1.

Introduction

DEFINITION AND EPIDEMIOLOGY OF OSTEOSARCOMA

Osteosarcoma is a high-grade, intra-osseous malignancy in which the neoplastic cells produce bone (Fig. 1.1) [89]. It is the most common primary bone sarcoma. The World Health Organisation (WHO) has defined several major histological subtypes of osteosarcoma (Table 1.1) [217]. High-grade osteosarcoma occurs predominantly in children and young adults (Fig 1.2, incidence rate up to 8.4 per million in children aged 15 to 19 years), with a second peak of incidence in the elderly (incidence rate of 4.2 per million in the over 60 years age group) [177;248]. Incidence rates in adults aged 25 to 59 years are lowest at 1.7 per million. Males are affected more often than females at a ratio of 1.3 to 1.

PATHOGENESIS

Several lines of evidence suggest that osteosarcoma originates from mesenchymal stromal cells (MSCs) or early osteoblast precursor cells [181]. High-grade osteosarcoma commonly develops at an age and anatomical site of rapid proliferation and differentiation of MSCs, i.e. intramedullary near the growth plate of the long bones during or after the pubertal growth spurt. Long term *in vitro* expansion of murine MSCs results in oncogenic transformation of the cultured cells which form osteosarcoma-like tumors *in vivo* [113;116;179;183;259]. A patient transplanted with bone marrow (containing hematopoietic stem cells and MSCs) from a sibling was diagnosed with an osteosarcoma originating from donor cells 17 years later [19]. These data support the hypothesized mesenchymal stromal cell origin of osteosarcoma.

Most osteosarcomas arise sporadically, but some genetic or environmental factors increase the risk for developing osteosarcoma. Germ line mutations in the tumor suppressor genes *TP53* and *RB1* are associated with the Li-Fraumeni and hereditary retinoblastoma syndromes, respectively, both of which are associated with an increased risk to develop osteosarcoma and other types of cancer [231]. Mutations in the helicase genes *RECQL2*, *RECQL3* and *RECQL4* respectively cause Werner, Bloom and Rothmund-Thompson syndrome, all of which are associated with an increased risk of developing osteosarcoma [88;117;276]. In several other

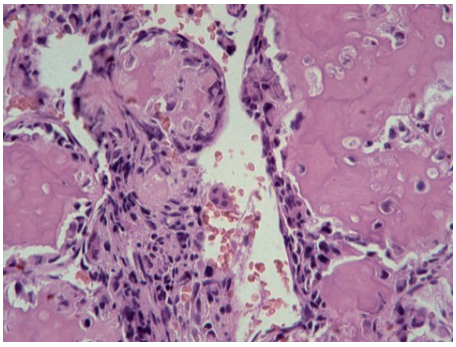

Fig. 1.1 Histology of conventional osteoblastic osteosarcoma. Hematoxylin and eosin stained section of diagnostic biopsy demonstrating osteoid production by neoplastic cells.

Table 1.1 Osteosarcoma classification according to the World Health Organisation [217]

Conventional osteosarcoma
- Osteoblastic (including sclerosing)
- Chondroblastic
- Fibroblastic
- Giant cell rich
- Osteoblastoma-like
- Epithelioid
- Clear cell
- Chondroblastoma-like
Telangiectatic osteosarcoma
Small cell osteosarcoma
Low grade central osteosarcoma
Parosteal osteosarcoma (low grade)
Periosteal osteosarcoma (intermediate grade)
High grade surface osteosarcoma

cancer syndromes an association with osteosarcoma has been reported, but is less well established. For example, according to Chowdry *et al.*, neurofibromatosis type 1 patients have a higher than expected incidence of bone sarcomas including osteosarcoma [43].

In a small percentage of osteosarcoma cases pre-malignant conditions can be identified, as has been reviewed in [105]. Well known precursor lesions are Paget's disease, fibrous dysplasia, dedifferentiated chondrosarcoma and giant cell tumor of bone [96;105;206]. Patients presenting with an osteosarcoma in the context of these conditions are often older than patients in whom no precursor lesion is identified.

Radiation exposure, for example in the treatment of other cancers, is the best known environmental risk factor in osteosarcoma, accounting for 0.5-5% of all new osteosarcoma cases [124;280].

