In vitro models of bone-forming tumours: from target to treatment

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Chapter 1

General introduction

Bone-forming tumours of the skeleton: clinical presentation, histology and molecular pathology

Bone-forming tumours of the skeleton comprise a group of tumours of mesenchymal origin. These tumours are characterized by bone deposition and include osteoma, osteoid osteoma, osteoblastoma and osteosarcoma. The current knowledge of the clinical presentation, histology and molecular pathology of these tumours is reviewed in detail in **chapter 2**. In general, the group of bone tumours can be divided into two groups based on the molecular pathology (1). There are tumours with a simple or a complex karyotype. Simple karyotype tumours are driven by specific gene mutations or translocations. In contrast, complex karyotype tumours typically do not harbor specific genetic alterations, but instead show many chromosomal alterations, copy number alterations and aneuploidy. Based on these criteria, osteoma, osteoid osteoma and osteoblastoma are tumours with a simple karyotype, whereas osteosarcoma is an example of a tumour with a complex karyotype.

Osteoid osteoma and osteoblastoma are bone forming tumours with an indistinguishable histology (2, 3). In both entities, trabeculae with woven bone are present, which are lined by active osteoblasts (4, 5). Both tumours are not malignant, but osteoblastomas can be locally aggressive. The distinction between osteoid osteoma and osteoblastoma is mostly based on size, where osteoid osteoma is smaller than 2 cm and osteoblastoma is larger (2). Osteoid osteoma and osteoblastoma are tumours with a simple karyotype, with frequent translocations in *FOS* (87%) and to a lesser extent *FOSB* (2%) (6). The translocation of *FOS* leads to a truncation of the FOS protein. This rearrangement has previously been discovered in epithelioid hemangioma (7, 8), and more recently also in cementoblastoma (9). The identification of *FOS* rearrangements in osteoid osteoma and osteoblastoma has led to the discovery of a novel diagnostic tool, where FOS immunohistochemical expression can be detected and osteoid osteoma and osteoblastoma can be more easily distinguished from other bone-forming lesions (10, 11). FOS and FOS B can form a heterodimer with JUN proteins to form the AP-1 transcription factor, regulating proliferation and differentiation (12, 13). The exact role of FOS or FOS B in the pathogenesis of osteoid osteoma or osteoblastoma is not completely understood.

Osteosarcoma is a malignant tumour of the bone, and typically diagnosed in children and adolescents (2). Histologically, it is characterized by osteoid production. Different histological subtypes are recognized, including the most common high-grade conventional osteosarcoma and low-grade osteosarcoma such as parosteal osteosarcoma (2). Tumours usually arise in the long bones. Osteosarcoma is a tumour with a complex karyotype: it is characterized by massive chromosomal abnormalities, copy number alterations and aneuploidy. Chromoanagenesis, including chromothripsis where chromosomes shatter into fragments and are randomly stitched together, is relatively frequent compared to other cancer types (14, 15). Given the molecular complexity of osteosarcoma, recurrent alterations are not often

identified. Among the most commonly altered genes are *TP53* and *RB1*, and genes involved in cell cycle regulation and genome stability, such as *MDM2*, *CDK4*, and *ATRX* (15-19).

Therapeutic strategies for bone-forming tumours: current strategies and advancements

Osteoid osteoma and osteoblastoma

Patients with osteoid osteoma typically present with nocturnal pain, which can be relieved by salicylates or NSAIDs (20). Some tumours spontaneously regress within 6 years (21). Removal of the tumour is necessary when symptoms of pain persist. Where removal of the tumour in the past has mostly involved open excision, in recent years techniques have become less invasive, for example using image-guided techniques such as radiofrequency ablation (22). For osteoblastoma, patients also present with pain, although NSAIDS usually do not relief pain (23). Instead, osteoblastoma requires surgical removal (24).

