Advances in Experimental Medicine and Biology 804

Eugenie S. Kleinerman *Editor*

Current Advances in Osteosarcoma

Springer

Advances in Experimental Medicine and Biology

Volume 804

For further volumes:
http://www.springer.com/series/5584

Eugenie S. Kleinerman
Editor

Current Advances in Osteosarcoma

Springer

Editor
Eugenie S. Kleinerman, M.D.
Division of Pediatrics
Department of Cancer Biology
The Mary V. and John A. Reilly
Distinguished Chair
University of Texas M.D. Anderson
Cancer Center
Houston, TX, USA

ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-3-319-04842-0 ISBN 978-3-319-04843-7 (eBook)
DOI 10.1007/978-3-319-04843-7
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014938103

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

To my father, Jerome I. Kleinerman, M.D., who was Chair of Pathology at Mt. Sinai Medical School in New York City and later MetroHealth Medical Center, Case Western Reserve University School of Medicine. He inspired me to love the discipline of medicine, laboratory research, and the importance of education. During his distinguished career as a pulmonary pathologist, his research integrated clinical medicine and experimental models of lung disease in an effort to improve the health of people with occupational and chronic obstructive lung disease and lung cancer. He was my first role model, a man of ethics and conviction. His memory and words of wisdom continue to guide and inspire me.

This book is also dedicated to my mother, Seretta Miller Kleinerman, who preached and fought for equal opportunity for women in the work place; a woman way ahead of her time. She pounded into my head that career and family were not mutually exclusive. Yet, she also stressed the importance of maintaining a lady-like decorum, insisting on perfect manners and gracious behavior.

My sisters and I often said we would write a book entitled "Seretta Says."

Finally, I thank my husband, Dr. Leonard Zwelling, for supporting, encouraging, and believing in me. He gave me the strength I needed to push through the insecurities that stem from being a professional woman and mother.

Eugenie S. Kleinerman, M.D.

Oseosarcoma: The State of Affairs Dictates a Change. What Do We Know?

Can We Apply These Discoveries and Alter Clinical Research Practices to Achieve Success?

Osteosarcoma continues to claim the lives of too many children, adolescents, and young adults. Being a rare cancer *and* a pediatric cancer, the resources allocated to finding a cure and improving outcomes have been and will continue to be sparse. This is why, as we move forward, we must be judicious and strategic in the selection of which new agents we incorporate into our clinical treatment regimens and the clinical trial design constructed to assess the activity of these new agents. Experience and multiple clinical trials have defined an accepted 3-drug chemotherapy regimen that results in a 65–70 % overall survival at 5 years. However, clinical trial after clinical trial adding additional chemotherapeutic agents to this 3-drug backbone failed to have an impact with no improvement in outcome since 1987. This is an unacceptable statistic. We need to recognize that we have achieved what we can with combination chemotherapy and move on.

The era of "targeted therapy" based on genomics and proteomics of the tumor cells has emerged. Genomic analysis of tumor tissue has identified potential targets for other solid tumors. However, the genetic signatures from individual osteosarcoma patient samples and even different metastatic tumor nodules in the same patient are not consistent. Furthermore, tumor cells do not grow in isolation. In my opinion, this approach will fail therapeutically unless we also understand (a) the interactions between the osteosarcoma cells and the lung microenvironment (the most common site of metastases); (b) which molecular pathways are altered epigenetically that permit bone cells to grow in the lung; and (c) how the osteosarcoma cells circumvent the immune response. We also need to understand how the osteosarcoma cells adapt to the lung microenvironment.

Recognizing the success of using chemotherapy to treat newly diagnosed osteosarcoma patients but also admitting that we have reached a plateau using this approach dictates that we must incorporate non-chemotherapy agents into our current 3-drug regimen to improve patient outcomes. Such new agents can include

those that target the dysregulated pathways that have been identified in the tumor cells, the tumor microenvironment, and the immune response.

How best to combine the new agent with chemotherapy and how to interdigitate it into the treatment schema based on our knowledge of the agent's target and whether chemotherapy can help or interfere must be a primary focus. This book has been compiled to bring the latest findings in regard to these three areas. National and international authorities have summarized the historical perspectives and their own laboratory research in an effort to provide a single resource to serve as the starting point as we move forward in designing novel therapeutic strategies. We cannot continue to merely add one new agent and measure success by evaluating response in the setting of bulky, visible relapsed disease. This has been our approach for the last 40 years. It was successful in identifying the active chemotherapy agents, but it is not appropriate for assessing immunostimulatory agents, agents that target the tumor microenvironment, or even agents that target specific pathways. In addition, we cannot continue to assess activity by tumor shrinkage. Agents that activate an immune response resulting in immune cell infiltration into the tumor may be interpreted as tumor progression if response is judged by radiographic measurements. Without histologic evaluation, we cannot decipher whether an enlarged mass is growing tumor or the result of immune cell infiltration, dead amorphous tissue, and edema. We must incorporate histologic evaluation and biologic measures that confirm that the chosen agent's target is being affected. Proper resources must be devoted and carefully designed clinical trials must be implemented. It is imperative that we use the discoveries made by the authors in this book to design our clinical trials, keeping in mind the biology of the tumor. If we do not implement such changes in our clinical research practice, we will continue to struggle and fail.

In this spirit, I express my gratitude to all of my distinguished colleagues for their willingness to contribute to this book. Without their assistance and their expertise, this project would not have been possible. It is my hope that the information in this book will provide inspiration, data, and the rationale needed to change the way we practice clinical research and design our clinical trials for patients with newly diagnosed and relapsed osteosarcoma.

Houston, TX, USA

Eugenie S. Kleinerman, M.D.
V. Mary
A. John

Acknowledgement

With gratitude, I acknowledge the expertise and dedicated hard work of my assistant, Ms. Jeanette Quimby, who was essential to the completion of this book.

Contents

Historical Perspective on the Introduction and Use of Chemotherapy for the Treatment of Osteosarcoma

Norman Jaffe

Abstract Chemotherapy for treatment of osteosarcoma was demonstrated to be effective in eradicating primary tumor and pulmonary metastases in the mid-twentieth century. The first agents that held promise were doxorubicin and high-dose methotrexate with leucovorin (citrovorin factor) in the mid-1970s. Since then, other agents that can eliminate or cause regression of tumor have been discovered: cis-diamminedichloroplatinum II (cisplatin) and the oxazaphosphorines ifosfamide and cyclophosphamide. Additional agents await further study to define their potential. The effective agents have been utilized in various combination regimens and have escalated the survival rate from <10 to 75 %. They have also enabled pulmonary metastectomy in patients with persistent and/or recurrent pulmonary metastases and tumor ablation and limb salvage in 80 % of newly diagnosed patients. Unfortunately, however, despite these impressive advances no change in survival expectancy of patients with osteosarcoma during the past 40 years has occurred. There have been no new chemotherapeutic agents effective in addressing disease that is resistant to current agents; the few that have been introduced await further study to substantiate their efficacy. This also includes attempts at alternate administration of chemotherapy (intra-arterial and inhalation therapy.) In this chapter, we provide an account of the sequential introduction of the chemotherapeutic agents, review the results of their application in selected regimens, and discuss the role of neoadjuvant chemotherapy.

Keywords Osteosarcoma • Chemotherapy • Neoadjuvant therapy • Limb salvage • Current status

N. Jaffe, M.D., Dip., Paed, D.Sc. (✉)
Division of Pediatrics, Children's Cancer Hospital, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
e-mail: njaffe@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_1,

Introduction

Major progress in the treatment of osteosarcoma was achieved in the last century. Developments took wing as a consequence of converging discoveries in disciplines deployed to address the treatment of this disease. Innovations evolved after it was shown that a variety of chemotherapeutic agents were effective in treating the tumor. Chemotherapy acted as a catalyst for a dynamic change in perspective. Disciplines were effectively integrated into multidisciplinary strategies and a new outlook for cure and limb preservation emerged. This chapter chronicles the discoveries in chemotherapy and the standard of care today.

Rationale for the Application of Chemotherapy

In 1879, Samuel Gross of Philadelphia published a manuscript entitled "Sarcoma of the long-bones: based upon a study of 165 cases" [1]. The majority of the cases, if not all, were probably osteosarcomas. Gross advocated early amputation although the operative mortality rate at that time was 30 %: limb salvage inevitably led to local recurrence, distant pulmonary metastases, and death. Despite this aggressive approach survival rates did not improve. Amputation remained the "standard and accepted" form of treatment for the ensuing 60–70 years. This led to the realization that silent pulmonary micrometastases were present in the majority of patients at diagnosis. These metastases were not evident on conventional radiological imaging but surfaced 6–9 months after amputation and were responsible for the patients' demise. The survival rate was invariably under 20 % (usually 5–10 %). This observation was supported by a review of the medical records of 78 patients treated at the Dana Farber Cancer Center and by several other publications [2–4]. It constituted the rationale for the hypothesis that osteosarcoma, despite its local presentation, should be considered a local *and* systemic disease and as such requires local *and* systemic treatment for cure. Systemic treatment generally implied the administration of chemotherapy.

Effective Chemotherapeutic Agents

Prior to the 1960s, osteosarcoma was considered a chemoresistant tumor. The agents investigated had yielded inconsistent, mediocre, or unconfirmed results. They included L-phenylalanine mustard (response, 16 %) [5, 6], mitomycin C (response 24 %) [7, 8], cyclophosphamide (response, 15 %) [9–12], vincristine [12, 13], 5-fluorouracil (complete remission reported in one of two patients) [14], and nitrogen mustard (Jaffe N, unpublished data, Dana Farber Cancer Institute). A regimen comprising mitomycin C, phenylalanine mustard, and vincristine proved fruitless [15]. However, commencing in the mid-1970s, a glimmer of hope emerged with the introduction

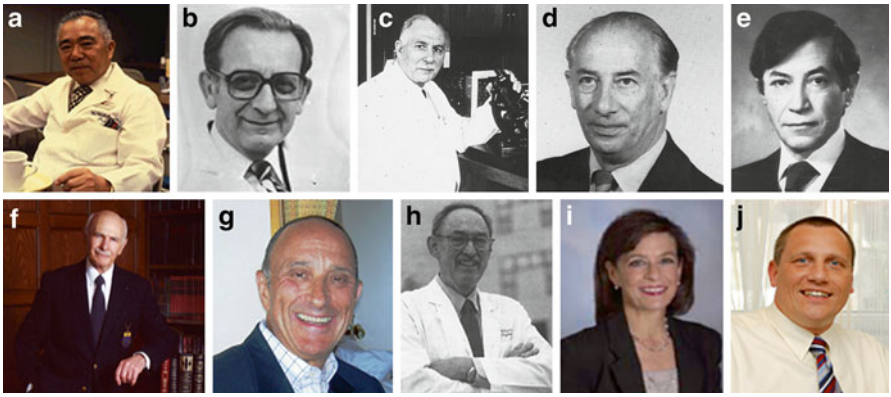


Fig. 1 Investigators and Outline of their contributions. (**a**) Wataru Sutow introduced Compadri regimen. (**b**) James Holland investigated doxorubicin. (**c**) Sidney Farber formulated methotrexate. (**d**) Abraham Goldin designed methotrexate "rescue." (**e**) Isaac Djerassi investigated methotrexate rescue in leukemia. (**f**) Norman Jaffe investigated methotrexate rescue in osteosarcoma. (**g**) Gerry Rosen pioneered neoadjuvant chemotherapy (T10). (**h**) Charles Pratt. Investigator. (**i**) Eugenie Kleinerman pioneered MTP-PE. (**j**) Stefan Bielack. Principal investigator EURAMOS

of combination chemotherapy regimens initially designated "Conpadri" [16, 17]. The major components of the regimens were an agent that had been recently discovered to be effective, doxorubicin, and later high-dose methotrexate with leucovorin "rescue." This advance was followed by the discovery of other active agents: cisplatin and, when given at higher doses than conventionally administered at that particular time, the oxazaphosphorines ifosfamide and cyclophosphamide. Administration of the higher doses was facilitated by the introduction of the adjuvant mesna to reduce their toxicity. The agents were integrated and combined into a variety of chemotherapeutic regimens.

Conpadri and Compadri Regimens

Historical Perspective

The Conpadri regimens, devised by Wataru Sutow (Fig. 1a) [16, 17], originated as follows. Cyclophosphamide was initially shown to be effective in rhabdomyosarcoma [11]. In the early 1970s, it was incorporated into a combination regimen, VAC (*v*incristine, *a*ctinomycin D, and *c*yclophosphamide), for the treatment of soft tissue sarcoma [18]. The combination was found to be highly effective, which prompted its investigation in osteosarcoma. The use of cyclophosphamide probably also derived from a report by Pinkel in 1962 that documented partial regression of an osteosarcoma lung metastasis with the administration of this agent [11] (The published radiograph of the response is suggestive that radiation therapy may also have

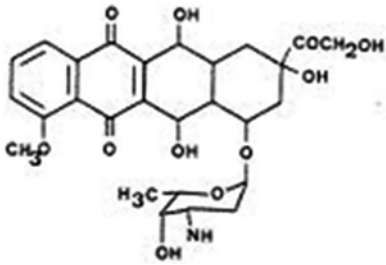


Fig. 2 Doxorubicin (Adriamycin)

been given). An intensive pulsed cyclophosphamide regimen was adopted based upon the report by Finkelstein et al. [9]. To the VAC combination, phenylalanine mustard was added based upon the studies of Sutow and Sullivan [5, 6]. With the demonstration that doxorubicin was highly effective in osteosarcoma, actinomycin D was replaced by doxorubicin. Thus, the resulting regimen, Conpadri, comprised *c*yclophosphamide, vincristine (brand name *Onc*ovorin), *p*henylalanine mustard, and doxorubicin (brand name *Adri*amycin) [16]. Later, when methotrexate and leucovorin were shown to be effective, methotrexate was added (Co*m*padri) [17]. The regimen yielded a 41 % disease-free survival rate 5 years following their discovery when used as postoperative adjuvant therapy after ablation of the primary tumor [19].

Doxorubicin

Historical Perspective

Doxorubicin was discovered by the Italian research company Farmitalia in the 1950s (Fig. 2). The mechanism of action is probably multifactorial. A major form of activity is its ability to intercalate into DNA and induce topoisomerase II-mediated single- and double-strand breaks in DNA. Doxorubicin may also induce topoisomerase II-mediated DNA cleavage by nonintercalative mechanisms. Through intercalation, doxorubicin can induce histone eviction from chromatin. As a result, DNA damage response at the epigenome and transcriptome levels is deregulated in doxorubicin-exposed cells.

Doxorubicin was found to be active in disseminated osteosarcoma [20]. Investigations in osteosarcoma were conducted principally under the direction of James Holland (Fig. 1b). Initial clinical reports of its activity alone and in association with dacarbazine (dimethyl triazeno imidazole carboxamide, or DTIC) produced responses in 35–40 % of patients with pulmonary metastases [21–25]. The responses developed within 1–2 months with doses of 30–35 mg/m^2/day administered for 3 days at 3- to 4-week intervals. When administered as the sole agent after ablation of the primary tumor, doxorubicin also improved survival rates [26]. It could also potentiate the effect of radiation therapy [27].

Current Practice

Doxorubicin may be administered as a single-bolus injection, short infusion (e.g., 6 h), or continuous infusion over 24, 48, or 72 h. It is a frequent component of combination regimens. The schedule of administration may vary particularly if the dose is divided: daily for 2–3 days, weekly, or tri-weekly. The conventional dose is 60–75 mg/m^2, every 3 weeks and may be administered as a single pulse or divided into three daily doses. The drug is administered intravenously; extravasation may cause ulceration. In a study of its intra-arterial administration over 24 h in combination with radiation (3.5 Gy) to treat the primary tumor, over 75 % tumor destruction was reported in 24 of 36 patients [28]. The procedure was complicated by erythema and ulceration of the skin and subcutaneous tissue in several patients. Selective entry of the drug into a small vessel was implicated, and it was suggested that the complication might be averted by positioning the catheter in a large-caliber vessel proximal to the tumor. However, in view of the possibility of ulceration, which may preclude a limb-salvage procedure, the intra-arterial approach has generally been abandoned.

Because doxorubicin may cause cardiac failure, several strategies have been used to minimize the likelihood of this complication. The cumulative dose is generally limited to 300 mg/m^2 in children under 6 years of age and in patients exposed to precordial radiation; in older patients, it is limited to 400–500 mg/m^2. It has been suggested that this complication is less likely to occur if the medication is administered over a more prolonged period (24–48–72 h) as opposed to a "pulse" infusion (e.g., 30 min–2 h). However, prolongation of the infusion, while possibly reducing the incidence of cardiac toxicity (as well as nausea and vomiting), may enhance mucositis and myelosuppression. Dexrazoxane has been used as a cardioprotective agent apparently with some success [29]. A liposomal formulation of the drug for a more prolonged infusion is available for use in patients with breast cancer. Despite its potential to cause cardiac failure, doxorubicin has been accepted as an integral component of most chemotherapeutic regimens for osteosarcoma. It has been claimed to be the most effective agent for the treatment of this tumor type [30].

High-Dose Methotrexate

Historical Perspective

The discovery of methotrexate as an anticancer agent (initially in leukemia) is generally attributed to observations by Lucy Wills and Sidney Farber. Lucy Wills, working in India in 1937, studied the effects of folic acid; it appeared to stimulate the proliferation of leukemic cells in children with acute lymphoblastic leukemia. Sidney Farber (Fig. 1c), a pathologist at the Children's Hospital Medical Center in Boston, MA, noted a similar phenomenon and considered the possibility of administering folate analogues antagonistic to folic acid to block the function of

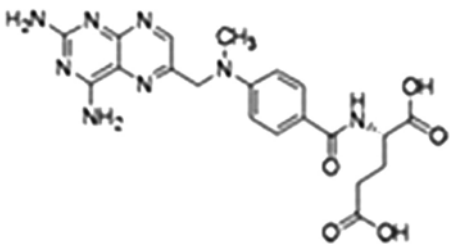


Fig. 3 Methotrexate

folate-requiring enzymes. This was an example of rational drug design rather than accidental discovery. Farber, in collaboration with Harriet Kiltie and Yellapragada Subbarao, director of Lederle Laboratories, produced a folate analogue, aminopterin. Remission was induced in children with acute lymphoblastic leukemia [31]. The rationale proved sound and successful. Aminopterin was somewhat toxic and was modified to amethopterin (methotrexate).

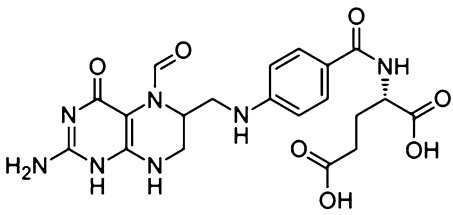
Methotrexate is a structural analogue of folic acid, a cofactor in the synthesis of purines and pyrimidines (Fig. 3). It exerts its activity during the S phase of the cell cycle and binds stoichiometrically and irreversibly to dihydrofolate reductase (DHFR). The latter is the enzyme responsible for converting folates into their chemically active reduced form, tetrahydrofolate. 10-formyltetrahydrofolate acts as a single carbon donor in the de novo purine synthetic pathway. 5,10-methylene tetrahydrofolate donates its single carbon group and is oxidized to dihydrofolate in the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) by thymidylate synthase. Through this process, methotrexate depletes intracellular tetrahydrofolate pools. This causes depletion of purines and thymidylate and inhibition of DNA synthesis. Accumulation of partially oxidized dihydrofolic acid resulting from the inhibition of DHFR also appears to contribute to the inhibition of de novo purine synthesis.

Upon entry into a cell methotrexate is rapidly and tightly bound to DHFR, the uptake into the target cell essentially being unidirectional. Once the binding sites are saturated, methotrexate is metabolized to polyglutamated derivatives. Methotrexate polyglutamate is also a potent inhibitor of DHFR and is capable of inhibiting other enzymes in the synthesis of thymidine and purine, which are required for DNA synthesis.

Resistance to methotrexate may be innate or acquired. The mechanisms of resistance include decreased membrane transport (into the cell); increased levels of, and altered affinity for, DHFR; decreased polyglutamination of methotrexate; decreased thymidylate activity; and amplification of gene encoding for DHFR.

In an effort to surmount the problem of decreased transport activity, investigators increased the drug concentration and exposure time in the extracellular environment. This practice was based on the premise that intracellular concentration is a function of extracellular concentration (C) and time (T), ($C \times T$). It was surmised that increased intracellular concentration could possibly be attained (in time) by passive diffusion across the cell membrane. Fyfe and Goldman also suggested that methotrexate uptake by tumor cells could be enhanced by pretreatment with vincristine [32]. This was the rationale for pretreatment with vincristine in the early clinical trials in osteosarcoma; however because it was suspected to have caused toxicity in several

Fig. 4 Leucovorin



patients it was later abandoned. Occasional toxicity notwithstanding was still encountered after discontinuing vincristine but was prevented, aborted or ameliorated with monitoring serum methotrexate levels.

Leucovorin (5-formyltetrahydrofolate, formerly known as citrovorin factor or citrovorum factor), is the antidote to methotrexate (Fig. 4). It is available in levo (L) and dextro (D) forms. The "L" form is biologically active and is utilized. It competes with methotrexate for entry into the cell using the same transport mechanism. Within the cell, it is converted to 5,10-methylenetetrahydrofolate and 5-methyltetrahydrofolate, thereby replenishing folate pools. By this process, it supplies the product surceased by methotrexate activity.

High-Dose Methotrexate "Rescue" in Osteosarcoma

The concept of leucovorin rescue is derived principally from experiments by Goldin et al. (Fig. 1d) [33]. They administered large doses of methotrexate, followed by leucovorin after a delayed interval, to leukemia-bearing mice. This destroyed the leukemic cells and simultaneously protected normal host tissues, yielding an improved therapeutic ratio. Rescue may also be related to homeostatic mechanisms. For example, methotrexate causes temporary inhibition of DNA synthesis for variable periods. Normal tissues may tolerate such inhibitions better than tumor cells, while tumor cell viability becomes severely compromised. Also, normal bone marrow cells may reenter the cycle rapidly and replenish original cell numbers. In contrast, tumor cells may have a delayed recovery after methotrexate perturbation, permitting enhanced activity by immune mechanisms or other chemotherapeutic agents. Selective rescue may also be related to the reduced amount of folate. Some tumor cells may require more of this substance than do normal cells.

Osteosarcoma is resistant to conventional doses of methotrexate because of an absent transport binding mechanism. However, it may still be susceptible to high doses of methotrexate. The exact mechanism of action is not fully established, but is postulated to be related to the transport mechanism that methotrexate shares with naturally occurring folates. The extracellular concentrations attained with the massive doses of methotrexate over time ($C \times T$) are apparently sufficient to force methotrexate to enter the osteosarcoma cell by passive diffusion. In contrast, methotrexate enters the normal cell by passive diffusion *and* the binding mechanism. The antidote, leucovorin, also enters normal cells via the common binding mechanism but, in the absence of a transport mechanism, is unable to enter osteosarcoma cells.

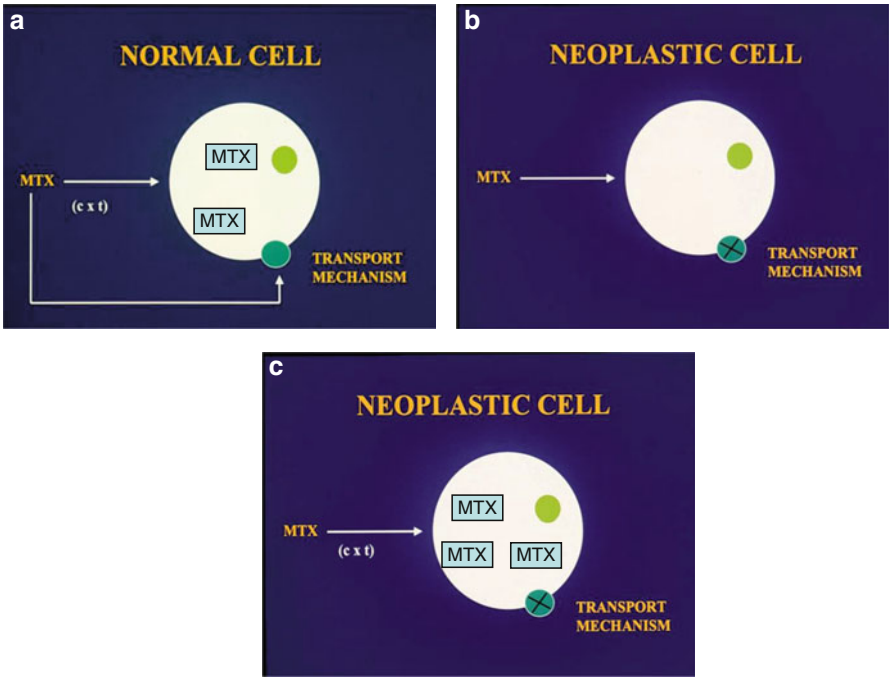


Fig. 5 Putative mechanism for methotrexate rescue. (**a**) Normal cell can absorb methotrexate and leucovorin through an inherent transport mechanism. (**b**) Absent transport mechanism in osteosarcoma cell. (**c**) Methotrexate enters neoplastic cell via passive diffusion ($C \times T$). Leucovorin unable to enter neoplastic cell and self destructs

The high intracellular methotrexate concentration in the absence of antidote causes the osteosarcoma cell to self-destruct. Concurrently the normal cell with an active transport mechanism for folate (leucovorin) is rescued (Fig. 5).

Clinical Application of High-Dose Methotrexate in Osteosarcoma

High-dose methotrexate with leucovorin rescue was first investigated clinically by Isaac Djerassi (Fig. 1e) in children with resistant leukemia and lymphoma reportedly with some success [34]. This intriguing and novel approach prompted the author (N. Jaffe Fig. 1f) to consider the possibility of investigating the high dose methotrexate regimen in osteosarcoma. The saga of the trials and tribulations in attempting to introduce this treatment in osteosarcoma, including the controversy related to historical controls and finally establishing proof of its efficacy are worthy of note. A brief chronicle of the events is presented. It originated in the Children's Cancer Research Foundation complex adjacent to the Children's Hospital Medical Center in Boston, MA.

Sidney Farber held a weekly tumor board conference on the fourth floor of the Jimmy Fund building in the Children's Cancer Research Foundation complex.

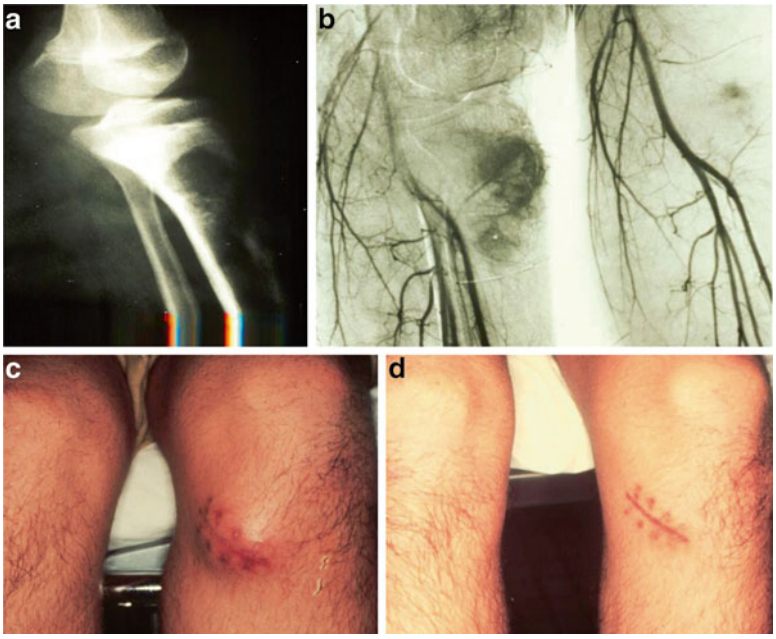


Fig. 6 Methotrexate–leucovorin effect on osteosarcoma of tibia. (**a**) Primary tumor proximal tibia. (**b**) Angiogram with tumor vascularity and disappearance after treatment with seven courses of therapy. (**c**) Clinical appearance of tumor at presentation. (**d**) Disappearance of tumor mass at completion of therapy

Cases were presented, management was discussed, and decisions were reached in a setting of mixed modern contemporary and anachronistic old world formality. The members of his staff always arrived early and rose when he entered the meeting room. They would then take their seats at his request. As Clinic Administrator, the author prepared the agenda of cases for discussion. In 1967, with Dr Farber's prior consent, he invited Isaac Djerassi to present his data on patients with resistant leukemia and lymphoma treated with high-dose methotrexate. The presentation was well received.