Osteosarcoma is characterized by gross chromosomal instability with very complex polyploid karyotypes and marked cell-to-cell heterogeneity [231]. This is in contrast to many other sarcomas which can be defined by specific translocations, resulting in specific fusion transcripts, such as the *EWS-FLI1* transcript in Ewing sarcoma [230]. The highly complex chromosomal rearrangements as are present in osteosarcoma can occur as a result of a single catastrophic event, termed chromothripsis [72]. However, this probably has to occur in a susceptible background, either as a genetic predisposition or acquired as a *de novo* event. If chromothripsis occurs in the development of osteosarcoma and which somatic genetic or epigenetic aberration would confer susceptibility in osteosarcoma patients is as yet unknown. No specific chromosomal aberrations can be identified in osteosarcoma, but gain of chromosome 1 (present in 22% of examined karyotypes) and loss of chromosomes 9, 10, 13, and 17 (in 29%, 37%, 36% and 30% of specimens, respectively) occur in a significant proportion of osteosarcomas [30]. On

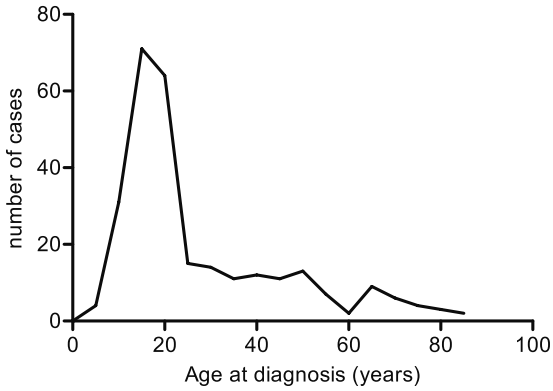

Fig. 1.2 Most osteosarcomas occur in children and young adults. Age distribution of 274 high-grade osteosarcoma patients diagnosed from 1990 to 2009 and treated at the Leiden University Medical Center. A second peak of incidence is often reported in the above sixty year age group, but is not apparent in our tertiary referral clinic, possibly reflecting referral bias.

a molecular genetic level, the TP53 and RB1 tumor suppressor pathways are often inactivated, resulting in loss of cell cycle control and unchecked cell proliferation [95;183;281].

DIAGNOSIS, TREATMENT AND PROGNOSIS

Most osteosarcomas are localized near the metaphyseal ends of the long bones, particularly in the distal femur, proximal tibia and proximal humerus. Patients often present with a history of pain and swelling of a few months duration. Symptoms sometimes seem to be precipitated by minor trauma. Rarely, patients present with a pathological fracture or functional impairment. Systemic symptoms are almost always absent. Radiographic examination usually reveals an osteolytic or osteosclerotic lesion with cortical involvement, a periosteal reaction and marked soft-tissue involvement (Fig. 1.3) [175]. Diagnostic biopsy of the lesion is required for a definitive diagnosis of osteosarcoma and for subtype classification (Fig. 1.1, Table 1.1). Staging studies include magnetic resonance imaging of the primary tumor to evaluate soft tissue expansion, computed tomography of the chest to evaluate the presence of pulmonary metastases and radionuclide bone scanning with technetium to evaluate the presence of bone metastases.

Prognosis for patients with osteosarcoma is mainly determined by the presence or absence of metastatic disease. About fifteen percent of patients have detectable metastatic disease at diagnosis and about 40 to 50 percent of initially non-metastatic patients subsequently develop detectable metastatic disease [172]. Overall survival for patients with metastatic disease is very poor at about twenty percent. Another major prognostic factor in osteosarcoma is the histological response to neo-adjuvant chemotherapy in the primary tumor, with less than ninety percent necrotic tumor area defined as prognostically unfavorable.

