, Trovas G (2014) A review on osteoporosis in men. Hormones (Athens) 13: 441-457. doi:10.14310/horm.2002.1550.

[4] Reginster JY, Burlet N (2006) Osteoporosis: a still increasing prevalence. Bone 38: S4-9. doi:10.1016/j.bone.2005.11.024.

[5] Ahlborg HG, Rosengren BE, Jarvinen TL, Rogmark C, Nilsson JA, Sernbo I, Karlsson MK (2010) Prevalence of osteoporosis and incidence of hip fracture in women--secular trends over 30 years. BMC Musculoskelet Disord 11: 48. doi:10.1186/1471-2474-11-48.

[6] Riggs BL, Khosla S, Melton LJ, 3rd (2002) Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23: 279-302. doi:10.1210/edrv.23.3.0465.

[7] Cauley JA (2015) Estrogen and bone health in men and women. Steroids 99: 11-15. doi:10.1016/j.steroids.2014.12.010.

[8] Khundmiri SJ, Murray RD, Lederer E (2016) PTH and Vitamin D. Compr Physiol 6: 561-601. doi:10.1002/cphy.c140071.

[9] Ahlborg HG, Nguyen ND, Nguyen TV, Center JR, Eisman JA (2005) Contribution of hip strength indices to hip fracture risk in elderly men and women. J Bone Miner Res 20: 1820-1827. doi:10.1359/JBMR.050519.

[10] Tan LO, Lim SY, Vasanwala RF (2017) Primary osteoporosis in children. BMJ Case Rep 2017. doi:10.1136/bcr-2017-220700.

[11] Stein E, Shane E (2003) Secondary osteoporosis. Endocrinol Metab Clin North Am 32: 115-134, vii.

[12] Sheu A, Diamond T (2016) Secondary osteoporosis. Aust Prescr 39: 85-87. doi:10.18773/austprescr.2016.038.

[13] Emkey GR, Epstein S (2014) Secondary osteoporosis: pathophysiology & diagnosis. Best Pract Res Clin Endocrinol Metab 28: 911-935. doi:10.1016/j.beem.2014.07.002.

[14] Panday K, Gona A, Humphrey MB (2014) Medication-induced osteoporosis: screening and treatment strategies. Ther Adv Musculoskelet Dis 6: 185-202. doi:10.1177/1759720X14546350.

[15] Mazziotti G, Canalis E, Giustina A (2010) Drug-induced osteoporosis: mechanisms and clinical implications. Am J Med 123: 877-884. doi:10.1016/j.amjmed.2010.02.028.

[16] Weng MY, Lane NE (2007) Medication-induced osteoporosis. Curr Osteoporos Rep 5: 139-145.

[17] Pscherer S, Nussler A, Bahrs C, Reumann M, Ihle C, Stockle U, Ehnert S, Freude T, Ochs BG, Flesch I, et al. (2016) [Retrospective Analysis of Diabetics with Regard to Treatment Duration and Costs]. Z Orthop Unfall. doi:10.1055/s-0042-116328.

[18] Pscherer S, Sandmann GH, Ehnert S, Nussler AK, Stockle U, Freude T (2015) Delayed Fracture Healing in Diabetics with Distal Radius Fractures. Acta Chir Orthop Traumatol Cech 82: 268-273.

[19] Ihle C, Freude T, Bahrs C, Zehendner E, Braunsberger J, Biesalski HK, Lambert C, Stockle U, Wintermeyer E, Grunwald J, et al. (2017) Malnutrition - An underestimated factor in the inpatient treatment of traumatology and orthopedic patients: A prospective evaluation of 1055 patients. Injury 48: 628-636. doi:10.1016/j.injury.2017.01.036.

[20] Ehnert S, Aspera-Werz RH, Ihle C, Trost M, Zirn B, Flesch I, Schroter S, Relja B, Nussler AK (2019) Smoking Dependent Alterations in Bone Formation and Inflammation Represent Major Risk Factors for Complications Following Total Joint Arthroplasty. J Clin Med 8. doi:10.3390/jcm8030406.

[21] Wintermeyer E, Ihle C, Ehnert S, Schreiner AJ, Stollhof L, Stockle U, Nussler A, Fritsche A, Pscherer S (2018) [Assessment of the Influence of Diabetes mellitus and Malnutrition on the Postoperative Complication Rate and Quality of Life of Patients in a Clinic Focused on Trauma Surgery]. Z Orthop Unfall. doi:10.1055/a-0654-5504.

[22] Rizzoli R, Biver E (2015) Glucocorticoid-induced osteoporosis: who to treat with what agent? Nat Rev Rheumatol 11: 98-109. doi:10.1038/nrrheum.2014.188.

[23] Briot K, Roux C (2015) Glucocorticoid-induced osteoporosis. RMD Open 1: e000014. doi:10.1136/rmdopen-2014-000014.

[24] Civitelli R, Ziambaras K (2008) Epidemiology of glucocorticoid-induced osteoporosis. J Endocrinol Invest 31: 2-6.

[25] Berris KK, Repp AL, Kleerekoper M (2007) Glucocorticoid-induced osteoporosis. Curr Opin Endocrinol Diabetes Obes 14: 446-450. doi:10.1097/MED.0b013e3282f15407.

[26] De Nijs RN (2008) Glucocorticoid-induced osteoporosis: a review on pathophysiology and treatment options. Minerva Med 99: 23-43.

[27] Canalis E, Delany AM (2002) Mechanisms of glucocorticoid action in bone. Ann N Y Acad Sci 966: 73-81.

[28] Canalis E, Pereira RC, Delany AM (2002) Effects of glucocorticoids on the skeleton. J Pediatr Endocrinol Metab 15 Suppl 5: 1341-1345.

[29] van Staa TP, Leufkens B, Cooper C (2002) Bone loss and inhaled glucocorticoids. N Engl J Med 346: 533-535.

[30] Sambrook PN (2000) Inhaled corticosteroids, bone density, and risk of fracture. Lancet 355: 1385. doi:10.1016/S0140-6736(00)02134-6.

[31] Fraser LA, Adachi JD (2009) Glucocorticoid-induced osteoporosis: treatment update and review. Ther Adv Musculoskelet Dis 1: 71-85. doi:10.1177/1759720X09343729.

[32] Tufano A, Coppola A, Contaldi P, Franchini M, Minno GD (2015) Oral anticoagulant drugs and the risk of osteoporosis: new anticoagulants better than old? Semin Thromb Hemost 41: 382-388. doi:10.1055/s-0034-1543999.

[33] Francucci CM, Ceccoli L, Rilli S, Fiscaletti P, Caudarella R, Boscaro M (2009) Skeletal effects of oral anticoagulants. J Endocrinol Invest 32: 27-31.

[34] Chin KY (2017) A Review on the Relationship between Aspirin and Bone Health. J Osteoporos 2017: 3710959. doi:10.1155/2017/3710959.

[35] Hernandez RK, Do TP, Critchlow CW, Dent RE, Jick SS (2012) Patient-related risk factors for fracture-healing complications in the United Kingdom General Practice Research Database. Acta Orthop 83: 653-660. doi:10.3109/17453674.2012.747054.

[36] Geusens P, Emans PJ, de Jong JJ, van den Bergh J (2013) NSAIDs and fracture healing. Curr Opin Rheumatol 25: 524-531. doi:10.1097/BOR.0b013e32836200b8.

[37] Vuolteenaho K, Moilanen T, Moilanen E (2008) Non-steroidal anti-inflammatory drugs, cyclooxygenase-2 and the bone healing process. Basic Clin Pharmacol Toxicol 102: 10-14. doi:10.1111/j.1742-7843.2007.00149.x.

[38] Suzuki Y, Mizushima Y (1997) Osteoporosis in rheumatoid arthritis. Osteoporos Int 7 Suppl 3: S217-222.

[39] Vestergaard P (2008) Skeletal effects of drugs to treat cancer. Curr Drug Saf 3: 173-177.

[40] Smith MR (2003) Management of treatment-related osteoporosis in men with prostate cancer. Cancer Treat Rev 29: 211-218.