Osteosarcoma

The current standard therapeutic strategy for high-grade conventional osteosarcoma is a combination of surgery and (neo)adjuvant chemotherapy. Currently used chemotherapeutic agents include doxorubicin, cisplatin, methotrexate and ifosfamide (25). Although the introduction of chemotherapy in the 1970s has greatly improved the outcome for osteosarcoma patients, the last five decades have not shown improvements in overall survival (26, 27). Since high-grade conventional osteosarcoma is mainly diagnosed in children and young adolescents, with an incidence of almost 15 per 100,000 in the United States, the burden of highly toxic chemotherapy for this vulnerable patient group is high (28). This emphasizes the need for novel therapeutic strategies, in particular for patients with metastasis or resistance to chemotherapy. In recent years an exponential increase in publications of osteosarcoma could be observed, in particular *in vitro* studies (29). A large part of these studies explore traditional Chinese medicine as novel therapeutic options, but also drugs targeting recurrent genetic alterations have been described. An overview of currently ongoing clinical trials where novel therapeutic targets are being tested in osteosarcoma is shown in **Table 1**.

Table 1. Overview of currently ongoing registered clinical trials for novel therapeutic options in osteosarcoma patients.

Category	Target	Drug	Clinical Trial Number	Clinical Trial Phase
Therapies targeting cell cycle / genome maintenance	CDK4/CDK6	Palbociclib	NCT03526250 NCT03709680	2 1
		Abemaciclib	NCT02389244 NCT02644460	2 1
	PARP	Talazoparib	NCT04901702	2
		Olaparib	NCT03233204	1
Therapies targeting receptor tyrosine kinases	RTK	Lenvatinib	NCT04154189 NCT02432274 NCT03742193 NCT04690231 NCT04824352	1 1 1 1 1
		Sunitinib	NCT03900793 NCT03277924	1 1
		Regorafenib	NCT04698785 NCT04055220 NCT04803877 NCT02389244 NCT02048371	1 2 2 2 2
		Apatinib	NCT03742193 NCT04690231 NCT04824352	2 2 2
		Cabozantinib	NCT05019703 NCT04661852 NCT02867592	2 2 2
Therapies targeting the mTOR pathway	PI3K (mTOR)	Samotolisib	NCT03213678	2
Immunotherapy	PDL-1/PD-1	ZKAB001	NCT03676985 NCT04359550	2 3
		Avelumab	NCT03006848	2
		Camreluzimab	NCT04294511	2
		Nivolumab	NCT03628209 NCT02500797	1 2
		Durvalumab	NCT04668300	2
	GD2	Dinutuximab	NCT02484443	2
	HER2	Trastuzumab	NCT04616560	2

Therapies targeting cell cycle and genome maintenance

The most common alterations include *TP53* and *RB1*. TP53 and RB1 are key players in genome maintenance and controlling cell cycle. Drugs that target recurrent alterations should be based on a distinction between cancerous and normal cells. Although mutations that lead to overexpression of *TP53* can be used to distinguish between cancerous and normal cells, the nuclear localization and lack of enzymatic activity of p53 makes it a rather difficult target for drugs (30). However, drugs that can reactivate the wild-type state of p53 mutant forms by induction of conformational changes, such as APR-246, seem promising and have progressed to clinical trials (31-33), although not in osteosarcoma.

RB1, when dephosphorylated and activated, blocks cell cycle progression by blocking cells from entering S-phase of the cell cycle (34). Proteins within the Rb-pathway are often affected in osteosarcoma patients, which include alterations in *CDK4*, *CDK6*, *CDKN2A* and *CDKN2B*. Drugs that prevent the phosphorylation, and thus inactivation of Rb, are currently strategies for targeting the Rb-pathway. CDK4 inhibitors block Rb phosphorylation and thereby cell proliferation. Currently clinical trials are ongoing to test CDK4 inhibitors in sarcomas, including osteosarcoma, with altered CDK4 expression (35, 36).

Recently, whole exome sequencing revealed that osteosarcoma shows a mutational profile that is reminiscent of tumours that are deficient in *BRCA*1/2, genes that are involved in the DNA homologous repair pathway (19). Osteosarcoma shows features of BRCAness, which is a mutational profile consisting of loss-of-heterozygosity, a specific combination of single nucleotide alterations and genomic instability. BRCAness could suggest sensitivity to PARP inhibitors such as talazoparib (19). Talazoparib has shown success *in vitro* where it reduced osteosarcoma cell viability, although this effect was less compared to *BRCA* negative breast cancer cell lines for which PARP inhibitor treatment is already the standard therapeutic option (37, 38). Clinical trials are currently ongoing to test PARP inhibitors olaparib and talazoparib in osteosarcoma (39, 40) (NCT04901702; NCT03233204).