After the meeting the author approached Farber for permission to administer the high dose methotrexate regimen to a patient with osteosarcoma who had developed pulmonary metastases after a hemipelvectomy. Surveillance committees and Institutional Review Boards had not been mandated or established at that time. Permission was granted. A response was obtained: the pulmonary metastases were eradicated [35]. This result provided justification for administering high-dose methotrexate postoperatively alone and in combination with other agents after ablation of the primary tumor. The rationale was based upon studies reported by Skipper et al., Laster et al., and Schabel [36–38]. Several publications ensued demonstrating the role, toxicity, and efficacy of the regimen alone and in combination regimens for osteosarcoma [39–44]. Improved survival was achieved. When administered preoperatively to the primary tumor, the regimen also enhanced the opportunity for limb salvage by destroying tumor and downstaging its status (Fig. 6).

Unfortunately, the introduction of high-dose methotrexate was confronted by controversy [45]. Historical-control survival rates (<10 %) had been used as a comparison to demonstrate methotrexate's efficacy, and their use was impugned: investigators at the Mayo Clinic suggested that there had been a "spontaneous" improvement in the survival of patients with osteosarcoma due to early diagnosis and other factors [46]. The argument was bolstered by a Mayo Clinic trial in which concurrent controls were used comparing amputation and methotrexate with leucovorin rescue versus amputation alone [47]. There was no improvement with the administration of methotrexate (survival ~40 % in both arms). That report gained traction with publication of an editorial refuting the role of methotrexate in osteosarcoma [48]. In contrast, several publications were presented in defense of adjuvant therapy and the use of historical controls: historical controls were reported from the MD Anderson Cancer Center in Houston, TX [49], St Jude's Children's Research Hospital in Memphis, TN [43], Memorial Sloan-Kettering Cancer Center in New York, NY [50], Dana Farber Cancer Center [51], and the Rizzoli Institute in Italy [52], and the comprehensive review compiled by Friedman and Carter [4]. The reports all confirmed an historical survival of <20 % (usually 5–10 %). Reference was also made to the successful use of preoperative chemotherapy to facilitate limb salvage. Many patients were offered (and accepted) the opportunity to undergo limb salvage under this strategy instead of amputation. However, it was claimed that the effects of such chemotherapy were "conjectural" [53].

The atmosphere became contentious, controversial, and emotionally charged. Neither friend nor foe at that stage gave succor to the use of the newly discovered chemotherapeutic agent. A call was made to conduct a prospectively randomized study utilizing concurrent as opposed to historical controls to demonstrate the asserted benefits of chemotherapy [54].

Resolution of the controversy was finally attained with the publication of two studies in which the efficacy of postoperative adjuvant therapy was indeed confirmed in comparison with a concurrent control group [55, 56]. The first study was the Multi-institutional Osteosarcoma Study (MIOS). Several of the investigators of the trial included physicians who had previously been coauthors of the methotrexate communications attesting to its efficacy. It was published first [55]. The second study by Eilber et al. was presented at a meeting of the American Society of Clinical Oncology (ASCO) and then published officially in the Society's journal [56]. The survival rates in both studies utilizing postoperative adjuvant chemotherapy that included methotrexate were ~65 %; in contrast the concurrent control curves (no postoperative chemotherapy) were superimposable on those of historical controls: survival rates of <10 %! A follow-up of Eilber's et al.'s trial was presented at the 2012 meeting of the Connective Tissue Oncology Society in Prague [125]. The patients who received chemotherapy had sustained improved survival over the past 30 years.

The author in a 1968 letter to the *New England Journal of Medicine* censured the implementation of the MIOS trial [57]. The letter emphasized the consistency of survival rates in a large number of historical controls published by different

institutions over many years: the biological behavior of the disease in the absence of effective treatment had been consistent and firmly established. The MIOS study had been forged on the anvil of scientific rigor that denied treatment claimed and demonstrated to be effective, to patients in the concurrent control arm. Several parents experienced self-recrimination and grief because they had given consent for their children to be randomized to the untreated control arm followed by demise from pulmonary metastases.

An analysis of Eilber et al.'s trial was presented by James Holland at the ASCO meeting and later addressed in an editorial in the *Journal of Clinical Oncology* when the trial was published [58]. He berated the authors at the meeting for conducting a trial with a "no-treatment arm." In the editorial, he recommended implementation of minimum standards in embarking on a major trial. These included a thorough review of the literature and an in-depth study of the raw data on which the proposal rested. He emphasized the need to assess past experience with the candidate disease in one's own clinical setting and to assess the feasibility of a pilot study of the candidate regimen. Finally, he emphasized the costs in human and economic terms.

An unsolicited letter sent to the author from Charles Pratt, a respected investigator who was present at the ASCO meeting, refers to Holland's criticism and the problems and controversy extant during this period (Fig. 7).

Following publication of the two trials, high-dose methotrexate with "rescue" and other effective agents were adopted for the treatment of patients with osteosarcoma. As adjuvant therapy, alone and in combination with other agents, it produced disease-free survival rates of 40–65 % [59]. A composite graph outlining published results over the ensuing period and comparative historical controls is depicted in Fig. 8.

Methotrexate was the first active agent formally subjected to a randomized trial to test its efficacy against another effective agent (cisplatin) in the treatment of the primary tumor [60, 61]. Cisplatin was more effective: its response rate was 60 % compared with 30 % for methotrexate. The response rate in the treatment of overt (pulmonary) metastases with methotrexate is ~25 %.

Current Practice

Methotrexate with leucovorin rescue is optimally administered as four courses in 10- to 14-day intervals. The dose is 10–12.5 g/m^2 administered over 4–6 h in 600 cm^3 of fluid followed by leucovorin rescue 10 mg as a loading dose at 6 h after initiation of treatment. Leucovorin 10 mg at 6 h intervals is again administered 24 h after commencement of therapy and maintained at the same dose at 6 h intervals. It may be discontinued when a serum methotrexate level of 0.1–0.3 mol/L is obtained. Intravenous fluids should be maintained at 3 L/m^2/24 h. In some centers, the dose of leucovorin is calculated according to the weight or body surface area.

Attempts should be made to obtain an optimum serum Methotrexate level of 1,500 μmol/L or higher at 6 h. The Safety of administration may be enhanced by monitoring the decay curve and increasing hydration and the leucovorin dose when higher than

Dear Norman:

I requested a copy of the interview that you had with Margaret Pierson (sic), so that I could read what your historical perspective on the treatment of osteosarcoma would contain.

While I was not at the Children's Cancer Research Foundation with you, I do count our relationships that started in 1972 at a meeting at the National Cancer Institute. At that time I had waiting for me a patient with osteosarcoma of the frontal bone which had enlarged as to almost cover the eyes. I was stopped on the way to the plane in Washington to return to Memphis and had to go to Richmond because of the death in the family. Accordingly while I was away, Don Pinkel treated the patient with 50 mg/kg of high dose methotrexate, a daring first step.

I agree with you as to the sentiments related to the MIOS, but remember the yelling throughout the auditorium in some city by Jim Holland and others.

I think that the bottom line is that we have come a long way and been reasonably productive in our careers and I think that the results are manifested by our survivors by their numbers and quality of life.

With all best regards and wishes.

Yours sincerely,

Charles B. Pratt, M.D.

Fig. 7 Unsolicited letter received from Charles Pratt, a respected investigator and Southern gentleman. He diplomatically characterizes the controversy as "yelling." The interview refers to Pearson M (1998) Historical perspective of the treatment of osteosarcoma: an interview with Dr Norman Jaffe J Pediatr Oncol Nurs 15: 90–94

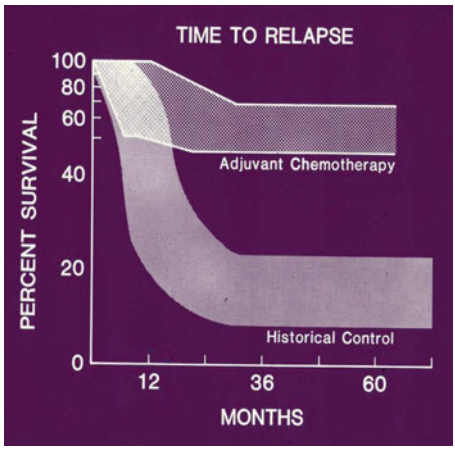


Fig. 8 Composite graph of reports of survival of patients treated with chemotherapy. Historical controls depicted as a comparison

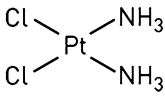


Fig. 9 Cis-diamminedichloroplatinum II

generally encountered serum methotrexate levels are detected [62]. Renal dialysis and Caboxypetidase-2 should be considered for decay levels which are abnormal or excessive and impending signs of renal and hepatic problems. Side effects of methotrexate with inadequate rescue or failure include gastrointestinal mucositis, myelosuppression, hepatic and renal dysfunction, and (rarely) neurological abnormalities.

Cis-Diamminodichloroplatinum

Historical Perspective

In 1965, reminiscent of the discovery of penicillin, Barnett Rosenberg et al. of Michigan State University discovered that electrolysis of platinum electrodes generated a soluble geographic platinum complex that inhibited binary fission in *E. coli* bacteria [63]. Subsequent investigations revealed that cis-$PtCl_2(NH_3)_2$ (cis-diamminedichloroplatinum II, commonly known as CDDP or cisplatin) was highly effective in causing regression in the majority of sarcomas in rats. Confirmation of this discovery and extension of testing to other tumor cell lines launched the clinical investigation and application of cisplatin (Fig. 9).

Cisplatin cross-links DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits repair mechanisms, which in turn activate apoptosis when repair proves impossible. Most notable among the changes in DNA are the 1,2-intrastrand cross-links with purine bases. These include 1,2-intrastrand d(GpG) adducts, which constitute nearly 90 % of the adducts, and the less common 1,2-intrastrand d(ApG) adducts. Other adducts include interstrand cross-links and nonfunctional adducts that have been postulated to contribute to cisplatin's activity. Interaction with cellular proteins, particularly HMG-domain proteins, has also been advanced as a mechanism by which cisplatin interferes with mitosis, although this is probably not its primary method of action. Cisplatin is frequently designated an alkylating agent, however it has no alkyl group and so cannot carry out alkylating reactions. It is more correctly classified as alkylating-like.

Studies of cisplatin in osteosarcoma were first published in the late 1970s [64–67]. It was effective in treating the primary tumor and pulmonary metastases. The response rate for intravenous administration was approximately 30 %. This rate included responses in patients with unresectable or metastatic disease; in these circumstances, cisplatin was administered alone or in combination with doxorubicin. However, enhanced activity in regionally confined tumors could be attained by administering the drug intra-arterially [68, 69]. Complete and partial responses for

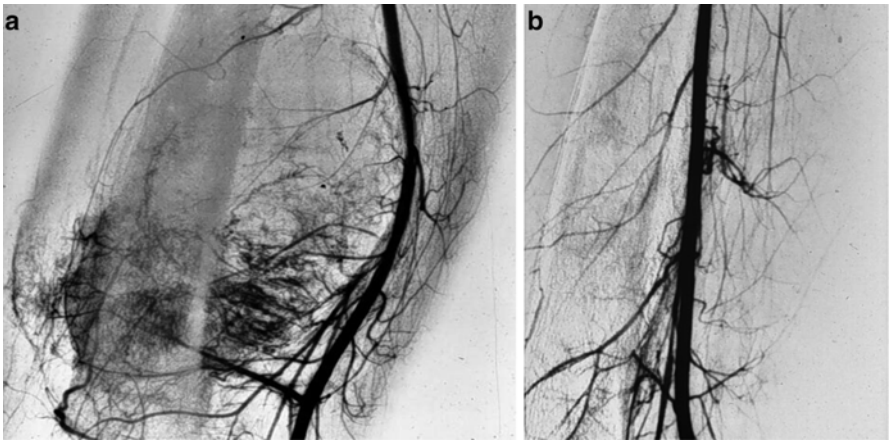


Fig. 10 Angiogram of tumor of the tibia before (**a**) and after treatment with cisplatin (**b**). Disappearance of tumor neovascularity after therapy

this route were 60 %; when cisplatin was combined with doxorubicin, the response approached 80–90 %. Responses obtained with intra-arterial cisplatin in two patients with osteosarcomas in the distal femur are illustrated in Figs. 10 and 11.

The concentration entering the systemic circulation with intra-arterial administration is sufficiently tumoricidal to destroy pulmonary metastases and cause systemic side effects (Figs. 12 and 13) [68]. The effects are enhanced by cumulative courses of cisplatin [69]. The extent of tumor destruction is proportional to the degree of cisplatin tumor deposition [70]. Repeat treatments with intra-arterial cisplatin are also successful in destroying recurrent tumor (Fig. 14) [70]. The majority of the Treatment and Investigation of Osteosarcoma (TIOS) studies in the Pediatrics department at the MD Anderson Cancer Center utilized intra-arterial cisplatin as the foundation of treatment [71].

Intra-arterial administration of chemotherapy is labor intensive. The procedure is performed under general anesthesia or conscious sedation in a radiological suite. Similar results may be obtained after a more prolonged period (weeks) by intravenously administering several courses of combination chemotherapy. As a result, over the past few years, enthusiasm has waned for deploying intra-arterial cisplatin in the pediatric population; however, in combination with doxorubicin, intra-arterial cisplatin still constitutes the primary therapeutic strategy for the adult population at the MD Anderson Cancer Center [72]. The strategy is particularly useful in obtaining a rapid response in tumors complicated by pathologic fractures or tumor infiltrating into, or directly adjacent to, the neurovascular bundle. The therapeutic potential of cisplatin may also be enhanced with radiation therapy.

Current Practice

The intravenous dose of cisplatin is 100–120 mg/m^2 generally administered over 2–4 h. It is administered every 3–4 weeks; the intra-arterial dose is 150 mg/m^2

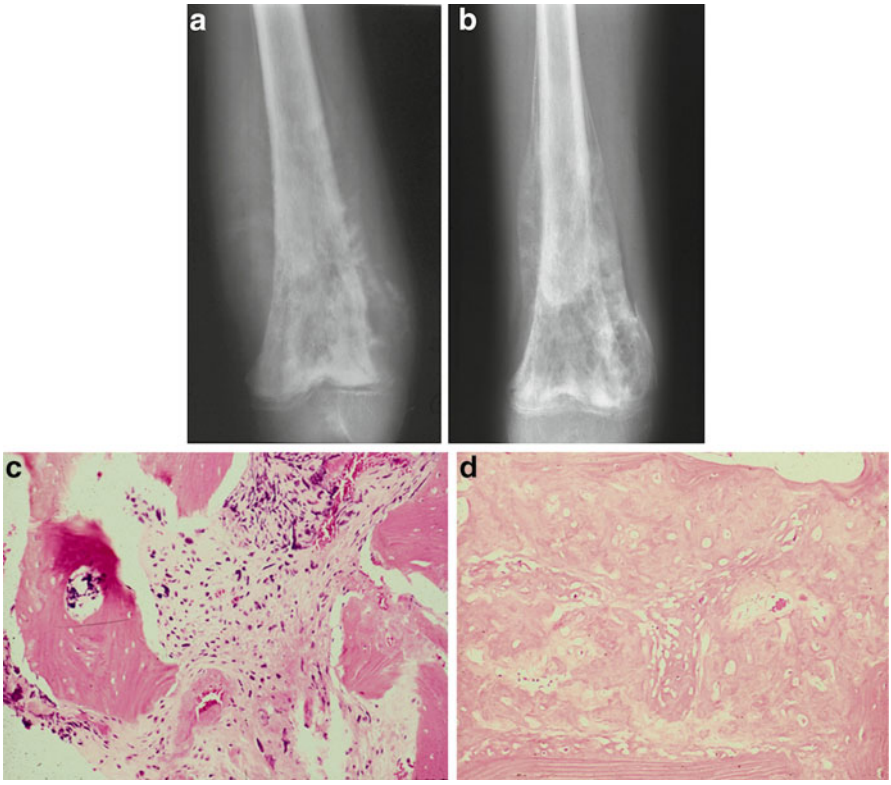


Fig. 11 Image of femoral osteosarcoma before (**a**) and after therapy (**b**) with four courses of intra-arterial cisplatin. The corresponding pathology sections of the untreated (**c**) and treated tumor (**d**) are depicted

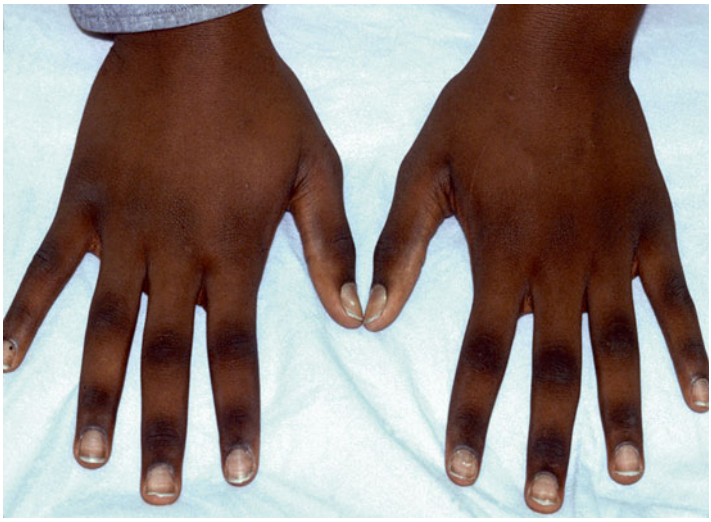


Fig. 12 Photomicrograph of nail beds depicting lines of growth arrest following treatment with four courses of intra-arterial treatment with cisplatin consistent with attainment of high systemic tumoricidal concentrations of cisplatin with intra-arterial administration

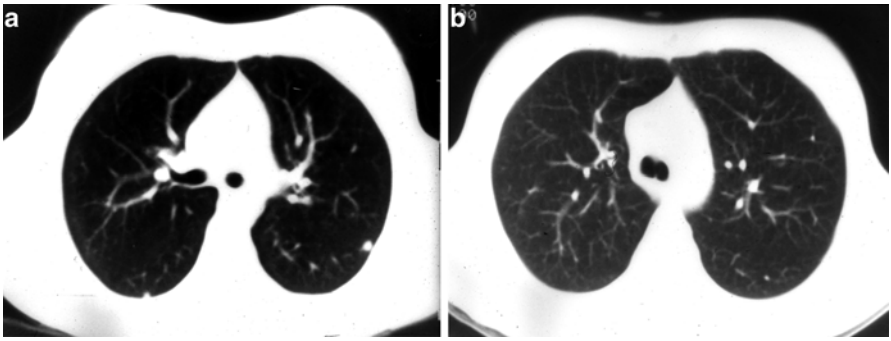


Fig. 13 Computer lung scan depicting tumor nodules (**a**) and disappearance following four courses of intra-arterial cisplatin (**b**)

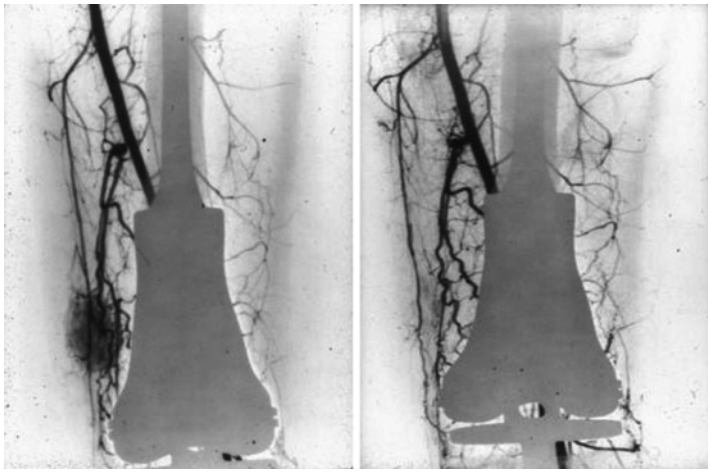


Fig. 14 Angiogram demonstrating recurrent tumor at distal end of femur following limb-salvage procedure. Primary tumor treated with several courses intra-arterial cisplatin, doxorubicin, and methotrexate. Complete response obtained and tumor resected with insertion of internal prosthesis. Tumor recurrences detected 1 year later and responded to repeat intra-arterial administration of cisplatin. Reproduced with permission of Springer from Pediatric and Adolescent Osteosarcoma [70]

administered over 2 h every 4 weeks for four courses. Mannitol is utilized to augment diuresis. Side effects include nausea and vomiting, nephrotoxicity, ototoxocity, electrolyte disturbances, and development of second malignant neoplasms.

Carboplatin has also been investigated as a mechanism of treatment for osteosarcoma [73]. It has been administered intravenously and intra-arterially. However, it does not appear to be as effective as cisplatin.

Fig. 15 Ifosfamide

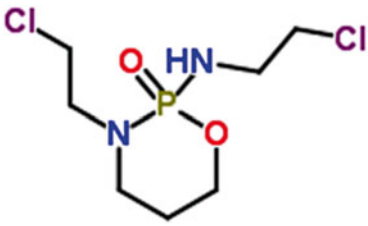
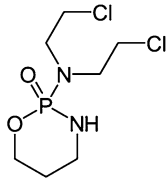


Fig. 16 Cyclophosphamide



Oxazaphosphorines

Historical Perspective

Alkylating antineoplastic agents attach alkyl groups (C_nH_{2n+1}) to DNA. Alkylating agents were initially known for their use as sulfur mustard ("mustard gas") and related chemical weapons in World War I. The nitrogen mustards were the first alkylating agents and the first modern cancer chemotherapies. Goodman, Gilman, and others at Yale began studying nitrogen mustards in 1942 [74]. Since cancer cells, in general, proliferate faster and with less error-correcting than healthy cells, cancer cells are more sensitive to DNA damage—such as being alkylated.

The two major alkylating agents employed in osteosarcoma today are cyclophosphamide and ifosfamide (Figs. 15 and 16). They require hepatic microsomes for activation. Cyclophosphamide was investigated in the early 1960s by Pinkel, who reported a response in a patient with pulmonary metastases (vide supra) [11]. Ifosfamide was introduced approximately 20 years later. It is an analogue of cyclophosphamide and may be used in its stead. They are both highly active in the treatment of osteosarcoma [6, 11, 75–78].

Cyclophosphamide and ifosfamide are metabolized to acrolein, which can cause hemorrhagic cystitis. This side effect can be aborted with the concurrent administration of mesna (2-*m*ercapto*e*thane *s*ulfonate *Na* [Na being the symbol for sodium]. Mesna binds to acrolein—its sulfhydryl group reacts with acrolein's vinyl group—with a detoxifying effect. Mesna permits oxazaphosphorine drugs to be administered in extremely high doses. As a consequence, higher response rates may be achieved. Mesna also increases urinary excretion of cysteine.

These drugs possess moderate to high efficiency in the treatment of osteosarcoma (response rate, approximately 40 %).

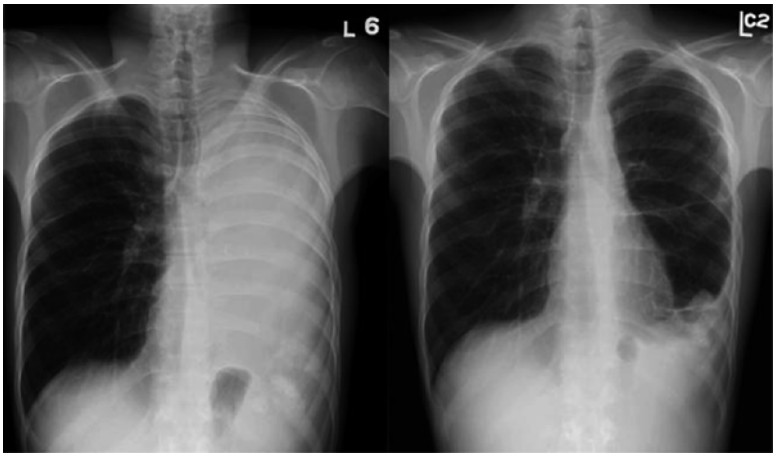


Fig. 17 Chest radiograph of a patient with pulmonary metastases and pleural effusion responding to ifosfamide 17.5 g/m^2. Prior therapy (with sequential response and relapse) comprised methotrexate, doxorubicin, cisplatin, and ifosfamide 9 and 14 g/m^2. Reproduced with permission by Springer from Jaffe N (2009) Pediatric and Adolescent Osteosarcoma [70]

Current Practice

Ifosfamide is administered in doses of 6–9 g/m^2. Patients who relapse may again achieve a response by escalating the dose to 14 g/m^2 (2 g/m^2/day × 7) or 17.5 g/m^2. Goorin et al., in a "therapeutic window," achieved a response rate of 59 % with a combination of ifosfamide (3.5 g/m^2/day for 5 days, total 17.5 g/m^2) and etoposide (100 mg/m^2/day for 5 days, total 500 mg/m^2) in newly diagnosed patients in a Children's Cancer Group Study [79] The experience was duplicated at the MD Anderson Cancer Center; however, in contrast to the patients treated by Goorin et al., the patients at MD Anderson were heavily pretreated and had received prior ifosfamide at lower doses (9 g/m^2). The number of courses of the high dose of ifosfamide, each (17.5 g/m^2), should be limited to four because of the potential for renal failure. An example of a patient who was previously treated and responded to ifosfamide (9 and 14 g/m^2) and relapsed and again responded to 17.5 g/m^2 is presented in Fig. 17.

Liberal amounts of intravenous fluids should accompany the administration of cyclophosphamide and ifosfamide as an additional measure to circumvent hemorrhagic cystitis. The agents, particularly ifosfamide, are effective in treating both pulmonary metastases and the primary tumor and are incorporated into many preoperative and postoperative combination regimens. The side effects include myelosuppression, hemorrhagic cystitis, and development of second malignant neoplasms.

Ifosfamide and cyclophosphamide are not cross resistant. If a tumor appears resistant to one it may still be responsive to the other.

Immunostimulatory Compounds and Chemotherapy

An additional tactic to compliment chemotherapy to prevent the emergence of metastases is the use of immunostimulatory drugs. Kleinerman (Fig. 1i) initiated investigations along these lines approximately 12 years ago with Liposome-encapsulated muramyl tripeptide-phosphatidyl ethanolamine (L-MTP-PE) [80]. This is an immune compound capable of stimulating pulmonary macrophages to destroy metastases. Clinical investigations to test the efficacy of L-MTP-PE in preventing pulmonary metastases comprised a 2×2 Factorial design randomized trial in which patients received chemotherapy alone or chemotherapy with L-MTP-PE [81]. Patients who received 3-drug (doxorubicin, methotrexate, and cisplatin) or 4-drug (3 drug + ifosfamide) chemotherapy plus L-MTP-PE had an overall survival rate of 78 % at 6 years, whereas patients who received 3 or 4 drug chemotherapy *without* L-MTP-PE had an overall survival rate of 70 % ($p \geq 0.03$). L-MTP-PE has been licensed in Europe, Israel, and Mexico. It was available as a compassionate investigational new drug therapy in the United States from 2008 to 2012. Full details of investigations with L-MTP-PE are presented in a separate chapter.

New Chemotherapeutic Agents

With the understanding that osteosarcoma can no longer be considered a chemoresistant tumor, intense investigations are currently in progress to identify other chemotherapeutic agents that may be active in this disease. Among more recently investigated agents are gemcitabine [82] and cedilanid [83].

Inhalation Chemotherapy

Although inhalation therapy has been utilized extensively for over a century in anesthesia, it is relatively new as a therapeutic option in osteosarcoma. Its application is being investigated as a potential conduit for the treatment of pulmonary metastases [84]. In this context, pioneering studies were initiated by Kleinerman with liposomal 9-nitro captothecin. The study did not yield any response in two patients and unfortunately could not be completed because of limited supplies of the agent (Kleinerman E, pers com). Granulocyte–monocyte colony stimulating factor (GM-CSF) was investigated by the Pediatric Oncology Group [85]. No detectable immunostimulatory effect against pulmonary metastases or improved outcome after relapse was seen. Inhaled lipid cisplatin for the treatment of patients with relapse or progressive osteosarcoma metastatic to the lung has recently been reported to achieve responses in some patients [86].