[41] Smith MR, Boyce SP, Moyneur E, Duh MS, Raut MK, Brandman J (2006) Risk of clinical fractures after gonadotropin-releasing hormone agonist therapy for prostate cancer. J Urol 175: 136-139; discussion 139. doi:10.1016/S0022-5347(05)00033-9.

[42] Smith MR, Lee WC, Brandman J, Wang Q, Botteman M, Pashos CL (2005) Gonadotropin-releasing hormone agonists and fracture risk: a claims-based cohort study of men with nonmetastatic prostate cancer. J Clin Oncol 23: 7897-7903. doi:10.1200/JCO.2004.00.6908.

[43] Tuchendler D, Bolanowski M (2014) The influence of thyroid dysfunction on bone metabolism. Thyroid Res 7: 12. doi:10.1186/s13044-014-0012-0.

[44] Tarraga Lopez PJ, Lopez CF, de Mora FN, Montes JA, Albero JS, Manez AN, Casas AG (2011) Osteoporosis in patients with subclinical hypothyroidism treated with thyroid hormone. Clin Cases Miner Bone Metab 8: 44-48.

[45] Vestergaard P, Mosekilde L (2002) Fractures in patients with hyperthyroidism and hypothyroidism: a nationwide follow-up study in 16,249 patients. Thyroid 12: 411-419. doi:10.1089/105072502760043503.

[46] Sendak RA, Sampath TK, McPherson JM (2007) Newly reported roles of thyroid-stimulating hormone and follicle-stimulating hormone in bone remodelling. Int Orthop 31: 753-757. doi:10.1007/s00264-007-0417-7.

[47] Cohen A, Shane E (2003) Osteoporosis after solid organ and bone marrow transplantation. Osteoporos Int 14: 617-630. doi:10.1007/s00198-003-1426-z.

[48] Rubert M, Montero M, Guede D, Caeiro JR, Martin-Fernandez M, Diaz-Curiel M, de la Piedra C (2015) Sirolimus and tacrolimus rather than cyclosporine A cause bone loss in healthy adult male rats. Bone Rep 2: 74-81. doi:10.1016/j.bonr.2015.05.003.

[49] Kulak CA, Borba VZ, Kulak Junior J, Custodio MR (2014) Bone disease after transplantation: osteoporosis and fractures risk. Arq Bras Endocrinol Metabol 58: 484-492.

[50] Lee J, Kim JH, Kim K, Jin HM, Lee KB, Chung DJ, Kim N (2007) Ribavirin enhances osteoclast formation through osteoblasts via up-regulation of TRANCE/RANKL. Mol Cell Biochem 296: 17-24. doi:10.1007/s11010-006-9293-5.

[51] Narayana K, D'Souza UJ, Seetharama Rao KP (2002) The genotoxic and cytotoxic effects of ribavirin in rat bone marrow. Mutat Res 521: 179-185. doi:S1383571802002395 [pii].

[52] Solis-Herruzo JA, Castellano G, Fernandez I, Munoz R, Hawkins F (2000) Decreased bone mineral density after therapy with alpha interferon in combination with ribavirin for chronic hepatitis C. J Hepatol 33: 812-817. doi:S0168-8278(00)80314-1 [pii].

[53] Petty SJ, Wilding H, Wark JD (2016) Osteoporosis Associated with Epilepsy and the Use of Anti-Epileptics-a Review. Curr Osteoporos Rep 14: 54-65. doi:10.1007/s11914-016-0302-7.

[54] Farhat G, Yamout B, Mikati MA, Demirjian S, Sawaya R, El-Hajj Fuleihan G (2002) Effect of antiepileptic drugs on bone density in ambulatory patients. Neurology 58: 1348-1353.

[55] Brigo F, Igwe SC, Del Felice A (2016) Melatonin as add-on treatment for epilepsy. Cochrane Database Syst Rev: CD006967. doi:10.1002/14651858.CD006967.pub4.

[56] Sheth RD (2002) Bone health in epilepsy. Epilepsia 43: 1453-1454.

[57] Ribaya-Mercado JD, Blumberg JB (2007) Vitamin A: is it a risk factor for osteoporosis and bone fracture? Nutr Rev 65: 425-438.

[58] Barker ME, McCloskey E, Saha S, Gossiel F, Charlesworth D, Powers HJ, Blumsohn A (2005) Serum retinoids and beta-carotene as predictors of hip and other fractures in elderly women. J Bone Miner Res 20: 913-920. doi:10.1359/JBMR.050112.

[59] Michaelsson K, Lithell H, Vessby B, Melhus H (2003) Serum retinol levels and the risk of fracture. N Engl J Med 348: 287-294. doi:10.1056/NEJMoa021171.

[60] Wang CY, Fu SH, Wang CL, Chen PJ, Wu FL, Hsiao FY (2016) Serotonergic antidepressant use and the risk of fracture: a population-based nested case-control study. Osteoporos Int 27: 57-63. doi:10.1007/s00198-015-3213-z.

[61] Moura C, Bernatsky S, Abrahamowicz M, Papaioannou A, Bessette L, Adachi J, Goltzman D, Prior J, Kreiger N, Towheed T, et al. (2014) Antidepressant use and 10-year incident fracture risk: the population-based Canadian Multicentre Osteoporosis Study (CaMoS). Osteoporos Int 25: 1473-1481. doi:10.1007/s00198-014-2649-x.

[62] Vestergaard P, Prieto-Alhambra D, Javaid MK, Cooper C (2013) Fractures in users of antidepressants and anxiolytics and sedatives: effects of age and dose. Osteoporos Int 24: 671-680. doi:10.1007/s00198-012-2043-5.

[63] Vestergaard P (2008) Skeletal effects of central nervous system active drugs: anxiolytics, sedatives, antidepressants, lithium and neuroleptics. Curr Drug Saf 3: 185-189.

[64] Hamilton C, Seidner DL (2004) Metabolic bone disease and parenteral nutrition. Curr Gastroenterol Rep 6: 335-341.

[65] Zhu S, Ehnert S, Rouss M, Haussling V, Aspera-Werz RH, Chen T, Nussler AK (2018) From the Clinical Problem to the Basic Research-Co-Culture Models of Osteoblasts and Osteoclasts. Int J Mol Sci 19. doi:10.3390/ijms19082284.

[66] Aerssens J, Boonen S, Lowet G, Dequeker J (1998) Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research. Endocrinology 139: 663-670. doi:10.1210/endo.139.2.5751.

[67] Foster AD (2019) The impact of bipedal mechanical loading history on longitudinal long bone growth. PLoS One 14: e0211692. doi:10.1371/journal.pone.0211692.

[68] Lessmollmann U, Hinz N, Neubert D (1976) In vitro system for toxicological studies on the development of mammalian limb buds in a chemically defined medium. Arch Toxicol 36: 169-176. doi:10.1007/bf00351978.

[69] Barrach HJ, Neubert D (1980) Significance of organ culture techniques for evaluation of prenatal toxicity. Arch Toxicol 45: 161-187. doi:10.1007/bf02418996.

[70] Houston DA, Staines KA, MacRae VE, Farquharson C (2016) Culture of Murine Embryonic Metatarsals: A Physiological Model of Endochondral Ossification. Journal of visualized experiments : JoVE. doi:10.3791/54978.

[71] Smith EL, Rashidi H, Kanczler JM, Shakesheff KM, Oreffo RO (2015) The effects of 1α, 25-dihydroxyvitamin D3 and transforming growth factor-β3 on bone development in an ex vivo organotypic culture system of embryonic chick femora. PLoS One 10: e0121653. doi:10.1371/journal.pone.0121653.

[72] Smith EL, Kanczler JM, Oreffo RO (2013) A new take on an old story: chick limb organ culture for skeletal niche development and regenerative medicine evaluation. Eur Cell Mater 26: 91-106; discussion 106. doi:10.22203/ecm.v026a07.

[73] Paradis FH, Yan H, Huang C, Hales BF (2019) The Murine Limb Bud in Culture as an In Vitro Teratogenicity Test System. Methods Mol Biol 1965: 73-91. doi:10.1007/978-1-4939-9182-2_6.