Therapies targeting receptor tyrosine kinases (RTKs)

Tyrosine kinase inhibitors (TKIs) are a class of drugs that target molecules essential for cell signaling pathways. Tyrosine kinases that have been reported to be affected in osteosarcoma, include EGFR, VEGFR, PDGFR and IGF1R, (41). In particular, the genomic region 4q12 that contains tyrosine kinases *KIT*, *KDR* and *PDGFRA* showed amplification in 20% of osteosarcoma patients, indicating that broad spectrum TKIs could be a promising candidate drug for osteosarcoma patients (42). Previous clinical trials in which TKIs were administered have already shown promise in osteosarcoma patients, and currently 13 more registered clinical trials are ongoing (43). Insulin growth factor receptor 1 (IGF1R) is also considered a receptor tyrosine kinase. The IGF signaling pathway, that plays a role in bone homeostasis, was shown to be overexpressed in osteosarcoma (15, 44). Although a clinical trial that included osteosarcoma patients did not show significant benefit of treatment with IGF 1R inhibitor

Robatumumab (45), selecting patients based on IGF1R expression levels was hypothesized to increase the response (46).

Therapies targeting the mTOR pathway

The mTOR pathway plays a key role in energy metabolism, which is highly active in cancer cells (47), often through PI3K and Akt signaling. Activation of the mTOR pathway leads to increased cell proliferation and cell cycle progression, and a decrease in autophagy, thereby promoting tumour growth. In osteosarcoma, recurrent alterations have been identified in the mTOR/PI3K/Akt pathway (18). It was discovered that inhibiting mTOR, in combination with 3-phosphoglycerate dehydrogenase (PHGDH) inhibition, attenuated cell proliferation in osteosarcoma cells and could serve as a novel therapeutic target (48). Rapamycin, an inhibitor of the mTOR signaling pathway, was found to inhibit osteosarcoma cell proliferation in an *in vitro* model, and decreased tumour growth in a mouse model (49). Combination therapies with chemotherapeutics have also been investigated for osteosarcoma: in osteosarcoma cells, the combination of cisplatin together with rapamycin increased apoptosis and autophagy activity (50). mTOR inhibition has also been tested in a clinical trial for osteosarcoma patients, in which the mTOR inhibitor temsirolimus was administered together with IGF1R inhibitor cixutumumab, but this did not improve outcome (51).

Immunotherapy

In recent years immunotherapy has shown success in various cancer types. Immunotherapy is mainly aimed at stimulating the activity of the immune system or inhibiting the anti-immune activity of cancer cells. Many studies have been published in which immunotherapy was investigated in osteosarcoma and several attempts have been made to test immunotherapies in clinical trials in osteosarcoma, but until now with limited success (52, 53). For example, in the EURAMOS-1 clinical trial in which immunostimulatory IFN-α2b was tested, this did not lead to improvement in overall survival (54). However, a phase 3 clinical study in osteosarcoma was successful in which muramyl tripeptide (MTP), a drug that activates monocytes and macrophages, was tested in osteosarcoma patients. MTP in combination with conventional chemotherapy improved overall survival (53). One method to inhibit the anti-immune response of cancer cells is to inhibit the immune checkpoint response, in which the immune system is prevented to target cancerous cells. PD-1 and PD-L1 are immune checkpoint molecules, and are often the target of immune checkpoint inhibitors. PD-L1 is expressed in osteosarcomas, which suggests sensitivity to immune checkpoint inhibitors (55). Immune checkpoint inhibitors targeting PD-1 and PD-L1 include ZKAB001, avelumab, camreluzimab, nivolumab and durvalumab and are currently being tested in clinical trials (**Table 1**). Other checkpoints may also be active in osteosarcoma, e.g. TIM3 which is overexpressed in osteosarcoma tissue (56), and thereby potential targets for therapy.

Immunotherapy may also involve the use of antibodies targeting cell surface proteins that are overexpressed in cancer cells. The binding of antibodies to cell surface proteins can recruit immune cells leading to cytotoxicity of cancer cells. An example is GD2, which is overexpressed in osteosarcoma (57). Dinutuximab, a monoclonal antibody targeting GD2 that induces immune cell-mediated cytotoxicity, is currently being tested in a phase 2 clinical trial for osteosarcoma patients (NCT02484443). Another example is trastuzumab, a monoclonal antibody targeting HER2, which is currently also being tested in a phase 2 clinical trial (NCT04616560).

