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Chapter 8

Summary and future perspectives
Osteosarcoma and chondrosarcoma are the first and second most common primary bone cancers, respectively. The aim of this thesis was to explore therapeutic strategies by unravelling cellular pathways that are essential for chondrosarcoma and osteosarcoma cell survival, taking the insulin-like growth factor pathway as a starting point. Chapter 1 gives an introduction to these two sarcoma subtypes and to the cellular pathways that have been studied in this thesis.

**Potential targets for treatment of osteosarcoma**

The large heterogeneity in osteosarcoma patients is reflected by the heterogeneity in osteosarcoma cell lines, which provide adequate models to study mechanisms of osteosarcoma genesis, cell biology, and drug responsiveness (1). It cannot have escaped the notice of researchers working in the field of osteosarcoma that the number of publications on *in vitro* studies of this relatively rare disease has increased considerably in the past five years. On the one hand, this is desirable, as such studies may lead to the identification of new treatment options, which are urgently required for a deadly disease occurring in young patients and for which no improvement of survival is observed since the eighties. On the other hand, an increase in quantity of low quality studies does more harm than good, as it is a waste of time and resources. Chapter 2 systematically identified osteosarcoma *in vitro* studies performed between 1996 and 2015, and demonstrates an almost exponential increase in the number of these studies in the last few years. Unfortunately, the majority of these studies have limited scientific value as they use questionable study designs. While in osteosarcoma *in vitro* studies the use of multiple cell lines is essential to represent the heterogeneity in patients, many studies were performed with only one or two cell lines, i.e. U-2 OS or MG-63. Furthermore, approximately 1/3 of all drugs described in the past three years could be classified as traditional medicine, for which the evidence of specific intracellular targets is lacking. The huge increase in osteosarcoma *in vitro* studies can be attributed by publications from Chinese institutes. This can be explained by the fast growing economy of China, combined with the evaluation system of Chinese medical doctors (2). In China, the Science Citation Index is used as the main indicator for medical career evaluation,
leading to a huge amount of pressure to publish articles each year. 53% of the publications in 2015 were published by Chinese institutes. The increase in publications from China is not restricted to osteosarcoma research. However, the easiness to grow osteosarcoma cell lines and their extremely high growth rate that boosts their drug responsiveness can probably explain why the amount of reports on osteosarcoma is relatively high compared to other research areas. Therefore, chapter 2 sketches the current situation in the osteosarcoma field, and emphasizes the general idea that there is a need to change the evaluation system of medical research (3).

In chapter 3, genome-wide gene expression data was analysed to identify new possibilities for targeted treatment of osteosarcoma. A difference in mRNA expression levels of genes involved in insulin-like growth factor receptor 1 (IGF1R) signalling between osteosarcoma and osteosarcoma progenitors was identified. In osteosarcoma cell lines and pre-treatment biopsies, IGFBP4 and GAS6 showed the highest significant downregulation, which are upstream inhibitors of IGF1R signalling (4). We therefore hypothesized that we could inhibit osteosarcoma growth by inhibiting this pathway. Osteosarcoma cell lines were treated with OSI-906, a dual inhibitor of the IGF1R and the insulin receptor (IR), as signalling via the IR can take over in case of IGF1R blockage. OSI-906 inhibited osteosarcoma cell viability in three out of four osteosarcoma cell lines tested. Furthermore, OSI-906 resulted in a decreased phosphorylation of insulin-receptor substrate 1, a direct downstream target of the IGF1R, thereby validating on target inhibition. Therefore, chapter 3 identified the IGF pathway as a potential target for treatment of osteosarcoma.