[74] Parivar K, Kouchesfehani MH, Boojar MM, Hayati RN (2006) Organ culture studies on the development of mouse embryo limb buds under EMF influence. Int J Radiat Biol 82: 455-464. doi:10.1080/09553000600863056.

[75] Muzic V, Katusic Bojanac A, Juric-Lekic G, Himelreich M, Tupek K, Serman L, Marn N, Sincic N, Vlahovic M, Bulic-Jakus F (2013) Epigenetic drug 5-azacytidine impairs proliferation of rat limb buds in an organotypic model-system in vitro. Croat Med J 54: 489-495. doi:10.3325/cmj.2013.54.489.

[76] Proffit WR, Ackerman JL (1964) Fluoride: Its Effects on 2 Parameters of Bone Growth in Organ Culture. Science 145: 932-934. doi:10.1126/science.145.3635.932.

[77] Abubakar AA, Ibrahim SM, Ali AK, Handool KO, Khan MS, Noordin Mustapha M, Azmi Ibrahim T, Kaka U, Mohamad Yusof L (2019) Postnatal ex vivo rat model for longitudinal bone growth investigations. Animal models and experimental medicine 2: 34-43. doi:10.1002/ame2.12051.

[78] Uribe V, Rosello-Diez A (2019) Culturing and Measuring Fetal and Newborn Murine Long Bones. Journal of visualized experiments : JoVE. doi:10.3791/59509.

[79] Abubakar AA, Noordin MM, Azmi TI, Kaka U, Loqman MY (2016) The use of rats and mice as animal models in ex vivo bone growth and development studies. Bone & joint research 5: 610-618. doi:10.1302/2046-3758.512.bjr-2016-0102.r2.

[80] Kunimoto T, Okubo N, Minami Y, Fujiwara H, Hosokawa T, Asada M, Oda R, Kubo T, Yagita K (2016) A PTH-responsive circadian clock operates in ex vivo mouse femur fracture healing site. Sci Rep 6: 22409. doi:10.1038/srep22409.

[81] Okubo N, Minami Y, Fujiwara H, Umemura Y, Tsuchiya Y, Shirai T, Oda R, Inokawa H, Kubo T, Yagita K (2013) Prolonged bioluminescence monitoring in mouse ex vivo bone culture revealed persistent circadian rhythms in articular cartilages and growth plates. PLoS One 8: e78306. doi:10.1371/journal.pone.0078306.

[82] Okubo N, Fujiwara H, Minami Y, Kunimoto T, Hosokawa T, Umemura Y, Inokawa H, Asada M, Oda R, Kubo T, et al. (2015) Parathyroid hormone resets the cartilage circadian clock of the organ-cultured murine femur. Acta Orthop 86: 627-631. doi:10.3109/17453674.2015.1029393.

[83] Sathi GA, Kenmizaki K, Yamaguchi S, Nagatsuka H, Yoshida Y, Matsugaki A, Ishimoto T, Imazato S, Nakano T, Matsumoto T (2015) Early initiation of endochondral ossification of mouse femur cultured in hydrogel with different mechanical stiffness. Tissue Eng Part C Methods 21: 567-575. doi:10.1089/ten.TEC.2014.0475.

[84] Madsen SH, Goettrup AS, Thomsen G, Christensen ST, Schultz N, Henriksen K, Bay-Jensen AC, Karsdal MA (2011) Characterization of an Ex vivo Femoral Head Model Assessed by Markers of Bone and Cartilage Turnover. Cartilage 2: 265-278. doi:10.1177/1947603510383855.

[85] Batushansky A, Lopes EBP, Zhu S, Humphries KM, Griffin TM (2019) GC-MS method for metabolic profiling of mouse femoral head articular cartilage reveals distinct effects of tissue culture and development. Osteoarthritis Cartilage 27: 1361-1371. doi:10.1016/j.joca.2019.05.010.

[86] Mohammad KS, Chirgwin JM, Guise TA (2008) Assessing new bone formation in neonatal calvarial organ cultures. Methods Mol Biol 455: 37-50. doi:10.1007/978-1-59745-104-8_3.

[87] Garrett IR (2003) Assessing bone formation using mouse calvarial organ cultures. Methods Mol Med 80: 183-198. doi:10.1385/1-59259-366-6:183.

[88] Marino S, Bishop RT, Carrasco G, Logan JG, Li B, Idris AI (2019) Pharmacological Inhibition of NFκB Reduces Prostate Cancer Related Osteoclastogenesis In Vitro and Osteolysis Ex Vivo. Calcified tissue international 105: 193-204. doi:10.1007/s00223-019-00538-9.

[89] Curtin P, Youm H, Salih E (2012) Three-dimensional cancer-bone metastasis model using ex-vivo co-cultures of live calvarial bones and cancer cells. Biomaterials 33: 1065-1078. doi:10.1016/j.biomaterials.2011.10.046..

[90] Salih E (2019) Ex-Vivo Model Systems of Cancer-Bone Cell Interactions. Methods Mol Biol 1914: 217-240. doi:10.1007/978-1-4939-8997-3_11.

[91] Choudhary S, Ramasundaram P, Dziopa E, Mannion C, Kissin Y, Tricoli L, Albanese C, Lee W, Zilberberg J (2018) Human ex vivo 3D bone model recapitulates osteocyte response to metastatic prostate cancer. Sci Rep 8: 17975. doi:10.1038/s41598-018-36424-x.

[92] Salamanna F, Borsari V, Brogini S, Giavaresi G, Parrilli A, Cepollaro S, Cadossi M, Martini L, Mazzotti A, Fini M (2016) An in vitro 3D bone metastasis model by using a human bone tissue culture and human sex-related cancer cells. Oncotarget 7: 76966-76983. doi:10.18632/oncotarget.12763.

[93] Sloan AJ, Taylor SY, Smith EL, Roberts JL, Chen L, Wei XQ, Waddington RJ (2013) A novel ex vivo culture model for inflammatory bone destruction. Journal of dental research 92: 728-734. doi:10.1177/0022034513495240.

[94] Srinivasaiah S, Musumeci G, Mohan T, Castrogiovanni P, Absenger-Novak M, Zefferer U, Mostofi S, Bonyadi Rad E, Grün NG, Weinberg AM, et al. (2019) A 300 μm Organotypic Bone Slice Culture Model for Temporal Investigation of Endochondral Osteogenesis. Tissue Eng Part C Methods 25: 197-212. doi:10.1089/ten.TEC.2018.0368.

[95] Marino S, Staines KA, Brown G, Howard-Jones RA, Adamczyk M (2016) Models of ex vivo explant cultures: applications in bone research. BoneKEy reports 5: 818. doi:10.1038/bonekey.2016.49.

[96] Colombo JS, Howard-Jones RA, Young FI, Waddington RJ, Errington RJ, Sloan AJ (2015) A 3D ex vivo mandible slice system for longitudinal culturing of transplanted dental pulp progenitor cells. Cytometry A 87: 921-928. doi:10.1002/cyto.a.22680.

[97] Alfaqeeh SA, Tucker AS (2013) The slice culture method for following development of tooth germs in explant culture. Journal of visualized experiments : JoVE: e50824. doi:10.3791/50824.

[98] Smith EL, Locke M, Waddington RJ, Sloan AJ (2010) An ex vivo rodent mandible culture model for bone repair. Tissue Eng Part C Methods 16: 1287-1296. doi:10.1089/ten.TEC.2009.0698.

[99] Tecles O, Laurent P, Aubut V, About I (2008) Human tooth culture: a study model for reparative dentinogenesis and direct pulp capping materials biocompatibility. J Biomed Mater Res B Appl Biomater 85: 180-187. doi:10.1002/jbm.b.30933.

[100] Melin M, Joffre-Romeas A, Farges JC, Couble ML, Magloire H, Bleicher F (2000) Effects of TGFbeta1 on dental pulp cells in cultured human tooth slices. Journal of dental research 79: 1689-1696. doi:10.1177/00220345000790090901.