Neoadjuvant (Preoperative or Presurgical) Chemotherapy

Agents administered preoperatively to treat the primary tumor, as well as to determine their potential use as postoperative treatment, are designated neoadjuvant chemotherapy. This term was coined by Emil Frei III in discussing a presentation by Gerry Rosen at an ASCO meeting in the 1980s and was first formally published in *Cancer* [87]. Neoadjuvant chemotherapy is essentially preoperative chemotherapy administered to treat the primary tumor; however, its application is given additional semeiology by utilizing the results to design a rational optimum regimen for postoperative adjuvant therapy. Thus, preoperative treatment that produced acceptable tumor necrosis (generally established at >90 % tumor necrosis) would be continued in the postoperative phase, whereas intermediate or poor necrosis (<90 % tumor necrosis) would call for an adjustment in the regimen. The strategy employed a grading system for necrosis devised by Huvos et al. [88].

Theoretically, preoperative chemotherapy could confer several important systemic and local advantages. Systemically, it would initiate immediate treatment against micrometastases. It could also reduce the opportunity for development of early or spontaneous emergence of drug-resistant clones of tumor cells [89, 90]. Further, as indicated earlier, it could serve as an in vivo/in vitro trial to determine the sensitivity of the tumor and the selection of "customized" postoperative treatment on the basis of extent of necrosis. This strategy is consistent with the assumption that chemotherapy having an effect on bulk tumor would also be operative on micrometastases, yielding optimum disease-free survivals. Necrosis induced by chemotherapy could conceivably also reduce the potential for escape of viable tumor cells during the operation to extirpate the primary tumor.

The benefits accrued with effective neoadjuvant chemotherapy include its potential to downstage the primary tumor, e.g., Stage 2B to 2A [91]. It can also lead to formation of a firm pseudocapsule or "carapace," enhancing the safety of limb-salvage procedures. This could reduce the opportunity for local recurrence. The latter is a function not only of the surgical procedure but also the degree of necrosis induced by chemotherapy [92, 93]. Better preservation of muscle tissue could also be achieved. Effective chemotherapy is not to be considered a substitute for the application of sound surgical principles in the procedure.

Preoperative chemotherapy can also cause healing of pathologic fractures, and not infrequently, tumors initially considered inoperable can be rendered operable [94]. Preoperative chemotherapy also provides an interval for the manufacture of a custom-made prosthesis and planning of the definitive procedure. Early extirpation of bulk (primary) tumor has occasionally been followed by an unexplained explosion of overt metastases. Theoretically, this spread could be prevented with neoadjuvant chemotherapy.

However, when neoadjuvant chemotherapy was introduced in the early 1980s, it was not without controversy. A primary opposing rationale was that preoperative chemotherapy might not be entirely effective, and a delay in extirpating an uncontrolled primary tumor could jeopardize survival if adequate tumor destruction was not attained.

In the absence of a response, the tumor in situ constituted an uninhibited source of pulmonary metastases. This could ultimately affect survival, particularly in the small number of patients who were initially free of pulmonary metastases. Prolonged exposure to preoperative chemotherapy might also select for development of drug-resistant tumor cells that might metastasize before surgical extirpation. It was also possible that the sensitivity of malignant cells in the bulk tumor (the primary) might not necessarily predict the response in microscopic tumor (tumor vascularity and other factors could partially contribute to its antineoplastic effect). Notwithstanding, experimentally, the kinetics of tumor growth suggested that unlike primary tumors, micrometastases were more sensitive to effective anticancer agents and could be eradicated when the total tumor burden was minimal [95, 96].

Additional concerns resided in the possibility that drugs discarded on the basis of modest activity against macroscopic disease (the primary tumor) might still be highly effective against microscopic disease in an adjuvant setting. Prolonged exposure to preoperative chemotherapy might also select for the development of drug-resistant cells that could metastasize before definitive surgery. Tumor heterogeneity could possibly also contribute to this hazard. This was supported by a report of disparate histologic responses in patients in whom the primary tumor and pulmonary metastases were treated simultaneously with preoperative chemotherapy [97]. The disparity was further supported by studies in which the anticipated improved results were not observed with "custom-tailored" chemotherapy [98, 99], contradicting the recommendation that active agents be withheld initially and only introduced after in vivo/in vitro studies [87]. Notwithstanding, neoadjuvant chemotherapy was adopted almost universally as an integral component of chemotherapeutic regimens for the management of osteosarcoma. A study comparing metastasis-free survival between patients who had immediate surgery and those who had neoadjuvant chemotherapy and a delay in extirpating the primary tumor revealed that the delay in surgery did not jeopardize ultimate survival time and rate [100].

Neoadjuvant chemotherapy pioneered by Rosen et al. as the T-10 protocol at Memorial Sloan-Kettering was adopted as the "gold standard" [87]. However, long-term results suggested that survival of patients with <90 % tumor necrosis was the same whether they did or did not have an adjustment (addition of cisplatin) in the postoperative phase of their chemotherapy regimen [98, 99]. Subsequent trials performed by other groups also failed to demonstrate improved event-free survival rates when drugs not included in the preoperative regimen were added to postoperative therapy [101, 102]. Notwithstanding, the degree of preoperative necrosis (and to a lesser extent, reduction in tumor size) was found to be powerful prognostic factors [71, 103]. Patients with an unfavorable histologic response were more likely to develop metastases.

As a consequence of the dilemma of the role of neoadjuvant chemotherapy, a multidisciplinary European and American Osteosarcoma study group was organized as a collaborative venture: EURAMOS (INT-0133) [104]. The intent was to identify an optimum regimen and define the role of neoadjuvant treatment. Initially, all patients were treated with neoadjuvant chemotherapy comprising methotrexate, doxorubicin, and cisplatin. Poor responders received the same therapy

postoperatively but were further randomized to receive (or not receive) ifosfamide and etoposide as well. Good responders received the same preoperative therapy postoperatively but were further randomized to receive (or not receive) maintenance interferon. Preliminary results of the EURAMOS study were reported at the recent ASCO meeting (June 2013) [105]. A sustained disease-free survival rate of 60–65 % at 3 years with chemotherapy was confirmed. However, an additional 2–3 years would be required to determine the benefit (if any) that would accrue with the alteration of postoperative therapy in hopes of improving survival.

Treatment of Pulmonary Metastases

Pulmonary recurrence occurs in approximately 25–30 % of patients. No standard second-line chemotherapy for such pulmonary metastases has been established. Patients with relapses are usually treated with gemcitabine (often in combination with docetaxel), higher doses of established agents, or newly discovered agents [79–82]. The efficacy of such agents in advancing the cure of such patients remains to be determined. Pulmonary metastasectomy constitutes the best therapeutic option for relapse [106]. The strategy is not new; the first resection was performed in 1940 [107]. Accelerated adoption of metastasectomy increased with the successes reported with the newly discovered effective chemotherapeutic agents. Contributing to the enthusiasm was a change in the pattern of metastases that emerged in patients who had relapses following apparently successful therapy: delay in the appearance and a reduction in the size and number of metastases [108]. Fifteen to twenty percent of patients are probably rendered disease free by metastasectomy, yielding an overall cure rate of 75–80 % [109].

Limb Salvage

The discovery of effective chemotherapeutic agents in osteosarcoma heralded a seminal milestone in the pursuit of a cure for the disease. It provided an enhanced impetus to consider *en bloc* resection and wide local excision for limb salvage. Limb salvage had been investigated and attempted previously by American orthopedic surgeons, particularly Phemister and Parrish, in the early and mid-twentieth century [110, 111]. However, local recurrence was a frequent complication and discouraged its general acceptance. This complication diminished dramatically with the effective deployment of preoperative (neoadjuvant) chemotherapy. Implementation of this procedure was predicated by the understanding that tumor-free margins would be achieved and that the principles of cancer surgery would not be compromised. Poor cancer surgery could not be excused on the grounds that chemotherapy would be an adequate substitute for incomplete or inadequate surgical tumor ablation. Improvements in limb-salvage prostheses followed. It is estimated that currently

approximately 80 % of newly diagnosed patients undergo limb-salvage procedures. Criteria for eligibility have been established.

Limb salvage involves the administration of preoperative chemotherapy and excision of the tumor-bearing bone, followed by replacement by a metal prosthesis or autologous or allogenic bone. Preoperative chemotherapy produced some exceptional results, leading to a unique study investigating the possibility of achieving limb salvage exclusively with chemotherapy and abrogation of surgery [112]. Among 31 patients entered into the study, prolonged remission with chemotherapy was successful in only in 3 patients. They have remained tumor free for over 30 years. In the total cohort, 15 patients have survived (~50 %). Among these are four patients who underwent delayed surgical resection and no evidence of tumor was detected. The other eight surviving patients developed local recurrence and pulmonary metastases and were rendered tumor free with additional chemotherapy and surgical intervention. Sixteen patients succumbed to tumor recurrence. In view of the high possibility of local recurrence and pulmonary metastases exclusive treatment of the primary tumor with current forms of chemotherapy does not appear to be a justified strategy.

Therapeutic Regimens and Strategies

Successes forged with the discovery of effective chemotherapeutic agents spawned the creation of innovative regimens. Protocols were devised for pre and postoperative treatment. The results demonstrated improvement in survival and the ability to offer limb salvage as an alternative to amputation. A brief outline of the components of selected protocols adopted by institutions and cooperative groups is presented in Table 1 to illustrate the diversity of treatment. Significant is the fact that the overall event free survival rates attained with these protocols have stabilized at approximately 60 % over the past 30 years. The informed investigator and rational observer will readily perceive that although protocols have been altered and redesigned in an attempt to improve the outcome, unfortunately there has been uniformly limited or minimal success. Several current strategies incorporating the established effective agents described above would appear to be consistent with the generally accepted term “standard of care.”

Drawbacks of Chemotherapy

Unfortunately, the dividends accrued with chemotherapy have not always been salutary. While chemotherapy has eradicated pulmonary metastases or changed the pattern of their development in a significant number of patients, it has permitted emergence of resistant clones in pulmonary and extra pulmonary sites [113]. Many of these have created complications requiring intensive palliation. Radiation therapy,

Table 1 Institutions/groups and chemotherapeutic agents utilized in their protocols

Institution/group	MTX	DOX	CDP	IFX	Other	References
EURAMOS	+	+	+	+	ETP, INF	[104, 105]
COSS studies	+	+		+	BCD, INF	[114]
MSKCC	+	+		+	BCD	[87, 98, 115]
Rizzoli Institute		+	+			[116]
MD Anderson (Pediatrics)	+	+	+			[71]
MD Anderson (Adult)	+	+	+			[72]
Scandinavia series (SSG)	+	+		+		[117]
EOI	+	+		+		[11, 126]
French series	+	+		+	ETP	[118, 119]
St Jude	+	+		+	CARBO	[43, 67, 120–122]
COG	+	+		+	MTP-PE	[81]
CCG	+	+		+	VCR	[101, 123]
Turkish series			+	+	EPI	[124]
Brazilian series		+			EPI, CARBO	[73]

The recorded chemotherapeutic agent may not necessarily have been incorporated in each protocol. If not utilized in one protocol, it was employed in another

COSS Cooperative German–Austrian–Swiss Osteosarcoma Study Group, *MSKCC* Memorial Sloan Kettering Cancer Center, *EOI* European Osteosarcoma Intergroup, St Jude St Jude Research Hospital, *COG* Children's Oncology Group, *CCG* Children's Cancer Group, *ETP* etoposide, *INF* interferon; *BCD* Bleomycin, Cyclophosphamide, Dactinomycin, *CARBO* carboplatin, *MTP-PE* Muramyl triphosphate Phosphatidyl ethanolamine, *VCR* vincristine, *EPI* epirubicin

often in association with methotrexate or cisplatin, or samarium therapy has been found useful as palliative measures.

Ancillary Discoveries

In parallel with the chemotherapeutic discoveries during the last century, new antibiotic therapy, new imaging studies, and improvements in prostheses have been introduced. Superb supportive care accompanied the discovery of chemotherapy, improving the safety of its administration. These developments have contributed to hope and new expectations for an improved outcome.

Comment

The doses and administration of the chemotherapeutic agents depicted in this communication are provided in the record of the chronicle of their discovery and their role in the management of osteosarcoma. Only a superficial account of their dosages and administration is provided. They are properly administered according to guidelines depicted in protocols. As such protocols should be consulted to ensure the indications, safety, and accuracy for their administration.

Conclusion

During the past century, the treatment and outlook for osteosarcoma changed dramatically. The discovery of effective chemotherapeutic agents heralded a seminal advance in the pursuit of cure. Whereas in the past, over 90 % of patients died of the disease, currently 75–80 % survive. Many are treated with limb-salvage procedures. These salutary events were aided and abetted by parallel improvements in disciplines devoted to patient care. However, the seminal advances have reached an impasse. There has been little advance in the curability over the past 40 years, and new agents and strategies are urgently required. Notwithstanding, disappointment is often a companion to progress and the journey traversed in the past century offers confidence and perspective for future discoveries.

References

1. Gross SW (1879) Sarcomas of the long bones: based on a study of one hundred and sixty five cases. Am J Med Sci 8:338–377
2. Jaffe N, Frei E III, Traggis D et al (1974) Adjuvant methotrexate and citrovorin factor in osteogenic sarcoma. N Engl J Med 291:994–997
3. Jeffree GM, Price CH, Sissons HA (1975) The metastatic patterns of osteosarcoma. Br M Cancer 32(1):87–107
4. Friedman M, Carter SK (1972) The therapy of osteogenic sarcoma: current status and thoughts for the future. J Surg Oncol 4(5):482–510
5. Sullivan MP, Sutow WW, Taylor G (1963) L-phenylalanine mustard as treatment for metastatic osteogenic sarcoma in children. J Ped 63:227–237
6. Sutow WW, Sullivan MP, Fernbach DJ et al (1975) Adjuvant chemotherapy in primary treatment of osteogenic sarcoma. Cancer 36:1598–1602
7. Evans AE (1961) Mitomycin C. Cancer Chemother Rep 14:1–9
8. Frank W, Osterberg A (1971) Mitomycin C" (NSC-26980) clinical brochure. Cancer Chemother Rep 55:285–291
9. Finkelstein JZ, Hittle RE, Hammond CD (1969) Evaluation of a high dose cyclophosphamide regimen in childhood tumors. Cancer 23:1239–1242
10. Haggard M (1967) Cyclophosphamide (NSC-26271) in the treatment of children with malignant neoplasms. Cancer Chemother Rep 51:403–405
11. Pinkle D (1962) Cyclophosphamide in children with cancer. Cancer 15:42–49
12. Sutow WW, Vietti TJ, Fernbach DJ et al (1971) Evaluation of chemotherapy in children with metastatic Ewing's sarcoma and osteogenic sarcoma. Cancer Chemother Rep 55:67–81
13. Selawry O, Holland J, Wolman J (1988) Effect of vincristine (NSC-67574) on malignant solid tumors in children. Cancer Chemother Rep 52:497–560
14. Groesbeck H, Budmore J (1963) Evaluation of 5-fluouracil (5-FU) in surgical practice. Am Surg 29:683–691
15. Jaffe N, Traggis D, Enriquez C (1971) Evaluation of mitomycin C (NSC-26980), phenylalanine mustard (NSC-16210) and vincristine (NSC-67574) in the treatment of osteogenic sarcoma. Cancer Chemother Rep 55:181–191
16. Sutow WW (1975) Combination chemotherapy with Adriamycin (NSC-123127) in primary treatment of osteosarcoma (Part III). Cancer Chemother Rep 6:315–317
17. Sutow WW, Gehan EA, Dyment PC et al (1978) Multi-drug adjuvant chemotherapy in osteosarcoma: interim report of the Southwest Oncology Group Studies. Cancer Treat Rep 62(2):265–269

18. Wilbur J (1974) Combination chemotherapy for rhabdomyosarcoma. Cancer Chemother Rep 58(2):281–284
19. Herson J, Sutow WW, Elder K (1980) Adjuvant chemotherapy in non-metastatic osteosarcoma: a Southwest Oncology Group Study. Med Pediatr Oncol 8:343–352
20. Cortez EP, Holland JF, Wang JJ et al (1972) Doxorubicin in disseminated osteosarcoma. JAMA 221:1132–1137
21. Bonnaonna G, Monifardi S, Douglass RC et al (1970) Phase I and preliminary phase II evaluation of Adriamycin (NSC-123127). Cancer Res 30:2572–2582
22. Cortez EP, Holland JF, Wang JJ et al (1972) Chemotherapy of adjuvant osteosarcoma. In: Proceedings of the 24th Symposium of the Colston Research Society, April, 1972. Butterworths, London
23. Gottlieb JA, Baker LH, O'Bryan RM et al (1975) Adriamycin (NSC-123127) used alone and in combination with soft tissue bone sarcoma. Cancer Chemother Rep 3:271–282
24. Middleman E, Luce L, Frei E (1971) Clinical trials with Adriamycin. Cancer 28:844–850
25. O'Bryan RM, Luce JK, Talley R et al (1973) Phase II evaluation of Adriamycin in human neoplasia. Cancer 32:1–7
26. Cortez EP, Holland JF, Wang JJ et al (1974) Amputation and Adriamycin in primary osteosarcoma. N Engl J Med 291:998–1000
27. Cassady JR, Richter MP, Piro AJ et al (1975) Radiation Adriamycin interactions-preliminary clinical observations. Cancer 36:946–949
28. Eilber FR, Grant T, Morton DC (1978) Adjuvant chemotherapy for osteosarcoma. Cancer Treat Rep 62:213–216
29. Seifert CF, Nesser ME, Thompson DF (1994) Dexrazoxane in the prevention of doxorubicin-induced cardiotoxicity. Ann Pharmacother 28:1063–1072
30. Smith MA, Ungerleider RS, Horowitz ME et al (1991) Influence of doxorubicin dose intensity on response and outcome for patients with osteogenic sarcoma and Ewing's sarcoma. J Nat Cancer Inst 83:1460–1470
31. Farber S, Diamond LK, Mercer RD et al (1946) Temporary remissions in children in acute leukemia produced by folic acid antagonist 4-aminopteroyl-glutamic acid (aminopterin). N Engl J Med 238:787–793
32. Fyfe MJ, Goldman ID (1973) Characteristics of the vincristine augmentation of methotrexate uptake in Ehrlich ascites tumor cells. J Biol Chem 248:5067–5073
33. Goldin A, Mantel N, Greenhouse SW et al (1954) Effect of delayed administration of citrovorum factor on the antileukemic effectiveness of aminopterin in mice. Cancer Res 14:43–48
34. Djerassi I, Abir E, Boyer GL et al (1966) Long-term remissions in childhood acute leukemia: use of infrequent infusions of methotrexate; supportive roles of platelet transfusions and citrovorum factor. Clin Pediatr 5:502–509
35. Jaffe N (1972) Recent advances in the chemotherapy of osteogenic sarcoma. Cancer 30:1627–1631
36. Skipper HE, Schabel FM, Wilcox WS (1964) Experimental evaluation of potential anticancer agents. XIV Further study of certain basic concepts underlying chemotherapy of leukemia. Cancer Chemother Rep 35:1–28
37. Laster WR Jr, Mayo JG, Simpson-Herrin L et al (1969) Success and failure in the treatment of solid tumors. II. Kinetic parameters and "cell cure" of moderately advanced carcinoma. Cancer Chemother Rep 53:169–188
38. Schabel FM Jr (1975) Concepts for systemic treatment of micro-metastases. Cancer 35:15–24
39. Jaffe N (1974) Progress report on high-dose methotrexate (NSC-740) with citrovorum rescue in the treatment of metastatic bone tumor. Cancer Chemother Rep 58:275–280
40. Jaffe N, Traggis D (1975) Toxicity of high-dose methotrexate (NSC-740) and citrovorum factor (NSC-3590) in osteogenic sarcoma. Cancer Chemother Rep 6:31–36
41. Jaffe N, Traggis D, Cassady JR et al (1976) Multidisciplinary treatment for macrometastatic osteogenic sarcoma. Br Med J 2:1039–1041
42. Frei E III, Jaffe N, Tattersall MHN, Pitman S, Parker L (1975) New approaches to cancer chemotherapy with methotrexate. N Engl J Med 292:846–851

43. Pratt C, Shanks E, Hustu O et al (1977) Adjuvant multiple drug chemotherapy for osteosarcoma of the extremity. Cancer 39:51–57
44. Rosen G, Tefft M, Martinez A et al (1975) Combination chemotherapy and radiation therapy in the treatment of metastatic osteosarcoma. Cancer 35:622–630
45. Muggia FM, Louis AC (1978) Five years of adjuvant chemotherapy: more questions than answers. Cancer Treat Rep 62:30–36
46. Taylor WF, Ivins JC, Prichard DJ et al (1985) Trends and variability in survival among patients with osteosarcoma: a 7-year update. Mayo Clin Proc 60(2):91–104
47. Edmonson JH, Green SJ, Ivins JC et al (1984) A controlled pilot study of high-dose methotrexate as postsurgical adjuvant treatment for primary osteosarcoma. J Clin Oncol 2:152–156
48. Carter SK (1984) Adjuvant chemotherapy in osteogenic sarcoma: the triumph that isn't? J Clin Oncol 2:147–148
49. Gehan EA, Sutow WW, Urube Botero T et al (1978) Osteosarcoma. The MD Anderson Experience 1850-1974. In: Terry WD, Windhorst D (eds) Immunotherapy of cancer. Present status of trials in man. Raven, New York, NY, pp 271–282
50. Mike V, Marcove RC (1978) Osteogenic sarcoma under the age of 21. Experiences at Memorial Sloan Kettering Cancer Center. In: Terry WD, Windhorst D (eds) Immunotherapy of cancer. Present status of trials in man. Raven, New York, NY, pp 283–292
51. Frei E III, Blum R, Jaffe N (1978) Sarcoma: natural history and treatment. In: Terry WD, Windhorst D (eds) Immunotherapy of cancer. Present status of trials in man. Raven, New York, NY, pp 248–255
52. Campanacci M, Bacci G, Bertoni F et al (1981) The treatment of osteosarcoma of the extremities: twenty years' experience at the Instituto Ortopedico Rizzoli. Cancer 48:1569–1581
53. Link MP, Vietti TJ (1983) Role of adjuvant chemotherapy in the treatment of osteosarcoma. Cancer Treat Rep 67:744–745
54. Lang EB, Levin AS (1982) Is it ethical not to conduct a prospectively controlled trial of adjuvant chemotherapy in osteosarcoma? Cancer Treat Rep 66:1699–1704
55. Link MP, Goorin AM, Miser A et al (1986) The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med 314:1600–1606
56. Eilber FR, Douglass HI Jr, Mendel ER et al (1986) Adjuvant Adriamycin and cisplatin in newly diagnosed nonmetastatic osteosarcoma of the extremity. J Clin Oncol 4:353–362
57. Jaffe N (1986) Osteosarcoma. N Engl J Med 314:1513
58. Holland JF (1987) Adjuvant chemotherapy of osteosarcoma: no runs, no hits, two men left on base. J Clin Oncol 5:4–6
59. Jaffe N, Gorlick R (2008) High-dose methotrexate in osteosarcoma: let the questions surcease—time for final acceptance. J Clin Oncol 24:4365–4366
60. Van Dalen EC, van As JW, de Camargo B Methotrexate for high-grade osteosarcoma in children and young adults. Cochrane Database of Systematic Reviews 2011, Issue 5. Art. No.: CD006325. doi: 10.1002/14651858.CD006325.pub3
61. Jaffe N, Robertson R, Ayala A, Wallace S (1985) Comparison of intra-arterial CDP II with high-dose methotrexate and citrovorum factor rescue in the treatment of primary osteosarcoma. J Clin Oncol 3:1101–1104
62. Jaffe N (1979) Antifolate rescue use of high-dose methotrexate and citrovorin factor. In: Rossowsky A (ed) Advances in chemotherapy. Marcel Decker Inc., New York, NY, pp 111–141
63. Rosenberg B, Van Camp L, Krigas T (1965) Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature 205(4972):698–699
64. Baum E, Greenberg L, Gaynon P et al (1978) Use of cis–diamminedichloroplatinum–II (CDDP) in osteogenic sarcoma in children (Abstract C-315). Proc AACR-ASCO 19:385
65. Nitschke R, Starling KA, Vats T et al (1978) Cis-diamminedichloroplatinum II (NSC119875) In: Childhood malignancies. A Southwest Oncology Group study. Med Pediatr Oncol 4:127–132
66. Ochs JJ, Freeman AI, Douglass HO Jr et al (1978) Cis-diamminedichloroplatinum (II) in advanced osteogenic sarcoma. Cancer Treat Rep 62:239–245

67. Pratt CB, Champion JE, Senzer N et al (1985) Treatment of unresectable or metastatic osteosarcoma with cisplatin or cisplatin–doxorubicin. Cancer 56:1930–1933
68. Jaffe N, Knapp J, Chuang VP et al (1983) Osteosarcoma intra-arterial treatment of the primary tumor with cis-diamminedichloroplatinum-II (CDP). Angiographic, pathologic and pharmacologic studies. Cancer 51:402–407
69. Jaffe N, Raymond AK, Ayala A et al (1989) Effect of cumulative courses of intra-arterial cis-diamminedichloroplatinum II on the primary tumor in osteosarcoma. Cancer 63:63–68
70. Jaffe N (2009) Osteosarcoma: review of the past, impact on the future. The American experience. In: Jaffe N, Bruland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 239–262
71. Hudson M, Jaffe MR, Jaffe N et al (1990) Pediatric osteosarcoma: therapeutic strategies, results and prognostic factors derived from a 10-year experience. J Clin Oncol 8:1988–1997
72. Lin PP, Patel S (2013) Osteosarcoma. In: Lin PP, Patel S (eds) Bone sarcoma. Springer, New York, NY, pp 75–97
73. Petrilli AS, de Camargo B, Filho VO et al (2006) Results of the Brazilian Osteosarcoma Treatment Group Studies III and IV: prognostic factors and impact on survival. J Clin Oncol 24:1161–1168
74. Goodman LS, Wintrobe MM, Dameshek W et al (1946) Nitrogen mustard therapy. JAMA 132(3):126–132
75. Sutow WW, Sullivan MP, Wilbur JR et al (1975) The study of adjuvant chemotherapy in osteogenic sarcoma. J Clin Pharmacol 15:530–533
76. Antman KH, Montella D, Rosenbaum C et al (1985) Phase II trial of ifosfamide with mesna in previously treated metastatic osteosarcoma. Cancer Treat Rep 69:499–504
77. Bowman LG, Mayer WN, Douglass EC et al (1987) Activation of ifosfamide in metastatic and unresectable osteosarcoma (Abstract). Proc ASCO C-214:844
78. Meyer WH, Pratt CB, Farham D et al (1988) The activity of ifosfamide (IFOS) in previously pretreated patients with osteosarcoma (OS): preliminary results of SJCRH OS-86 Study. Proc ASCO 7:26180
79. Goorin AM, Harris MB, Bernstein M et al (2002) Phase II/III trial of etoposide and high–dose ifosfamide in newly diagnosed metastatic osteosarcoma: a Pediatric Oncology Group Trial. J Clin Oncol 20:426–433
80. Kleinerman ES, Jia S-F, Griffin J (1992) Phase II study of liposomal muramyl tripeptide in osteosarcoma: the cytokine cascade and monocyte activation following administration. J Clin Onc 10(8):1310–1316
81. Meyers P, Schwartz CL, Krailo M et al (2008) Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival – a report from the Children's Oncology Group. J Clin Oncol 26:633–688
82. Navid F, Willert JR, McCarville MB et al (2008) Combination of gemcitabine and docetaxel in the treatment of children and young adults with refractory bone sarcoma. Cancer 113:419–425
83. Fox E, Aplenc R, Bagatell R (2010) A phase 1 trial and pharmacokinetic study of cediranib, an orally bioavailable pan-vascular endothelial growth factor receptor inhibitor in children and adolescents with refractory solid tumors. J Clin Oncol 28:5174–5181
84. Andrews NA (2013) Osteosarcoma therapy: what is the way forward? IBMS BoneKEy, doi 1038/bonekey.2013.106
85. Arndt CAS, Koshkina NV, Inwards CY (2010) Inhaled granulocyte macrophage colony stimulating factor for first pulmonary recurrence of osteosarcoma on disease-free survival and immunomodulation. A report from the Children's Oncology Group. Cancer Clin Res 16:4024–4030
86. Chou AJ, Gupta R, Bell MD et al (2013) Inhaled lipid cisplatin (ILC) in the treatment of patients with relapsed progressive osteosarcoma metastatic to the lung. Ped Blood Can 60(4):580–586
87. Rosen G, Caparros B, Huvos AG et al (1982) Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. Cancer 49:1221–1230