Before the introduction of chemotherapy in the management of osteosarcoma in the 1970s, long term survival rates were about 10% following complete resection of the tumor,

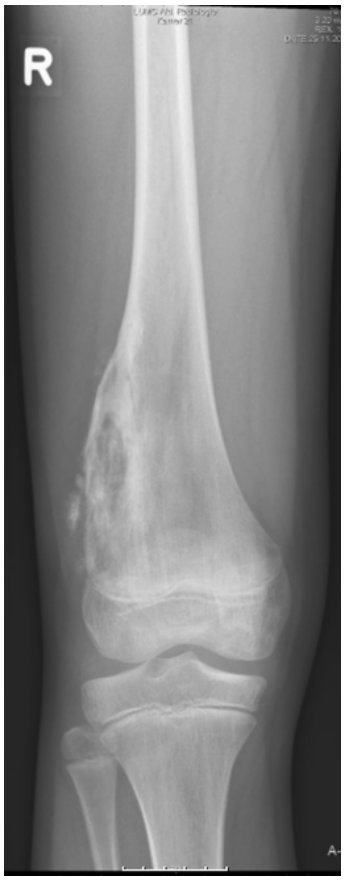

Fig. 1.3 Conventional radiograph demonstrating an osteosarcoma of the right femur in a 13 year old boy. Note the irregular structure of the bony matrix in metaphysis and diaphysis, the periostal reaction and the extension of the lesion into the soft tissue.

implicating that macroscopic or microscopic metastases are present in almost all patients at diagnosis. Since the introduction of chemotherapeutic treatment, overall long term survival of osteosarcoma has improved to about sixty percent. However, the overall survival rate has not improved substantially in the last twenty years, despite clinical trials investigating higher dosages and higher dose intensity chemotherapy regimens (Fig. 1.4). Currently, treatment consists of several rounds of neo-adjuvant and adjuvant chemotherapy in addition to radical surgical treatment of the primary tumor and metastases whenever feasible. Chemotherapeutic regimens have typically consisted of doxorubicin and cisplatin with or without high-dose methotrexate. Addition of etoposide and high-dose ifosfamide have been used in salvage regimens for non-resectable metastatic disease. A recent meta-analysis suggests that treatment with four or five drugs may not confer a survival advantage to treatment with a three drug regimen [8]. The addition of etoposide and ifosfamide for patients with poor histological response to the standard (neo-)adjuvant three drug regimen of methotrexate, adriamycin and doxorubicin is currently being evaluated in European and American Osteosarcoma Study Group (EURAMOS)-1 trial, for which results are due in 2015-2016 [160].

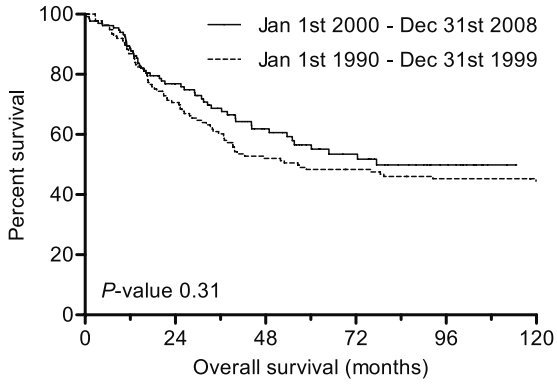

Fig. 1.4 Overall survival of osteosarcoma patients has not improved significantly since the 1990s (data of 274 high-grade osteosarcoma patients diagnosed from 1990 to 2008, treated at the Leiden University Medical Center)

INNATE IMMUNITY IN OSTEOSARCOMA PATIENTS

The innate immune system is the first-line defense system against pathogens and consists of physical barriers, cellular components and humoral components. Cellular components of the innate immune system such as granulocytes and macrophages are able to recognize and phagocytose pathogens through interaction of intracellular and surface membrane receptors with pathogen associated molecular patterns (PAMPs). Natural killer (NK) cells recognize and lyse virus infected cells when the balance of signals transduced via inhibitory and activating NK cell receptors is shifted towards activation [213]. There is increasing evidence that cells of the innate immune system such as macrophages and NK cells are able to not only detect and kill pathogens, but also tumor cells. In this thesis, we have studied the interaction between cells of the innate immune system -in particular macrophages and NK cells- and osteosarcoma cells, with the aim to provide preclinical data to guide future trials employing immunotherapeutic strategies.