[101] Rawlinson SC, el-Haj AJ, Minter SL, Tavares IA, Bennett A, Lanyon LE (1991) Loading-related increases in prostaglandin production in cores of adult canine cancellous bone in vitro: a role for prostacyclin in adaptive bone remodeling? J Bone Miner Res 6: 1345-1351. doi:10.1002/jbmr.5650061212.

[102] Knothe Tate ML, Knothe U (2000) An ex vivo model to study transport processes and fluid flow in loaded bone. J Biomech 33: 247-254. doi:10.1016/s0021-9290(99)00143-8.

[103] Kluter T, Hassan R, Rasch A, Naujokat H, Wang F, Behrendt P, Lippross S, Gerdesmeyer L, Eglin D, Seekamp A, et al. (2020) An Ex Vivo Bone Defect Model to Evaluate Bone Substitutes and Associated Bone Regeneration Processes. Tissue Eng Part C Methods 26: 56-65. doi:10.1089/ten.TEC.2019.0274.

[104] Templeton ZS, Bachmann MH, Alluri RV, Maloney WJ, Contag CH, King BL (2015) Methods for culturing human femur tissue explants to study breast cancer cell colonization of the metastatic niche. Journal of visualized experiments : JoVE. doi:10.3791/52656.

[105] Vivanco J, Garcia S, Ploeg HL, Alvarez G, Cullen D, Smith EL (2013) Apparent elastic modulus of ex vivo trabecular bovine bone increases with dynamic loading. Proc Inst Mech Eng H 227: 904-912. doi:10.1177/0954411913486855.

[106] Endres S, Kratz M, Wunsch S, Jones DB (2009) Zetos: a culture loading system for trabecular bone. Investigation of different loading signal intensities on bovine bone cylinders. J Musculoskelet Neuronal Interact 9: 173-183.

[107] Simpson AE, Stoddart MJ, Davies CM, Jahn K, Furlong PI, Gasser JA, Jones DB, Noble BS, Richards RG (2009) TGFbeta3 and loading increases osteocyte survival in human cancellous bone cultured ex vivo. Cell Biochem Funct 27: 23-29. doi:10.1002/cbf.1529.

[108] Davies CM, Jones DB, Stoddart MJ, Koller K, Smith E, Archer CW, Richards RG (2006) Mechanically loaded ex vivo bone culture system 'Zetos': systems and culture preparation. Eur Cell Mater 11: 57-75; discussion 75. doi:10.22203/ecm.v011a07.

[109] Stoddart MJ, Furlong PI, Simpson A, Davies CM, Richards RG (2006) A comparison of non-radioactive methods for assessing viability in ex vivo cultured cancellous bone: technical note. Eur Cell Mater 12: 16-25; discussion 16-25. doi:10.22203/ecm.v012a02.

[110] David V, Guignandon A, Martin A, Malaval L, Lafage-Proust MH, Rattner A, Mann V, Noble B, Jones DB, Vico L (2008) Ex Vivo bone formation in bovine trabecular bone cultured in a dynamic 3D bioreactor is enhanced by compressive mechanical strain. Tissue Eng Part A 14: 117-126. doi:10.1089/ten.a.2007.0051.

[111] Bellido T, Delgado-Calle J (2020) Ex Vivo Organ Cultures as Models to Study Bone Biology. JBMR Plus 4. doi:10.1002/jbm4.10345.

[112] Owen R, Reilly GC (2018) In vitro Models of Bone Remodelling and Associated Disorders. Frontiers in bioengineering and biotechnology 6: 134. doi:10.3389/fbioe.2018.00134.

[113] Schulze S, Wehrum D, Dieter P, Hempel U (2018) A supplement-free osteoclast-osteoblast co-culture for pre-clinical application. Journal of cellular physiology 233: 4391-4400. doi:10.1002/jcp.26076.

[114] Jimi E, Nakamura I, Amano H, Taguchi Y, Tsurukai T, Tamura M, Takahashi N, Suda T (1996) Osteoclast function is activated by osteoblastic cells through a mechanism involving cell-to-cell contact. Endocrinology 137: 2187-2190. doi:10.1210/endo.137.5.8612568.

[115] Itoh K, Udagawa N, Matsuzaki K, Takami M, Amano H, Shinki T, Ueno Y, Takahashi N, Suda T (2000) Importance of membrane- or matrix-associated forms of M-CSF and RANKL/ODF in osteoclastogenesis supported by SaOS-4/3 cells expressing recombinant PTH/PTHrP receptors. J Bone Miner Res 15: 1766-1775. doi:10.1359/jbmr.2000.15.9.1766.

[116] Green J, Schotland S, Stauber DJ, Kleeman CR, Clemens TL (1995) Cell-matrix interaction in bone: type I collagen modulates signal transduction in osteoblast-like cells. Am J Physiol 268: C1090-1103. doi:10.1152/ajpcell.1995.268.5.C1090.

[117] Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simoes MJ, Cerri PS (2015) Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. Biomed Res Int 2015: 421746. doi:10.1155/2015/421746.

[118] Forte L, Torricelli P, Boanini E, Gazzano M, Rubini K, Fini M, Bigi A (2016) Antioxidant and bone repair properties of quercetin-functionalized hydroxyapatite: An in vitro osteoblast-osteoclast-endothelial cell co-culture study. Acta Biomater 32: 298-308. doi:10.1016/j.actbio.2015.12.013.

[119] Wu L, Feyerabend F, Schilling AF, Willumeit-Romer R, Luthringer BJ (2015) Effects of extracellular magnesium extract on the proliferation and differentiation of human osteoblasts and osteoclasts in coculture. Acta Biomater 27: 294-304. doi:10.1016/j.actbio.2015.08.042.

[120] Heinemann S, Heinemann C, Wenisch S, Alt V, Worch H, Hanke T (2013) Calcium phosphate phases integrated in silica/collagen nanocomposite xerogels enhance the bioactivity and ultimately manipulate the osteoblast/osteoclast ratio in a human co-culture model. Acta Biomater 9: 4878-4888. doi:10.1016/j.actbio.2012.10.010.

[121] Heinemann C, Heinemann S, Worch H, Hanke T (2011) Development of an osteoblast/osteoclast co-culture derived by human bone marrow stromal cells and human monocytes for biomaterials testing. Eur Cell Mater 21: 80-93.

[122] Tortelli F, Pujic N, Liu Y, Laroche N, Vico L, Cancedda R (2009) Osteoblast and osteoclast differentiation in an in vitro three-dimensional model of bone. Tissue Eng Part A 15: 2373-2383. doi:10.1089/ten.tea.2008.0501.

[123] Penolazzi L, Lolli A, Sardelli L, Angelozzi M, Lambertini E, Trombelli L, Ciarpella F, Vecchiatini R, Piva R (2016) Establishment of a 3D-dynamic osteoblasts-osteoclasts co-culture model to simulate the jawbone microenvironment in vitro. Life Sci 152: 82-93. doi:10.1016/j.lfs.2016.03.035.

[124] Papadimitropoulos A, Scherberich A, Guven S, Theilgaard N, Crooijmans HJ, Santini F, Scheffler K, Zallone A, Martin I (2011) A 3D in vitro bone organ model using human progenitor cells. Eur Cell Mater 21: 445-458; discussion 458.

[125] Kadow-Romacker A, Duda GN, Bormann N, Schmidmaier G, Wildemann B (2013) Slight changes in the mechanical stimulation affects osteoblast- and osteoclast-like cells in co-culture. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie 40: 441-447. doi:10.1159/000356284.

[126] Kadow-Romacker A, Hoffmann JE, Duda G, Wildemann B, Schmidmaier G (2009) Effect of mechanical stimulation on osteoblast- and osteoclast-like cells in vitro. Cells, tissues, organs 190: 61-68. doi:10.1159/000178022.

[127] Ruoss M, Kieber V, Rebholz S, Linnemann C, Rinderknecht H, Haussling V, Hacker M, Olde Damink LHH, Ehnert S, Nussler AK (2019) Cell-Type-Specific Quantification of a Scaffold-Based 3D Liver Co-Culture. Methods Protoc 3. doi:10.3390/mps3010001.