88. Huvos AG, Rosen G, Marcove RC (1997) Primary osteogenic sarcoma: pathologic aspects in 20 patients after treatment with chemotherapy, en bloc resection, and prosthetic bone replacement. Arch Pathol Lab Med 101:14–18
89. DeVita VT (1983) The relationship between tumor mass and resistance to chemotherapy. Implications for surgical adjuvant treatment of cancer. Cancer 51:1209–1220
90. Goldie JH, Coleman AJ (1979) A mathematical model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treat Rep 63:1727–1733
91. Murray JA, Jessup K, Romsdahl M et al (1985) Limb salvage surgery in osteosarcoma: early experience at MD Anderson Hospital and Tumor Institute. Cancer Treat Symp 3:131–137
92. Picci P, Bacci G, Campanna R et al (1988) Neoadjuvant chemotherapy for osteosarcoma - results of a prospective study. In: Ryan JR, Baker LO (eds) Recent concepts in sarcoma treatment. Kluver Academic Publishers, Dordrecht, pp 291–295
93. Picci P, Bacci G, Neff JR (1990) The influence of preoperative chemotherapy (POC) in the surgical planning in patients (PTS) with osteosarcoma (OS): a histopathological study on 205 PTS. Proc ASCO 9:310
94. Jaffe N, Spears R, Eftekhari F et al (1987) Pathologic fracture in osteosarcoma: effect of chemotherapy in primary tumor and survival. Cancer 59:701–709
95. Schabel FM Jr (1969) The use of tumor growth kinetics in planning "curative" chemotherapy of advanced solid tumors. Cancer Res 29:2384–2389
96. Schabel FM (1977) Rationale for adjuvant chemotherapy. Cancer 39:2875–2882
97. Nachman J, Simon MA, Dean L et al (1987) Disparate histologic prognosis in simultaneously resected primary and metastatic osteosarcoma following intravenous neoadjuvant chemotherapy. J Clin Oncol 5:1185–1190
98. Meyers PA, Heller G, Healy J et al (1992) Chemotherapy for non metastatic osteogenic sarcoma. The Memorial Sloan Kettering Experience. J Clin Oncol 10:5–13
99. Winkler K, Beron G, Dellin G et al (1988) Neoadjuvant chemotherapy for osteosarcoma: results of a randomized cooperative trial (COSS-82) with salvage chemotherapy based on histological tumor response. J Clin Oncol 6:329–337
100. Goorin AM, Schwartzentruber DJ, Devidas M et al (2003) Presurgical chemotherapy compared with immediate surgery and adjuvant chemotherapy for non metastatic osteosarcoma. Pediatric Oncology Group Study POG-8651. J Clin Oncol 21:1574–1580
101. Proviser AJ, Ettinger LJ, Nachman JB et al (1997) Treatment of non metastatic osteosarcoma of the extremity with preoperative and postoperative chemotherapy: a report from the Children's Cancer Group. J Clin Oncol 15(1):1076–1084
102. Smeland B, Muller C, Alvegard TA et al (2003) Scandinavian Sarcoma Group Osteosarcoma Study SSG VIII: prognostic factors for outcome and the role of replacement salvage chemotherapy for poor histological responders. Eur J Cancer 30(4):488–494
103. Benjamin RS, Chawala SP, Murray J et al (1984) Preoperative chemotherapy for osteosarcoma: a treatment approach facilitating limb salvage with major prognostic indications. In: Jones SF, Salmon SE (eds) Adjuvant therapy of cancer IV. Grune and Stratton, Philadelphia, PA, pp 601–610
104. Marina N, Bielack S, Whelan S et al (2009) International collaboration is feasible in trials for rare conditions: the EURAMOS experience. In: Jaffe N, Bruland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 339–353
105. Bielack SS, Smeland S, Whelan J et al (2013) MAP plus maintenance pegylated interferon α-2b (MAPIfn) versus MAP alone in patients with resectable high-grade osteosarcoma and good histologic response to preoperative MAP: first results of the EURAMOS-"good response" randomization. J Clin Oncol 31 (suppl; abstr LBA10504)
106. Briccoli A, Rocca M, Salone M et al (2005) Resection of recurrent pulmonary metastases in patients with osteosarcoma. Cancer 104(8):1721–1725
107. Martini N, McCormack PM (1988) Pulmonary resection in sarcoma metastases. In: Ryan JR, Baker LO (eds) Recent concepts in sarcoma treatment. Kluwer Academic Publishers, Dordrecht, pp 197–200
108. Jaffe N, Smith E, Abelson HT et al (1983) Osteogenic sarcoma: alterations in the pattern of pulmonary metastases with adjuvant chemotherapy. J Clin Oncol 1:252–254

109. Harting MT, Blakely ML, Jaffe N et al (2006) Long-term survival after aggressive resection of pulmonary metastases among children and adolescents with osteosarcoma. J Pediatr Surg 41(1):194–199
110. Phemister DB (1940) Conservative surgery in the treatment of bone tumors. Surg Gynecol Obstet 70:355–364
111. Parrish FF (1968) Treatment of bone tumors by total excision and replacement with massive autologous and homologous grafts. J Bone Joint Surg 48A:968–990
112. Jaffe N, Carrasco H, Raymond K et al (2002) Can cure in patients with osteosarcoma be achieved exclusively with chemotherapy and abrogation of surgery? Cancer 95:2202–2210
113. McCarten KM, Jaffe N, Kirkpatrick JA (1980) The changing radiographic appearance of osteosarcoma. Ann Radiol 23:203–208
114. Bielack S, Jurgens H, Jundt G et al (2009) Osteosarcoma: the COSS experience. In: Jaffe N, Bruland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 289–308
115. Myers PA, Gorlick R, Heller G et al (1998) Intensification of preoperative chemotherapy for osteogenic sarcoma: results of the Memorial Sloan Kettering (T12) protocol. J Clin Oncol 16(7):2452–2458
116. Ferrari S, Palmerini E, Staals EL et al (2009) The treatment of nonmetastatic high-grade osteosarcoma of the extremity: review of the Italian Rizzoli experience. Impact on the future experience. In: Jaffe N, Bruland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 275–287
117. Bruland OS, Bauer H, Alvegard T et al (2009) Treatment of osteosarcoma. The Scandinavia Sarcoma Group experience. In: Jaffe N, Bruland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 309–318
118. Delepine N, Delepine G, Jasmine C et al (1988) Importance of age and methotrexate dosage: prognosis in children and young adults with high-grade osteosarcoma. Biomed Pharmacother 42:257–262
119. Le Deley M-C, Guinebretiere J-M, Gentet J-C (2007) A randomized trial comparing preoperative high-methotrexate plus doxorubicin to high dose methotrexate plus etoposide and ifosfamide in osteosarcoma patients. Eur J Cancer 43(4):751–761
120. Pratt CB, Luo X, Fay L et al (1996) Response of pediatric malignant solid tumors following ifosfamide or ifosfamide/carboplatin/etoposide. A single hospital experience. Med Pediatr Oncol 27:145–148
121. Daw NE, Neel MD, Rao BN (2011) Frontline treatment of localized osteosarcoma without methotrexate: results of the St Jude Children's Research Hospital OS99 trial. Cancer 117(12):2770–2778
122. Meyer WH, Pratt CB, Poquette CA et al (2001) Carboplatin/ifosfamide window therapy for osteosarcoma: results of the St Jude Children's Research Hospital OS-91 trial. J Clin Oncol 19:171–182
123. Krailo M, Ertel I, Makley J et al (1987) A randomized study comparing high dose methotrexate with moderate dose methotrexate as components of adjuvant therapy in childhood nonmetastatic osteosarcoma: a report from the Children's Cancer Study Group. Med Pediatr Oncol 15:69–77
124. Ayan I, Kebudi R, Ozger H (2009) Childhood osteosarcoma: multimodal therapy in a single-institution Turkish series. In: Jaffe N, Bruland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 319–338
125. Bernthal N, Federman N, Nelson S (2012) Early pathologic response to neoadjuvant therapy predictive of long term (>25 year) survival in osteosarcoma. Paper presented at 2012 annual meeting of the Connective Tissue Oncology Society, Prague, Czech Republic, and 14–17 November 2012
126. Craft AW (2009) Osteosarcoma: the European Osteosarcoma Intergroup (EOI) perspective. In: Jaffe N, Rutland O, Bielack S (eds) Pediatric and adolescent osteosarcoma. Springer, New York, NY, pp 263–274

Part I
Novel Therapeutic Targets

Wnt Signaling in Osteosarcoma

Carol H. Lin, Tao Ji, Cheng-Fong Chen, and Bang H. Hoang

Abstract Osteosarcoma (OS) is the most common primary bone malignancy diagnosed in children and adolescents with a high propensity for local invasion and distant metastasis. Despite current multidisciplinary treatments, there has not been a drastic change in overall prognosis within the last two decades. With current treatments, 60–70 % of patients with localized disease survive. Given a propensity of Wnt signaling to control multiple cellular processes, including proliferation, cell fate determination, and differentiation, it is a critical pathway in OS disease progression. At the same time, this pathway is extremely complex with vast arrays of cross-talk. Even though decades of research have linked the role of Wnt to tumorigenesis, there are still outstanding areas that remain poorly understood and even controversial.

C.H. Lin
The Hyundai Cancer Institute, CHOC Children's Hospital, Orange, CA USA

Department of Orthopaedic Surgery and Chao Family Comprehensive Cancer Center, University of California, 101 The City Drive South, Irvine, Orange, CA 92868, USA

T. Ji
Department of Orthopaedic Surgery and Chao Family Comprehensive Cancer Center, University of California, 101 The City Drive South, Irvine, Orange, CA 92868, USA

Musculoskeletal Tumor Center, People's Hospital, Peking University, Beijing, People's Republic of China

C.-F. Chen
Department of Orthopaedic Surgery and Chao Family Comprehensive Cancer Center, University of California, 101 The City Drive South, Irvine, Orange, CA 92868, USA

Department of Orthopaedics, Therapeutical and Research Center of Musculoskeletal Tumor, Taipei Veterans General Hospital, Taipei, Taiwan

B.H. Hoang, M.D. (✉)
Department of Orthopaedic Surgery and Chao Family Comprehensive Cancer Center, University of California, 101 The City Drive South, Irvine, Orange, CA 92868, USA
e-mail: bhhoang@uci.edu

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_2,

The canonical Wnt pathway functions to regulate the levels of the transcriptional co-activator β-catenin, which ultimately controls key developmental gene expressions. Given the central role of this mediator, inhibition of Wnt/β-catenin signaling has been investigated as a potential strategy for cancer control. In OS, several secreted protein families modulate the Wnt/β-catenin signaling, including secreted Frizzled-related proteins (sFRPs), Wnt inhibitory protein (WIF), Dickkopf proteins (DKK-1,2,3), sclerostin, and small molecules. This chapter focuses on our current understanding of Wnt/β-catenin signaling in OS, based on recent in vitro and in vivo data. Wnt activates noncanonical signaling pathways as well that are independent of β-catenin which will be discussed. In addition, stem cells and their association with Wnt/β-catenin are important factors to consider. Ultimately, the multiple canonical and noncanonical Wnt/β-catenin agonists and antagonists need to be further explored for potential targeted therapies.

Keywords Osteosarcoma • Wnt • β-Catenin • Dickkopf • Wnt inhibitory protein • Frizzled-related proteins

Introduction

Osteosarcoma is the most common primary bone malignancy which occurs frequently in a bimodal distribution, with peak incidences in the second decade of life and after the age of 60 [1]. With the current multidisciplinary treatments, 60–70 % of patients with localized disease survive [2]. OS has a high tendency for local invasion and early metastasis. Unfortunately, with metastatic disease, the rate of 5 year overall survival is greatly reduced to 20–30 %, and the 5-year event-free survival for patients with relapse is 20 % [3, 4]. Metastasis occurs primarily to the lungs and bones. Even though initial scans may not show evidence of pulmonary disease, it is thought that micrometastasis is extremely common, making it difficult to successfully eradicate this tumor. Despite aggressive efforts to strengthen and modify chemotherapy, the outcome of patients with OS has not significantly improved over the past few decades [5].

The exact molecular mechanism leading to the development of OS is not fully understood. Research endeavors have focused on the Wnt signaling pathway since the discovery of the WNT1 gene (originally named Int-1) in 1982 [6, 7]. The discovery of the Drosophila segment polarity gene Wingless and the mouse proto-oncogene Int-1 initiated the advancement of this signaling pathway now commonly referred to as the canonical Wnt signaling pathway [8]. There are currently 19 Wnt proteins which have been identified in the human genome [9, 10]. A good portion of them are considered target genes of Wnt signaling and play a critical role in development and tumorigenesis [11–15] (see http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). Aberrant signaling by Wnt pathways is linked to a wide spectrum of diseases, including neurodegenerative, bone, cardiovascular, and especially cancer. Notably, colon cancer has been associated with mutations of the

Wnt-regulating gene, adenomatous polyposis coli (APC) [16, 17]. Several studies have also suggested that this particular signaling pathway plays an important role in the pathophysiology of bone tumors [18, 19].

Overview of Wnt/β-Catenin Signaling Pathway and Cancer

The Wnt family is a group of secreted glycolipoproteins that initiate a signaling cascade to direct cell proliferation, cell fate determination, and differentiation in numerous developmental stages, from embryogenesis to adult tissue homeostasis [15, 20–23]. Aberrant Wnt signaling plays a role in multiple cancers, such as colon, gastric, lung, breast, prostate, skin cancers and osteosarcoma [19, 24–28]. Given the power of this central mediator, understanding the mechanisms to inhibit the Wnt/β--catenin signaling pathway is a potential strategy for cancer therapy.

In order to understand this pathway, the components of the signaling system are important to grasp. In a non-proliferative state, there is an absence or inhibition of Wnt, which enables the cytoplasmic β-catenin to form a complex with multiple entities, including Axin, adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β) [9, 20, 21, 29, 30] (see Fig. 1). Once this complex forms, CK1 and GSK3β act in conjunction to phosphorylate β-catenin, which is then recognized by the β-Trcp, an E3 ubiquitin ligase subunit. β-Trcp targets β-catenin for proteasomal degradation.

When the signaling cascade is "on," in the presence of Wnt, binding to targeted receptors, comprising Frizzleds (seven-span transmembrane receptor proteins)/low-density lipoprotein receptor-related protein 5 and 6 (single-span transmembrane co-receptor proteins) and cytoplasmic disheveled (Dvl), causes phosphorylation of the complex, leading to inhibition of GSK3β. This creates a cytoplasmic accumulation of non-phosphorylated β-catenin, inhibiting its degradation and promoting translocation to the nucleus. Within the nucleus, it creates a complex with transcription factors, including T-cell transcription factor (TCF) and lymphoid enhancer-binding factor (LEF), and transcriptional co-activators, causing transcriptional activity of multiple downstream target oncogenes, such as c-myc, cyclin-D1, and Axin2, thus enhancing cellular proliferation [9, 20]. Other secreted factors, such as WIF-1 and Frzb/sFRP3 inhibit Wnt binding to frizzled receptors, and Dickkopf (Dkk) proteins antagonize the Wnt/LRP interaction. Wnt antagonists will be further explained in the latter part of the chapter.

The Wnt pathway has been extensively studied in colon cancer. Mutation of the APC gene leads to the activation of the Wnt pathway via stabilization of the β-catenin. This pathway was first associated with cancer development when it was discovered to be activated in both inherited familial adenomatous polyposis (FAP) and colorectal cancer. Approximately 90 % of sporadic colon cancers display mutations in APC leading to aberrant Wnt signaling activity [31, 32]. Since this time, multiple investigators have sought to uncover the role of the Wnt signaling pathway in other malignancies, including OS.

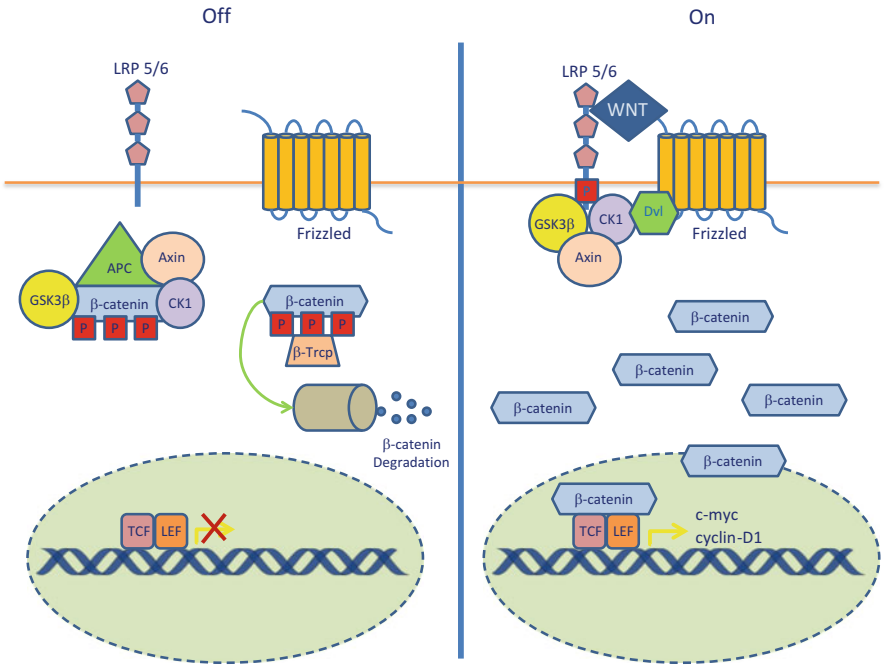


Fig. 1 Overview of Wnt/β-catenin signaling. In the absence or inhibition of Wnt, the cytoplasmic β-catenin forms a complex with Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β). CK1 and GSK3β phosphorylate β-catenin. β-Trcp (E3 ubiquitin ligase subunit) recognizes this complex and targets β-catenin for proteosomal degradation. In the presence of Wnt binding to targeted receptors frizzleds, low-density lipoprotein receptor-related protein 5 and 6 (LPR 5/6), and disheveled (Dvl), the complex becomes phosphorylated, leading to the inhibition of GSK3β. Cytoplasmic non-phosphorylated β-catenin accumulates, inhibiting its degradation and promoting translocation to the nucleus. A complex with transcription factors, including T-cell transcription factor (TCF), Lymphoid enhancer-binding factor (LEF), and transcriptional co-activators, lead to transcriptional activity of multiple downstream target oncogenes

Overview of Wnt/β-Catenin Signaling Pathway and Osteosarcoma

Clinical tissue samples from osteosarcoma patients have been used to correlate various entities of the Wnt pathway and clinical outcome. In our 2004 study, RNA isolated from fresh-frozen osteosarcoma tissue was used to examine the expression of the Wnt receptor LRP-5 by polymerase chain reaction. LRP-5 RNA expression statistically correlated with worse event-free survival in patients [33, 34], and dominant negative LRP-5 decreased tumorigenicity and metastasis of OS in vivo in nude mice experiments [35]. Furthermore, it appears that blocking Wnt/LRP-5 signaling can reduce tumor invasiveness by reversing the epithelial-to-mesenchymal transition [36].

Role of Wnt Antagonists in Osteosarcoma

Secreted Wnt antagonists have been observed to suppress tumorigenesis and metastatic potential in osteosarcoma. Two types of secreted Wnt antagonists are characterized by their mechanisms of inhibition. The first type directly binds to Wnt ligands, promoting an inhibitory response. Wnt inhibitory factor-1 (WIF-1), sFRP family and Cerberus are examples of Wnt antagonists that bind directly to Wnt ligands and inducing an inhibitory response. The second type of antagonist such as the Dickkopf family and sclerostin inhibit the Wnt pathway by binding to transmembrane receptors, thereby preventing Wnt signaling activation.

The Dickkopf family comprises of four secretory proteins, including Dkk-1, Dkk-2, Dkk-3, and Dkk-4. Human Dkk-1 inhibits Wnt signaling pathway by binding to the transmembrane receptors LRP5/6 [37]. Dickkopf 3(Dkk-3), also known as reduced expression in immortalized cells (REIC), has been shown to impede invasion and motility of osteosarcoma cells [38]. Dkk-3 expression is downregulated in multiple cancer cell lines although its exact oncogenic suppressive mechanism is still unknown. Dkk-3 has been shown to downregulate β-catenin nuclear translocation in OS cells leading to inhibition of downstream LEF/TCF activation [39]. The expression of Dkk-3 and dominant-negative LRP5 mutant in OS cell lines substantially decreases cell invasion and motility. We further demonstrated the ability of Dkk-3 to suppress tumorigenesis and pulmonary metastasis in nude mice via intratibial injection of Dkk-3 transfected OS cells [40].

Frzb, a member of secreted frizzled-related protein (sFRP) family, is another Wnt antagonist that has been associated with cancer. It has an amino-terminal cysteine-rich domain (CRD) that is homologous to the ligand-binding domain of Frizzled [41]. Frzb blocks receptor signaling by primarily binding to the extracellular Wnt ligands, preventing the ligand-receptor interaction [42]. Frzb re-expression has been shown to inhibit tumorigenesis and invasiveness in both prostate and fibrosarcoma cancer cells. In vitro studies demonstrated that Frzb can inhibit c-Met, a Wnt target gene that plays a key role in sarcoma progression [24, 43, 44]. Not only is Frzb expression downregulated in soft tissue sarcomas, but it is also less expressed in OS tissue and cell lines [45]. DeAlmeida et al. demonstrated that a secreted Wnt antagonist comprising of the CRD of Fz8 attached with human IgG showed antitumor activity in a teratocarcinoma animal model [46]. This suggests the possibility of creating antagonist fusion proteins as a potential class of therapeutic agent.

The antagonist Wnt inhibitor factor 1 (WIF-1) is frequently downregulated in cancer cells, including prostate, breast, lung, bladder and in osteosarcoma [47, 48]. This secreted protein comprises of a WIF domain for Wnt binding activity and epidermal growth factor repeats [49]. In various cancers, such as lung, breast, gastric, colorectal, and nasopharyngeal, silencing of the WIF-1 promoter by hypermethylation is associated with Wnt signaling activation [50–54]. Kansara et al. demonstrated that in primary OS, silencing of WIF-1 was also associated with in vivo acceleration of tumorigenesis in mice [55]. Recently, we demonstrated that

re-expressing WIF-1 in OS cells resulted in inhibition of anchorage-independent growth and cellular motility. With elevated WIF-1 expression, proteolytic enzyme matrix metalloproteinases (MMP-9 and MMP-14) were suppressed from degrading extracellular matrix. In vivo, injecting WIF-1 transfected OS cells into nude mice showed reduced tumorigenesis and pulmonary metastasis [48].

Besides naturally occurring antagonists, small molecule Wnt inhibitors are also being explored as a potential means to suppress tumorigenesis. Chen et al. examined several synthetic compounds via high-stringency cell-based screening and discovered two new classes of small molecules that perturb the Wnt pathway. The first class of compound inhibits the membrane-bound acyltransferase Porcupine, which is involved in Wnt protein production. The second class nullifies the destruction of Axin, which are known suppressors of the Wnt/β-catenin signaling pathway [56].

More specifically, it has been shown that OS progression can be affected by small molecule inhibitors that disrupt the Wnt/β-catenin pathway. Previous research on the natural compound curcumin showed an inhibitory effect against β-catenin/Tcf signaling amongst several cancer cell lines [57]. Hallet et al. found that PKIF118-310 (β-catenin/TCF inhibitor II) given to breast tumor-bearing syngeneic mice arrested tumor growth in vivo [58]. In OS, Leow et al. revealed that both curcumin and PKIF118-310 suppressed both intrinsic and activated β-catenin/Tcf transcriptional activities using luciferase reporter assays. They also showed significant reduction of nuclear β-catenin and inhibitory effects on OS cell migration and invasion in a dose-dependent manner. These anticancer effects correlated with decreased expression of downstream targeted oncogenes, such as cyclin D1, c-Myc, and survivin [59]. Other small molecule inhibitors, targeting Met, such as PF2362376 (targeting canine OS cell lines) and STA-1474 (heat-shock protein 90 inhibitor targeting both human and canine OS cell lines) have also shown to decrease proliferation and decrease phosphorylation of both Met and PKB/AKT [60, 61].

Grandy et al. recently revealed another small molecule inhibitor of Wnt via the PDZ domain of dishevelled [62]. Dishevelled (dvl) is an essential component of the Wnt signaling pathway, which transduces Wnt signals from the Frizzled receptor to downstream targeted components. Through structure-based ligand screening and NMR spectroscopy, these investigators were able to discover a small molecule inhibitor (3289-8625) with an affinity to the PDZ domain of dvl. It was shown to suppress the tumorigenesis of prostate cancer PC-3 cells, decrease Wnt signaling in the hyaloid vessel system, and may prove to have similar affects in OS cells.

Sclerostin is yet another glycoprotein that is known to antagonize the Wnt/β-catenin signaling in osteoblasts by binding to LRP5/LRP6 and subsequently inhibiting osteoblast differentiation, activity, and survival [63, 64]. The SOST gene encodes for sclerostin, and its inhibition has been an area of interest for treatment of osteoporosis [65, 66].

Controversy of Inactivity of Wnt/β-Catenin Pathway in High-Grade OS

There is some controversy over the impact of Wnt/β-catenin pathway in high-grade osteosarcoma. Unlike previous data, Cai, et al. in 2010 published results suggesting that loss of Wnt/β-catenin pathway activity may contribute to osteosarcoma proliferation [67]. Nuclear β-catenin rather than cytoplasmic β-catenin was examined in osteosarcoma biopsies/cell lines and osteoblastomas by immunohistochemistry. Nuclear β-catenin was not detected in 90 % of the OS biopsies and cell lines and the rest of the cases showed weak nuclear staining. After treating OS cells with GSK3β inhibitor (Wnt pathway activator), immunofluorescence β-catenin nuclear staining was positive in all cell lines and cellular proliferation rates were reduced. These investigators noted that only nuclear staining, and not membranous/cytoplasmic staining of β-catenin, can determine the degree of Wnt activity, since it is within the nucleus that transcription occurs for target gene expression. On the contrary, other groups such as Goentoro et al. demonstrated that the fold change, and not absolute level of β-catenin, determines the impact of Wnt activity and transcriptional changes [68]. With limitations of in vitro models, the theory from Cai et al. has yet to be proven within the context of an in vivo environment.

Targeting Noncanonical Wnt Pathways (β-Catenin-Independent Pathways)

Besides the canonical pathway, Wnt has been known to affect β-catenin-independent pathways as well, including Wnt/calcium, Wnt/Rho GTPase, and Wnt/JNK pathways [10]. Over the past two decades, more noncanonical Wnt pathways have been described, although they are less understood and are initiated by Wnt/Frizzled signaling, rather than β-catenin transcriptional function. These signals are transduced via Frizzled family receptors and co-receptors ROR2 and RYK [69]. In the Wnt/calcium pathway, Wnt5a/Frizzled-2 modulates the calcium-sensitive proteins, calcium/calmodulin-dependent kinase II and protein kinase C, thus increasing the intracellular calcium flux [70]. Wnt/Frizzled activates cyclic GMP-specific phosphodiesterase (PDE6) leading to depletion of cellular cGMP and inactivation of protein kinase G (PKG), and subsequently causing increase intracellular calcium concentration. The calcium-dependent effector molecules of this pathway are Nemo-like kinase (NLK) and nuclear factor of activated T cells (NFAT). The NLK inhibits TCF/β-catenin signaling via phosphorylation of TCF/LEF family transcription factors, while the NFAT inhibits ventral patterning in Xenopus, respectively [71–73].

