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

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Chapter 8

Summary and concluding remarks

Summary

Bone-forming tumours are rare tumours characterized by bone deposition, and include osteoid osteoma, osteoblastoma and osteosarcoma (1). The molecular pathology differs greatly between osteosarcoma and osteoid osteoma/osteoblastoma. Osteoid osteoma and osteoblastoma are tumours with simple genomics and show translocations of the *FOS* gene in the majority of cases, whereas osteosarcoma is a tumour with complex genomics and not driven by a specific mutation or translocation. For osteoid osteoma and osteoblastoma the role of FOS in the pathogenesis is not completely understood. For osteosarcoma, the complex genomics hampers the identification of driver events and this is largely unknown. Thus, the main aim of this thesis was to study the pathogenesis of these bone-forming tumours. Furthermore, the aim was to investigate novel treatment options for osteosarcoma, since current treatment has not improved survival in the last decades. Good models are essential to study pathogenesis and therefore the first part of this thesis describes the development of *in vitro* models for functional analysis of molecular alterations in bone-forming tumours. In the second part of this thesis, in *vitro* models have been used to test novel therapeutic strategies for osteosarcoma.

Part 1: *in vitro* models for functional analysis of molecular alterations in bone-forming tumours

The *in vitro* models described in **chapter 3** and **chapter 4** of this thesis are cell-of-origin based models of bone-forming tumours, for which the mesenchymal stem cell was used as the cell-of-origin for functional analysis of molecular alterations. In part 2 we describe different *in vitro* models for drug testing. An overview of *in vitro* models most suitable for different research questions is presented in **Figure 1**.

Cell-of-origin based models for functional analysis of molecular alterations

In **chapter 3** we established a cell-of-origin based model for osteoid osteoma and osteoblastoma. These tumours are characterized by the deposition of immature woven bone (1). The underlying molecular alterations have recently been unraveled, and it was discovered that both osteoid osteoma and osteoblastoma show recurrent translocations in *FOS* leading to a truncation of the protein, and overexpression of truncated FOS due to loss of the C-terminal protein destabilizing motif (2, 3). Currently, cell models for osteoid osteoma and osteoblastoma are lacking. Thus, in this thesis a cell-based model for osteoid osteoma and osteoblastoma was generated, in which fetal mesenchymal stem cells have been transduced to overexpress a truncated form of FOS, lacking the last 90 amino acids of the C-terminal domain. These cells were characterized for their osteogenic differentiation potential and proliferation rate. It was demonstrated that mesenchymal stem cells overexpressing truncated FOS have slightly reduced osteogenic differentiation capacity, and a reduced proliferation rate. We have demonstrated that mesenchymal stem cells overexpressing

truncated FOS show similarities to osteoid osteoma and osteoblastoma, thus providing evidence that the presence of FOS translocations can be linked to the presence of immature woven bone in osteoid osteoma and osteoblastoma.

For osteosarcoma there is a plethora of cell-based models (4), and we have used a previously established model based on mesenchymal stem cells in **chapter 4**. Where previous studies have already demonstrated that murine mesenchymal stem cells undergo spontaneous transformation after long-term culture (5-7), we have now established that also canine mesenchymal stem cells may undergo spontaneous transformation, although less frequent compared to murine cells. Transformed murine and canine mesenchymal stem cells showed a myriad of genomic abnormalities, including aneuploidy, translocations, and copy number alterations, and are therefore genetically very similar to human osteosarcoma. More importantly, this thesis demonstrated that in our spontaneously transformed canine and murine mesenchymal stem cells model, *TP53* or *CDKN2A/CDKN2B* is lost, and this loss is a driving event in osteosarcomagenesis. Alterations in *TP53* and *CDKN2A/CDKN2B* are among the most common recurrent alterations in human osteosarcoma (8). These results support that our murine and canine mesenchymal stem cell model can be used as a model for osteosarcoma or other sarcomas with complex genomics. Furthermore, our model allows mapping the genetic events prior to transformation, which is particularly useful to study a tumour which is characterized by high genomic instability and for which no benign precursor is known.