[128] Tendulkar G, Grau P, Ziegler P, Buck A, Sr., Buck A, Jr., Badke A, Kaps HP, Ehnert S, Nussler AK (2016) Imaging Cell Viability on Non-transparent Scaffolds - Using the Example of a Novel Knitted Titanium Implant. Journal of visualized experiments : JoVE. doi:10.3791/54537.

[129] Greiner S, Kadow-Romacker A, Schmidmaier G, Wildemann B (2009) Cocultures of osteoblasts and osteoclasts are influenced by local application of zoledronic acid incorporated in a poly(D,L-lactide) implant coating. J Biomed Mater Res A 91: 288-295. doi:10.1002/jbm.a.32245.

[130] Heinemann C, Heinemann S, Rößler S, Kruppke B, Wiesmann HP, Hanke T (2019) Organically modified hydroxyapatite (ormoHAP) nanospheres stimulate the differentiation of osteoblast and osteoclast precursors: a co-culture study. Biomedical materials (Bristol, England) 14: 035015. doi:10.1088/1748-605X/ab0fad.

[131] Ehnert S, Häuser J, Kerler I, Schmitt A, Olderøy M, Freude T, Neumaier M, Egaña T, Schenck T, Stöckle U, et al. (2011) Mesenchymale Stammzellen aus Fettgewebe - Die bessere Alternative zu Knochenmarkszellen für das Tissue Engineering von Knochen? . Zeitschrift für Regenerative Medizin 1: 3-8.

[132] Zachos C, Steubesand N, Seekamp A, Fuchs S, Lippross S (2014) Co-cultures of programmable cells of monocytic origin and mesenchymal stem cells do increase osteogenic differentiation. J Orthop Res 32: 1264-1270. doi:10.1002/jor.22663.

[133] Zehnder T, Boccaccini A, Detsch R (2017) Biofabrication of a co-culture system in an osteoid-like hydrogel matrix. Biofabrication. doi:10.1088/1758-5090/aa64ec.

[134] Jablonski H, Rekasi H, Jager M (2016) The influence of calcitonin gene-related peptide on markers of bone metabolism in MG-63 osteoblast-like cells co-cultured with THP-1 macrophage-like cells under virtually osteolytic conditions. BMC Musculoskelet Disord 17: 199. doi:10.1186/s12891-016-1044-5.

[135] Lauvrak SU, Munthe E, Kresse SH, Stratford EW, Namlos HM, Meza-Zepeda LA, Myklebost O (2013) Functional characterisation of osteosarcoma cell lines and identification of mRNAs and miRNAs associated with aggressive cancer phenotypes. Br J Cancer 109: 2228-2236. doi:10.1038/bjc.2013.549.

[136] Mohseny AB, Machado I, Cai Y, Schaefer KL, Serra M, Hogendoorn PC, Llombart-Bosch A, Cleton-Jansen AM (2011) Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Lab Invest 91: 1195-1205. doi:10.1038/labinvest.2011.72.

[137] Lucero CM, Vega OA, Osorio MM, Tapia JC, Antonelli M, Stein GS, van Wijnen AJ, Galindo MA (2013) The cancer-related transcription factor Runx2 modulates cell proliferation in human osteosarcoma cell lines. Journal of cellular physiology 228: 714-723. doi:10.1002/jcp.24218.

[138] Trojani C, Weiss P, Michiels JF, Vinatier C, Guicheux J, Daculsi G, Gaudray P, Carle GF, Rochet N (2005) Three-dimensional culture and differentiation of human osteogenic cells in an injectable hydroxypropylmethylcellulose hydrogel. Biomaterials 26: 5509-5517. doi:10.1016/j.biomaterials.2005.02.001.

[139] Rochet N, Leroy P, Far DF, Ollier L, Loubat A, Rossi B (2003) CAL72: a human osteosarcoma cell line with unique effects on hematopoietic cells. Eur J Haematol 70: 43-52. doi:10.1034/j.1600-0609.2003.02766.x.

[140] Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, Milz S (2004) Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res 24: 3743-3748.

[141] Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF (1997) Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res 12: 2014-2023. doi:10.1359/jbmr.1997.12.12.2014.

[142] Yano M, Kawao N, Okumoto K, Tamura Y, Okada K, Kaji H (2014) Fibrodysplasia ossificans progressiva-related activated activin-like kinase signaling enhances osteoclast formation during heterotopic ossification in muscle tissues. J Biol Chem 289: 16966-16977. doi:10.1074/jbc.M113.526038.

[143] Tani-Ishii N, Penninger JM, Matsumoto G, Teranaka T, Umemoto T (2002) The role of LFA-1 in osteoclast development induced by co-cultures of mouse bone marrow cells and MC3T3-G2/PA6 cells. J Periodontal Res 37: 184-191. doi:10.1034/j.1600-0765.2002.00610.x.

[144] Park E, Kim J, Kim MC, Yeo S, Kim J, Park S, Jo M, Choi CW, Jin HS, Lee SW, et al. (2019) Anti-Osteoporotic Effects of Kukoamine B Isolated from Lycii Radicis Cortex Extract on Osteoblast and Osteoclast Cells and Ovariectomized Osteoporosis Model Mice. Int J Mol Sci 20. doi:10.3390/ijms20112784.

[145] Chen S, Ye X, Yu X, Xu Q, Pan K, Lu S, Yang P (2015) Co-culture with periodontal ligament stem cells enhanced osteoblastic differentiation of MC3T3-E1 cells and osteoclastic differentiation of RAW264.7 cells. International journal of clinical and experimental pathology 8: 14596-14607.

[146] Zhang S, Feng P, Mo G, Li D, Li Y, Mo L, Yang Z, Liang D (2017) Icariin influences adipogenic differentiation of stem cells affected by osteoblast-osteoclast co-culture and clinical research adipogenic. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 88: 436-442. doi:10.1016/j.biopha.2017.01.050.

[147] Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF (2002) MLO-Y4 osteocyte-like cells support osteoclast formation and activation. J Bone Miner Res 17: 2068-2079. doi:10.1359/jbmr.2002.17.11.2068.

[148] Hayashida C, Ito J, Nakayachi M, Okayasu M, Ohyama Y, Hakeda Y, Sato T (2014) Osteocytes produce interferon-β as a negative regulator of osteoclastogenesis. J Biol Chem 289: 11545-11555. doi:10.1074/jbc.M113.523811.

[149] Liao C, Cheng T, Wang S, Zhang C, Jin L, Yang Y (2017) Shear stress inhibits IL-17A-mediated induction of osteoclastogenesis via osteocyte pathways. Bone 101: 10-20. doi:10.1016/j.bone.2017.04.003.

[150] Heni H, Ebner JK, Schmidt G, Aktories K, Orth JHC (2018) Involvement of Osteocytes in the Action of Pasteurella multocida Toxin. Toxins (Basel) 10. doi:10.3390/toxins10080328.

[151] Middleton K, Al-Dujaili S, Mei X, Gunther A, You L (2017) Microfluidic co-culture platform for investigating osteocyte-osteoclast signalling during fluid shear stress mechanostimulation. J Biomech 59: 35-42. doi:10.1016/j.jbiomech.2017.05.012.

[152] Shu R, Bai D, Sheu T, He Y, Yang X, Xue C, He Y, Zhao M, Han X (2017) Sclerostin Promotes Bone Remodeling in the Process of Tooth Movement. PLoS One 12: e0167312. doi:10.1371/journal.pone.0167312.

[153] Sakamoto M, Fukunaga T, Sasaki K, Seiryu M, Yoshizawa M, Takeshita N, Takano-Yamamoto T (2019) Vibration enhances osteoclastogenesis by inducing RANKL expression via NF-κB signaling in osteocytes. Bone 123: 56-66. doi:10.1016/j.bone.2019.03.024.

[154] Li B, Lu D, Chen Y, Zhao M, Zuo L (2016) Unfractionated Heparin Promotes Osteoclast Formation in Vitro by Inhibiting Osteoprotegerin Activity. Int J Mol Sci 17. doi:10.3390/ijms17040613.