Tumor-associated macrophages and cancer immunology

Infiltration of tumors with macrophages is often associated with worse prognosis. Several mechanisms have been proposed to explain the pro-tumorigenic effect of tumor-associated macrophages (TAMs). First, TAMs can express matrix-degrading proteins and thus facilitate tumor cell evasion and metastasis. Matrix metalloproteinase (MMP) expression by tumor stromal cells is associated with worse prognosis in many tumor types and MMP-9 expression by hematopoietic cells was essential for tumor progression in a murine squamous cell carcinoma model [50;63]. Second, TAMs can support angiogenesis through expression of specific cytokines and growth factors, for example vascular endothelial growth factor (VEGF), urokinase plasminogen activator and C-X-C chemokine receptor (CXCR)-2 ligands such as C-X-C chemokine ligand (CXCL)-8 [3;5;48;142]. Third, TAMs excrete immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor- β (TGF-β), consequently hampering effective anti-tumor immunity [70;238]. These tumor promoting TAMs have many

characteristics in common with 'alternatively' IL-4 and IL-13 activated M2 type macrophages. M2 type macrophages express the scavenger receptor CD163 and have important tissue regenerative roles, for example in wound healing and angiogenesis. It is these homeostatic features that also promote growth and dissemination of tumor cells. Intratumoral infiltration with M2, or 'alternatively activated' macrophages is associated with increased angiogenesis, metastasis and decreased survival in many tumors, including sarcomas [78;138;266].

In contrast, 'classical activation' of macrophages by interferon-γ or microbial products results in expression of high levels of pro-inflammatory cytokines, such as IL-12, IL-1 and IL-6 [188]. These so-called M1 type macrophages have potent anti-tumor efficacy. They are able to kill tumor cells directly, through phagocytosis of tumor cells, reactive oxygen species and cytokine-induced cytotoxicity [109]. Additionally, they can recruit and activate NK and T cells and can initiate an adaptive immune cell response. In rare instances, high numbers of TAMs are associated with better survival of cancer patients [73;122]. It is presumed that the infiltrating macrophages in these tumors are polarized towards an M1 phenotype, but this has not been unequivocally proven.

M1 and M2 type activation of macrophages represent extremes of a spectrum (Fig 1.5). Macrophages demonstrate considerable phenotypic plasticity. Under the influence of environmental factors, polarization towards one or the other activation status can occur. To exploit this plasticity in an immunotherapeutic context, tumor resident M2 type macrophages may be classically activated towards an M1 phenotype by microbial cell products, or PAMPs. The successful utilization of immunostimulation in the treatment of sarcomas was first demonstrated by William B. Coley in 1891 [49;246]. Coley injected a mixture of streptococcal toxins into unresectable tumors and observed tumor regression in several sarcoma patients. More recently, post-operative infection has been shown to be associated with a better survival in osteosarcoma patients, possibly through activation of the innate immune system [114;136;242]. Treatment of osteosarcoma patients with the microbial cell wall product and macrophage activating agent muramyl tripeptide (MTP) in addition to standard chemotherapy improved overall survival in a recent clinical trial, although the exact mechanism remains unclear [170]. Another mechanism for classical activation of macrophages is through induction of immunogenic cell death. Often, chemotherapy induced cancer cell death is immunologically 'silent'. However, as a result of certain cytotoxic treatments, such as alkylating agents, oxaliplatin and ionizing radiation, immunogenic cell death can occur . The expression and cell surface translocation of damage-associated molecular patterns (DAMPs) such as calreticulin and high mobility group protein B1 trigger the activation of antigen presenting cells, similar to the response to PAMPs during infection [10;131;193].

NK cells

NK cells are innate immune cells that lack a clonally rearranged antigen-specific receptor. They have important regulatory functions through the expression of cytokines and chemokines and are potent effector cells. Cytolytic activity depends on the balance of between activating signals and inhibitory signals. High expression of ligands for the activating NK cell receptors Natural Killer Group 2, member D (NKG2D), DNAX accessory molecule-1 (DNAM-1) and the natural