In the gene set analysis performed in chapter 3, nineteen osteosarcoma cell lines were included. This broad panel of osteosarcoma cell lines optimally reflects the heterogeneity observed in osteosarcoma patients. The potency of targeting the IGF pathway in osteosarcoma sarcoma was subsequently explored in four osteosarcoma cell lines, which might question the degree in which the heterogeneity of osteosarcoma patients is captured in these experiments. However, these four cell lines demonstrated different potential to undergo osteo- chondro- and adipogenic differentiation in vitro, and showed different potency to induce tumour growth in vivo (1). Therefore, although
only four cell lines were used, the captured heterogeneity was considered sufficient for this study.

In chapter 4, a commentary on a study by Yang et al. (5) is given. Similar to our investigations described in chapter 3, the study by Yang et al. also compared gene expression between osteosarcoma (biopsies and cell lines), and osteosarcoma progenitor cells. Interestingly, they also identified downregulation of genes upstream of the IGF1R, and thereby pointed toward a role for the IGF pathway in osteosarcoma genesis. In the first section of our commentary, we describe where we are now. While preclinical models have shown promising results, evidence for the efficacy in large-scale randomized controlled trials is lacking, resulting in pharmaceutical companies discontinuing the production of all IGF pathway-targeting agents. In the second section, it is described where we need to go. Biomarkers should be identified, and trials with compounds that target both the IGF1R and the IR should be performed. The last section, we describe how we should get there. Biomarkers that will be identified in a clinical trial with OSI-906 in Ewing sarcoma (NCT02546544, clinicaltrials.gov) should be tested in other tumour types including osteosarcoma. As this trial incorporates a strong translational research program, it sets an example of how future trials with IGF1R-IR inhibitors in osteosarcoma should be performed.

Currently, no clinical trials with IGF1R inhibitors are open for osteosarcoma patients. The clinical trials that have been performed with IGF1R inhibitors in osteosarcoma patients are summarized in Table 1. As mentioned above, an important limitation of these trials is that they all involved IGF1R monoclonal antibodies. By targeting the IGF1R, the endocrine feedback loop is disrupted resulting in a strong increase in GH and IGF1 levels (6). These increased IGF1 levels can still activate the IGF pathway by stimulating IGF1 receptors that are not blocked by the treatment and by signalling via the IR, thereby stimulating tumour growth. The increased GH and IGF1 levels upon IGF1R directed therapy can potentially explain the difference in efficacy of IGF1R inhibitors in xenografts and human patients, as most antibodies are specific for human IGF1R binding and therefore do not disrupt the endocrine feedback loop in rodents (7).
Table 1. Overview of clinical trials and results of IGF inhibitors in osteosarcoma and chondrosarcoma patients. All inhibitors are monoclonal antibodies to the IGF1R.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Osteosarcoma patients</th>
<th>Efficacy</th>
<th>Phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVE1642</td>
<td>N=3 bone sarcomas, refractory disease</td>
<td>1 osteosarcoma unconfirmed partial response</td>
<td>I</td>
<td>(8)</td>
</tr>
<tr>
<td>Figitumumab</td>
<td>N=11, patients with no other treatment options</td>
<td>No responders</td>
<td>I</td>
<td>(9)</td>
</tr>
<tr>
<td>Figitumumab and Everolimus (mTOR)</td>
<td>N=3, no curative treatment options</td>
<td>1 prolonged stable disease</td>
<td>I</td>
<td>(10)</td>
</tr>
<tr>
<td>Cixutumumab</td>
<td>N=3, patients with relapsed/refractory tumours</td>
<td>No responders</td>
<td>I</td>
<td>(11)</td>
</tr>
<tr>
<td>Cixutumumab</td>
<td>N=11, elapsed refractory solid tumours</td>
<td>No responders</td>
<td>II</td>
<td>(12)</td>
</tr>
<tr>
<td>Cixutumumab and temsirolimus (mTOR)</td>
<td>N=24, received previous treatments</td>
<td>3 partial responses</td>
<td>II</td>
<td>(13)</td>
</tr>
<tr>
<td>RG1507</td>
<td>N=3, relapsed or refractory solid tumours</td>
<td>2 stable disease for &gt;52 weeks</td>
<td>I</td>
<td>(14)</td>
</tr>
<tr>
<td>R1507</td>
<td>N=38, recurrent or refractory osteosarcoma</td>
<td>2 partial responses</td>
<td>II</td>
<td>(6)</td>
</tr>
<tr>
<td>Cixutumumab and temsirolimus (mTOR)</td>
<td>N=11, relapsed or refractory osteosarcoma</td>
<td>No responders</td>
<td>II</td>
<td>(15)</td>
</tr>
<tr>
<td>Robatumumab</td>
<td>Group 1: N=68 relapsed, resectable recurrences &lt; 6 months after prior treatment Group 2: N=35, relapsed unresectable metastasis</td>
<td>Group 1: 3 partial responders, 17 stable disease Group 2: 6 stable disease</td>
<td>II</td>
<td>(16)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Chondrosarcoma patients</th>
<th>Efficacy</th>
<th>Phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cixutumumab and temsirolimus (mTOR)</td>
<td>N=17, received previous treatments</td>
<td>1 partial response</td>
<td>II</td>
<td>(13)</td>
</tr>
<tr>
<td>Figitumumab</td>
<td>N=1, myxoid chondrosarcoma* that received previous treatments</td>
<td>Small decrease in tumour size</td>
<td>I</td>
<td>(17)</td>
</tr>
<tr>
<td>Figitumumab and docetaxel</td>
<td>N=2, received previous treatments</td>
<td>1 stable disease after 6 months</td>
<td>lb</td>
<td>(18)</td>
</tr>
<tr>
<td>BIIB022</td>
<td>N=1, relapsed or refractory solid tumour</td>
<td>Not clear, results presented for all sarcomas combined</td>
<td>I</td>
<td>(19)</td>
</tr>
</tbody>
</table>