Chimeric Antigen Receptor (CAR) T-cell therapy is a type of immunotherapy in which the receptors of T-cells, derived from the patient or from a healthy donor, are genetically engineered to target cell surface proteins of cancer cells. CAR T-cell therapy has delivered promising results in leukemia, and could also be a novel therapeutic strategy for osteosarcoma patients (58).

In vitro models of bone-forming tumours

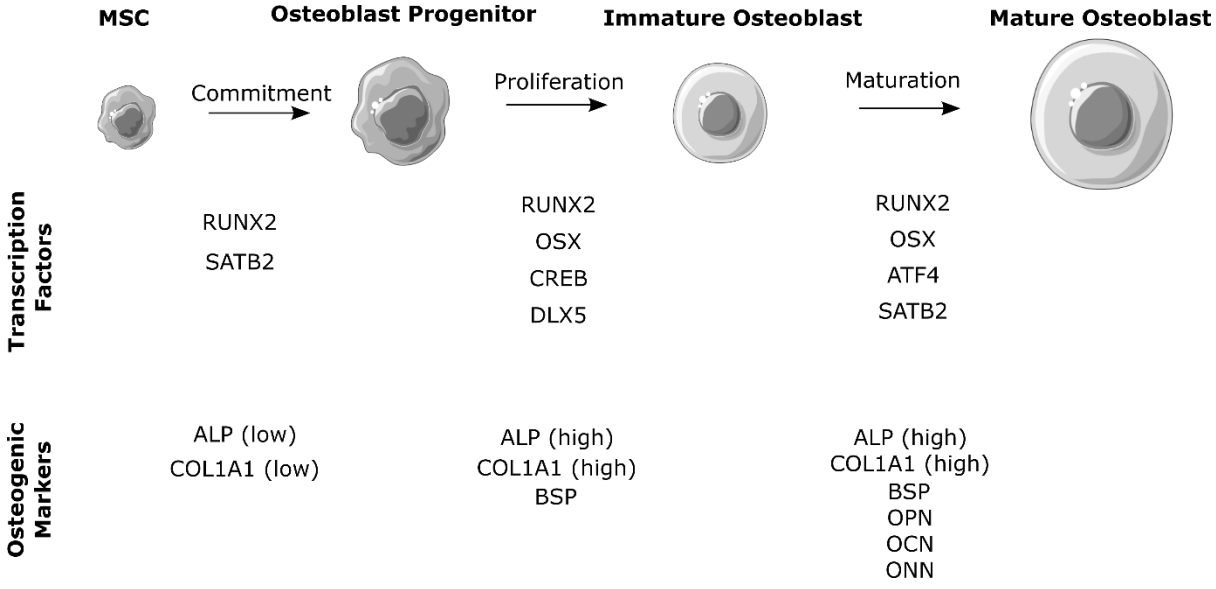
Good representative *in vitro* models are essential for pre-clinical testing of novel targeted treatments. There is a plethora of cell models available for osteosarcoma, including cell-of-origin based models, cell models derived from patient material, or from xenografts (59). Typically cell models involve the culture of cells, either directly derived from patient material or from animal tumor tissue, onto a plastic surface. The cells are cultured as a monolayer onto a two-dimensional surface and as such lack the three-dimensional environment the cells have originated from. Since the three-dimensional environment is lacking, it could be less representative for the *in vivo* situation. This could influence results obtained from drug testing in these cell models. In recent years an increase in studies was seen in which cells are instead cultured in a three-dimensional environment, where cells do have the interaction with other cells and the microenvironment (60).