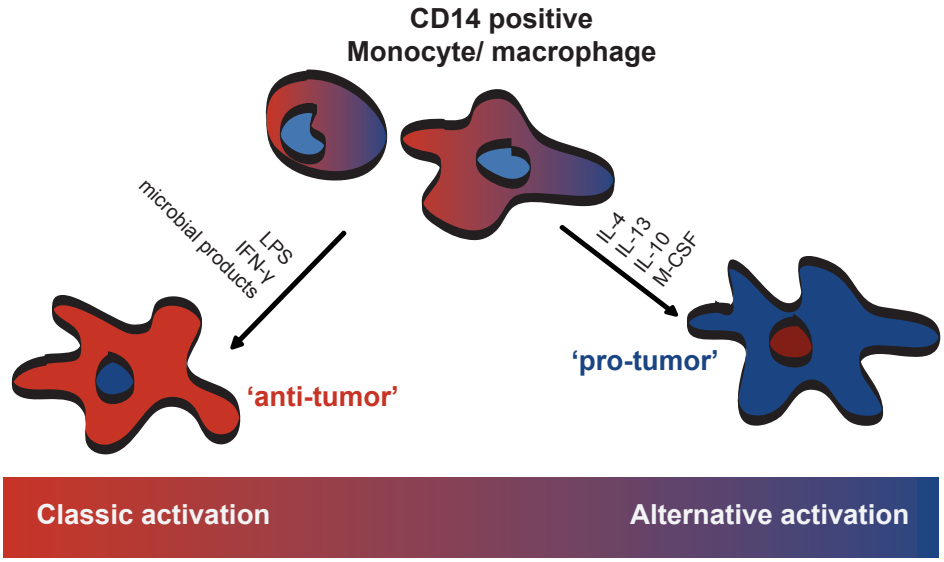

Fig. 1.5 M1 and M2 type macrophage activation represent extremes of a spectrum. Classically activated M1 type macrophages exhibit anti-tumor characteristics such as high expression of pro-inflammatory cytokines. Alternatively activated M2 type macrophages support tumor growth through tissue homeostatic features such as expression of matrix degrading proteins and support of angiogenesis. Adapted from [5] and [237].

cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 in conjunction with loss of expression of the ligands for the inhibitory NK cell receptors Killer Immunoglobulin-like Receptors (KIRs) and the C-type lectin heterodimer CD94/NKG2A on tumor cells can render them susceptible to NK cell mediated cytotoxicity (Fig. 1.6) [20;26;38;64;118;135;178;186;213;272].

Although NK cells have the natural ability to lyse tumor cells without the need for prior sensitization, NK cell mediated anti-tumor activity can be enhanced through the administration of NK cell activating cytokines such as IL-2 and IL-15, or the adoptive transfer of *ex vivo* activated NK cells [268;275]. There are several lines of evidence to suggest that treatment with NK cells or NK cell activating agents may be an effective adjunct to treatment of osteosarcoma patients. Previous pre-clinical studies have shown that osteosarcoma cell lines are sensitive to NK cell mediated cytolysis [104;132;143;156-159;165;191]. A murine model of post-operative osteomyelitis in osteosarcoma, in which anti-tumor activity was dependent on NK cells and monocytes, suggests that NK cell-mediated cytotoxicity also occurs *in vivo* [242]. A small cohort of osteosarcoma patients was treated with IL-2, which resulted in NK cell activation and a better outcome [151]. Interferon-α is an anti-proliferative and immunomodulatory cytokine,