[155] You L, Temiyasathit S, Lee P, Kim CH, Tummala P, Yao W, Kingery W, Malone AM, Kwon RY, Jacobs CR (2008) Osteocytes as mechanosensors in the inhibition of bone resorption due to mechanical loading. Bone 42: 172-179. doi:10.1016/j.bone.2007.09.047.

[156] Warabi S, Tachibana Y, Kumegawa M, Hakeda Y (2001) Dexamethasone inhibits bone resorption by indirectly inducing apoptosis of the bone-resorbing osteoclasts via the action of osteoblastic cells. Cytotechnology 35: 25-34. doi:10.1023/A:1008159332152.

[157] Kim YH, Jun JH, Woo KM, Ryoo HM, Kim GS, Baek JH (2006) Dexamethasone inhibits the formation of multinucleated osteoclasts via down-regulation of beta3 integrin expression. Arch Pharm Res 29: 691-698. doi:10.1007/bf02968254.

[158] Andersson G, Johansson EK (1996) Adhesion of human myelomonocytic (HL-60) cells induced by 1,25-dihydroxyvitamin D3 and phorbol myristate acetate is dependent on osteopontin synthesis and the alpha v beta 3 integrin. Connect Tissue Res 35: 163-171. doi:10.3109/03008209609029188.

[159] Bocker W, Yin Z, Drosse I, Haasters F, Rossmann O, Wierer M, Popov C, Locher M, Mutschler W, Docheva D, et al. (2008) Introducing a single-cell-derived human mesenchymal stem cell line expressing hTERT after lentiviral gene transfer. J Cell Mol Med 12: 1347-1359. doi:10.1111/j.1582-4934.2008.00299.x.

[160] Mulholland BS, Forwood MR, Morrison NA (2019) Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) Drives Activation of Bone Remodelling and Skeletal Metastasis. Curr Osteoporos Rep 17: 538-547. doi:10.1007/s11914-019-00545-7.

[161] Ono T, Nakashima T (2018) Recent advances in osteoclast biology. Histochem Cell Biol 149: 325-341. doi:10.1007/s00418-018-1636-2.

[162] Zhu S, Ehnert S, Rouß M, Häussling V, Aspera-Werz RH, Chen T, Nussler AK (2018) From the Clinical Problem to the Basic Research-Co-Culture Models of Osteoblasts and Osteoclasts. Int J Mol Sci 19. doi:10.3390/ijms19082284.

[163] Dolci LS, Panzavolta S, Torricelli P, Albertini B, Sicuro L, Fini M, Bigi A, Passerini N (2019) Modulation of Alendronate release from a calcium phosphate bone cement: An in vitro osteoblast-osteoclast co-culture study. International journal of pharmaceutics 554: 245-255. doi:10.1016/j.ijpharm.2018.11.023.

[164] Knowles HJ, Athanasou NA (2008) Hypoxia-inducible factor is expressed in giant cell tumour of bone and mediates paracrine effects of hypoxia on monocyte-osteoclast differentiation via induction of VEGF. J Pathol 215: 56-66. doi:10.1002/path.2319.

[165] Schroder HC, Wang XH, Wiens M, Diehl-Seifert B, Kropf K, Schlossmacher U, Muller WE (2012) Silicate modulates the cross-talk between osteoblasts (SaOS-2) and osteoclasts (RAW 264.7 cells): inhibition of osteoclast growth and differentiation. J Cell Biochem 113: 3197-3206. doi:10.1002/jcb.24196.

[166] Clark JC, Akiyama T, Thomas DM, Labrinidis A, Evdokiou A, Galloway SJ, Kim HS, Dass CR, Choong PF (2011) RECK in osteosarcoma: a novel role in tumour vasculature and inhibition of tumorigenesis in an orthotopic model. Cancer 117: 3517-3528. doi:10.1002/cncr.25757.

[167] Yamakawa Y, Tazawa H, Hasei J, Osaki S, Omori T, Sugiu K, Komatsubara T, Uotani K, Fujiwara T, Yoshida A, et al. (2017) Role of zoledronic acid in oncolytic virotherapy: Promotion of antitumor effect and prevention of bone destruction. Cancer Sci 108: 1870-1880. doi:10.1111/cas.13316.

[168] Matsuzaki K, Katayama K, Takahashi Y, Nakamura I, Udagawa N, Tsurukai T, Nishinakamura R, Toyama Y, Yabe Y, Hori M, et al. (1999) Human osteoclast-like cells are formed from peripheral blood mononuclear cells in a coculture with SaOS-2 cells transfected with the parathyroid hormone (PTH)/PTH-related protein receptor gene. Endocrinology 140: 925-932. doi:10.1210/endo.140.2.6573.

[169] Zauli G, Rimondi E, Corallini F, Fadda R, Capitani S, Secchiero P (2007) MDM2 antagonist Nutlin-3 suppresses the proliferation and differentiation of human pre-osteoclasts through a p53-dependent pathway. J Bone Miner Res 22: 1621-1630. doi:10.1359/jbmr.070618.

[170] Tokukoda Y, Takata S, Kaji H, Kitazawa R, Sugimoto T, Chihara K (2001) Interleukin-1beta stimulates transendothelial mobilization of human peripheral blood mononuclear cells with a potential to differentiate into osteoclasts in the presence of osteoblasts. Endocr J 48: 443-452. doi:10.1507/endocrj.48.443.

[171] Orlandini SZ, Formigli L, Benvenuti S, Lasagni L, Franchi A, Masi L, Bernabei PA, Santini V, Brandi ML (1995) Functional and structural interactions between osteoblastic and preosteoclastic cells in vitro. Cell and tissue research 281: 33-42. doi:10.1007/bf00307956.

[172] Masi L, Malentacchi C, Benvenuti S, Amedei A, Bigozzi U, Montali E, Brandi ML (2000) In vitro expression of proalpha1(I) collagen mRNA by human pre-osteoclastic cells. J Endocrinol Invest 23: 1-4. doi:10.1007/bf03343667

[173] Kostenuik PJ, Shalhoub V (2001) Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. Curr Pharm Des 7: 613-635. doi:10.2174/1381612013397807.

[174] Kang IS, Kim C (2019) Taurine Chloramine Inhibits Osteoclastic Differentiation and Osteoclast Marker Expression in RAW 264.7 Cells. Adv Exp Med Biol 1155: 61-70. doi:10.1007/978-981-13-8023-5_6.

[175] Yang J, Bi X, Li M (2019) Osteoclast Differentiation Assay. Methods Mol Biol 1882: 143-148. doi:10.1007/978-1-4939-8879-2_12.

[176] Xu H, Liu T, Li J, Xu J, Chen F, Hu L, Zhang B, Zi C, Wang X, Sheng J (2019) Oxidation derivative of (-)-epigallocatechin-3-gallate (EGCG) inhibits RANKL-induced osteoclastogenesis by suppressing RANK signaling pathways in RAW 264.7 cells. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 118: 109237. doi:10.1016/j.biopha.2019.109237.

[177] Song R, Liu X, Zhu J, Gao Q, Wang Q, Zhang J, Wang D, Cheng L, Hu D, Yuan Y, et al. (2015) RhoV mediates apoptosis of RAW264.7 macrophages caused by osteoclast differentiation. Mol Med Rep 11: 1153-1159. doi:10.3892/mmr.2014.2817

[178] Kong L, Smith W, Hao D (2019) Overview of RAW264.7 for osteoclastogensis study: Phenotype and stimuli. J Cell Mol Med 23: 3077-3087. doi:10.1111/jcmm.14277.

[179] Song C, Yang X, Lei Y, Zhang Z, Smith W, Yan J, Kong L (2019) Evaluation of efficacy on RANKL induced osteoclast from RAW264.7 cells. Journal of cellular physiology 234: 11969-11975. doi:10.1002/jcp.27852.

[180] Kim B, Lee KY, Park B (2018) Icariin abrogates osteoclast formation through the regulation of the RANKL-mediated TRAF6/NF-kappaB/ERK signaling pathway in Raw264.7 cells. Phytomedicine 51: 181-190. doi:10.1016/j.phymed.2018.06.020.