Molecular alterations driving bone-forming tumours

The cell-of-origin based models that are described in this thesis, have been generated to better unravel the underlying mechanism of genetic alterations in bone-forming tumours. The aim of **chapter 3** was to study the role of FOS in osteoid osteoma and osteoblastoma. It is still not completely understood how translocations in *FOS* lead to the formation of osteoid osteoma or osteoblastoma. In **chapter 3**, we have demonstrated that mesenchymal stem cells overexpressing truncated FOS had slower proliferation rates compared to full length FOS expressing mesenchymal stem cells, and that overexpression of both truncated and full length FOS showed changes in osteogenic differentiation compared to non-manipulated wild-type mesenchymal stem cells. The reduced proliferation observed in fetal mesenchymal stem cells that overexpress truncated FOS compared to cells overexpressing full length FOS could explain the non-malignant nature of osteoid osteoma and osteoblastoma. Interestingly, in contrast to osteoid osteoma and osteoblastoma, osteosarcoma does not show frequent recurrent alterations of *FOS*, although overexpression of full length *FOS* can transform cells into osteosarcoma in c-fos transgenic mice (9-11). Indeed, our model shows that mesenchymal stem cells overexpressing full length FOS has the highest proliferation rate. Thus, the mesenchymal stem cell model expressing truncated or full length FOS can

recapitulate the phenotype observed in bone-forming tumours and suggests that the definitive phenotype is a careful balance between differentiation and proliferation.

The genetic alterations leading to the formation of osteosarcoma have been studied in **chapter 4** and **chapter 5** and here we demonstrated that both *TP53* and *CDKN2A*/*CDKN2B* are driving events in osteosarcoma, as loss of either gene results in spontaneous transformation *in vitro*. This observation is in line with literature, where loss of *TP53* or genes within the *RB*-pathway are most often affected (8, 12). Previous studies have also demonstrated that alterations in *TP53* or the *RB*-pathway are early events in osteosarcomagenesis (13, 14). Although alterations in *TP53* are common in different cancer types, mutations in *TP53* are often not an early event in tumorigenesis of epithelial tumours, but instead occur later in the process of tumorigenesis (15). Complicating the question what is driving osteosarcomagenesis is chromothripsis, the event in which one or a few chromosomes shatter in a random order or orientation. The percentage of osteosarcomas in which chromothripsis occurs varies between 30% and 90% (16, 17). The discrepancy may be attributed to the uncertain definition of chromothripsis (18). Chromothripsis and other catastrophic cellular events, such as chromoanagenesis or chromoplexy, are closely related and lead to genomic instability. Such catastrophic events could lead to the generation of alterations in genes such as *TP53* or *CDKN2A/CDKN2B* (17), which are genes that regulate genome maintenance pathways. Thus, it is not completely understood what the driving event is for osteosarcomagenesis, an event such as chromothripsis, or a direct alteration in *TP53* or *CDKN2A/CDKN2B,* and this warrants further investigation. Nevertheless, the transformed murine MSC model that was used in this thesis provides a tool to investigate which genetic alterations occur prior to an event such as chromothripsis.

In our model the transformed murine mesenchymal stem cells formed not only osteosarcoma but also undifferentiated pleomorphic sarcoma after subcutaneous injection in mice. Moreover, not all mesenchymal stem cells with loss of *TP53* or *CDKN2A*/*CDKN2B* were able to form colonies in soft agar, indicating only a specific combination of alterations lead to malignant transformation and osteosarcoma formation. However, it must be noted that osteosarcoma formation still occurred after injection of one transformed murine mesenchymal stem cell line, that did not show colonies in soft ager (B6_4). Thus, to answer the question which alterations lead to osteosarcoma formation, the ability to form *in vivo* tumors should be tested. The order of genetic alterations also determines which tumour (sub)type is formed (19). Furthermore, not only genetic alterations, but also epigenetic changes could play a role in determining the subtype of sarcoma (20). In this thesis, the MSC model was used to identify which mutations or copy number alterations occur prior to transformation, but further mapping of RNA expression profiles and protein networks would allow complete understanding of the transformation process (21). Thus, further research should be done to identify which combination of (epi)genetic changes, in what order these genetic alterations occur, and which stage of differentiation the cells are in, lead to the formation of different (osteo)sarcoma subtypes.