The Wnt/planar cell polarity (PCP) pathway, consisting of Wnt5a and Wnt11, initiates signaling through its interaction with frizzled (Fz), activating dishevelled (Dvl) and forming Dvl/effector complexes [71, 74, 75]. The Dvl downstream

pathway, including both Ras homolog gene family A (RhoA) and Jun Kinase (JNK), regulates actin cytoskeleton, cell motility, and adhesion [9, 76]. It impacts temporal and spatial control of embryonic development seen in both Xenopus embryos and cuticular hairs in Drosophila.

The JNK pathway is also a mediator of noncanonical Wnt signaling which is activated via Wnt-Ror2 signaling. By using siRNA to suppress Wnt5a or Ror2, Enomoto et al. demonstrated reduced invasiveness and invadopodia formation in OS cells [77].

Wnt/B-Catenin Signaling and Stem Cells

The Wnt/β-catenin pathway not only has a role in tumorigenesis but has also been suggested to exert diverse regulatory effects on cancer stem cells (CSC) [78]. Stem cells in general are defined as having the ability to self-renew along with creating specialized cells. Several groups of investigators have examined the Wnt pathway and its effects on specific stem cell functions [6]. As early as the 1990s, Korinek et al. demonstrated the association between mutated TCF4 and subsequent depletion of intestinal stem cells. Studies on the role of stem cells in hair follicle formation have suggested that Wnt inhibitors play a prominent role in regulating the stem cell microenvironments [79]. The transgenic overexpression of LEF1 resulted in not only follicle stem cell growth but also differentiation of the hair lineage [80].

In OS cell lines, Tirino et al. identified a subpopulation of CD133+ cells with self-renewal properties, higher proliferation, spherical formation, and expression of the stem cell-associated gene OCT3/4 [81]. In addition, elevated aldehyde dehydrogenase (ALDH) activity in normal stem cells and solid tumor CSC has led to the use of ALDH as a means of identifying CSC in sarcomas. Wang et al. found that OS cell lines containing a subpopulation of cells with high ALDH activity possess increased tumorigenic capacity, proliferative capacities, self-renewal, and expression of stem cell markers [82].

Therapy Against Wnt Target Genes in Osteosarcoma

Given an abundance of data suggesting Wnt/β-catenin involvement in tumorigenesis, there is a need to discover ways to mitigate the Wnt transcriptional activation [29, 83]. Several strategies have been proposed to exploit the Wnt pathway for cancer therapy [22, 84, 85]. The challenge to some of these strategies is that the Wnt pathway is a vast network that also regulates normal cell functions, tissue regeneration, and stem cell differentiation. Depending on how this pathway is targeted (extracellular, cytoplasmic, nuclear), detrimental side effects may be incurred.

Targeting the Wnt/β-catenin signaling at the extracellular level is a strategy that focuses on secreted Wnt antagonists, including WIF-1, Dkk, and sFRPs. Restoring

the expression of these antagonists in the antagonist-deficient tumors may prove to be helpful in reducing the proliferation of OS cells. Another strategy that simulates the mechanisms of Wnt antagonists is to create anti-Wnt monoclonal antibodies that can induce apoptosis of OS cells by blocking Wnt-Frizzled interaction. Therapeutic monoclonal antibodies against Wnt-1 and Wnt-2 have demonstrated inhibition of Wnt signaling and suppression of tumor growth in hepatocellular carcinoma and melanoma, respectively [86, 87]. This model can also be explored and potentially replicated for OS. Besides the extracellular level, we can aim to target the cytoplasmic components, such as β-catenin-binding domain of APC, for tumor suppression. Shih et al. showed that in colon cancer cells, re-expression of a recombinant adenovirus with APC (with known β-catenin binding repeats) can inhibit nuclear translocation of β-catenin as well as β-catenin/Tcf-mediated transactivation [88]. At the nuclear level, targeting the β-catenin/Tcf transcriptional activity along with key downstream mediators, such as c-Myc, cyclin D1, survivin, needs to be further explored. In OS, the hepatocyte growth factor receptor c-Met is another Wnt target gene that is frequently overexpressed. Recent evidence suggests that c-Met can transform normal human osteoblasts into OS cells [44]. Therefore, c-Met is a candidate Wnt-related gene that can explored for OS therapeutics.

Nonsteroidal anti-inflammatory drugs (NSAIDS) have been studied and thought to impact the Wnt pathway by inhibiting the accumulation of prostaglandin E2, which ultimately decreases degradation of the β-catenin. NSAIDs have mainly shown chemopreventative effects against colon cancer [89, 90]. Xia et al. demonstrated the effects of celecoxib (cyclo-oxygenase-2 inhibitor) on inhibiting β-catenin-dependent survival of human OS cell line (MG-63). Not only did β-catenin protein decrease in the cytosol and nucleus following celecoxib treatment, but there was also a significant reduction of the Wnt target gene c-Myc and CCND1 [91]. As mentioned previously, using small molecule inhibitors identified by high-throughput screens can be helpful to make an impact on OS therapy. These inhibitors are known to target β-catenin/TCF antagonists, transcriptional co-activator modulators along with the PDZ domain of Dvl [92].

Conclusion

Given the complexity of the Wnt signaling network, it is not an easy task to determine which group of components is responsible for the interactions that drives OS progression. With a large permutation of Wnt signaling (given 19 human Wnt family members, 11 human Fz receptors, 4 human Dkks, along with multiple regulatory proteins), it is challenging to identify specific combinations of interaction that may be clinically relevant to OS. Although our understanding of the Wnt pathway has improved over the last few decades, there are certainly many regulatory mechanisms yet to be discovered. From this standpoint, the Wnt pathway offers a plethora of targeting potentials for cancer drug development. By understanding the pathophysiology of aberrant Wnt signaling in OS, we are getting closer to designing much more precise and personalized treatment for this disease.

References

1. Mirabello L, Troisi RJ, Savage SA (2009) Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer 115(7):1531–1543
2. Bacci G et al (2000) Long-term outcome for patients with nonmetastatic osteosarcoma of the extremity treated at the istituto ortopedico rizzoli according to the istituto ortopedico rizzoli/ osteosarcoma-2 protocol: an updated report. J Clin Oncol 18(24):4016–4027
3. Mialou V et al (2005) Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome – the French pediatric experience. Cancer 104(5):1100–1109
4. Hayden JB, Hoang BH (2006) Osteosarcoma: basic science and clinical implications. Orthop Clin North Am 37(1):1–7
5. Lewis IJ et al (2007) Improvement in histologic response but not survival in osteosarcoma patients treated with intensified chemotherapy: a randomized phase III trial of the European Osteosarcoma Intergroup. J Natl Cancer Inst 99(2):112–128
6. Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. Cell 149(6):1192–1205
7. Nusse R, Varmus HE (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31(1):99–109
8. Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature 434(7035):843–850
9. Lustig B, Behrens J (2003) The Wnt signaling pathway and its role in tumor development. J Cancer Res Clin Oncol 129(4):199–221
10. van Amerongen R, Mikels A, Nusse R (2008) Alternative wnt signaling is initiated by distinct receptors. Sci Signal 1(35):re9
11. Waltzer L, Bienz M (1999) The control of beta-catenin and TCF during embryonic development and cancer. Cancer Metastasis Rev 18(2):231–246
12. Polakis P (2000) Wnt signaling and cancer. Genes Dev 14(15):1837–1851
13. Polakis P (1999) The oncogenic activation of beta-catenin. Curr Opin Genet Dev 9(1):15–21
14. Behrens J (2000) Control of beta-catenin signaling in tumor development. Ann N Y Acad Sci 910:21–33, discussion 33-5
15. Moon RT (2005) Wnt/beta-catenin pathway. Sci STKE 2005(271):cm1
16. Yeh JR, Peterson RT (2009) Novel Wnt antagonists target porcupine and Axin. Nat Chem Biol 5(2):74–75
17. Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87(2): 159–170
18. Thomas DM (2010) Wnts, bone and cancer. J Pathol 220(1):1–4
19. McQueen P et al (2011) The Wnt signaling pathway: implications for therapy in osteosarcoma. Expert Rev Anticancer Ther 11(8):1223–1232
20. MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 17(1):9–26
21. Jamieson C, Sharma M, Henderson BR (2012) Wnt signaling from membrane to nucleus: beta-catenin caught in a loop. Int J Biochem Cell Biol 44(6):847–850
22. Luu HH et al (2004) Wnt/beta-catenin signaling pathway as a novel cancer drug target. Curr Cancer Drug Targets 4(8):653–671
23. Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810
24. Zi X et al (2005) Expression of Frzb/secreted Frizzled-related protein 3, a secreted Wnt antagonist, in human androgen-independent prostate cancer PC-3 cells suppresses tumor growth and cellular invasiveness. Cancer Res 65(21):9762–9770
25. Mohinta S et al (2007) Wnt pathway and breast cancer. Front Biosci 12:4020–4033
26. Tomita H et al (2007) Development of gastric tumors in Apc(Min/+) mice by the activation of the beta-catenin/Tcf signaling pathway. Cancer Res 67(9):4079–4087
27. Najdi R, Holcombe RF, Waterman ML (2011) Wnt signaling and colon carcinogenesis: beyond APC. J Carcinog 10:5

28. Larue L, Delmas V (2006) The WNT/Beta-catenin pathway in melanoma. Front Biosci 11:733–742
29. Takahashi-Yanaga F, Sasaguri T (2007) The Wnt/beta-catenin signaling pathway as a target in drug discovery. J Pharmacol Sci 104(4):293–302
30. Nusse R (2012) Wnt signaling. Cold Spring Harb Perspect Biol 4(5)
31. Korinek V et al (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 275(5307):1784–1787
32. Morin PJ et al (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275(5307):1787–1790
33. Hoang BH (2012) Wnt, osteosarcoma, and future therapy. J Am Acad Orthop Surg 20(1):58–59
34. Hoang BH et al (2004) Expression of LDL receptor-related protein 5 (LRP5) as a novel marker for disease progression in high-grade osteosarcoma. Int J Cancer 109(1):106–111
35. Guo Y et al (2008) Dominant negative LRP5 decreases tumorigenicity and metastasis of osteosarcoma in an animal model. Clin Orthop Relat Res 466(9):2039–2045
36. Guo Y et al (2007) Blocking Wnt/LRP5 signaling by a soluble receptor modulates the epithelial to mesenchymal transition and suppresses met and metalloproteinases in osteosarcoma Saos-2 cells. J Orthop Res 25(7):964–971
37. Hsieh SY et al (2004) Dickkopf-3/REIC functions as a suppressor gene of tumor growth. Oncogene 23(57):9183–9189
38. Veeck J, Dahl E (2012) Targeting the Wnt pathway in cancer: the emerging role of Dickkopf-3. Biochim Biophys Acta 1825(1):18–28
39. Hoang BH et al (2004) Dickkopf 3 inhibits invasion and motility of Saos-2 osteosarcoma cells by modulating the Wnt-beta-catenin pathway. Cancer Res 64(8):2734–2739
40. Lin CH et al (2013) Dkk-3, a secreted wnt antagonist, suppresses tumorigenic potential and pulmonary metastasis in osteosarcoma. Sarcoma 2013:147541
41. Hoang B et al (1996) Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis. J Biol Chem 271(42): 26131–26137
42. Leyns L et al (1997) Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell 88(6):747–756
43. Guo Y et al (2008) Frzb, a secreted Wnt antagonist, decreases growth and invasiveness of fibrosarcoma cells associated with inhibition of Met signaling. Cancer Res 68(9): 3350–3360
44. Patane S et al (2006) MET overexpression turns human primary osteoblasts into osteosarcomas. Cancer Res 66(9):4750–4757
45. Mandal D et al (2007) Severe suppression of Frzb/sFRP3 transcription in osteogenic sarcoma. Gene 386(1–2):131–138
46. DeAlmeida VI et al (2007) The soluble wnt receptor Frizzled8CRD-hFc inhibits the growth of teratocarcinomas in vivo. Cancer Res 67(11):5371–5379
47. Wissmann C et al (2003) WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer. J Pathol 201(2):204–212
48. Rubin EM et al (2010) Wnt inhibitory factor 1 decreases tumorigenesis and metastasis in osteosarcoma. Mol Cancer Ther 9(3):731–741
49. Hsieh JC et al (1999) A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature 398(6726):431–436
50. Lin YC et al (2006) Wnt signaling activation and WIF-1 silencing in nasopharyngeal cancer cell lines. Biochem Biophys Res Commun 341(2):635–640
51. Mazieres J et al (2004) Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. Cancer Res 64(14):4717–4720
52. Ai L et al (2006) Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. Carcinogenesis 27(7):1341–1348
53. Taniguchi H et al (2005) Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. Oncogene 24(53):7946–7952

54. Lee BB et al (2009) Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. Clin Cancer Res 15(19):6185–6191
55. Kansara M et al (2009) Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcomagenesis in mice. J Clin Invest 119(4):837–851
56. Chen B et al (2009) Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat Chem Biol 5(2):100–107
57. Park CH et al (2005) The inhibitory mechanism of curcumin and its derivative against beta-catenin/Tcf signaling. FEBS Lett 579(13):2965–2971
58. Hallett RM et al (2012) Small molecule antagonists of the Wnt/beta-catenin signaling pathway target breast tumor-initiating cells in a Her2/Neu mouse model of breast cancer. PLoS One 7(3):e33976
59. Leow PC et al (2010) Antitumor activity of natural compounds, curcumin and p KF118–310, as Wnt/beta-catenin antagonists against human osteosarcoma cells. Invest New Drugs 28(6):766–782
60. Liao AT et al (2007) A novel small molecule Met inhibitor, PF2362376, exhibits biological activity against osteosarcoma. Vet Comp Oncol 5(3):177–196
61. McCleese JK et al (2009) The novel HSP90 inhibitor STA-1474 exhibits biologic activity against osteosarcoma cell lines. Int J Cancer 125(12):2792–2801
62. Grandy D et al (2009) Discovery and characterization of a small molecule inhibitor of the PDZ domain of dishevelled. J Biol Chem 284(24):16256–16263
63. Li X et al (2005) Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. J Biol Chem 280(20):19883–19887
64. Holdsworth G et al (2012) Characterization of the interaction of sclerostin with the low density lipoprotein receptor-related protein (LRP) family of Wnt co-receptors. J Biol Chem 287(32): 26464–26477
65. Lewiecki EM (2011) Sclerostin: a novel target for intervention in the treatment of osteoporosis. Discov Med 12(65):263–273
66. Lewiecki EM (2011) Sclerostin monoclonal antibody therapy with AMG 785: a potential treatment for osteoporosis. Expert Opin Biol Ther 11(1):117–127
67. Cai Y et al (2010) Inactive Wnt/beta-catenin pathway in conventional high-grade osteosarcoma. J Pathol 220(1):24–33
68. Goentoro L, Kirschner MW (2009) Evidence that fold-change, and not absolute level, of beta-catenin dictates Wnt signaling. Mol Cell 36(5):872–884
69. Oishi I et al (2003) The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells 8(7):645–654
70. Kohn AD, Moon RT (2005) Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 38(3–4):439–446
71. Semenov MV et al (2007) SnapShot: noncanonical Wnt signaling pathways. Cell 131(7):1378
72. Ishitani T et al (2003) The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol Cell Biol 23(1): 131–139
73. Dejmek J et al (2006) Wnt-5a/Ca2+-induced NFAT activity is counteracted by Wnt-5a/Yes-Cdc42-casein kinase 1alpha signaling in human mammary epithelial cells. Mol Cell Biol 26(16):6024–6036
74. Veeman MT, Axelrod JD, Moon RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell 5(3):367–377
75. Wang Y, Nathans J (2007) Tissue/planar cell polarity in vertebrates: new insights and new questions. Development 134(4):647–658
76. Seifert JR, Mlodzik M (2007) Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility. Nat Rev Genet 8(2):126–138
77. Enomoto M et al (2009) Autonomous regulation of osteosarcoma cell invasiveness by Wnt5a/Ror2 signaling. Oncogene 28(36):3197–3208

78. Dravid G et al (2005) Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem Cells 23(10):1489–1501
79. Fuchs E, Tumbar T, Guasch G (2004) Socializing with the neighbors: stem cells and their niche. Cell 116(6):769–778
80. Zhou P et al (1995) Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. Genes Dev 9(6):700–713
81. Tirino V et al (2011) Human primary bone sarcomas contain CD133+ cancer stem cells displaying high tumorigenicity in vivo. FASEB J 25(6):2022–2030
82. Wang L et al (2011) Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity. Int J Cancer 128(2):294–303
83. Barker N, Clevers H (2006) Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov 5(12):997–1014
84. Luo J et al (2007) Wnt signaling and human diseases: what are the therapeutic implications? Lab Invest 87(2):97–103
85. Moon RT et al (2004) WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 5(9):691–701
86. Wei W et al (2009) Blockade of Wnt-1 signaling leads to anti-tumor effects in hepatocellular carcinoma cells. Mol Cancer 8:76
87. You L et al (2004) An anti-Wnt-2 monoclonal antibody induces apoptosis in malignant melanoma cells and inhibits tumor growth. Cancer Res 64(15):5385–5389
88. Shih IM et al (2000) The beta-catenin binding domain of adenomatous polyposis coli is sufficient for tumor suppression. Cancer Res 60(6):1671–1676
89. Thun MJ, Henley SJ, Patrono C (2002) Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 94(4):252–266
90. Chan TA (2002) Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. Lancet Oncol 3(3):166–174
91. Xia JJ et al (2010) Celecoxib inhibits beta-catenin-dependent survival of the human osteosarcoma MG-63 cell line. J Int Med Res 38(4):1294–1304
92. Takahashi-Yanaga F, Kahn M (2010) Targeting Wnt signaling: can we safely eradicate cancer stem cells? Clin Cancer Res 16(12):3153–3162

Receptor Tyrosine Kinases in Osteosarcoma: Not Just the Usual Suspects

Ashley N. Rettew, Patrick J. Getty, and Edward M. Greenfield

Abstract Despite aggressive surgical and chemotherapy protocols, survival rates for osteosarcoma patients have not improved over the last 30 years. Therefore, novel therapeutic agents are needed. Receptor tyrosine kinases have emerged as targets for the development of new cancer therapies since their activation leads to enhanced proliferation, survival, and metastasis. In fact, aberrant expression and activation of RTKs have been associated with the progression of many cancers. Studies from our lab using phosphoproteomic screening identified RTKs that are activated and thus may contribute to the signaling within metastatic human osteosarcoma cells. Functional genomic screening using siRNA was performed to distinguish which of

A.N. Rettew
Department of Orthopaedics, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Department of Pathology, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA
e-mail: anr14@case.edu

P.J. Getty
Department of Orthopaedics, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Seidman Cancer Center, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA
e-mail: Patrick.Getty@UHhospitals.org

E.M. Greenfield (✉)
Department of Orthopaedics, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Department of Pathology, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Case Comprehensive Cancer Center, Case Medical Center, Case Western Reserve University, Cleveland, OH, USA
e-mail: emg3@case.edu

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_3,

the activated RTKs contribute to in vitro phenotypes associated with metastatic potential (motility, invasion, colony formation, and cell growth). The resulting RTK hits were then validated using independent validation experiments. From these results, we identified four RTKs (Axl, EphB2, FGFR2, and Ret) that have not been previously studied in osteosarcoma and provide targets for the development of novel therapeutics.

Keywords Receptor tyrosine kinases • Axl • EphB2 • FGFR2 • Ret • IGF-1R • Targeted therapy • Phosphoproteomic screen • Osteosarcoma

Receptor Tyrosine Kinases

Protein tyrosine kinases are important signaling molecules with highly regulated activity. Their main function is to catalyze the transfer of the γ phosphate of ATP to tyrosine residues on protein substrates [1]. This creates binding sites for adaptor proteins and downstream signaling molecules leading to changes in cell proliferation, differentiation, migration, survival, or other metabolic activities. In most situations, autophosphorylation is associated with increased enzymatic activity [1].

There are 90 protein tyrosine kinases encoded in the human genome [2]. They can further be divided into two subtypes. Receptor tyrosine kinases (RTKs) are transmembrane glycoproteins that undergo dimerization and autophosphorylation upon ligand binding [3]. Most RTKs consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular kinase domain [4]. Many receptors for growth factors are included in the RTK family. Additionally, there are non-receptor tyrosine kinases (NRTKs) that are downstream signaling molecules of various receptors including RTKs. NRTKs lack transmembrane domains and are located in either the cytoplasm or the nucleus. NRTKs can be trans-autophosphorylated upon dimerization or phosphorylated by a different tyrosine kinase [1]. The remainder of this chapter will focus on RTKs.

RTKs are divided into families based on the composition of their extracellular domains as well as shared ligands [4]. Upon ligand binding, the receptor undergoes oligomerization and other conformational changes that result in autophosphorylation of their intracellular domain [3]. This initiates downstream signaling cascades in which cytoplasmic substrates are recruited and sequentially phosphorylated, ultimately leading to signaling outputs such as proliferation, survival, migration, apoptosis, and cell adhesion [5] (Fig. 1).

Regulation of RTKs

The activity of RTKs is tightly regulated on multiple levels. Ligand binding reverses autoinhibition of the RTK by altering its conformation and inducing autophosphorylation of key tyrosine residues. For example, the activation loop, situated next to

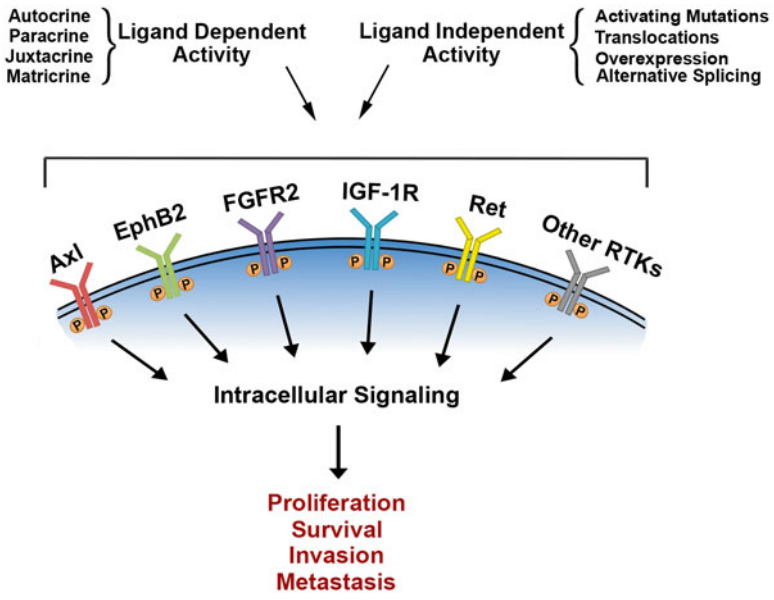


Fig. 1 Mechanisms of pathological receptor tyrosine kinase signaling in osteosarcoma. Dysregulation of RTK signaling can occur through ligand-dependent mechanisms such as autocrine production of the ligand, paracrine signaling from the tumor environment, or juxtacrine signaling between cells. Aberrant signaling may also occur through ligand-independent mechanisms such as activating mutations, translocations leading to fusion proteins, gene amplification resulting in overexpression, or alternative splicing. Autophosphorylation and activation of the receptor induces intracellular signaling. When unregulated, which is common in cancer, RTK activation can lead to aberrant proliferation, survival and ultimately invasion and metastasis

the active site in the kinase domain, contains tyrosine residues that stabilize the active configuration when autophosphorylated after receptor dimerization [6]. The juxtamembrane region is outside of the kinase domain and may make contact with several areas within the tyrosine kinase domain, including the activation loop, to stabilize the autoinhibited configuration [4, 7]. The juxtamembrane region also contains tyrosine residues that are transphosphorylated upon ligand binding and receptor dimerization. In turn, the autoinhibitory interactions of the juxtamembrane region are disrupted, promoting activation of the receptor.

RTK signaling is also regulated by positive and negative feedback loops. Protein tyrosine phosphatases may be inhibited as part of a positive feedback loop or induced during negative feedback loops. For example, reactive oxygen species, produced after prolonged epidermal growth factor receptor (EGFR) activation, inhibit phosphatase activity by oxidizing a cysteine in the phosphatase active domain. On the other hand, EGFR activation can also recruit Shp2, an SH domain-containing phosphatase, which leads to dephosphorylation of the receptor in a negative feedback loop [8]. Another positive feedback loop occurs when receptor activation induces production of its cognate ligand resulting in autocrine signaling [4]. Receptor activation may also lead to downstream expression of negative regulators of the signaling cascade representing a negative feedback mechanism.

RTK Dysregulation in Cancer

Since key physiological functions of a cell are regulated by RTK signaling, aberrant activation of RTKs may result in adverse effects, such as induction of tumorigenesis and metastasis. In fact, dysregulation of RTK activity has been shown to contribute to the progression of many cancers [9]. There are many mechanisms that can induce pathological signaling by an RTK, most leading to activation of the kinase domain (Fig. 1). Such mechanisms can be split into ligand-dependent and ligand-independent categories. The ligand-independent mechanisms include chromosomal translocations, activating mutations, and overexpression of the RTK. Chromosomal translocations lead to fusion proteins containing the c-terminal tyrosine kinase domain of an RTK juxtaposed with an n-terminal oligomerization domain of another protein [10]. Such fusion proteins dimerize and become constitutively activated without ligand stimulation. Historically, BCR-Abl is one of the most well-known fusion proteins associated with cancer [11]. It has been predominantly described in chronic myeloid leukemia patients positive for the Philadelphia chromosome [12, 13]. Chromosomal translocations involving the RTK Ret are associated with papillary thyroid carcinoma [14]. Translocations have also been described in sarcomas. In fact, 85 % of Ewing's sarcoma cases are attributed to a translocation between chromosome 11 and 22 that produces the EWS/FLI-1 fusion protein [15]. Unlike the previous fusion proteins that function as constitutively active tyrosine kinases, EWS/FLI-1 acts as an aberrant transcription factor directly regulating the expression of a range of oncogenes and tumor suppressors [16]. There have been no characteristic chromosomal translocations described for osteosarcoma due to complex karyotypes and the high degree of genetic variation between patients.

The genetic instability in cancer may also give rise to constitutively active RTKs. Some of the best characterized mutations occur in the EGFR and fibroblast growth factor receptor (FGFR) RTKs. A specific deletion in the extracellular domain of EGFR (EGFRvIII) has been described in non-small-cell lung carcinoma (NSCLC), glioblastomas, ovarian and breast carcinomas [17]. In bladder cancer, a point mutation in the extracellular domain of FGFR3 results in the formation of an abnormal disulfide bridge allowing ligand-independent dimerization [18]. Mutations have also been found in the catalytic and juxtamembrane domains.

Gene amplification of the receptor itself may give rise to overexpression of the RTK in cancer cells. This produces a greater number of receptor molecules on the surface of the cell that are available for ligand binding and stimulation of downstream signaling cascades. Overexpression may also lead to receptor molecules in greater proximity to each other, facilitating ligand-independent dimerization and phosphorylation of the receptors [19]. The overexpression of many different RTKs has been reported in a wide array of cancers. Moreover, the overexpression of some of those RTKs has been correlated with patient survival.

Constitutive activation of RTKs can also occur through ligand-dependent mechanisms, including autocrine, paracrine, juxtacrine, or matricrine [18, 20, 21].

Targeting RTKS in Cancer

Inhibitors of RTKs represent a promising class of novel targeted anticancer therapy. Different strategies are currently being developed to either target the extracellular ligand-binding domain or the intracellular tyrosine kinase domain [22].

The number of tyrosine kinase inhibitors (TKIs) in phase III clinical trials or approved by the FDA for cancer therapies continues to grow. Most are small molecule inhibitors (SMIs) designed to interfere with the binding of ATP or substrates and thereby inhibit the catalytic activity of the kinase [5, 9, 23]. Since the ATP binding pocket is relatively conserved between RTKs, many of the SMIs inhibit multiple kinases. This may, in fact, be clinically advantageous by targeting multiple different phenotypes activated by different kinases. Based on their mechanism of inhibition, the SMIs are categorized into type I–IV inhibitors.