[181] Sumi K, Abe T, Kunimatsu R, Oki N, Tsuka Y, Awada T, Nakajima K, Ando K, Tanimoto K (2018) The effect of mesenchymal stem cells on chemotaxis of osteoclast precursor cells. J Oral Sci 60: 221-225. doi:10.2334/josnusd.17-0187.

[182] Abe T, Sumi K, Kunimatsu R, Oki N, Tsuka Y, Nakajima K, Ando K, Tanimoto K (2019) The effect of mesenchymal stem cells on osteoclast precursor cell differentiation. J Oral Sci 61: 30-35. doi:10.2334/josnusd.17-0315.

[183] Murillo A, Guerrero CA, Acosta O, Cardozo CA (2010) Bone resorptive activity of osteoclast-like cells generated in vitro by PEG-induced macrophage fusion. Biol Res 43: 205-224. doi:/S0716-97602010000200009.

[184] Coxon FP, Thompson K, Roelofs AJ, Ebetino FH, Rogers MJ (2008) Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. Bone 42: 848-860. doi:10.1016/j.bone.2007.12.225.

[185] Luckman SP, Coxon FP, Ebetino FH, Russell RG, Rogers MJ (1998) Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure-activity relationships in J774 macrophages. J Bone Miner Res 13: 1668-1678. doi:10.1359/jbmr.1998.13.11.1668.

[186] Hughes A, Rogers MJ, Idris AI, Crockett JC (2007) A comparison between the effects of hydrophobic and hydrophilic statins on osteoclast function in vitro and ovariectomy-induced bone loss in vivo. Calcified tissue international 81: 403-413. doi:10.1007/s00223-007-9078-1.

[187] Cicco SR, Vona D, Leone G, De Giglio E, Bonifacio MA, Cometa S, Fiore S, Palumbo F, Ragni R, Farinola GM (2019) In vivo functionalization of diatom biosilica with sodium alendronate as osteoactive material. Mater Sci Eng C Mater Biol Appl 104: 109897. doi:10.1016/j.msec.2019.109897.

[188] Li ZH, Si Y, Xu G, Chen XM, Xiong H, Lai L, Zheng YQ, Zhang ZG (2017) High-dose PMA with RANKL and MCSF induces THP1 cell differentiation into human functional osteoclasts in vitro. Mol Med Rep 16: 8380-8384. doi:10.3892/mmr.2017.7625.

[189] Teixeira LN, de Castro Raucci LM, Alonso GC, Coletta RD, Rosa AL, de Oliveira PT (2016) Osteopontin expression in co-cultures of human squamous cell carcinoma-derived cells and osteoblastic cells and its effects on the neoplastic cell phenotype and osteoclastic activation. Tumour Biol 37: 12371-12385. doi:10.1007/s13277-016-5104-0.

[190] Yoneda T, Alsina MM, Garcia JL, Mundy GR (1991) Differentiation of HL-60 cells into cells with the osteoclast phenotype. Endocrinology 129: 683-689. doi:10.1210/endo-129-2-683.

[191] Hillstrom Shapiro L, Venta PJ, Yu YS, Tashian RE (1989) Carbonic anhydrase II is induced in HL-60 cells by 1,25-dihydroxyvitamin D3: a model for osteoclast gene regulation. FEBS Lett 249: 307-310. doi:10.1016/0014-5793(89)80647-7.

[192] Kaji Y, Ikeda K, Ikeda T, Kawakami K, Sasaki K, Shindo M, Hatake K, Harada M, Motoyoshi K, Mori S, et al. (2000) IL-4, but not vitamin D(3), induces monoblastic cell line UG3 to differentiate into multinucleated giant cells on osteoclast lineage. Journal of cellular physiology 182: 214-221. doi:10.1002/(SICI)1097-4652(200002)182:2<214::AID-JCP10>3.0.CO;2-F.

[193] Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH (2010) The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One 5: e8668. doi:10.1371/journal.pone.0008668.

[194] Padilla PI, Wada A, Yahiro K, Kimura M, Niidome T, Aoyagi H, Kumatori A, Anami M, Hayashi T, Fujisawa J, et al. (2000) Morphologic differentiation of HL-60 cells is associated with appearance of RPTPbeta and induction of Helicobacter pylori VacA sensitivity. J Biol Chem 275: 15200-15206. doi:10.1074/jbc.275.20.15200.

[195] Hassanpour S, Jiang H, Wang Y, Kuiper JW, Glogauer M (2014) The actin binding protein adseverin regulates osteoclastogenesis. PLoS One 9: e109078. doi:10.1371/journal.pone.0109078.

[196] Miyamoto T (2011) Regulators of osteoclast differentiation and cell-cell fusion. Keio J Med 60: 101-105. doi:10.2302/kjm.60.101.

[197] Vallet S, Raje N, Ishitsuka K, Hideshima T, Podar K, Chhetri S, Pozzi S, Breitkreutz I, Kiziltepe T, Yasui H, et al. (2007) MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. Blood 110: 3744-3752. doi:10.1182/blood-2007-05-093294.

[198] Yu X, Huang Y, Collin-Osdoby P, Osdoby P (2004) CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. J Bone Miner Res 19: 2065-2077. doi:10.1359/JBMR.040910.

[199] Michael H, Harkonen PL, Kangas L, Vaananen HK, Hentunen TA (2007) Differential effects of selective oestrogen receptor modulators (SERMs) tamoxifen, ospemifene and raloxifene on human osteoclasts in vitro. Br J Pharmacol 151: 384-395. doi:10.1038/sj.bjp.0707232.

[200] Michael H, Harkonen PL, Vaananen HK, Hentunen TA (2005) Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption. J Bone Miner Res 20: 2224-2232. doi:10.1359/JBMR.050803.

[201] Keremu A, Aini A, Maimaitirexiati Y, Liang Z, Aila P, Xierela P, Tusun A, Moming H, Yusufu A (2019) Overcoming cisplatin resistance in osteosarcoma through the miR-199a-modulated inhibition of HIF-1alpha. Biosci Rep 39. doi:10.1042/BSR20170080.

[202] El Naggar A, Clarkson P, Zhang F, Mathers J, Tognon C, Sorensen PH (2012) Expression and stability of hypoxia inducible factor 1alpha in osteosarcoma. Pediatr Blood Cancer 59: 1215-1222. doi:10.1002/pbc.24191.

[203] Taylor RM, Kashima TG, Knowles HJ, Athanasou NA (2012) VEGF, FLT3 ligand, PlGF and HGF can substitute for M-CSF to induce human osteoclast formation: implications for giant cell tumour pathobiology. Lab Invest 92: 1398-1406. doi:10.1038/labinvest.2012.108.

[204] Richter E, Ventz K, Harms M, Mostertz J, Hochgrafe F (2016) Induction of Macrophage Function in Human THP-1 Cells Is Associated with Rewiring of MAPK Signaling and Activation of MAP3K7 (TAK1) Protein Kinase. Front Cell Dev Biol 4: 21. doi:10.3389/fcell.2016.00021.

[205] Ehnert S, Heuberger E, Linnemann C, Nussler AK, Pscherer S (2017) TGF-β1-Dependent Downregulation of HDAC9 Inhibits Maturation of Human Osteoblasts. J Funct Morphol Kinesiol 2: 41.

[206] Aspera-Werz RH, Chen T, Ehnert S, Zhu S, Frohlich T, Nussler AK (2019) Cigarette Smoke Induces the Risk of Metabolic Bone Diseases: Transforming Growth Factor Beta Signaling Impairment via Dysfunctional Primary Cilia Affects Migration, Proliferation, and Differentiation of Human Mesenchymal Stem Cells. Int J Mol Sci 20. doi:10.3390/ijms20122915.

[207] Maria SM, Prukner C, Sheikh Z, Mueller F, Barralet JE, Komarova SV (2014) Reproducible quantification of osteoclastic activity: characterization of a biomimetic calcium phosphate assay. J Biomed Mater Res B Appl Biomater 102: 903-912. doi:10.1002/jbm.b.33071.