Cell of origin

Osteoid osteoma, osteoblastoma and osteosarcoma are tumours of mesenchymal origin. For osteoid osteoma and osteoblastoma no cell models have been described. The presence of trabeculae of woven bone surrounding active osteoblasts, could indicate that it arises from osteoblast, or its progenitor the mesenchymal stem cell (61). Mesenchymal stem cells are undifferentiated cells but have the capacity to differentiate into osteoblasts, adipocytes, chondrocytes or myocytes. For osteosarcoma the proposed cell of origin is a topic of debate (62). There are studies that suggest osteoblasts are the cell of origin, or that osteosarcoma originates from mesenchymal stem cells.
The evidence from studies that suggest that osteoblasts are the cell of origin of osteosarcoma is mostly based on mouse models. Studies have shown that mice develop osteosarcoma when

Trp53 is deleted in osteoblast cells, throughout different stages of differentiation, by utilizing promotors that are specific to cells of the osteoblast lineage (63, 64).
Other studies have been published that mesenchymal stem cells rather than osteoblast are the cell-of-origin. Since mesenchymal stem cells are the progenitor cells of bone-forming osteoblasts, it is likely that bone-forming tumours arise during the differentiation process of mesenchymal stem cells. An overview of the differentiation of mesenchymal stem cells towards the osteogenic lineage is shown in **Figure 1**. Mesenchymal stem cells originating from mice are reported to spontaneously transform *in vitro*, which showed aneuploidy and loss of *Cdkn2a* similar to human osteosarcoma (65, 66). Furthermore, mice injected with transformed murine mesenchymal stem cells formed undifferentiated pleomorphic sarcoma or osteosarcoma (65, 67, 68). In human mesenchymal stem cells *in vitro* spontaneous transformation towards osteosarcoma has not been reported, but genetic manipulation of these cells by deletion of *TP53* or *RB1* has led to malignant transformation (69). In mouse studies, injection of mesenchymal stem cells carrying a deletion of *TP53* and RB1, or a combination of *MYC* overexpression and *CDKN2A* loss led to the formation of osteosarcoma (70, 71).

Figure 1. Mesenchymal stem cells differentiate towards osteoblast. During each stage of osteogenic differentiation different transcription factors and osteogenic markers play a role. Figure is adapted from reference (72). Templates adapted from Servier Medical Art, licensed under a Creative Commons attribution 3.0 Unported License.



Cell lines

Among the most often used cell models are cultured cell lines, which have originated from patient material. Osteosarcoma cell lines are widely used not only to study osteosarcoma but also for general cell biology research applications, as osteosarcoma cell lines are among the human cell lines that are easily transfected (59). The ease of culture and high growth speed of osteosarcoma cell lines could also explain the exponential rise in publications using osteosarcoma cell lines (29). Among the most commonly used osteosarcoma cell lines there is high heterogeneity in metastatic potential, differentiation capacity and *in vivo* tumorigenicity, which also reflects the heterogeneity observed in osteosarcoma patients (73).

Cell lines can either be derived from human osteosarcoma or from animals. One animal model in particular is of great interest for studying human osteosarcoma: dogs. The incidence of osteosarcoma in dogs is 10 times higher compared to humans, and most often occur in large dog breeds (74). Since osteosarcoma is a rare bone tumour in humans, this makes dogs an attractive alternative model to study pathogenesis and for pre-clinical drug testing. Previous studies have already shown that osteosarcoma cell lines and tumours derived from dogs show similar genetic alterations compared to human osteosarcoma, including alterations in *TP53* (75, 76).