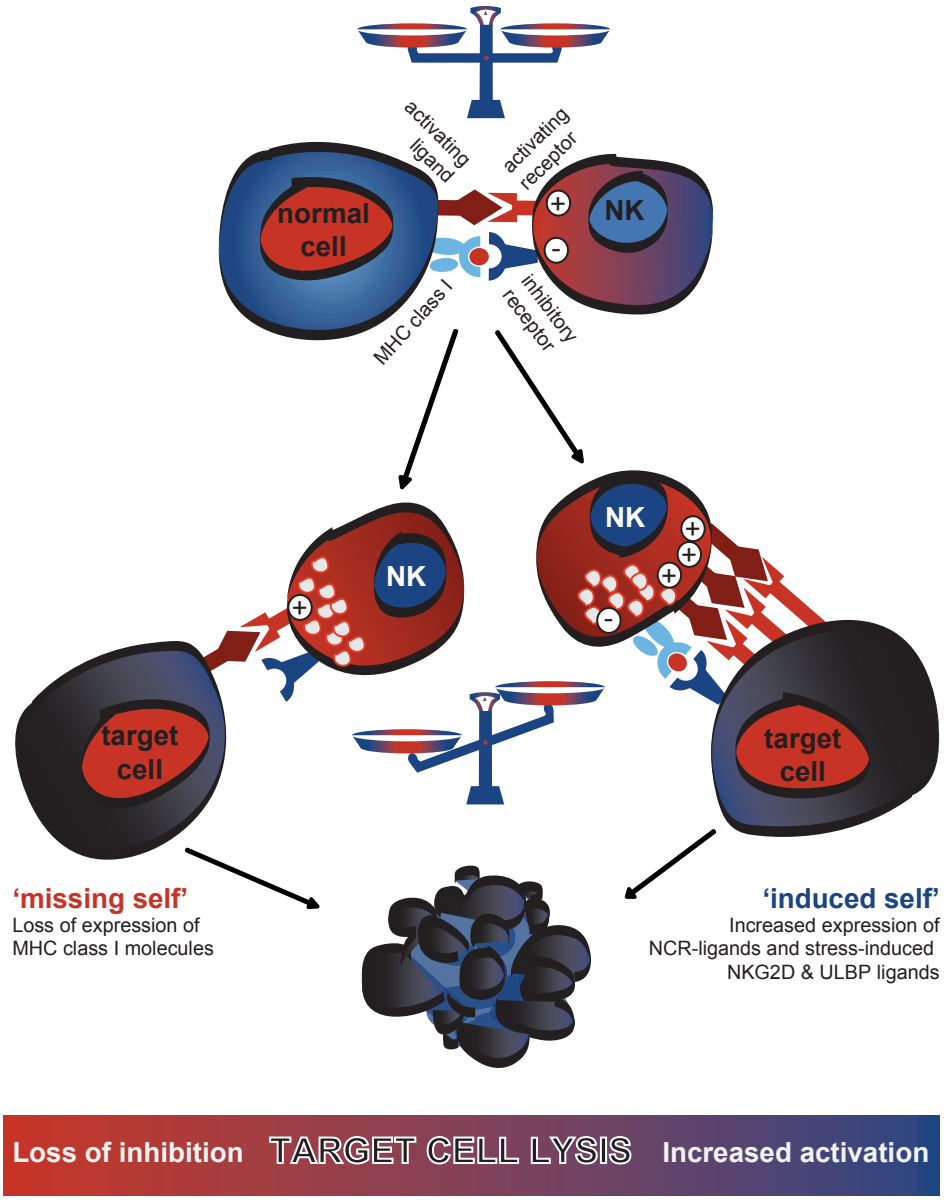

Fig. 1.6 NK cells engage in target cell lysis when the balance between inhibitory and activating signals is shifted towards activation. Adapted from [213].

administration of which results in activation of immune cell subsets such as monocytes and NK cells, in addition to direct anti-proliferative effects on tumor cells [31;62;154]. In Scandinavia, the addition of interferon to the treatment regimen of osteosarcoma patients resulted in a better outcome as compared to historical controls [249]. The efficacy of adjuvant interferon (IFN)-

α-2b is currently under study in the EURAMOS-1 trial. Preliminary data suggests no benefit, but follow-up of patients is ongoing [25;160;278].

AIMS AND OUTLINE OF THIS THESIS

A better understanding of the pathogenesis of high-grade osteosarcoma has the potential to identify novel targets for (immuno-)therapeutic interventions. MSCs are the proposed cell of origin of osteosarcoma. In **chapter 2**, the results of long term *in vitro* culture and genetic analyses of MSCs from osteosarcoma patients and healthy donors is described, to determine if MSCs from osteosarcoma patients are predisposed to malignant transformation. The main cause of death for osteosarcoma patients is pulmonary metastatic disease. In **chapter 3**, prognostic factors in pulmonary metastasized osteosarcoma patients are identified. This will aid in determining which patients are most likely to benefit from novel treatment modalities. In the next chapters, the constitutive *in vivo* interactions between the innate immune system and high-grade osteosarcoma are studied. Possible avenues for therapeutic exploitation of anti-osteosarcoma immunopotency are studied through *in vitro* manipulation of immune and target cells. In **chapter 4** the prognostic significance of intratumoral infiltration with macrophages is presented. In **chapter 5** cytotoxic activity of NK cells towards (chemotherapy resistant) osteosarcoma cells is studied. The recently closed EURAMOS-1 trial randomizes for treatment with interferon-α in patients with good histological response to neo-adjuvant chemotherapy [160]. In **chapter 6**, the molecular and functional effects of interferon treatment on the activation and anti-tumor activity of peripheral blood lymphocytes and monocytes of osteosarcoma patients, both *in vivo* and *ex vivo* is shown. In **chapter 7**, the findings of this thesis are summarized and discussed, and a view towards the direction of further studies is presented.