[208] Hayden RS, Vollrath M, Kaplan DL (2014) Effects of clodronate and alendronate on osteoclast and osteoblast co-cultures on silk-hydroxyapatite films. Acta Biomater 10: 486-493. doi:10.1016/j.actbio.2013.09.028.

[209] Hayden RS, Quinn KP, Alonzo CA, Georgakoudi I, Kaplan DL (2014) Quantitative characterization of mineralized silk film remodeling during long-term osteoblast-osteoclast co-culture. Biomaterials 35: 3794-3802. doi:10.1016/j.biomaterials.2014.01.034.

[210] Hayden RS, Fortin JP, Harwood B, Subramanian B, Quinn KP, Georgakoudi I, Kopin AS, Kaplan DL (2014) Cell-tethered ligands modulate bone remodeling by osteoblasts and osteoclasts. Advanced functional materials 24: 472-479. doi:10.1002/adfm.201302210.

[211] Griffith LG, Swartz MA (2006) Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol 7: 211-224. doi:10.1038/nrm1858.

[212] Ding D, Xu H, Liang Q, Xu L, Zhao Y, Wang Y (2012) Over-expression of Sox2 in C3H10T1/2 cells inhibits osteoblast differentiation through Wnt and MAPK signalling pathways. Int Orthop 36: 1087-1094. doi:10.1007/s00264-011-1368-6.

[213] Marcellini S, Henriquez JP, Bertin A (2012) Control of osteogenesis by the canonical Wnt and BMP pathways in vivo: cooperation and antagonism between the canonical Wnt and BMP pathways as cells differentiate from osteochondroprogenitors to osteoblasts and osteocytes. Bioessays 34: 953-962. doi:10.1002/bies.201200061.

[214] Park SB, Seo KW, So AY, Seo MS, Yu KR, Kang SK, Kang KS (2012) SOX2 has a crucial role in the lineage determination and proliferation of mesenchymal stem cells through Dickkopf-1 and c-MYC. Cell death and differentiation 19: 534-545. doi:10.1038/cdd.2011.137.

[215] Seo, E., Basu-Roy, U., Gunaratne, P. H., Coarfa, C., Lim, D.-S., Basilico, C., & Mansukhani, A. (2013). SOX2 Regulates YAP1 to Maintain Stemness and Determine Cell Fate in the Osteo-Adipo Lineage. Cell Reports, 3(6), 2075–2087. doi:10.1016/j.celrep.2013.05.029.

[216] Zhao W, Li X, Liu X, Zhang N, Wen X (2014) Effects of substrate stiffness on adipogenic and osteogenic differentiation of human mesenchymal stem cells. Mater Sci Eng C Mater Biol Appl 40: 316-323. doi:10.1016/j.msec.2014.03.048.

[217] Sun M, Chi G, Xu J, Tan Y, Xu J, Lv S, Xu Z, Xia Y, Li L, Li Y (2018) Extracellular matrix stiffness controls osteogenic differentiation of mesenchymal stem cells mediated by integrin alpha5. Stem Cell Res Ther 9: 52. doi:10.1186/s13287-018-0798-0.

[218] Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. Cell 126: 677-689. doi:10.1016/j.cell.2006.06.044.

[219] Dawson JI, Oreffo RO (2008) Bridging the regeneration gap: stem cells, biomaterials and clinical translation in bone tissue engineering. Arch Biochem Biophys 473: 124-131. doi:10.1016/j.abb.2008.03.024.

[220] Frost HM (1994) Wolff's Law and bone's structural adaptations to mechanical usage: an overview for clinicians. Angle Orthod 64: 175-188. doi:10.1043/0003-3219(1994)064<0175:WLABSA>2.0.CO;2.

[221] Ponzetti M, Rucci N (2019) Updates on Osteoimmunology: What's New on the Cross-Talk Between Bone and Immune System. Front Endocrinol (Lausanne) 10: 236. doi:10.3389/fendo.2019.00236.

[222] Ehnert, S., Linnemann, C., Aspera-Werz, R., Bykova, D., Biermann, S., Fecht, L., … Stuby, F. (2018). Immune Cell Induced Migration of Osteoprogenitor Cells Is Mediated by TGF-β Dependent Upregulation of NOX4 and Activation of Focal Adhesion Kinase. International Journal of Molecular Sciences, 19(8), 2239. doi:10.3390/ijms19082239.

[223] Ehnert S, van Griensven M, Unger M, Scheffler H, Falldorf K, Fentz AK, Seeliger C, Schroter S, Nussler AK, Balmayor ER (2018) Co-Culture with Human Osteoblasts and Exposure to Extremely Low Frequency Pulsed Electromagnetic Fields Improve Osteogenic Differentiation of Human Adipose-Derived Mesenchymal Stem Cells. Int J Mol Sci 19. doi:10.3390/ijms19040994.

[224] Pepe J, Cipriani C, Cilli M, Colangelo L, Minisola S (2016) Adipokines and bone metabolism: an interplay to untangle. J Endocrinol Invest 39: 1359-1361. doi:10.1007/s40618-016-0549-y.

[225] Palermo A, Tuccinardi D, Defeudis G, Watanabe M, D'Onofrio L, Lauria Pantano A, Napoli N, Pozzilli P, Manfrini S (2016) BMI and BMD: The Potential Interplay between Obesity and Bone Fragility. Int J Environ Res Public Health 13. doi:10.3390/ijerph13060544.

[226] Grassel SG (2014). The role of peripheral nerve fibers and their neurotransmitters in cartilage and bone physiology and pathophysiology. Arthritis Res Ther 16: 485. doi:10.1186/s13075-014-0485-1.

[227] Garcia-Castellano JM, Diaz-Herrera P, Morcuende JA (2000) Is bone a target-tissue for the nervous system? New advances on the understanding of their interactions. Iowa Orthop J 20: 49-58.

[228] Kirkpatrick CJ, Fuchs S, Unger RE (2011) Co-culture systems for vascularization--learning from nature. Adv Drug Deliv Rev 63: 291-299. doi:10.1016/j.addr.2011.01.009.

[229] James Kirkpatrick C, Fuchs S, Iris Hermanns M, Peters K, Unger RE (2007) Cell culture models of higher complexity in tissue engineering and regenerative medicine. Biomaterials 28: 5193-5198. doi:10.1016/j.biomaterials.2007.08.012.

[230] Ehnert S, Fentz AK, Schreiner A, Birk J, Wilbrand B, Ziegler P, Reumann MK, Wang H, Falldorf K, Nussler AK (2017) Extremely low frequency pulsed electromagnetic fields cause antioxidative defense mechanisms in human osteoblasts via induction of *O2(-) and H2O2. Sci Rep 7: 14544. doi:10.1038/s41598-017-14983-9.

[231] Ehnert S, Sreekumar V, Aspera-Werz RH, Sajadian SO, Wintermeyer E, Sandmann GH, Bahrs C, Hengstler JG, Godoy P, Nussler AK (2017) TGF-beta1 impairs mechanosensation of human osteoblasts via HDAC6-mediated shortening and distortion of primary cilia. J Mol Med (Berl) 95: 653-663. doi:10.1007/s00109-017-1526-4.

[232] Ehnert S, Falldorf K, Fentz AK, Ziegler P, Schroter S, Freude T, Ochs BG, Stacke C, Ronniger M, Sachtleben J, et al. (2015) Primary human osteoblasts with reduced alkaline phosphatase and matrix mineralization baseline capacity are responsive to extremely low frequency pulsed electromagnetic field exposure - Clinical implication possible. Bone Rep 3: 48-56. doi:10.1016/j.bonr.2015.08.002.

[233] Reumann MK, Linnemann C, Aspera-Werz RH, Arnold S, Held M, Seeliger C, Nussler AK, Ehnert S (2018) Donor Site Location Is Critical for Proliferation, Stem Cell Capacity, and Osteogenic Differentiation of Adipose Mesenchymal Stem/Stromal Cells: Implications for Bone Tissue Engineering. Int J Mol Sci 19. doi:10.3390/ijms19071868.