3D models for mesenchymal tumours

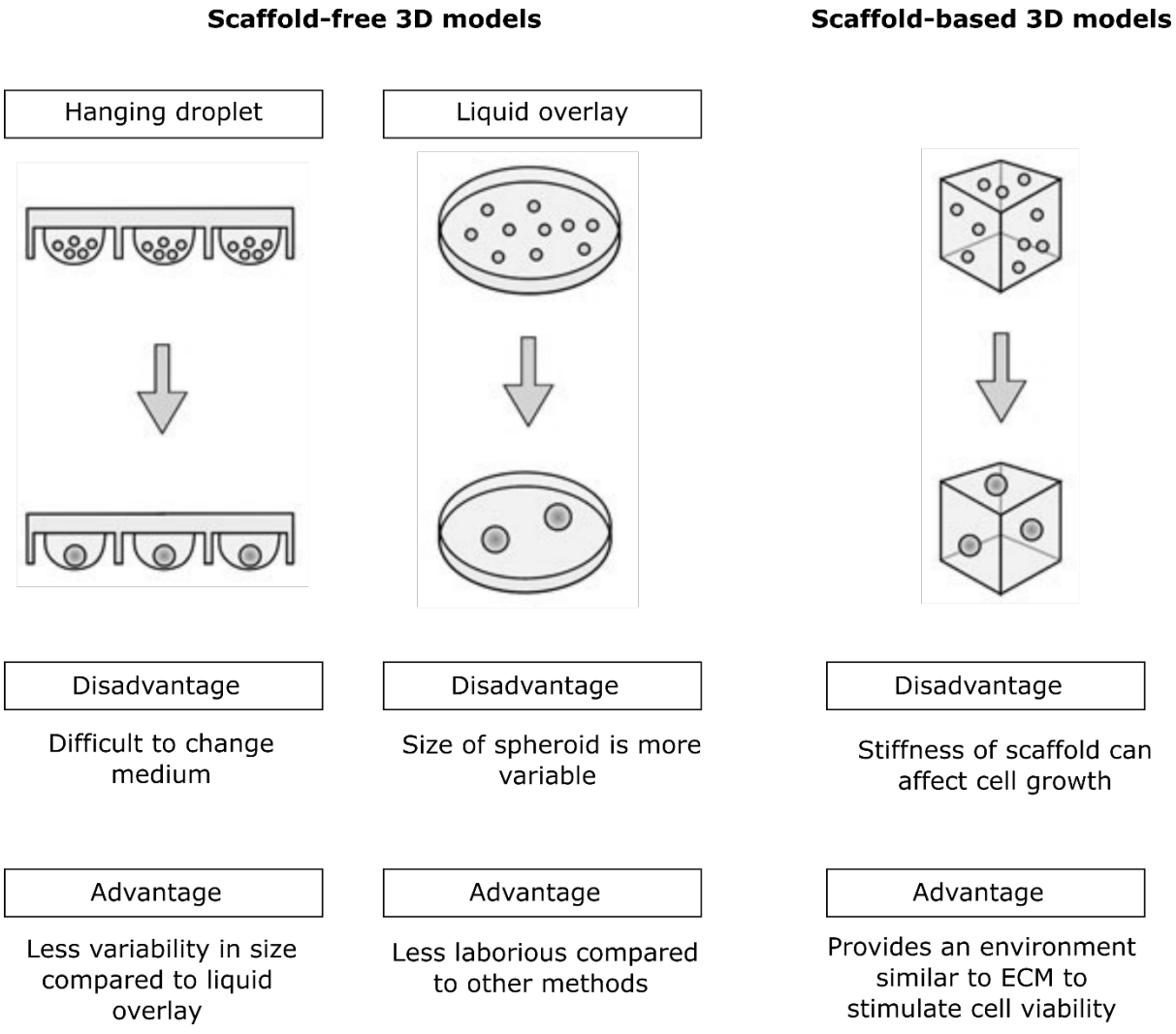
3D models are cell models in which cultured cells are not in direct contact with a plastic culture surface. The cells are not cultured in monolayer, and therefore cells can interact with other cells and produce extra-cellular matrix, which characterizes mesenchymal tumours and distinguishes them from epithelial tumours. It was previously shown that response to drugs also heavily relies on these interactions (77), illustrating the importance of 3D models. Moreover, larger 3D cultures show oxygen and nutrient gradients which resemble the oxygen and nutrient gradients found in tumours.

Many different types of 3D cell models for tumour cells have been developed. In general, 3D tumour models can be grown as organoids or as spheroids, also called multicellular tumour spheroids (MCTS) (78) or in scaffolds. Tumour organoids are clusters of different cell types that are present in the original tumour, for example cancer associated fibroblasts and tumour cells, whereas tumour spheroids only consist of tumour cells. Although tumour organoid models have gained popularity as a 3D model, in the case of mesenchymal tumours the tumour cells are mostly surrounded by self-produced extra-cellular matrix, which makes the organoid model less suitable. Instead, the spheroid model in which tumour cells can be grown on their own is most often used (60). Multicellular tumour spheroids have successfully been generated for osteosarcoma cells, and shown response to chemotherapeutic drugs, such as doxorubicin, although they were more resistant compared to 2D cultures (79). The transition

of 2D cultured cells into 3D by generating multicellular tumour spheroids, in which cells form aggregates without touching the culture plastic, can be done with different methods. An overview is shown in **Figure 2,** and can be divided by scaffold-free models or scaffold-based models. Scaffold-free models include liquid overlay culture or hanging droplet culture (80). With liquid overlay cultures, cells are seeded onto a non-adherent surface and form spheroids. The hanging droplet method involves the generation of droplets of cells on a surface that is placed upside-down, resulting in the formation of spheroids by gravitational forces. In 3D cultures that make use of a scaffold, the scaffolds are generated from biomaterials and typically contain elements that are also found in the extra-cellular matrix. Studies have been published where scaffolds have successfully been generated based on collagen, alginate, hyaluronic acid, hydroxyapatite or a combination of aforementioned materials (81, 82).