* unclear whether this was an extraskeletal myxoid chondrosarcoma or a chondrosarcoma of bone
A recent whole genome sequencing study by Behjati et al. reported alterations in IGF receptor genes in 7% of the osteosarcoma cases, and additional driver mutation in pathways downstream of IGF1R in an additional 20% of the cases (20). Therefore, 27% of the tumours had perturbed IGF1R signalling due to somatic mutations. In addition, 14% of the osteosarcoma cases had 15 copies or more of the IGF1R gene. These findings suggest that a substantial subgroup of osteosarcoma patients may give a significant response to IGF-directed therapy and that we might be able to select patients based on their genotype in the future. Due to driver mutations downstream of the IGF1R, resistance to IGF1R-IR inhibitors might occur. Therefore, these inhibitors should potentially be combined with agents that target the IGF pathway at different levels.

**Potential targets for treatment of chondrosarcoma**

In chapter 5 it is reported whether, in addition to osteosarcoma, the IGF pathway is a potential target for chondrosarcoma therapy. Mediators of IGF1R signalling were heterogeneously expressed in chondrosarcoma cell lines, and phosphorylated IRS1 was identified in 2 out of 3 cell lines tested. Although these results indicated that the IGF1R pathway was active in a subset of chondrosarcoma cell lines, treating chondrosarcoma cell lines with three different IGF1R inhibitors did not influence chondrosarcoma cell line viability, migration, or chemoresistance. To elucidate the discrepancy between the absence of an effect of IGF1R inhibition and pathway activity in chondrosarcoma cell lines, IGF1R expression level was assessed in chondrosarcoma cell lines and primary tumours using immunohistochemistry. In contrast to chondrosarcoma cell lines, we found no expression (66%) or weak expression (34%) in the primary tumours. Moreover, determining expression in four patients with matched cell lines and primary tumours suggested that chondrosarcoma cells had upregulated IGF1R upon culturing, thereby questioning the validity of using monolayer-cultured cells as a model to study IGF pathway activity. In conclusion, chapter 5 demonstrates that the IGF pathway is not expected to be an effective therapeutic target for chondrosarcoma.