Early drug discovery sought to design ATP mimics and used enzymatic kinase assays. Therefore, most SMIs are type I ATP-competitive inhibitors that bind to the active conformation of the kinase [24]. Specificity for these inhibitors is gained by targeting the gatekeeper residue located in the ATP-binding pocket [25]. Type I inhibitors include erlotinib, dasatinib, and sunitinib. In contrast, type II compounds are ATP-competitive inhibitors that bind the inactive form of RTKs [24]. These inhibitors recognize and stabilize a hydrophobic region exposed next to the ATP site when the RTK is in its inactive conformation [25]. Specificity is gained by identifying the RTKs that preferentially adopt this conformation. Common type II inhibitors include imatinib, sorafenib, and nolotinib. Type III inhibitors target allosteric sites outside of the ATP-binding pocket. These inhibitors offer the highest amount of specificity by targeting unique binding sites and regulatory mechanisms of a particular kinase [24]. No type III inhibitors have been described for RTKs, but the type III Abl inhibitor, GNF2, has been studied as an alternative to imatinib [26]. Type IV inhibitors form covalent bonds in the kinase domain. Most target a cysteine residue, irreversibly blocking ATP binding [24]. Many are cautious about this class of inhibitors, as the irreversible nature could lead to increased toxicity from binding off-target molecules.

Monoclonal antibodies have also been effective at neutralizing RTKs or their cognate ligands to prevent signaling [9]. The antibodies may either bind to the extracellular ligand-binding domain of the RTK or the ligand itself to block receptor–ligand interaction. RTK-specific antibodies may also induce receptor internalization and degradation. Trastuzumab (Herceptin) was one of the first monoclonal antibodies developed for cancer therapies that targeted an RTK. It is approved for use in breast cancer patients positive for the receptor Her2/neu [27]. Bevacizumab (Avastin) is an FDA approved monoclonal antibody targeting the RTK ligand vascular endothelial growth factor (VEGF). It was originally developed for colorectal cancer but has since been approved for renal and certain types of lung cancers as well as glioblastoma [28]. Finally, Cetuximab (Erbitux) is an anti-EGFR antibody approved for the treatment of colorectal cancer and squamous cell carcinoma of the head and neck [29, 30].

Acquired resistance to TKIs is a growing concern. One of the most common mechanisms of resistance is a mutation in the gatekeeper residue that modulates the binding of the inhibitor to the ATP binding pocket of the RTK [31]. However such mutations do not alter the ability of ATP to bind and therefore have little effect on kinase activity in the absence of the inhibitor. Gatekeeper mutations have been identified in EGFR conferring resistance to gefitinib and erlotinib [32, 33]. Other mechanisms of resistance involve upregulation of alternative RTK pathways or downstream signaling cascades [25]. Several strategies are being developed to overcome resistance such as new inhibitors that avoid the gatekeeper position [24].

RTK-targeted therapies have not yet been approved for osteosarcoma since studies examining the roles of RTKs are limited, both in quality and quantity. Clinical trials that include osteosarcoma patients are rare and usually have too few patients to accurately predict a response. Therefore, a thorough investigation identifying RTKs that are activated and contribute to the metastatic potential of osteosarcoma is needed to expand the number of potential targets for novel therapies.

RTKs in Osteosarcoma

RTKs contribute to the progression of many cancers but remain relatively unstudied in osteosarcoma. Overexpression of several RTKs and their ligands occurs in osteosarcoma, including EGFR, ErbB2, IGF-1R, *met*, NGFR, PDGFR, VEGFR and their ligands [34–41]. Specifically, overexpression of ErbB2, PDGF, PDGFR, VEGF, and VEGFR correlates with metastasis and overall poor prognosis in osteosarcoma [39, 40, 42–46]. However, the functional contribution of RTKs to the underlying biology of osteosarcoma has not been well established.

Screening and Validation Study

To begin to understand the role of RTKs in osteosarcoma, we performed a screening and validation study to identify novel RTKs that are activated and promote the in vitro phenotypes of metastatic osteosarcoma cell lines (Fig. 2). Two established families of genetically related human osteosarcoma cell lines were used. The 143B cell line was created from the parental weakly metastatic TE85 cell line by overexpressing oncogenic KRAS [47]. The LM7 cell line was isolated by cycling the parental weakly metastatic Saos2 cells through the lungs of nude mice seven times [48].

Since phosphorylation events occur during the activation of most receptor tyrosine kinases, we performed phosphoproteomic screening to identify RTKs that are activated and potentially contribute to signaling within the highly metastatic human LM7 or 143B cells. This also reduced the number of RTKs examined in the functional genomic screen making it feasible to investigate multiple in vitro phenotypes instead of focusing on one high throughput phenotype, such as proliferation, as is common in large-scale screens. For our study, we measured migration, invasion,

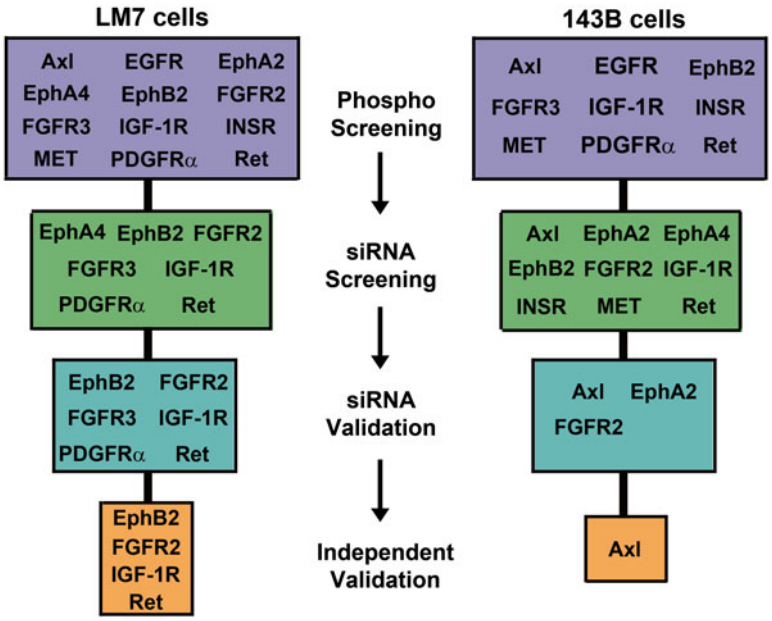


Fig. 2 Summary of screening and validation approaches. Phosphoproteomic screening was performed on 42 of the RTKs encoded in the human genome. Functional genomic screening using siRNA against the activated RTKs was performed. Finally, validation of the hits from the functional screen identified specific novel RTKs that promoted the in vitro phenotypes of metastatic human osteosarcoma cell lines [49]

colony formation, and cell growth. In our screen, the siRNAs affected cell growth the least out of the four assays performed, indicating that many important effects would have been missed had we focused solely on cell growth. Results from the phosphoproteomic screening identified 12 RTKs that are phosphorylated in one or both of the metastatic cell lines [49].

Functional genomic screening using siRNA was performed to distinguish which of the activated RTKs contribute to in vitro phenotypes associated with metastatic potential (motility, invasion, colony formation, and cell growth). Finally, validation of the screen was necessary since siRNA has the potential for off-target effects. The validation experiments confirmed that five RTKs (Axl, EphB2, FGFR2, IGF-1R, Ret) promote the in vitro behavior of the metastatic osteosarcoma cell lines [49]. This was the first study to demonstrate a role for Axl, EphB2, FGFR2 or Ret in the in vitro phenotype of human metastatic osteosarcoma cell lines.

IGF-1R

IGF-1R is one of the few RTKs that have been extensively studied in osteosarcoma [50] and therefore acts as a positive control to support our screening and validation strategies for identifying RTKs important to the in vitro behavior of osteosarcoma.

IGF-1R is a member of the insulin receptor family, which also includes the insulin receptor (IR). Their ligands include IGF-1 and IGF-2. IGF-1R is ubiquitously expressed and is involved in proliferation, differentiation and motility. It is also important for anchorage independent growth [51].

In our study, IGF-1R was phosphorylated in all cell lines tested. Inhibition of IGF-1R potently reduced motility and colony formation in the LM7 cell line. These results are consistent with previous studies in osteosarcoma. Inhibition of IGF-1R reduced proliferation [52], growth, and invasiveness and induced apoptosis in vitro [53]. IGF-1R neutralizing antibodies inhibited tumor growth in murine xenograft models of osteosarcoma [54–57]. Lung metastasis was inhibited in hypophysectomized mice, which lack IGF-1R signaling [58]. Finally, overexpression of IGF-1R has been reported in osteosarcoma patient samples [34]. Additionally, our lab previously demonstrated that an IGF-1R small molecule inhibitor significantly reduced motility and colony formation in LM7 cells [59]. The promising results from the above studies have led to phase I clinical trials using IGF-1R inhibitors in osteosarcoma patients [60].

There are a number of small molecule inhibitors that target IGF-1R. BMS-754807 and OSI-906, SMIs selective for both IGF-1R and IR, demonstrated antitumor activity in osteosarcoma xenografts and cell lines [61, 62]. Stable disease was achieved in osteosarcoma patients in a phase I trial using BMS-854807 [63]. There are also a number of neutralizing antibodies that target IGF-1R and are currently under development for targeted therapies. SCH717454, R1507, and cixutumumab (IMC-A12) have demonstrated antitumor effects in preclinical testing of osteosarcoma [55, 56, 64] and are currently being tested in phase 2 clinical trials including patients with recurrent osteosarcoma.

Axl

Axl (also known as Ufo or Ark) is a member of the TAM family of RTKs, which also includes Tyro3 and Mer [65]. Axl was first identified as a transforming gene in chronic myeloid leukemia. The name was derived from the Greek word ***anexelekto*** which means uncontrolled [65]. Activation of Axl can occur through binding of its ligand Gas6. Gas6 is a 75 kDa vitamin K-dependent protein containing a modified γ-carboxyglutamic acid residue, EGF-like repeats and a sex hormone binding globulin (SHBG)-like region [66]. The latter region directly binds Axl and activates signaling. Gas6 can bind the other family members but has been shown to have a three to tenfold lower affinity for Mer than Axl [67]. Axl can also be activated through ligand-independent dimerization [68]. This can be through binding of Axl molecules on different cells, which can modulate cell aggregation, or through cross talk with other RTKs such as EGFR [69]. Axl is ubiquitously expressed and has been implicated in cell survival, proliferation, endothelial cell migration, and vascular network formation [67]. Complete knockout of Axl in transgenic mice results in a normal phenotype, which indicates that inhibition of Axl will likely have mild adverse effects [70].

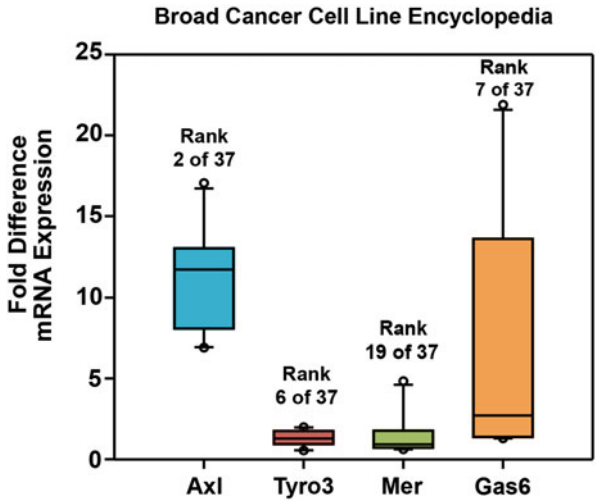


Fig. 3 Fold differences in mRNA expression in osteosarcoma samples compared to all other cancer types based on the CCLE. The median mRNA expression in osteosarcoma samples was compared to the median mRNA expression in all other cancer cell lines for each member of the TAM family of RTKs and their ligand Gas6. The data was based on the Broad Institutes CCLE [85]. The rank represents the level of expression in osteosarcoma samples compared to the other 36 cancer cell line types

Consistent with this, the Axl SMI BGB324 or Axl-Fc is effective at inhibiting Axl signaling with little to no side effects in vivo [71, 72].

Recent studies have demonstrated that Axl is important to the progression of many different cancers. Specifically, dysregulation of Axl activation can lead to enhanced invasion, migration, and survival ([73], and references therein). There is growing evidence that Axl is one of the common genes found to be upregulated and responsible for resistance to targeted or conventional chemotherapies [73–78]. Moreover, Axl overexpression has been associated with shorter overall survival in many different cancers including acute myeloid leukemia, pancreatic, lung, and breast carcinoma [79–82]. Overexpression of Axl has also been found in sarcomas such as synovial sarcoma and liposarcoma [83–86]. Prior to our work, there were a few studies that reported Axl overexpression in osteosarcoma. In a microarray study, Axl mRNA expression was upregulated the greatest (~40 fold) in metastatic osteosarcoma cell lines compared to their parental cell line and related non-metastatic cells [87]. Another microarray study demonstrated that Axl expression was upregulated in osteosarcoma tissue samples compared to other cancer tissues (www.ebi.ac.uk/arrayexpress, accession number E-MTAB-62). More recently, a compilation of gene expression data for 947 cell lines (Cancer Cell Line Encyclopedia (CCLE), http://www.broadinstitute.org/ccle) has revealed that Axl expression was highest in chondrosarcoma and osteosarcoma cell lines [85]. Specifically, Axl expression was 11-fold higher in osteosarcoma cell lines compared to all other cancer cell types (Fig. 3). Further analysis of the CCLE data indicated that Axl was the

predominant TAM family receptor expressed in osteosarcoma cell lines (Fig. 3). These results are consistent with a recently published paper that evaluated total Axl expression in a human osteosarcoma tissue microarray (TMA) [88]. The authors found no statistical difference between patients with metastases and patients with localized disease. This is likely due to the fact that the majority of patients (75 %) evaluated were positive for Axl expression [88].

Through our phosphoproteomic screening, we demonstrated that Axl is activated in all osteosarcoma cell lines tested [49]. These results are consistent with other recently published reports in which Axl was phosphorylated in additional osteosarcoma cell lines and patient samples. Activating mutations and translocations have not been found in osteosarcoma or in other cancers [89, 90]. Therefore, it is unlikely activating mutations or translocations are causing Axl activation in our cell lines [91, 92]. In contrast, overexpression of Axl and Gas6 has been reported for many different cancers [67]. Further studies are needed to determine whether receptor overexpression or autocrine signaling by Gas6 is the underlying mechanism of Axl activation in osteosarcoma.

Our siRNA screening and validation results indicate that Axl promotes the motility and colony formation of 143B cells in vitro. Preliminary results from recently published studies show that Axl knockdown resulted in reduced proliferation and increased apoptosis and Gas6 stimulation increased invasion and migration of osteosarcoma cell lines [88, 92]. We also demonstrated that BGB324 (also known as R428), a selective Axl SMI [72], reduced Axl phosphorylation, motility, and colony formation in a dose-dependent manner. BGB324, which is orally bioavailable and well tolerated in mice, reduces invasion, migration, and colony formation in esophageal adenocarcinoma [93] and reduces metastasis in murine models of breast cancer [72]. This supports the investigation of BGB324 as a potential lead compound for targeted therapy in osteosarcoma.

Another promising approach is the anti-Axl neutralizing monoclonal antibody YW327.6S2, which blocked Axl activity and growth of lung cancer cells and metastasis of breast cancer cells in murine xenograft models [67]. It was also effective at sensitizing lung carcinoma cells to erlotinib [67]. Aptamers, which are short single-stranded RNA or DNA, are able to bind to cell surface proteins with high affinity, are relatively stable, and produce little immunogenicity. For these reasons, they have become the focus of novel cancer therapeutic drug development. GL21.T is a recently developed aptamer targeting Axl and has been shown to inhibit migration and invasion in vitro and tumor growth in vivo in human non-small-cell lung cancer cells [94].

EphB2

The Eph receptors represent the largest family of RTKs, consisting of ten EphA and six EphB receptors. The receptors were initially subdivided based on similarities in their extracellular domain and their binding preferences to the two classes of ligands,

ephrin-A and ephrin-B ligands. Nonetheless, there is promiscuity in binding between ephrins and Eph receptors. Since ephrins are membrane bound ligands requiring cell-to-cell contact, interactions between Eph receptors and their ligands can induce bidirectional signaling, forward signaling in the Eph-expressing cell as well as reverse signaling in the ephrin-expressing cell [95]. EphB2 signaling has been implicated in the progression of many cancers including synovial sarcoma [95–97] and colorectal carcinoma [98]. In our study, EphB2 was phosphorylated in both the 143B and LM7 cells. Motility was significantly inhibited in LM7 cells after EphB2 knockdown by either siRNA or antisense. This result is consistent with the finding that Eph Receptor signaling is involved in actin cytoskeleton organization thus modulating cell morphology and migration [95, 96]. Ephrin expression profiling in osteosarcoma demonstrated that ephrin-A5 and ephrin-B1, ligands for Eph receptors including EphB2, are expressed in osteosarcoma specimens but not in normal bone [99, 100]. Our study is the first to demonstrate that EphB2 is important to the in vitro phenotype of osteosarcoma and may be a valuable target for the development of new treatments.

Peptides that bind specifically to Eph receptors and block the binding of ephrins have been identified through phage display. Among them, SNEW (SNEWIQPRLPQH) selectively binds EphB2 in the ephrin-binding pocket and inhibits EphB2 signaling in cells [101]. However, preclinical studies are needed to determine whether the peptide is effective at inhibiting tumor growth and metastasis in vivo.

Dasatinib and nilotinib are two multikinase inhibitors that have been shown to inhibit EphB2, along with BCR-abl, c-Kit, PDGFR-β, other Eph receptors, and members of the src family [102]. The effect of nilotinib in osteosarcoma has not been investigated. Dasatinib inhibited the in vitro phenotypes of osteosarcoma cell lines but failed to inhibit metastasis in vivo [103].

FGFR2

The fibroblast growth factor (FGF) family consists of four receptors, FGFR1, FGFR2, FGFR3, and FGFR4, and 18 different FGF ligands. FGF signaling is involved in embryogenesis, as well as angiogenesis and wound healing. It is also critical for limb development, with mutations in FGFR2 and FGFR3 resulting in skeletal dysplasias [18]. Germ-line and somatic mutations in FGFRs are associated with many different cancers, including bladder, breast, lung, gastric, and cervical cancer, rhabdomyosarcoma, and glioblastoma [104]. In osteosarcoma, several genetic alterations of FGFRs have been found including single nucleotide polymorphisms [105], overexpression [106], and germ-line amplifications [107]. Oncomine (Compendia Biosciences, Ann Arbor, MI) was also used for the analysis and visualization of osteosarcoma studies showing FGFR2 mRNA overexpression osteosarcoma biopsy samples compared to other sarcomas [108, 109]. Furthermore, a previous study demonstrated that FGF2, an FGFR ligand, upregulated migration in an osteosarcoma cell line [110]. This is consistent with our results indicating that

FGFR2 is phosphorylated and promotes the motility and colony formation of the metastatic LM7 cells. Taken together with the previous studies, our results indicate that FGFR2 may be important to the underlying biology of osteosarcoma. In contrast, FGFR1 and FGFR4 were not phosphorylated in either cell line. FGFR3 was phosphorylated in both cell lines tested. The individual siRNAs and antisense targeting FGFR3 had a small effect on the in vitro phenotypes.

Due to the similarity in their kinase domains, there are no small molecule inhibitors able to target a single FGFR. However, there are pan-FGFR inhibitors that have shown promising results. PD173074 has been shown to be effective at inhibiting the in vitro and in vivo phenotypes of many different cancer cell types including glioblastoma and rhabdomyosarcoma [111, 112]. Preliminary results from our lab demonstrate that treatment of metastatic osteosarcoma cell lines with PD173074 significantly reduces motility and colony formation in a dose dependent manner. Moreover, PD173074 causes preformed colonies to undergo rapid morphological changes consistent with cell death. Additional studies using PD173074 have demonstrated a role of FGFRs in the resistance of cancer cells to other small molecule inhibitors, such as gefitinib resistance in non-small-cell lung cancer [113] and cetuximab resistance in squamous cell carcinoma [114]. NVP-BGJ398, another pan-FGFR inhibitor, has recently been used in a preclinical study of 500 cancer cell lines. Cell viability assays demonstrated that FGFR1 amplification is a powerful predictor of sensitivity to NVP-BGJ398 in osteosarcoma [115]. Due to the structural similarity in the kinase domains, several of the FGFR inhibitors also target VEGFRs. Two different SMIs targeting FGFRs and VEGF-Rs reduced colony formation or motility by osteosarcoma cell lines [116, 117].

Ret

Ret, which stands for *re*arranged during *t*ransfection, was one of the first RTKs that was shown to play a role in cancer. Ret is primarily expressed in urogenital and neural crest-derived cells and is important for the development of the peripheral nervous system and kidney. The glial cell line-derived neurotrophic factor (GDNF) family of growth factors are the main ligands for Ret. Genetic alterations involving Ret, such as chromosomal translocations and activating mutations, are most commonly associated with thyroid cancers but have also been reported in colon cancer and lung adenocarcinoma. Chromosomal translocations between Ret and at least ten different fusion partners make up the class of Ret/PTC fusion proteins associated with papillary thyroid carcinoma (PTC) [14, 118]. Furthermore, Ret contributes to the migration of neuroblastoma, thyroid, pancreatic, and non-small-cell lung cancer cells in vitro [119–121], and metastasis of breast cancer in vivo. These studies are consistent with our results demonstrating that Ret is activated and promotes the migration of the metastatic LM7 cells. While ours is the first study to investigate Ret in osteosarcoma, overexpression has been correlated with poor prognosis in liposarcoma [122].

Currently there are no tyrosine kinase inhibitors specific for Ret. However, a growing number of multikinase inhibitors have demonstrated anti-Ret activity. These include sorafenib, vandetanib, sunitinib, regorafenib, and cabozantinib (XL-184), which are currently being tested in clinical trials for other cancers. A few preclinical studies have demonstrated antitumor activity in osteosarcoma after treatment with sorafenib [123–125]. A phase I clinical study testing the multikinase inhibitor regorafenib included two osteosarcoma patients [126]. One of the osteosarcoma patients achieved a partial response after treatment. Furthermore, progression free survival at 4 months was greatly improved when compared to the previously published outcomes in a phase II clinical trial of 37 osteosarcoma patients treated with sorafenib [127]. Further studies are required to determine whether the effects of the inhibitors are due to inhibition of Ret in osteosarcoma.

Other RTKs

Other groups have begun to determine the role of other RTKs in the pathogenesis of osteosarcoma. One chapter in this book focuses on the role of Her2 in osteosarcoma. Additionally, Met signaling has been investigated in osteosarcoma. While the RTK was highly phosphorylated in our cell lines, the individual siRNAs had little effect on the in vitro phenotypes. Nevertheless, in other studies using different cell lines, inhibition of Met signaling resulted in reduced invasion, motility, and proliferation in vitro and tumor growth in vivo [128, 129]. An expression analysis demonstrated Met overexpression in 60 % of human patient samples [38]. Osteoblast-specific overexpression of Met induced tumorigenesis demonstrating the transforming capacity of Met [130]. Finally, Met inhibition by overexpression of caveolin-1 (Cav-1), a major component of caveolae which compartmentalize membrane receptors, reduced lung metastasis in an experimental metastatic mouse model of osteosarcoma [131].

Summary

Cancer cells acquire specific characteristics that distinguish them from normal cells. These include enhanced proliferation, survival, adhesion, and migration, most of which are regulated by receptor tyrosine kinase signaling [132]. In fact, aberrant expression and activation of RTKs have been associated with the progression of many cancers [9]. Tyrosine kinase inhibitors may reverse or inhibit such progression and have already been successfully implemented in a number of other cancers. For these reasons we were compelled to examine RTK signaling in osteosarcoma.

Our systematic phosphoproteomic and siRNA screening study established a role for RTKs in the pathogenesis of osteosarcoma and highlights novel RTKs that may be promising targets for future therapies. However, indicative of the molecular complexity of osteosarcoma, none of the RTKs were validated in both the LM7 and

143B cell lines. Similar to osteosarcoma patients, the 143B and LM7 families of cell lines are genetically different from each other with varying chromosomal aberrations and mutations [133]. Due to this genetic instability, osteosarcoma patients will likely benefit from patient-tailored, targeted therapy. In our lab, the phosphoarray has only been tested on cell extracts. However, the same array was successfully used to evaluate RTK activity in glioma xenografts and human leiomyoma tumor samples [134, 135]. Studies are needed to evaluate the feasibility of using osteosarcoma tissue samples with the array. Nevertheless, this array could be very useful in the future for screening RTK activity in osteosarcoma tumor samples. Another method for personalizing targeted therapy has recently been reported in a canine osteosarcoma case [136]. Primary tumor cells were isolated prior to chemotherapy treatment and cell survival was screened using a panel of SMIs. Dasatinib was chosen since it was one of the most effective at inducing cell death in the primary tumor cells, and it is well tolerated in humans and is readily available. The dog was still in remission 24 months following the primary diagnosis [136]. It remains to be determined whether such method may be more broadly feasible in human patients.

It is well accepted that RTK signaling networks are highly complex. Cross talk and coactivation of RTKs may occur within a cell. In fact, with advances in high-throughput screening, cross talk may actually occur more often than previously thought [137]. In addition, chemoresistance to both conventional and targeted therapies is an ongoing challenge and may result from acquired mutations in the kinase or compensatory mechanisms, such as upregulation of other kinases [25]. Therefore, inhibition of multiple kinases may be necessary for successful outcomes.

The ultimate goal in osteosarcoma is to improve the overall survival for patients. However, no major improvement in survival has been made since the introduction of chemotherapy in the 1970s. This illustrates the necessity for a better understanding of the underlying biology of osteosarcoma to create improved therapies. Our results identified four RTKs (Axl, EphB2, FGFR2, and Ret) that have not been previously studied in osteosarcoma and provide novel targets for the development of new therapies.