Part 2: Utilizing in vitro models to identify novel treatment options in osteosarcoma

For osteosarcoma the current treatment strategy of (neo)adjuvant chemotherapy in combination with surgery has not improved the outcome in the last decades (22). This underlines the importance of discovering novel therapeutic options, which can be tested pre-clinically using cell-based models. In part 2 of this thesis 2D and 3D cell culture models were used with the aim of pre-clinical testing of novel therapeutic options for osteosarcoma.

Novel therapeutic options for osteosarcoma patients

In **chapter 5** murine mesenchymal stem cells were used to identify that loss of *CDKN2A* and *CDKN2B* are early events in the transformation towards osteosarcoma, which implicates sensitivity towards CDK4/CDK6 inhibitor treatment (23). Indeed, we discovered that transformed murine mesenchymal stem cells and osteosarcoma cell lines with a loss of p16 are more sensitive to the CDK4/CDK6 inhibitor palbociclib compared to wild-type cells, provided Rb function is intact. In **chapter 7**, we have demonstrated that a primary tumour derived 3D culture, with confirmed loss of *CDKN2A,* was indeed sensitive to palbociclib. Therefore these results suggest that palbociclib can be considered as a novel therapeutic option for osteosarcoma patients. We have shown that there is indeed a subgroup of osteosarcoma patients (20-23%) that have overexpression of CDK4 or CDK6 or have loss of p16 with intact Rb, that could possibly benefit from palbociclib treatment. Previous studies have been published that demonstrate that CDK4/CDK6 inhibitors show promise as a novel treatment option (24, 25). However, the current study and others have used a relatively higher dose of palbociclib than is now considered for other cancer types, such as breast cancer (26). The currently ongoing clinical trials in which CDK4/CDK6 inhibitors are tested in osteosarcoma patients, should demonstrate whether these inhibitors are promising *in vivo* as well (27, 28).

Chapter 6 describes another potential treatment for osteosarcoma patients. It was demonstrated that osteosarcoma cells are sensitive to NAMPT inhibitor FK866, which targets the NAD salvage synthesis pathway. Osteosarcoma cells with low *NAPRT* RNA expression, or high *NAPRT* promotor methylation, were the most sensitive to FK866 treatment. Although the exact mechanism of the NAMPT inhibitor FK866 needs to be elucidated further, the current study demonstrates that targeting the NAD salvage pathway can be considered as a novel treatment option for osteosarcoma patients.

The results in **chapters 5** and **chapter 6** demonstrate there is unfortunately no 'one size fits all' treatment option for osteosarcoma patients, as only a subgroup of osteosarcoma patients has the potential to benefit from a treatment option such as FK866. In **chapter 7** we performed whole exome sequencing of osteosarcomas and established 3D primary cultures with proven genetic alterations that are identical to the original tumour to identify novel targeted treatment options. The genetic alterations could point towards novel targeted treatment options, such as MYC-inhibition. However, the response was not predicted by the

genetic biomarker and indicates that these novel treatment strategies can be further explored for osteosarcoma patients.

2D culture vs 3D culture

Throughout **chapters 5**, **6** and **7** different *In vitro* models for pre-clinical drug testing have been used: from 2D cell lines to 3D cultured multicellular tumour spheroids and primary tissue derived hydrogels. 3D models are aimed at mimicking the context of tumour cells *in vivo*. In **chapter 1** the different methods for 3D culture have been explained in detail. Since osteosarcoma cells produce their own extra-cellular matrix, we have not chosen the organoid model that is frequently used for epithelial cancers, but instead used the liquid overlay method as a scaffold-free 3D model and collagen/alginate hydrogels as a scaffold-based 3D model. Throughout this thesis we have generated scaffold-free multi-cellular tumour spheroids and established for the first time a long-term 3D culture of patient-derived tumours, cultured in collagen/alginate hydrogels. In **chapter 7** we compared these different 3D culture methods and conventional 2D culture methods.
We showed that drug response differs greatly among the different methods, where 3D cultures are less sensitive compared to 2D cultures. Moreover, 2D cells cultured in a 3D environment were also less sensitive compared to 3D cultured cells that have never grown on a plastic surface and are cultured as hydrogels. Thus, it is important to carefully consider which model is the most representative for the *in vivo* situation. In chapter 7, the difference in sensitivity among the different methods was evident. However, to determine which method is the most representative of the *in vivo* situation can only be determined when a drug is tested in animal models and in clinical studies. In general, the consensus is that 3D cultures are more representative compared to 2D cultures (29). However, 2D cultures are still useful for initial screening of potential novel therapeutic options. This is in particular important for high-throughput screening. The reproducibility, material cost and labor intensiveness are important factors to consider in 3D cultures, as these remain challenging (30). Especially patient-derived primary tumour hydrogels are labor-intensive and costly. Nevertheless, 3D cultures can be a valuable addition to the pre-clinical drug testing pipeline and minimize the need of *in vivo* models.