Chapter 5 illustrates the need to develop more representative *in vitro* models for chondrosarcoma. In the last decade, models with cancer cell culture in a three-
dimensional (3D) environment have been developed. Our group has been using a spheroid model in which chondrosarcoma cells are injected into a collagen gel (21). Lhuissier et al. recently published a model in which chondrosarcoma cells were embedded in an alginate hydrogel (22). 3D in vitro models contain several in vivo features such as drug penetration, drug resistance, cell-cell interaction, a hypoxic gradient and the deposition of extracellular matrix (23). Therefore, 3D models should be the focus of future research.

As can be seen in Table 1, a few clinical trials with IGF1R inhibitors included chondrosarcoma patients, which in line with our preclinical study, do not support using IGF1R inhibitors in the clinic of chondrosarcoma. The largest is the study by Schwartz et al., which included 17 chondrosarcoma patients that did not respond to previous treatments. This reflects that despite the huge difference in aetiology between different sarcomas, clinical trials often combine patients from different subtypes. This can be explained by the rareness of these cancers. However, these trials do not fully evaluate the potency of a drug for a specific sarcoma subtype, potentially leading to incorrect conclusions. Large international research consortia can solve this problem, as by these consortia, enough patients of a particular sarcoma subtype can be included in a clinical study. Therefore, international collaborations should be facilitated even more in the future (24).

As chapter 5 demonstrates that IGF1R signalling is not a potential target for treatment of chondrosarcoma, other therapeutic strategies are needed. Therefore, in chapters 6 and 7, a different approach was taken to identify potential targets for chondrosarcoma therapy, exploiting the genetic properties of this tumour type. About 50% of the conventional chondrosarcomas carry an IDH1 or IDH2 hotspot mutation. It is acknowledged that the oncogenic activity of these mutations lies in the aberrant production of the oncometabolite D-2-hydroxyglutarate. However, as IDH1 and IDH2 are key enzymes in cell metabolism, these mutations potentially may lead to metabolic vulnerabilities that can be targeted.

**Chapter 6** investigated whether nicotinamide adenine dinucleotide (NAD+) depletion can be used to target IDH1/2 mutant chondrosarcoma cells, based
on a study from Tateishi et al. (25) who identified this vulnerability in IDH1/2 mutant glioma cells. Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT) are rate-limiting enzymes in the NAD+ synthesis pathway. Treating eleven chondrosarcoma cell lines with two NAMPT inhibitors revealed that chondrosarcoma cell lines showed a dose-dependent decrease in cell viability, 3D collagen invasion and colony formation upon treatment with NAMPT inhibitors. Nearly half of the cell lines demonstrated IC₅₀s in the low nM range. qRT-PCR analyses demonstrated that increasing IC₅₀s for NAMPT inhibitors correlated to increasing NAPRT expression levels, and datasets of genome-wide methylation arrays revealed that the increasing NAPRT expression levels were correlated to decreasing NAPRT methylation. Strikingly, higher methylation of the NAPRT promoter was observed in high-grade versus low-grade tumours. In contrast to our initial hypothesis, we did not observe a correlation between the IDH1/2 mutation status and sensitivity to NAMPT inhibitors, nor could we find a difference in NAPRT methylation between IDH1/2 mutant and wildtype primary tumours. Therefore, this study identified NAMPT as a potential target for treatment of chondrosarcoma, especially for those of high histological grade, irrespective of the IDH1/2 mutation status.