References

1. Hubbard SR, Till JH (2000) Protein tyrosine kinase structure and function. Annu Rev Biochem 69:373–398
2. Manning G, Whyte DB, Martinez R et al (2002) The protein kinase complement of the human genome. Science 298(5600):1912–1934
3. Chong PK, Lee H, Kong JW et al (2008) Phosphoproteomics, oncogenic signaling and cancer research. Proteomics 8(21):4370–4382
4. Lemmon MA, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. Cell 141(7):1117–1134
5. Gschwind A, Fischer OM, Ullrich A (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer 4(5):361–370
6. Huse M, Kuriyan J (2002) The conformational plasticity of protein kinases. Cell 109(3): 275–282

7. Hubbard SR, Miller WT (2007) Receptor tyrosine kinases: mechanisms of activation and signaling. Curr Opin Cell Biol 19(2):117–123
8. Bae YS, Kang SW, Seo MS et al (1997) Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J Biol Chem 272(1):217–221
9. Krause DS, Van Etten RA (2005) Tyrosine kinases as targets for cancer therapy. N Engl J Med 353(2):172–187
10. Kim H, Gillis LC, Jarvis JD et al (2011) Tyrosine kinase chromosomal translocations mediate distinct and overlapping gene regulation events. BMC Cancer 11:528
11. Kurzrock R, Kantarjian HM, Druker BJ et al (2003) Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. Annal Int Med 138(10): 819–830
12. Rowley JD (1973) Chromosomal patterns in myelocytic leukemia. N Engl J Med 289(4):220–221
13. Rowley JD (1973) Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243(5405): 290–293
14. Kim DW, Hwang JH, Suh JM et al (2003) RET/PTC (rearranged in transformation/papillary thyroid carcinomas) tyrosine kinase phosphorylates and activates phosphoinositide-dependent kinase 1 (PDK1): an alternative phosphatidylinositol 3-kinase-independent pathway to activate PDK1. Mol Endocrinol 17(7):1382–1394
15. May WA, Lessnick SL, Braun BS et al (1993) The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol Cell Biol 13(12):7393–7398
16. De Vito C, Riggi N, Suva ML et al (2011) Let-7a is a direct EWS-FLI-1 target implicated in Ewing's sarcoma development. PLoS One 6(8):e23592
17. Nishikawa R, Ji XD, Harmon RC et al (1994) A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc Natl Acad Sci U S A 91(16):7727–7731
18. Turner N, Grose R (2010) Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer 10(2):116–129
19. Zwick E, Bange J, Ullrich A (2002) Receptor tyrosine kinases as targets for anticancer drugs. Trend Mol Med 8(1):17–23
20. Singh AB, Harris RC (2005) Autocrine, paracrine and juxtacrine signaling by EGFR ligands. Cell Signal 17(10):1183–1193
21. Miyamoto S, Nakamura M, Yano K et al (2007) Matrix metalloproteinase-7 triggers the matricrine action of insulin-like growth factor-II via proteinase activity on insulin-like growth factor binding protein 2 in the extracellular matrix. Cancer Sci 98(5):685–691
22. Baselga J (2006) Targeting tyrosine kinases in cancer: the second wave. Science 312(5777):1175–1178
23. Noble ME, Endicott JA, Johnson LN (2004) Protein kinase inhibitors: insights into drug design from structure. Science 303(5665):1800–1805
24. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer 9(1):28–39
25. Fabbro D, Cowan-Jacob SW, Mobitz H et al (2012) Targeting cancer with small-molecular-weight kinase inhibitors. Methods Mol Biol 795:1–34
26. Adrian FJ, Ding Q, Sim T et al (2006) Allosteric inhibitors of Bcr-abl-dependent cell proliferation. Nat Chem Biol 2(2):95–102
27. Hudis CA (2007) Trastuzumab – Mechanism of action and use in clinical practice. N Engl J Med 357(1):39–51
28. Lambrechts D, Lenz HJ, de Haas S et al (2013) Markers of response for the antiangiogenic agent bevacizumab. J Clin Oncol 31(9):1219–1230
29. Karapetis CS, Khambata-Ford S, Jonker DJ et al (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359(17):1757–1765

30. Bonner JA, Harari PM, Giralt J et al (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 354(6):567–578
31. Sierra JR, Cepero V, Giordano S (2010) Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. Mol Cancer 9:75
32. Kobayashi S, Ji H, Yuza Y et al (2005) An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. Cancer Res 65(16):7096–7101
33. Pao W, Miller VA, Politi KA et al (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2(3):e73
34. Burrow S, Andrulis IL, Pollak M et al (1998) Expression of insulin-like growth factor receptor, IGF-1, and IGF-2 in primary and metastatic osteosarcoma. J Surg Oncol 69(1):21–27
35. Benini S, Baldini N, Manara MC et al (1999) Redundancy of autocrine loops in human osteosarcoma cells. Int J Cancer 80(4):581–588
36. Hughes DP, Thomas DG, Giordano TJ et al (2006) Essential erbB family phosphorylation in osteosarcoma as a target for CI-1033 inhibition. Pediatr Blood Cancer 46(5):614–623
37. Charity RM, Foukas AF, Deshmukh NS et al (2006) Vascular endothelial growth factor expression in osteosarcoma. Clin Orthop Relat Res 448:193–198
38. Ferracini R, Di Renzo MF, Scotlandi K et al (1995) The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. Oncogene 10(4):739–749
39. Lee YH, Tokunaga T, Oshika Y et al (1999) Cell-retained isoforms of vascular endothelial growth factor (VEGF) are correlated with poor prognosis in osteosarcoma. Eur J Cancer 35(7):1089–1093
40. Sulzbacher I, Birner P, Trieb K et al (2003) Expression of platelet-derived growth factor-AA is associated with tumor progression in osteosarcoma. Mod Pathol 16(1):66–71
41. Fukuda T, Ichimura E, Shinozaki T et al (1998) Coexpression of HGF and c-Met/HGF receptor in human bone and soft tissue tumors. Pathol Int 48(10):757–762
42. Gorlick R, Huvos AG, Heller G et al (1999) Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 17(9):2781–2788
43. Handa A, Tokunaga T, Tsuchida T et al (2000) Neuropilin-2 expression affects the increased vascularization and is a prognostic factor in osteosarcoma. Int J Oncol 17(2):291–295
44. Kaya M, Wada T, Kawaguchi S et al (2002) Increased pre-therapeutic serum vascular endothelial growth factor in patients with early clinical relapse of osteosarcoma. Br J Cancer 86(6):864–869
45. Oda Y, Yamamoto H, Tamiya S et al (2006) CXCR4 and VEGF expression in the primary site and the metastatic site of human osteosarcoma: analysis within a group of patients, all of whom developed lung metastasis. Mod Pathol 19(5):738–745
46. Onda M, Matsuda S, Higaki S et al (1996) ErbB-2 expression is correlated with poor prognosis for patients with osteosarcoma. Cancer 77(1):71–78
47. Luu HH, Kang Q, Park JK et al (2005) An orthotopic model of human osteosarcoma growth and spontaneous pulmonary metastasis. Clin Exp Meta 22(4):319–329
48. Jia SF, Worth LL, Kleinerman ES (1999) A nude mouse model of human osteosarcoma lung metastases for evaluating new therapeutic strategies. Clin Exp Meta 17(6):501–506
49. Rettew AN, Young ED, Lev DC et al (2012) Multiple receptor tyrosine kinases promote the *in vitro* phenotype of metastatic human osteosarcoma cell lines. Oncogenesis 1(e34):1–9
50. Kolb EA, Gorlick R (2009) Development of IGF-IR Inhibitors in Pediatric Sarcomas. Curr Oncol Rep 11(4):307–313
51. Larsson O, Girnita A, Girnita L (2005) Role of insulin-like growth factor 1 receptor signalling in cancer. Br J Cancer 92(12):2097–2101
52. Kappel CC, Velez-Yanguas MC, Hirschfeld S et al (1994) Human osteosarcoma cell lines are dependent on insulin-like growth factor I for in vitro growth. Cancer Res 54(10):2803–2807
53. Wang YH, Wang ZX, Qiu Y et al (2009) Lentivirus-mediated RNAi knockdown of insulin-like growth factor-1 receptor inhibits growth, reduces invasion, and enhances radiosensitivity in human osteosarcoma cells. Mol Cell Biochem 327(1–2):257–266

54. Dong J, Demarest SJ, Sereno A et al (2010) Combination of two insulin-like growth factor-I receptor inhibitory antibodies targeting distinct epitopes leads to an enhanced antitumor response. Mol Cancer Ther 9(9):2593–2604
55. Kolb EA, Gorlick R, Houghton PJ et al (2008) Initial testing (stage 1) of a monoclonal antibody (SCH 717454) against the IGF-1 receptor by the pediatric preclinical testing program. Pediatr Blood Cancer 50(6):1190–1197
56. Kolb EA, Kamara D, Zhang W et al (2010) R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts. Pediatr Blood Cancer 55(1):67–75
57. Wang Y, Lipari P, Wang X et al (2010) A fully human insulin-like growth factor-I receptor antibody SCH 717454 (Robatumumab) has antitumor activity as a single agent and in combination with cytotoxics in pediatric tumor xenografts. Mol Cancer Ther 9(2): 410–418
58. Pollak M, Sem AW, Richard M et al (1992) Inhibition of metastatic behavior of murine osteosarcoma by hypophysectomy. J Natl Cancer Inst 84(12):966–971
59. Messerschmitt PJ, Rettew AN, Brookover RE et al (2008) Specific tyrosine kinase inhibitors regulate human osteosarcoma cells in vitro. Clin Orthop Relat Res 466(9):2168–2175
60. Bagatell R, Herzog CE, Trippett TM et al (2011) Pharmacokinetically guided phase 1 trial of the IGF-1 receptor antagonist RG1507 in children with recurrent or refractory solid tumors. Clin Cancer Res 17(3):611–619
61. Kolb EA, Gorlick R, Lock R et al (2011) Initial testing (stage 1) of the IGF-1 receptor inhibitor BMS-754807 by the pediatric preclinical testing program. Pediatr Blood Cancer 56(4):595–603
62. Kuijjer ML, Peterse EF, van den Akker BE et al (2013) IR/IGF1R signaling as potential target for treatment of high-grade osteosarcoma. BMC Cancer 13:245
63. Desai J, Solomon J, Davis ID et al (2010) Phase I dose-escalation study of daily BMS-754807, an oral, dual IGF-1R/insulin receptor (IR) inhibitor in subjects with solid tumors. In: ASCO Annual Meeting Proceedings, 2010, p 3104
64. Houghton PJ, Morton CL, Gorlick R et al (2010) Initial testing of a monoclonal antibody (IMC-A12) against IGF-1R by the Pediatric Preclinical Testing Program. Pediatr Blood Cancer 54(7):921–926
65. Linger RM, Keating AK, Earp HS et al (2008) TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. Adv Cancer Res 100:35–83
66. Varnum BC, Young C, Elliott G et al (1995) Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature 373(6515):623–626
67. Verma A, Warner SL, Vankayalapati H et al (2011) Targeting Axl and Mer kinases in cancer. Mol Cancer Ther 10(10):1763–1773
68. Bellosta P, Costa M, Lin DA et al (1995) The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. Mol Cell Biol 15(2):614–625
69. Korshunov VA (2012) Axl-dependent signalling: a clinical update. Clin Sci 122(8):361–368
70. Linger RM, Keating AK, Earp HS et al (2010) Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. Exp Opin Therap Targ 14(10):1073–1090
71. Rankin EB, Fuh KC, Taylor TE et al (2010) AXL is an essential factor and therapeutic target for metastatic ovarian cancer. Cancer Res 70(19):7570–7579
72. Holland SJ, Pan A, Franci C et al (2010) R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. Cancer Res 70(4):1544–1554
73. Linger RM, Cohen RA, Cummings CT et al (2012) Mer or Axl receptor tyrosine kinase inhibition promotes apoptosis, blocks growth and enhances chemosensitivity of human non-small cell lung cancer. Oncogene 32(29):3420–3431
74. Liu L, Greger J, Shi H et al (2009) Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res 69(17):6871–6878

75. Hong J, Peng D, Chen Z et al (2013) ABL regulation by AXL promotes cisplatin resistance in esophageal cancer. Cancer Res 73(1):331–340
76. Keating AK, Kim GK, Jones AE et al (2010) Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. Mol Cancer Ther 9(5):1298–1307
77. Huang F, Hurlburt W, Greer A et al (2010) Differential mechanisms of acquired resistance to insulin-like growth factor-i receptor antibody therapy or to a small-molecule inhibitor, BMS-754807, in a human rhabdomyosarcoma model. Cancer Res 70(18):7221–7231
78. Zhang Z, Lee JC, Lin L et al (2012) Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. Nat Genet 44(8):852–860
79. Rochlitz C, Lohri A, Bacchi M et al (1999) Axl expression is associated with adverse prognosis and with expression of Bcl-2 and CD34 in de novo acute myeloid leukemia (AML): results from a multicenter trial of the Swiss Group for Clinical Cancer Research (SAKK). Leukemia 13(9):1352–1358
80. Koorstra JB, Karikari CA, Feldmann G et al (2009) The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. Cancer Biol Ther 8(7):618–626
81. Shieh YS, Lai CY, Kao YR et al (2005) Expression of axl in lung adenocarcinoma and correlation with tumor progression. Neoplasia 7(12):1058–1064
82. Gjerdrum C, Tiron C, Hoiby T et al (2010) Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. Proc Natl Acad Sci U S A 107(3):1124–1129
83. Fernebro J, Francis P, Eden P et al (2006) Gene expression profiles relate to SS18/SSX fusion type in synovial sarcoma. Int J Cancer 118(5):1165–1172
84. Peng T, Zhang P, Liu J et al (2011) An experimental model for the study of well-differentiated and dedifferentiated liposarcoma; deregulation of targetable tyrosine kinase receptors. Lab Invest 91(3):392–403
85. Barretina J, Caponigro G, Stransky N et al (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483(7391):603–607
86. Hoffman A, Ghadimi MP, Demicco EG et al (2013) Localized and metastatic myxoid/round cell liposarcoma: clinical and molecular observations. Cancer 119(10):1868–1877
87. Nakano T, Tani M, Ishibashi Y et al (2003) Biological properties and gene expression associated with metastatic potential of human osteosarcoma. Clin Exp Meta 20(7): 665–674
88. Zhang Y, Tang YJ, Man Y et al (2013) Knockdown of AXL receptor tyrosine kinase in osteosarcoma cells leads to decreased proliferation and increased apoptosis. Int J Immunopathol Pharmacol 26(1):179–188
89. Choy E, Hornicek F, MacConaill L et al (2012) High-throughput genotyping in osteosarcoma identifies multiple mutations in phosphoinositide-3-kinase and other oncogenes. Cancer 118(11):2905–2914
90. Seo JS, Ju YS, Lee WC et al (2012) The transcriptional landscape and mutational profile of lung adenocarcinoma. Genome Res 22(11):2109–2119
91. Bai Y, Li J, Fang B et al (2012) Phosphoproteomics identifies driver tyrosine kinases in sarcoma cell lines and tumors. Cancer Res 72(10):2501–2511
92. Han J, Tian R, Yong B et al (2013) Gas6/Axl mediates tumor cell apoptosis, migration and invasion and predicts the clinical outcome of osteosarcoma patients. Biochem Biophys Res Commun 435(3):493–500
93. Alvarez H, Montgomery EA, Karikari C et al (2010) The Axl receptor tyrosine kinase is an adverse prognostic factor and a therapeutic target in esophageal adenocarcinoma. Cancer Biol Ther 10(10):1009–1018
94. Cerchia L, Esposito CL, Camorani S et al (2012) Targeting Axl with an high-affinity inhibitory aptamer. Mol Ther 20(12):2291–2303
95. Pasquale EB (2010) Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer 10(3):165–180

96. Barco R, Hunt LB, Frump AL et al (2007) The synovial sarcoma SYT-SSX2 oncogene remodels the cytoskeleton through activation of the ephrin pathway. Mol Biol Cell 18(10):4003–4012
97. Surawska H, Ma PC, Salgia R (2004) The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 15(6):419–433
98. Mao W, Luis E, Ross S et al (2004) EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer. Cancer Res 64(3):781–788
99. Fritsche-Guenther R, Noske A, Ungethum U et al (2010) De novo expression of EphA2 in osteosarcoma modulates activation of the mitogenic signalling pathway. Histopathology 57(6):836–850
100. Varelias A, Koblar SA, Cowled PA et al (2002) Human osteosarcoma expresses specific ephrin profiles: implications for tumorigenicity and prognosis. Cancer 95(4):862–869
101. Koolpe M, Burgess R, Dail M et al (2005) EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. J Biol Chem 280(17):17301–17311
102. Noberini R, Lamberto I, Pasquale EB (2012) Targeting Eph receptors with peptides and small molecules: progress and challenges. Semin Cell Dev Biol 23(1):51–57
103. Hingorani P, Zhang W, Gorlick R et al (2009) Inhibition of Src phosphorylation alters metastatic potential of osteosarcoma in vitro but not in vivo. Clin Cancer Res 15(10):3416–3422
104. Kelleher FC, O'Sullivan H, Smyth E et al (2013) Fibroblast growth factor receptors, developmental corruption and malignant disease. Carcinogenesis 34(10):2198–2205
105. Mirabello L, Yu K, Berndt SI et al (2011) A comprehensive candidate gene approach identifies genetic variation associated with osteosarcoma. BMC Cancer 11(1):209
106. Sadikovic B, Yoshimoto M, Chilton-MacNeill S et al (2009) Identification of interactive networks of gene expression associated with osteosarcoma oncogenesis by integrated molecular profiling. Hum Mol Genet 18(11):1962–1975
107. Entz-Werle N, Lavaux T, Metzger N et al (2007) Involvement of MET/TWIST/APC combination or the potential role of ossification factors in pediatric high-grade osteosarcoma oncogenesis. Neoplasia 9(8):678–688
108. Baird K, Davis S, Antonescu CR et al (2005) Gene expression profiling of human sarcomas: insights into sarcoma biology. Cancer Res 65(20):9226–9235
109. Henderson SR, Guiliano D, Presneau N et al (2005) A molecular map of mesenchymal tumors. Gen Biol 6(9):R76
110. Datsis GA, Berdiaki A, Nikitovic D et al (2011) Parathyroid hormone affects the fibroblast growth factor-proteoglycan signaling axis to regulate osteosarcoma cell migration. FEBS J 278(19):3782–3792
111. Singh D, Chan JM, Zoppoli P et al (2012) Transforming fusions of FGFR and TACC genes in human glioblastoma. Science 337(6099):1231–1235
112. Crose LE, Etheridge KT, Chen C et al (2012) FGFR4 blockade exerts distinct antitumorigenic effects in human embryonal versus alveolar rhabdomyosarcoma. Clin Cancer Res 18(14):3780–3790
113. Terai H, Soejima K, Yasuda H et al (2013) Activation of the FGF2-FGFR1 autocrine pathway: a novel mechanism of acquired resistance to gefitinib in NSCLC. Mol Cancer Res 11(7):759–767
114. Oliveras-Ferraros C, Cufi S, Queralt B et al (2012) Cross-suppression of EGFR ligands amphiregulin and epiregulin and de-repression of FGFR3 signalling contribute to cetuximab resistance in wild-type KRAS tumour cells. Br J Cancer 106(8):1406–1414
115. Guagnano V, Kauffmann A, Wohrle S et al (2012) FGFR genetic alterations predict for sensitivity to NVP-BGJ398, a selective pan-FGFR inhibitor. Cancer Discov 2(12):1118–1133
116. Grigoriadis A, Patino-Garcia A, Zandueta C et al (2010) The role of FGFR signaling in osteosarcoma progression and metastasis. In: 16th annual meeting of the Connective Tissue Oncology Society, Paris, France, 2010, pp 155–156
117. Glen H, Mason S, Patel H et al (2011) E7080, a multi-targeted tyrosine kinase inhibitor suppresses tumor cell migration and invasion. BMC Cancer 11:309

118. Nikiforov YE (2002) RET/PTC rearrangement in thyroid tumors. Endoc Pathol 13(1):3–16
119. Cockburn JG, Richardson DS, Gujral TS et al (2010) RET-mediated cell adhesion and migration require multiple integrin subunits. J Clin Endocrinol Metab 95(11):E342–E346
120. Gil Z, Cavel O, Kelly K et al (2010) Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves. J Natl Cancer Inst 102(2):107–118
121. Tang JZ, Kong XJ, Kang J et al (2010) Artemin-stimulated progression of human non-small cell lung carcinoma is mediated by BCL2. Mol Cancer Ther 9(6):1697–1708
122. Cheng H, Dodge J, Mehl E et al (2009) Validation of immature adipogenic status and identification of prognostic biomarkers in myxoid liposarcoma using tissue microarrays. Hum Pathol 40(9):1244–1251
123. Wolfesberger B, Tonar Z, Gerner W et al (2010) The tyrosine kinase inhibitor sorafenib decreases cell number and induces apoptosis in a canine osteosarcoma cell line. Res Vet Sci 88(1):94–100
124. Pignochino Y, Grignani G, Cavalloni G et al (2009) Sorafenib blocks tumour growth, angiogenesis and metastatic potential in preclinical models of osteosarcoma through a mechanism potentially involving the inhibition of ERK1/2, MCL-1 and ezrin pathways. Mol Cancer 8:118
125. Pignochino Y, Dell'Aglio C, Basirico M et al (2013) The combination of sorafenib and everolimus abrogates mTORC1 and mTORC2 upregulation in osteosarcoma preclinical models. Clin Cancer Res 19(8):2117–2131
126. Mross K, Frost A, Steinbild S et al (2012) A phase I dose-escalation study of regorafenib (BAY 73-4506), an inhibitor of oncogenic, angiogenic, and stromal kinases, in patients with advanced solid tumors. Clin Cancer Res 18(9):2658–2667
127. Grignani G, Palmerini E, Dileo P et al (2012) A phase II trial of sorafenib in relapsed and unresectable high-grade osteosarcoma after failure of standard multimodal therapy: an Italian Sarcoma Group study. Ann Oncol 23(2):508–516
128. Coltella N, Manara MC, Cerisano V et al (2003) Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. FASEB J 17(9):1162–1164
129. Sampson ER, Martin BA, Morris AE et al (2011) The orally bioavailable met inhibitor PF-2341066 inhibits osteosarcoma growth and osteolysis/matrix production in a xenograft model. J Bone Miner Res 26(6):1283–1294
130. Patane S, Avnet S, Coltella N et al (2006) MET overexpression turns human primary osteoblasts into osteosarcomas. Cancer Res 66(9):4750–4757
131. Cantiani L, Manara MC, Zucchini C et al (2007) Cavelolin-1 reduces osteosarcoma metastases by inhibiting c-Src activity and met signaling. Cancer Res 67(16):7675–7685
132. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70
133. Forbes SA, Bindal N, Bamford S et al (2011) COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 39(Database issue): D945–D950
134. Stommel JM, Kimmelman AC, Ying H et al (2007) Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. Science 318(5848):287–290
135. Yu L, Saile K, Swartz CD et al (2008) Differential expression of receptor tyrosine kinases (RTKs) and IGF-I pathway activation in human uterine leiomyomas. Mol Med 14(5–6): 264–275
136. Davis LE, Hofmann NE, Li G et al (2013) A case study of personalized therapy for osteosarcoma. Pediatr Blood Cancer 60(8):1313–1319
137. Xu AM, Huang PH (2010) Receptor tyrosine kinase coactivation networks in cancer. Cancer Res 70(10):3857–3860

Understanding the Role of Notch in Osteosarcoma

Madonna M. McManus, Kurt R. Weiss, and Dennis P.M. Hughes

Abstract The Notch pathway has been described as an oncogene in osteosarcoma, but the myriad functions of all the members of this complex signaling pathway, both in malignant cells and nonmalignant components of tumors, make it more difficult to define Notch as simply an oncogene or a tumor suppressor. The cell-autonomous behaviors caused by Notch pathway manipulation may vary between cell lines but can include changes in proliferation, migration, invasiveness, oxidative stress resistance, and expression of markers associated with stemness or tumor-initiating cells. Beyond these roles, Notch signaling also plays a vital role in regulating tumor angiogenesis and vasculogenesis, which are vital aspects of osteosarcoma growth and behavior in vivo. Further, osteosarcoma cells themselves express relatively low levels of Notch ligand, making it likely that nonmalignant cells, especially endothelial cells and pericytes, are the major source of Notch activation in osteosarcoma tumors in vivo and in patients. As a result, Notch pathway expression is not expected to be uniform across a tumor but likely to be highest in those areas immediately adjacent to blood vessels. Therapeutic targeting of the Notch pathway is likewise expected to be complicated. Most pharmacologic approaches thus far have focused on inhibition of gamma secretase, a protease of the presenilin complex. This enzyme, however, has numerous other target proteins that would be expected

M.M. McManus, M.S.
The Children's Cancer Hospital at MD Anderson Cancer Center, Houston, TX, USA

The University of Texas Graduate School of Biomedical Sciences at Houston, Unit 853, MOD Lab 1.021d, 1515 Holcombe Blvd., Houston, TX 77030, USA

K.R. Weiss, M.D.
Department of Orthopedic Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

D.P.M. Hughes, M.D., Ph.D. (✉)
The Children's Cancer Hospital, University of Texas at MD Anderson Cancer Center, Houston, TX, USA
e-mail: dphughes@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_4,

to affect osteosarcoma behavior, including CD44, the WNT/β-catenin pathway, and Her-4. In addition, Notch plays a vital role in tissue and organ homeostasis in numerous systems, and toxicities, especially GI intolerance, have limited the effectiveness of gamma secretase inhibitors. New approaches are in development, and the downstream targets of Notch pathway signaling also may turn out to be good targets for therapy. In summary, a full understanding of the complex functions of Notch in osteosarcoma is only now unfolding, and this deeper knowledge will help position the field to better utilize novel therapies as they are developed.

Keywords Osteosarcoma • Notch • DLL4 • Jag1 • Angiogenesis • Metastasis • Dormancy • Cancer stem cells

Introduction: The Notch Signaling Pathway

The Notch signaling pathway, a key component in normal bone development that is implicated as a key mediator in a number of various cancers, is initiated when a membrane-bound ligand belonging to the Delta–Serrate–Lag (DSL) family (jagged 1/Jag1, Jag2, delta-like-1/DLL1, DLL3, and DLL4) on the surface of a cell interacts with a membrane-bound Notch receptor (Notch1–4) on another cell. This interaction induces a two-step proteolytic cleavage of the receptor, first by ADAM10 (also known as Kuz) or ADAM17 (also known as TACE) and then by the γ-secretase complex which is made up of at least four individual proteins: presenilin, nicastrin, anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2). These cleavage events release the *i*ntra*c*ellular domain of *N*otch (ICN). Now activated, ICN enters the nucleus where it forms a transcriptional complex with *Mastermind-like* 1 (MAM1) to regulate transcriptional complexes containing the DNA-binding protein *C*BF1/RBPjk/*S*u(H)/*L*ag1 (CSL). This complex initiates the transcription of *H*airy/*E*nhancer of *S*plit-1 (Hes1), Hes5, Hes7, HES-related with YRPW motif (Hey1/HERP2), Hey2 (HERP1), and HeyL which encode basic helix loop helix (bHLH) transcription factors that perform a range of cellular activities that include promoting progenitor cell survival and suppressing differentiation [1, 2]. This pathway is displayed schematically in Fig. 1. The Notch signaling pathway, via cell–cell contacts, highly regulated feedback loops, and lateral inhibition/induction mechanisms, has been shown to influence multiple cellular processes including cell fate decisions, proliferation, apoptosis, migration, angiogenesis, and plasticity. In terms of bone homeostasis, skeletal cells express Notch1, Notch2, and low levels of Notch3, although Notch1 and 2 are considered responsible for the effects of Notch in the skeleton [3] (Notch signaling reviewed in [4, 5]).

Role of Notch Signaling in Normal Osteoblast Development

Mesenchymal stem cells (MSCs) can give rise to multiple lineages in response to environmental molecular cues: the presence of MyoD leads to the differentiation of MSCs into myocytes, PPARγ leads to the generation of adipocytes, the Sox family

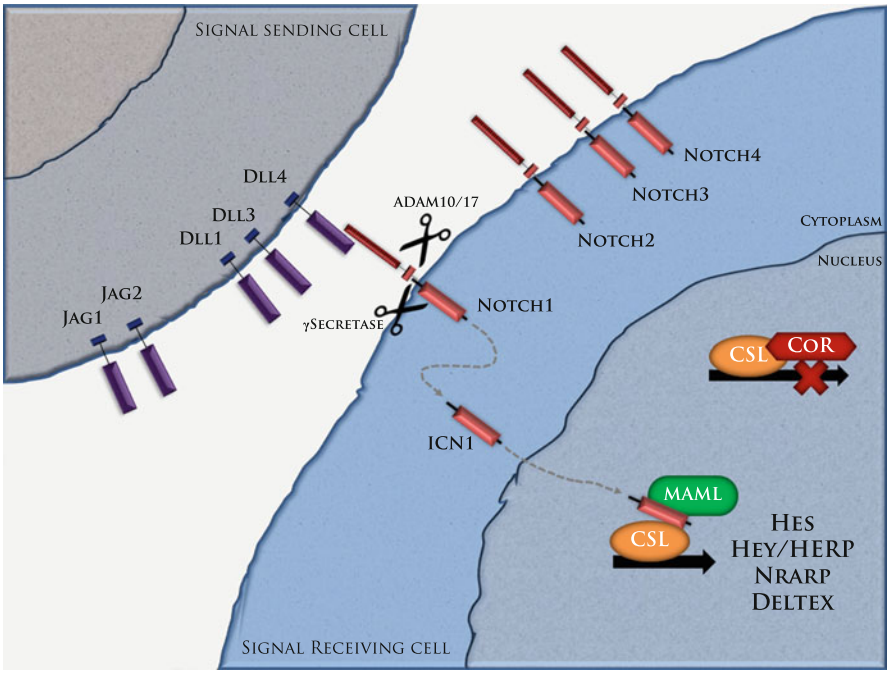


Fig. 1 Schematic diagram of Notch pathway signaling. Notch ligands, consisting of the jagged (Jag1 and Jag2) and delta-like (DLL1, DLL3, and DLL4) families, typically are presented on the surface of signal-sending cells, though these receptors can be shed by proteolytic cleavage in some circumstances. Prior to ligand binding, the Notch family receptors (Notch1, Notch2, Notch3, and Notch4) remain fixed at the plasma membrane, and the CSL transcription complex remains bound to corepressor elements, shutting off transcription of CSL target genes. Upon binding ligand, Notch1 is subject to a two-step proteolytic cleavage by ADAMS family protease and then γ-secretase. Cleavage by γ-secretase frees the cytoplasmic domain of the Notch1 from the plasma membrane; this fragment is termed *in*tracellular *N*otch*1* (ICN1). ICN1 binds to CSL, displacing corepressor elements and recruiting coactivator elements, including *Ma*ster*m*ind-*L*ike (MAML), turning on transcription of CSL target genes, including the Hes, Hey, Herp, NRARP, and Deltex families. Notch1 also mediates transcription of non-CSL target genes, which is termed the noncanonical Notch pathway. Regulation of Notch2, Notch3, and Notch4 is similar

of genes drive chondrocytogenesis, and runt-related transcription factor-2 (RunX2) and osterix lead to osteoblastogenesis [6–8]. Normal osteoblast development and subsequent bone formation are meticulously regulated not only by RunX2 and osterix but also by a cascade of regulatory signaling that includes morphogens, signaling molecules, and transcriptional regulators [9–16]. A partial list of these factors includes the Wnt/β-catenin, TGFβ/bone morphogenic protein (BMP), FGF, Notch and Hedgehog signaling pathways, ATF4, TAZ, RANKL, and NFATc1 transcription factors [16–19]. Signaling molecules like RunX2, BMPs, and the Wnt/β-catenin canonical pathway are conducive to osteoblastogenesis, while others, such as the Notch signaling pathway, obstruct osteoblast differentiation [20–22]. In osteoblasts, RunX2 regulates the transcription of genes including osteocalcin,

bone sialoprotein, osteopontin, type I collagen, fibronectin, galectin 3, MMP13, osteoprotegrin (OPG), Tram2, Lnx2 (an intracellular scaffolding protein that may play a role in Notch signaling), and Tnfrsf12a (a tumor necrosis factor receptor family member) by binding to sequences that resemble the 5′-ACACCA-3′ motif upstream from their transcription start sites [23–28]. Because of its importance in this process, RunX2 is labeled the "master regulator" of osteoblast differentiation; indeed homozygous RunX2 mutant mice have cartilaginous skeletons that fail to mineralize, owing to a complete arrest in osteoblast differentiation [24, 29, 30]. For further details of the role of RunX2 in osteoblast development and in osteosarcoma, please see the chapter on this book entitled "Developmental Pathways Hijacked by Osteosarcoma."