Biomarker identification for targeted treatment

The novel therapeutic strategies that have been tested in the studies presented in this thesis were targeted approaches, which is in contrast to the “one size fits all” strategy of chemotherapy. Therefore it is important to carefully consider the target prior to drug testing. We have discovered potential novel biomarkers to identify patients for which novel therapeutics might be promising. In **chapter 5** we identified that osteosarcoma cells with loss of p16, provided Rb is functional, are most sensitive to treatment with CDK4/CDK6 inhibitor palbociclib. Moreover, the patient subgroup with loss of p16 or overexpression of CDK6 showed worse overall survival and therefore novel therapeutic strategies could be valuable

to improve outcome. In **chapter 6** we have shown that osteosarcoma cells with low *NAPRT* RNA expression are particularly sensitive to NAMPT inhibitor treatment, FK866. Furthermore, *NAPRT* RNA expression positively correlated with promotor methylation, indicating that patients with low *NAPRT* RNA expression/high methylation of *NAPRT* could be selected for treatment. However, a clinical study is needed to investigate whether treatment of this group of patients with NAMPT inhibitor would also improve overall survival.
Once a novel biomarker has been discovered, it is important to consider how to identify the biomarker in patients. In **chapter 5** the p16, Rb, CDK4 and CDK6 status have been evaluated by immunohistochemical staining. Immunohistochemical expression of p16 and Rb has been shown to predict the genomic status (31, 32). Moreover, this method is easy to implement into daily clinical practice. This is in contrast with next-generation sequencing techniques, which we have used in **chapter 7** to identify targetable genomic alterations in osteosarcoma patients. Next-generation sequencing is costly and more time consuming compared to immunohistochemistry. However, more potential targets can be evaluated in each patient and deep sequencing is currently part of clinical practice in many institutes. Independent of the chosen detection method, the studies presented in this thesis demonstrate that valuable information from either immunohistochemistry or next-generation sequencing about potential targets can point to novel therapeutic options in osteosarcoma patients.

Concluding remarks

This thesis describes the generation of cell-of-origin based models, using mesenchymal stem cells, to further elucidate the underlying mechanism of molecular alterations of the bone-forming tumours osteoid osteoma, osteoblastoma and osteosarcoma. Furthermore, we describe the identification of novel treatment options for osteosarcoma using 2D and 3D *in vitro* models.

We have shown that the translocation of *FOS*, which is recurrent in osteoid osteoma and osteoblastoma, reduces osteogenic differentiation and proliferation rate of mesenchymal stem cells, thus providing a possible explanation of the phenotype observed in osteoid osteoma and osteoblastoma. For osteosarcoma, we have demonstrated that *TP53* and *CDKN2A/CDKN2B* are early events in osteosarcomagenesis. The loss of *CDKN2A/CDKN2B* can be exploited with CDK4/CDK6 inhibitor palbociclib, providing a potential novel treatment option for osteosarcoma patients. Another novel treatment option for osteosarcoma is targeting the NAD salvage pathway using NAMPT inhibitor FK866.

The investigation of the underlying mechanism of molecular alterations and the pre-clinical testing of novel treatment options described in this thesis, were performed using a variety of *in vitro* models. Not all models are suitable for every application. A suggestion for the different applications of each type of *in vitro* model described in this thesis is shown in **Figure 1**. Cell-of-origin based models are ideal for functional analysis of molecular alterations, whereas tumour cell lines cultured in 2D or in 3D are more suitable for pre-clinical drug testing.