The phase I clinical trials that tested the safety of NAMPT inhibition (also including FK866 and GMX1778) were discontinued due to dose-limiting toxicities (26). Co-administration with nicotinic acid (NA) has been proposed to increase the therapeutic index of NAMPT inhibitors, as NA can be used to synthesise NAD+ in NAPRT-proficient cells. In chondrosarcoma xenograft, it was shown that the inhibitory effect of NAMPT inhibitors was not affected by co-administration of NA (27), suggesting that this could be a suitable approach to decrease dose-limiting toxicities in chondrosarcoma patients. Therefore, NAMPT inhibitors (in combination with NA) should be evaluated in chondrosarcoma mouse models. In addition, a Phase I clinical trial with KPT-9274, a dual inhibitor of PAK4 and NAMPT, is currently recruiting patients with advanced solid malignancies (including sarcomas) and non-Hodgkin’s lymphoma (NCT02702492, clinicaltrials.gov). Interestingly, NAPRT1 and IDH1 tumour status will be determined prior to enrolment. Hopefully, this study will recruit (sufficient) chondrosarcoma
patients to be able to indicate whether NAMPT inhibitors can be used as a treatment of chondrosarcoma.

As multiple studies point towards an increased dependency on glutaminolysis in IDH1/2 mutant glioma cells, chapter 7 evaluated if there was preclinical rationale for targeting glutaminolysis in chondrosarcoma. By immunohistochemistry, it was demonstrated that increasing glutaminase expression levels correlated to increasing tumour grades, and qRT-PCR analyses of chondrosarcoma cell lines revealed a higher expression of glutaminase in chondrosarcoma cell lines compared to the controls (growth plate and cartilage). Treating chondrosarcoma cell lines with the glutaminase inhibitor CB-839 revealed that a subset of chondrosarcoma cell lines is dependent on glutaminase-mediated glutaminolysis to maintain cell viability. As the safety of CB-839 for patient treatment is still under investigation, the effects of the widely used anti-diabetic drug metformin, its lipophilic analogue phenformin, and the anti-malaria drug chloroquine were evaluated in vitro, as these drugs also inhibit glutaminolysis. These four metabolic compounds inhibited chondrosarcoma cell viability in a subset of chondrosarcoma cell lines tested. To further investigate the cellular mechanism by which these four metabolic drugs inhibit chondrosarcoma cell viability, cell apoptosis, mTOR activity and LC3B-II levels were determined. While the induction of apoptosis was limited, metformin and phenformin decreased mTOR activity in chondrosarcoma cells, and metformin decreased autophagy, which is counteracted by chloroquine. The mechanisms by which CB-839 decreased cell viability remain to be identified. In conclusion, chapter 7 suggests that targeting glutaminolysis with CB-839, metformin, phenformin or chloroquine is a potential therapeutic strategy for a subset of high-grade chondrosarcomas.

Similar to chapter 6, we could not confirm the prevailing hypothesis that IDH1/2 mutant chondrosarcoma cells are more dependent on glutaminolysis as compared to IDH1/2 wildtype cells, since there was also no correlation of the IDH1/2 mutation status of chondrosarcoma cells with sensitivity to these compounds, or with the expression levels of glutaminase in primary tumours. Two clinical trials that target glutaminolysis are currently recruiting patients, which select patients based on the IDH1/2 mutation status. These
so called “basket trials” recruit patients of a variety of tumour types at the same time, based on the presence of a specific mutation, thereby increasing the number of eligible patients. The first one is a trial with the glutaminase inhibitor CB-839 (NCT02071862, clinicaltrials.gov), and the second one is a trial that combined metformin and chloroquine (NCT02496741, clinicaltrials.gov). These trials already started before the results of our preclinical studies became available. Chapter 7 demonstrates that in contrast to our initial hypothesis based on findings in gliomas, there is limited preclinical rationale to only include chondrosarcoma patients that harbour an \textit{IDH1/2} mutation for these trials. This is in line with previous studies from our group, which demonstrated that in contrast to gliomas, \textit{IDH1/2} mutation status does not correlate to chondrosarcoma prognosis, \textit{IDH1/2} mutations do not affect immunohistochemical levels of 5-hmC, 5mC and trimethylation of H3K4, -9, and -27, and prolonged inhibition of the IDH1 mutant enzyme does not affect global gene expression, CpG island methylation nor histone H3K4, -9, and -27 trimethylation in chondrosarcoma cell lines. Therefore, together with other studies from our group, Chapter 6 and Chapter 7 suggest that \textit{IDH1/2} mutant chondrosarcomas do not require a different therapeutic approach than \textit{IDH1/2} wildtype chondrosarcomas. This can have two potential explanations. First, it might be that although the \textit{IDH1/2} mutation is involved in the initiation of a chondrosarcoma, it does not play a role in chondrosarcoma progression. This is supported by the lack of an \textit{in vitro} effect of IDH1 mutant inhibitors on the tumorigenic properties of chondrosarcoma cells (28). Second, it might be that chondrosarcomas without an \textit{IDH1/2} mutation have other aberrant intracellular routes that have the same effect as an \textit{IDH1/2} mutation on chondrosarcoma cell metabolism. A key regulator of cell metabolism is mTOR, a kinase that integrates input from many upstream pathways and passes the signal to multiple target proteins. Currently, one clinical trial that recruits \textit{IDH1/2} mutant and wildtype chondrosarcoma patients, run in the LUMC, uses the compound sirolimus (mTOR inhibitor) in combination with cyclophosphamide (NCT02821507, clinicaltrials.gov). As this trial incorporates a translational research program, it will help unravelling the intracellular biology of these tumours, which might provide useful insights regarding which of these potential explanations is accurate.
Chondrosarcoma and osteosarcoma model systems