Not only is the 3D culture method important, the type of cell to use for 3D culture is of equal importance. Typically, in 3D culture studies cells that have been previously cultured in 2D are transitioned into a 3D environment. However, cell lines can change over time after extensive culturing in 2D, including chromosomal rearrangement or mutations (83). To overcome this problem, cells can also be cultured into 3D straight from the source material, such as a primary tumour, and therefore has not come into contact with a plastic culture surface. Since many different types of 3D culture exist, it remains challenging to identify the best and most representative culture method for a complex and heterogenous tumour such as osteosarcoma (84).

Figure 2. Overview of different types of 3D culture. Cells can be cultured in 3D either with or without a scaffold. Scaffold-free models can be generated by the hanging droplet method, in which cells are dropped onto a surface and placed upside down to form spheroids, or by the liquid overlay method, where cells are seeded onto a non-adherent surface to form spheroids. In scaffold-based models, cells are cultured inside structures made from biomaterials, for example collagen. For each method, advantages and disadvantages are described. Figure adapted from https://cytosmart.com/resources/spheroids



Aim and outline of this thesis

The aim of this thesis is to study the pathogenesis of osteoid osteoma, osteoblastoma and osteosarcoma by generating *in vitro* models, and utilize these models to understand tumorigenesis and to discover novel therapeutic options for osteosarcoma. In **chapter 2** all novel insights within the molecular pathology, clinical presentation and histology of osteoid osteoma, osteoblastoma and osteosarcoma are summarized and reviewed.

In the first part of this thesis, the development of *in vitro* models to study the pathogenesis of bone-forming tumours of the skeleton is described. In **chapter 3** a mesenchymal stem cell-based model for osteoid osteoma and osteoblastoma has been generated. In this model, mesenchymal stem cells overexpress a truncated form of FOS protein, which is a recurrent alteration in osteoid osteoma and osteoblastoma. The proliferation rate and osteogenic differentiation capacity of these cells have been determined, in order to investigate the role of FOS in the pathogenesis of osteoid osteoma and osteoblastoma. In **chapter 4** another mesenchymal stem cell-based model is described, in which spontaneously transformed murine and canine mesenchymal stem cells show similarities to sarcomas with complex genomics, with many copy number alterations and aneuploidy. Furthermore, these transformed cells formed (osteo) sarcoma after subcutaneous injection in a mouse model. This model has been used to identify the driver events in sarcoma with complex genomics.

In the second part of this thesis, different *in vitro* models of osteosarcoma are used to identify novel therapeutic strategies. In **chapter 5** the murine mesenchymal stem cell model for osteosarcoma from chapter 4 is used in which loss of *CDKN2A* and/or *CDKN2B* has been discovered. In this chapter it was investigated whether loss of *CDKN2A* and/or *CDKN2B* is an early event in osteosarcoma genesis. Furthermore, since loss of this locus can indicate that cells are sensitive to CDK4/CDK6 inhibition, the sensitivity of osteosarcoma cells to CDK4/CDK6 inhibitor palbociclib was investigated, which could be used in osteosarcoma patients with intact Rb and that show loss of p16 or overexpression of CDK4/CDK6. **Chapter 6** describes a potential novel therapeutic strategy for osteosarcoma patients with low *NAPRT* expression. Using both 2D and 3D cultured osteosarcoma cell line models, the sensitivity of cells to NAMPT inhibitor FK866 was tested, which targets the NAD salvage synthesis pathway. **Chapter 7** shows the development of osteosarcoma patient-derived 3D cultures, which have been used to test genome-informed targeted therapy for osteosarcoma. Finally, the thesis is summarized and discussed in **chapter 8**.

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