Whether to choose 2D or 3D models should also be carefully considered. It was demonstrated that sensitivity to drugs differs greatly between 2D or 3D culture models, with 3D models the least sensitive, but perhaps the most representative of the *in vivo* situation. However, 2D models are more convenient for high-throughput screening given the ease of culture. In conclusion, in future research one should carefully consider which *in vitro* model is the most suitable given the research question.

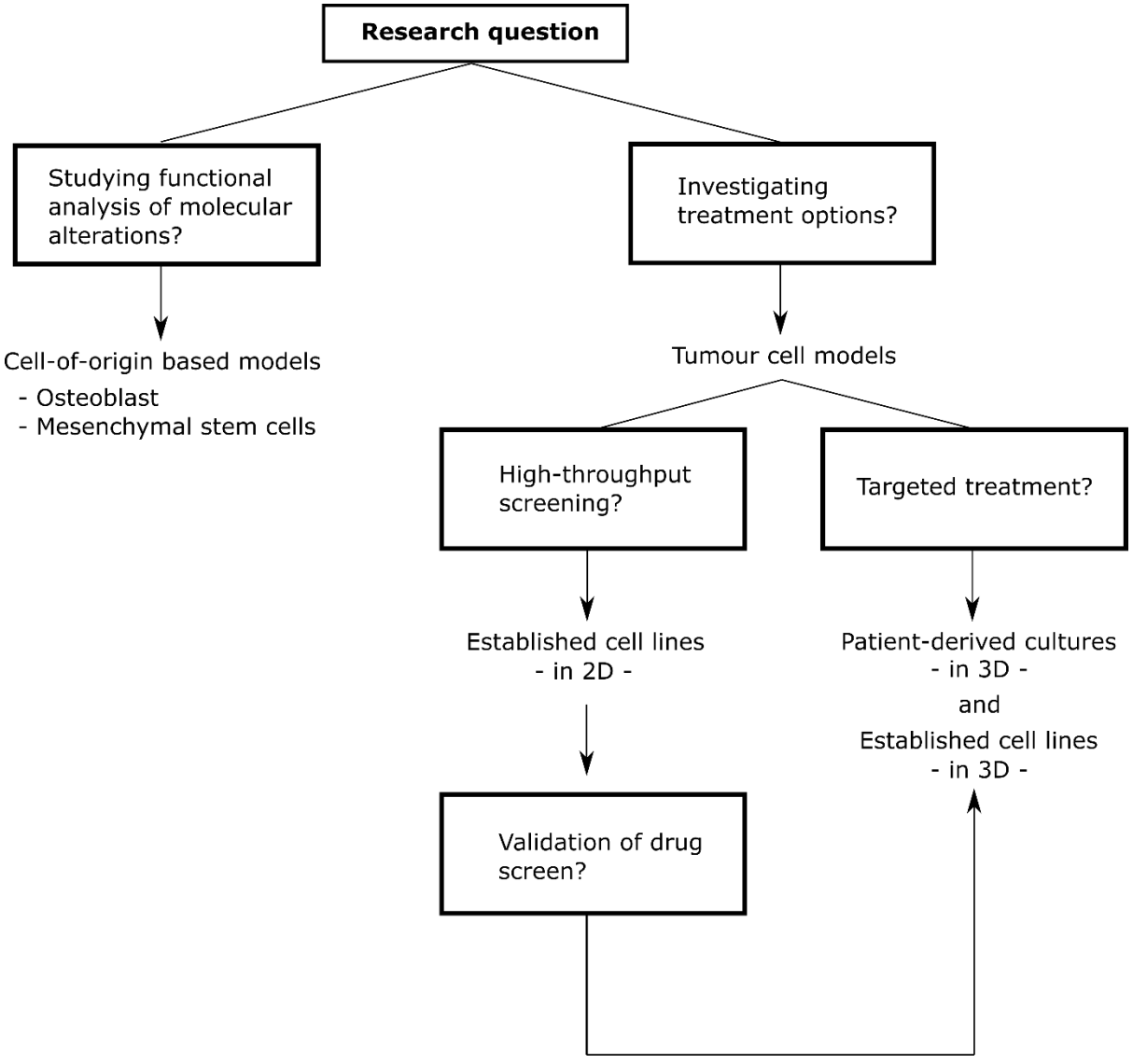

Figure 1. Flowchart of which in vitro model is most suitable for which research question.

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