In this thesis, mainly monolayer-cultured cells were used to study the drug responsiveness of osteosarcoma and chondrosarcoma cells. Testing the potency of a drug in this model system is a first step in developing new treatment options. However, monolayer-cultured cells are being criticized for two reasons. First, cell lines have repeatedly been discovered as being misidentified, which can be caused by cross-contaminations or mislabelling of samples. The international cell line authentication committee created a database of these cell lines, now containing 488 cell lines. It is estimated that 0.8% of the total literature on cells involved the use of these misidentified cell lines (29). Although techniques have been introduced for cell line authentication, misidentification of cell lines remains a substantial problem. Second, cell lines are grown without a reflective three-dimensional structure and microenvironment. Therefore, as discussed above, new in vitro models have been developed that better reflect the original tumours. These 3D models will become more and more important in future research.

In addition to in vitro models, in vivo models can be used the study the potency of new osteosarcoma and chondrosarcoma therapies. Ideally, this is the second step in the development of new therapies. Four groups of in vivo models can be distinguished:

- Spontaneous models, such as canine osteosarcoma.
- Induced models, such as the induction of murine osteosarcoma by radiation.
- PDX models, i.e. patient derived xenografts, in which osteosarcoma or chondrosarcoma cells from a patient are transplanted in mice or zebrafish.
- Transgenic mice, in which the (conditional) knock-out of a certain gene results in the development of a particular tumour, such as the osterix-cre-mediated deletion of p53 and pRb in murine osteosarcoma models.

As these models all have advantages and disadvantages, the best in vivo model depends on the research question (30). When subsequently in vitro and in vivo models demonstrate the potency of a drug, clinical trials can be initiated as a final step in the development of new therapies.
Summary

In conclusion, this thesis explored potential new therapeutic strategies by identifying cellular pathways that are essential for chondrosarcoma and osteosarcoma cell survival. Although clinical trials with IGF1R inhibitors have disappointing results in osteosarcoma, this thesis strengthens the view that the IGF pathway can be an effective target for osteosarcoma therapy if an appropriate selection of patients is treated with IGF1R/IR dual inhibitors. When optimized clinical trials targeting the IGF pathway will be performed in the future, chondrosarcoma patients should not be recruited, as there is limited preclinical rationale for the efficacy of IGF1R targeting agents in chondrosarcoma. We identified two promising pathways that can be used to target chondrosarcoma; the NAD+ synthesis pathway and glutaminolysis. Our results suggest that chondrosarcoma patients should be included in future studies with drugs that interfere in these pathways, regardless of their IDH1/2 mutation status.
References