The Notch signaling pathway plays an important and complex role in bone homeostasis [22, 31–34]. In bone marrow, Notch signaling normally acts to maintain a pool of mesenchymal progenitors by suppressing osteoblast differentiation by inhibiting RunX2 [3]. In osteoblasts, the Notch pathway has several mechanisms that inhibit osteoblastogenesis. Notch antagonizes Wnt signaling: ICN2 colocalizes with glycogen synthase kinase-3β (GSK3β) to mediate the degradation of β-catenin [22, 35]. It has been shown that NFATc1 and osterix form a complex that activates osterix-dependent transcription [36]; ICN and mastermind form a complex with Foxo1 which inhibits NFAT-mediated osteoblastogenesis, osteoblastic bone formation, as well as osteoclastogenesis and bone resorption [37, 38]. Furthermore, Engin et al. show that Notch both stimulates early osteoblastic proliferation by upregulating cyclin D, cyclin E, and osterix and represses osteoblast maturation through the binding of ICN to RunX2 [31]. Hilton et al. and others demonstrate an additional mechanism by which Notch signaling inhibits RunX2: RunX2 transcriptional activity is inhibited by its direct interactions with the HLH and Orange domains of Hey1 [3, 34]. The enzyme necessary for Notch receptor cleavage and activation, ADAM10, is expressed in cells of the osteoblast lineage and is localized at sites of active bone formation. Catalytically active ADAM10 was found to colocalize with Notch2 at these bone-forming sites [39]. This suggests that ADAM10 may play a role in controlling osteoblast differentiation; alternatively, it has been suggested that ADAM10 may work rather to cleave Notch receptor ligands to provide soluble activators of the receptor [39, 40].

Osteosarcoma and Differentiation. Osteosarcoma (OS) may be thought of as a disease of disrupted osteogenic differentiation [8, 10, 41–43]. With the prevention of the differentiation of MSCs into mature osteoblasts, there is an increased risk for malignant transformation as cells continue to proliferate uncontrollably [8, 44]. Osteosarcoma cells display similar characteristics to undifferentiated osteoblasts: early osteogenic markers like CTGF are high in OS cell lines, while markers of differentiation like RunX2, alkaline phosphatase, osteopontin, and osteocalcin are low [10, 41, 42, 45]. Interestingly, the aggressiveness of OS may depend on the stage of differentiation that was disrupted: more aggressive OS may develop from disruptions in the differentiation of early osteoblast progenitors, while benign tumors may arise from disruptions in late-stage osteoblasts [8, 41]. Considering the

importance of Notch in normal osteoblast development, the Notch signaling pathway has become increasingly interesting to those studying the progression of osteosarcoma [46–52].

Notch and the Vasculature

Introduction. Blood vessels comprise an extensive tubular network that delivers oxygen and nutrients to all organs and tissues. Vital processes such as embryogenesis, wound healing, body temperature stabilization, and homeostatic balance maintenance all require highly adjustable blood supply and nutrient delivery. These demands are met through the meticulously regulated growth and expansion of the vascular network by angiogenesis. The process of sprouting angiogenesis is highly dynamic and requires a multitude of individual processes such as the proliferation of endothelial cells (ECs), selection of leading cells that develop filopodia and promote endothelial motility, elongation of the new sprout, formation of new cell–cell junctions, conversion into endothelial tubules, specification into arteries, veins, and capillaries, recruitment of mural cells (smooth muscle cells, SMCs, and pericytes), anastomosis with other vessels, remodeling and pruning, perfusion, and stabilization of the newly formed vessel.

The Notch signaling pathway is evolutionarily conserved and is an important mediator of cell–cell communication during the formation of new blood vessels [53]. Major components of the Notch pathway are expressed in the vasculature [54, 55], and genetic deletion of Notch pathway components, including Notch1 [56–58], Notch2 [59], Jag1 [60], DLL1 [61, 62], DLL4 [63, 64], Hey1/Hey2 [65], CSL [66], or presenilins which make up the γ-secretase complex [67, 68], as well as the ectopic activation of Notch1/Notch 4 [69, 70], results in embryonic lethality associated with defects in sprouting angiogenesis, arterial/venous specification, vascular remodeling, and vascular SMC organization (Table 1).

Role of Notch Signaling in Normal Vascular Development

Notch and Arterial/Venous Specification

One of the earliest roles for Notch is in the developing embryo; Notch functions in early vascular development to drive endothelial identity while suppressing venous identity [64, 71]. Later in development, arterial endothelial cells have been shown to require DLL1 to maintain their cellular identity [61]. A more detailed review of this subject has been published recently [72].

Table 1 Notch signaling pathway knockout mice

Knockout	Major effect	Author, Year
Notch ligands		
Jagged 1	Embryonic lethal; severe vascular defects	Xue et al. [60]
Jagged 2	Defects in limb, craniofacial, thymic development	Jiang et al. [207]
Delta-like ligand 1	Embryonic lethal; defects in the formation of somite borders; defects in arterial identity	Hrabe de Angelis et al. [62]; Sorensen et al. [208]
Delta-like ligand 3	Highly disorganized vertebrae and costal defects; disruption of the segmentation clock	Dunwoodie et al. [209]
Delta-like ligand 4	Embryonic lethal; defects in arterial development	Duarte et al. [64]; Gale et al. [63]
Notch receptors		
Notch1	Embryonic lethal; severe defects in angiogenic vascular remodeling	Swiatek et al. [58]; Krebs et al. [57]; Limbourg et al. [56]
Notch2	Embryonic lethal; defects in postimplantation development	Hamada et al. [59]
Notch3	Defects in arterial identity and maturation of vascular smooth muscle cells	Domenga et al. [210]
Notch4	No apparent deficiencies	Krebs et al. [57]
Notch1 and 4	More severe than Notch1 KO only	Krebs et al. [57]
Downstream notch targets		
Hes1	Death occurs in utero or neonatally	Blake et al. [206]
Hey1	No apparent deficiencies	Fischer et al. [65]
Hey2	Postnatal lethality; cardiac defects	Fischer et al. [65]
Hey1 and 2	Embryonic lethal; global lack of vascular remodeling	Fischer et al. [65]
Notch-related genes		
γ-secretase complex		
Presenilin 1	Skeletal and CNS defects	Shen et al. [211]; Nakajima et al. [68]
Presenilin 2	Mild pulmonary fibrosis	Herreman et al. [67]
CSL	Vascular defects	Krebs et al. [66]

The major effects observed in mice with each of the Notch family ligands, receptors, and downstream signaling molecules are summarized, together with the relevant publication referenced

Notch and Sprouting Angiogenesis

Vascular endothelial growth factor (VEGF/VEGF-A) is the key regulator that promotes sprouting angiogenesis. In normal/physiologic angiogenesis, VEGF-A is secreted by astrocytes in the avascular region leading to the formation of a VEGF gradient [73, 74]. VEGF-A binds to the tyrosine kinase receptors VEGFR1 (Flt1) and VEGFR2 (KDR/Flk1/Flt2) expressed on the cell surface of nearby ECs.

VEGFR2 is the primary receptor transmitting VEGF signals in ECs [75, 76], while VEGFR1, with weaker kinase activity, acts as a VEGF decoy [77, 78]. Newly sprouting blood vessels are made up of two important endothelial cell types: *tip cells*, which initiate new sprouting, and *stalk cells* which maintain connection with the parent vessel [79–83]. In response to VEGF-A/VEGFR2-mediated signaling, ECs at the leading front of angiogenic sprouts develop protruding filopodia and become tip cells that extend toward sources of pro-angiogenic growth factors. These tip cells respond to positive/negative guidance cues to allow for directional growth while preventing unorganized and random vessel development [84, 85]. Once such negative guidance cue involves VEGF-mediated induction of DLL4 as a negative feedback regulator, which acts to prevent uncontrolled angiogenic sprouting while promoting the timely formation of a well-differentiated vascular network [83, 86]. Expression of DLL4 stimulates Notch1 activation in adjacent ECs that trail tip cells and form the base of the protrusion and become stalk cells [87]. Whereas tip cells mainly express DLL4, stalk cells primarily express Jag1 which consequentially antagonizes DLL4 activity by competing for Notch receptors via DLL4/Notch1/Jag1-mediated lateral inhibition [82–84, 88–90]. Stalk cells are important in that they proliferate when stimulated with VEGF-A, form the vascular lumen, establish adherins and tight junctions to maintain integrity of the new sprout, and maintain connection with parental vessels so as to establish luminal/abluminal polarity which leads to basal lamina deposition and mural cell recruitment and attachment [84, 91, 92]. In stalk cells, Notch signaling potently inhibits VEGFR3 [93, 94]; VEGFC/VEGFR3 signaling activates PI3K and its downstream target FoxC2, which results in the downregulation of DLL4 in the stalk cell [95, 96]. High levels of activated Notch (ICN) lead to the production of soluble VEFGR1 which acts to enhance the steepness of the VEGF-A signaling gradient by sequestering VEGF-A and inhibiting its action with VEGFR2 in stalk cells [97]. Stalk cells express Hes1 and Hey1 which act to downregulate the levels of VEGFR2, VEGFR3, and DLL4, thereby transiently decreasing the responsiveness to VEGF-A and further enhancing the stalk cell phenotype [81, 82, 93]. This allows new tip cells to form along the front to form branching vessels [98, 99]. Vessel branching within the developing vascular network is also the consequence of another downstream Notch target, Notch-regulated ankyrin repeat protein (Nrarp), which counteracts Notch signaling and is expressed in stalk cells at the branch points [100, 101].

Considering that local changes in VEGF/Notch signaling can trigger the conversion of stalk cells into tip cells, and that the Notch pathway can act in a highly transient and oscillating manner [102], tip and stalk cell phenotypes are remarkably transitory and interchangeable as ECs dynamically shuffle position along the angiogenic sprout competing for the tip cell position [103]. This leads to highly regulated and organized vessel formation. In normal vascular development, these mechanisms work together to balance the numbers of tip cells and stalk cells required for effective sprouting and network formation [82, 104–107]. Tissue oxygenation eventually downregulates paracrine VEGF-A production and thus helps establish a quiescent state for the new vessels [108]. This process has been reviewed in detail [87, 109]. The role of Notch pathway signaling in regulating normal vascular development is shown schematically in Fig. 2.

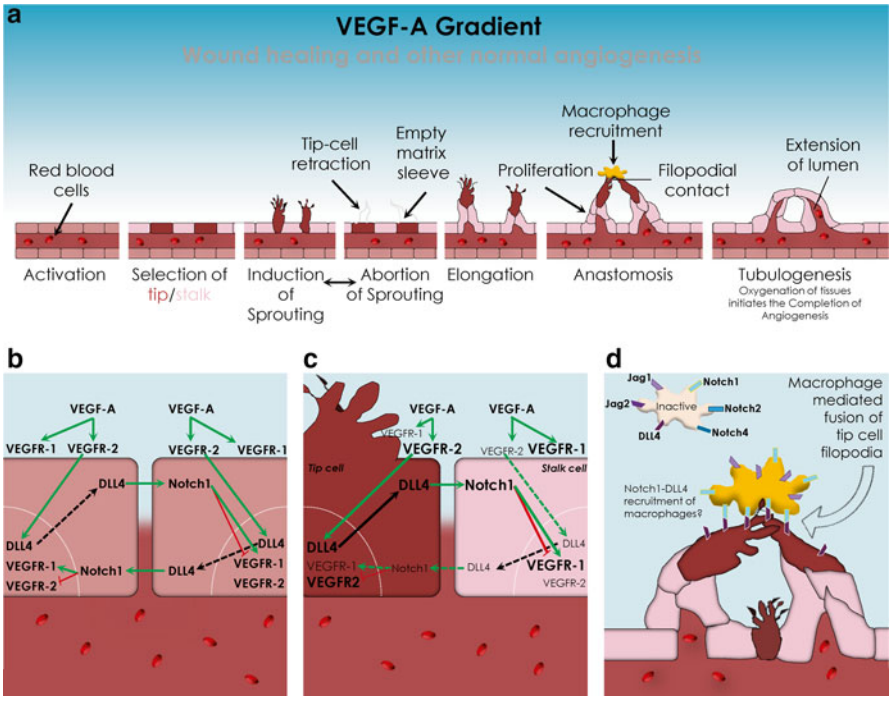


Fig. 2 Normal angiogenesis and the role of Notch pathway signaling. (**a**) Tip cell development through tubulogenesis. Upon exposure to VEGF-A, endothelial cells respond by taking on a tip cell signaling phenotype. The initial response is stochastic and cyclical, eventually allowing some cells to acquire the full tip cell phenotype, while adjacent cells are prevented from acquiring this phenotype through lateral inhibition, which is Notch mediated. Initial sprouting of tips is also a cyclic process, with individual tips extending and retracting back into the tip cell, leaving behind empty matrix sleeves that help to repattern the extracellular matrix needed in the sprouting blood vessel. Cells adjacent to tip cells become stalk cells, extending outwards toward the VEGF-A gradient, pushing the tip cell outward from the parent vessel. As the filopodia of nearby tip cells contact each other, macrophages are recruited to the site of anastomosis, facilitating fusion of tubes, with subsequent extension of these tubes. (**b** and **c**) Notch/VEGF signaling during activation, selection, and sprouting. VEGF-A binds to both VEGFR-1 and VEGFR-2 on adjacent endothelial cells, signaling through both receptors. Predominance of VEGFR-2 signaling favors a tip cell phenotype, which VEGFR-1 favors a stalk cell phenotype. VEGFR-2 signaling mediates upregulation of DLL4 which, in turn, activates Notch1 on the cells to either side of the endothelial cell. DLL4 reduces transcription of VEGFR-2 and promotes secretion of a soluble VEGFR-1 that serves as a ligand trap and reduces the ability of stalk cells to respond to VEGF-A. (**d**) Notch/VEGF signaling during anastomosis and the role of macrophages. Normal macrophages, without activation, express cell surface DLL4, Jag1, and Jag2 as well as Notch1, Notch2, and Notch4. Notch receptors, especially Notch2, allow macrophages to be recruited to the sites of tip cell anastomosis, where the high levels of DLL4 activate these macrophages. Through a process that is not fully understood, the activated macrophage then helps two tip cells to form a stable bridge that develops into a full vascular loop

Notch and Vascular Mural Functions

Notch signaling also plays an important role in vessel stability by regulating vascular mural cell function. Mural cells (SMCs and pericytes) are attached to the basal surface of certain vessels and help to stabilize the vessel wall, signal to ECs to inhibit their proliferation, promote survival, and regulate blood pressure [110, 111]. Mural cells express Notch1–3, Jag1, and DLL4 [112]. In vitro, it has been shown that endothelial Jag1 activates Notch3 on SMCs to induce Notch3 expression and regulate SMC differentiation [113]. Notch1 signaling is critical for mural cell recruitment to new vessels, whereas Notch3 plays a role in pericyte/SMC maturation once it arrives at its final destination. This process has been reviewed recently [72]. Notch pathway activity is essential for recruitment of bone marrow-derived pericytes to the blood vessels of Ewing sarcoma tumors, and inhibition of the Notch pathway with either shRNA or antibodies impeded Ewing sarcoma tumor growth in vivo and caused impaired vasculogenesis [114, 115]. Perivascular cells, in addition to the endothelium, also have been shown to play an important role in angiogenesis and are deregulated in pathological angiogenesis [110, 115].

Notch and Macrophage-Mediated Angiogenesis

Macrophages have been recognized as key angiogenic effector cells [116, 117]. Macrophages are closely associated with sprouting endothelial cells during retinal angiogenesis [118]. Importantly, tissue macrophages act as cellular chaperones during VEGF-mediated endothelial tip cell induction and anastomosis, allowing for the bridging of tip cells to form stable, perfused vessels [117, 119]. Inactive macrophages express Notch1, -2, and -4, DLL4, and Jag1-2; once activated, macrophages increase their expression of Notch1 and Jag1 [120–122]. Though it is known that VEGFR1 recruits macrophages to sites of inflammation and active angiogenesis [123], macrophage recruitment to sites of anastomosis remains an active area of research. It has been hypothesized that DLL4 expressed in tip cells attracts macrophages via Notch1–DLL4 signaling [117]. Mice with heterozygous mutations for Notch1 have decreased macrophage recruitment and, interestingly, also have decreased expression of VEGFR-1 [124]. Through these studies and others, it is clear that both VEGFR1 and Notch1 play an important role in macrophage recruitment to sites of angiogenesis. Recent publications are available with more complete reviews of the role of macrophages in angiogenesis [72, 125].

Role of Notch Signaling in Tumor Vascular

Notch Signaling at the Primary Tumor

Tumor angiogenesis relies on many of the same mechanisms involved in physiological angiogenesis. Tumors, restricted to 1–2 mm^3 without an oxygen and nutrient source, release large amounts of VEGF in response to their hypoxic environment. Unlike normal angiogenesis, however, tumors continuously release pro-angiogenic factors despite the ever-growing expansion of blood vasculature into the well-oxygenated portions of the tumor. This vasculature not only feeds the tumor and allows for uncontrolled proliferation, but it also allows for the metastatic spread of the disease to distant loci, since osteosarcoma spreads almost exclusively via the hematogenous route.

VEGF-A has been shown to be over-expressed in many tumor types [126–128]. Although not much is known about the process of vasculogenesis in osteosarcoma, multiple studies have shown that VEGF overexpression in osteosarcoma unfavorably impacts the overall survival [129–131]. Similarly, the role of Notch has been well documented in other tumor types [115, 132, 133] but continues to be an active area of study in osteosarcoma. In multiple tumor types, it has been shown that either blockade [105, 106, 134–136] or forced activation of the Notch pathway [137–142] can inhibit angiogenesis. Genetic or pharmacologic inactivation/inhibition of either DLL4 or Notch1 signaling leads to an increase in the number of filopodia and sprouting tip cells at the angiogenic front which, together with EC proliferation, results in the formation of a hyperdense vascular network with immature, hyperplastic, and nonfunctional characteristics [81, 83, 86, 104, 107, 143]. Chronic blockade of the pathway, however, results in the formation of vascular neoplasms [144]. Conversely, activation of Notch signaling leads to a reduced number of tip cells and less dense vascular network [86, 107]. A schematic model of the role of Notch in tumor angiogenesis is shown in Fig. 3.

Notch Signaling at the Metastatic Site

Judah Folkman first championed the concept that tumors require an "angiogenic switch" in the balance between pro- and anti-antigenic signals to establish a robust blood supply capable of supporting rapid tumor growth [145]. By extension, this model would suggest that, for dormant tumors, there is a balance between signals that increase angiogenesis and those that impede angiogenesis and that dormant micrometastases of osteosarcoma would be relatively poorly vascularized. Indraccolo and colleagues showed that expression of DLL4 on blood vessels in close proximity to colon cancer cells was necessary for these tumor cells to awaken from dormancy [146]. The same group had shown already that a short-term "spike" in angiogenesis was sufficient to awaken dormant tumors [147]. This awakening is associated with a transcriptional switch from expressing anti-angiogenic proteins to secreting

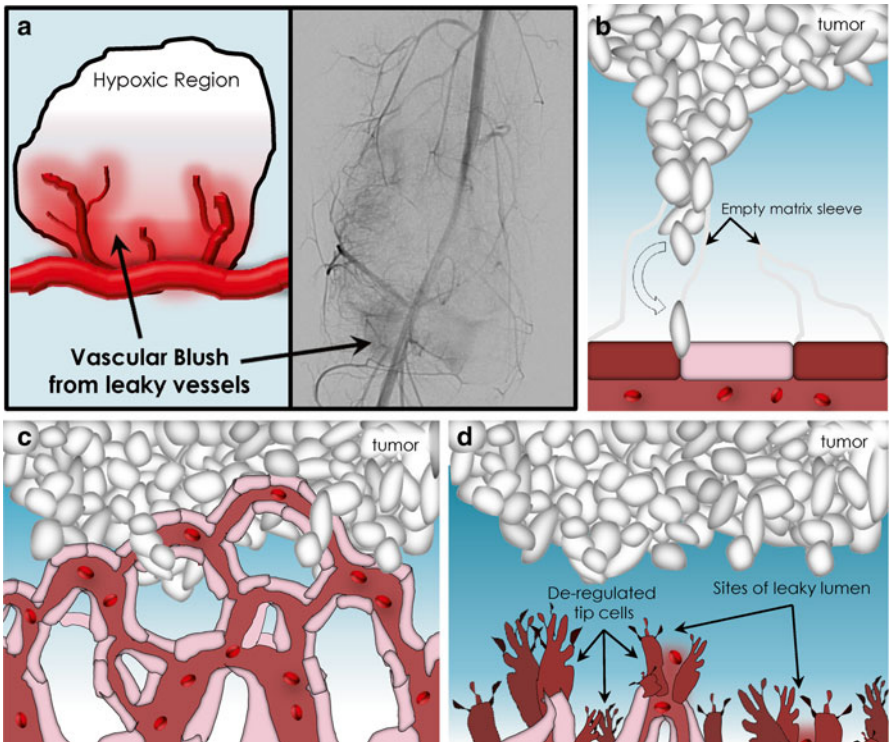


Fig. 3 Tumor angiogenesis. (**a**) Heterogenic distribution of vasculature and O_2 in a tumor. Because oxygen diffusion is limited in tissues to ~1 mm from capillaries, rapidly growing tumors will have regions of relative normoxia and other areas of profound hypoxia, with an oxygen gradient between these regions. The extremely high levels of VEGF-A secreted in the areas with the worst hypoxia override normal angiogenic controls, leading to large numbers of small, dysfunctional, and leaky blood vessels that can be observed on arteriograms (**a**, *right hand panel*) as a "vascular blush." Other areas of the tumor do not appear to have any blood supply at all and often are necrotic when examined pathologically. (The *right hand panel* in (**a**) is taken from an osteosarcoma patient receiving an arteriogram prior to the delivery of intra-arterial chemotherapy. The method is exactly as described previously [205].) (**b**) Tumors hijack empty matrix sleeves for migration/invasion. As described above and in Fig. 2, normal angiogenesis involves cyclical extension and retraction of tips, repatterning the extracellular matrix, including spreading laminin away from the basement membrane toward the VEGF-A source. In tumors, these empty sleeves left behind by tip cell extension and retraction become pathways in which the extracellular matrix ceases to be a barrier to tumor cell migration, but rather a guide for tumor cells to "find" blood vessels. (**c**) Tumors promote uncontrolled angiogenesis. Growing tumors provide a sustained source of VEGF-A, either directly through their own secretion or by inducing hypoxia, thereby promoting VEGF-A secretion from nonmalignant cells within the tumor, such as tumor-associated fibroblasts. Unlike normal angiogenesis, in which VEGF-A levels eventually decline and new vessels are allowed to mature and stabilize, the sustained VEGF-A secretion in the tumor microenvironment causes uncontrolled, sustained angiogenesis, without the maturation and stabilization found in normal angiogenesis. (**d**) High expression of VEGF promotes an all tip cell phenotype. In areas with the highest VEGF-A secretion, the concentration of VEGF-A is sufficient to override the cellular processes that induce lateral inhibition and organized vessel formation. In this environment, endothelial cells may take on an "all tip cell" phenotype, leading to vascular leak and highly disorganized blood vessels that completely lack vessel wall components. Note that in any given tumor, aspects of abnormal blood vessel development shown in panels A–D may all be taking place, each in different regions of the tumor

pro-angiogenic ones [148]. While there is no direct experimental evidence in osteosarcoma models to support this role of the vasculature in osteosarcoma metastasis, it certainly seems plausible that a similar effect operates in these patients' lungs.

There is a conception among some patients and families that major operations for osteosarcoma patients "spread the tumor." While this has not been scientifically validated, it has been observed that pulmonary metastasis sometimes develops shortly after resection of the primary tumor or lung metastases. Since the healing of large wounds results in high levels of circulating growth factors and cytokines such as EGF and its related ERBB family ligands, these growth factors and angiogenic cytokines could stimulate the expansion of tumor vessels in micrometastases. The transient upregulation of Notch ligands on vessels near the dormant micrometastases may initiate the angiogenic response that facilitates growth. A more comprehensive review of the putative roles of the Notch pathway in regulating tumor escape from dormancy in the metastatic site has been published recently [149].

Notch Signaling in Osteosarcoma

The Notch pathway has been called "the stem cell master switch" [53, 150] because it influences multiple processes that drive morphogenesis, lineage specification, apoptosis, and proliferation, not only in normal tissues but also in some cancers [151]. Notch dysregulation serves as an oncogene for many cancers including T-cell leukemia [152] and solid tumors of pancreas, breast, prostate, melanoma, and colon [151, 153–157]. In these malignancies, it contributes to malignancy by promoting growth, survival, motility, neo-angiogenesis, drug resistance, invasion, and metastasis [158–163]. In other cancers, Notch functions as a tumor suppressor, impeding growth or causing apoptosis in B-lineage ALL [164], myeloid malignancies [165], squamous cell carcinomas [166], neuroblastoma [167], other neural crest-derived cancers [168], and the GI stromal tumor [169]. It was recently suggested that Notch1 signaling is activated in human OS and may play a role in tumor invasion and metastasis [47, 52, 170]. One possible reason for this association is the reported link between Notch pathway activity and behavior of tumor-initiating cells or the putative cancer stem cells [171–177].

Two popular models for tumorigenesis include the stochastic model and the cancer stem cell model. The traditional stochastic model presumes that cancer arises from a single cell which has become genetically unstable and initiates tumor growth. The cancer stem cell model proposes that tumor-initiating cells share important properties with normal stem cells, including self-renewal and resistance to stress [42]. Over the past 5 years or more, the theory of cancer stem cells in osteosarcoma has gained a great deal of acceptance, with numerous publications in recent years describing phenotype, behavior, and therapeutic potential [178–194]. Logically, cells with stem cell-like properties should be superior at tumorigenesis and metastasis.

This concept was studied recently using two murine cell lines, K7M2 and K12, which were derived from the same spontaneously occurring murine osteosarcoma. K7M2 metastasizes with high frequency to the lung in mouse models, whereas K12 is much less metastatic [195].

Several groups have published that K7M2 and K12 cells produce different quantities of cytokines and that inhibition of these cytokines alters OS cell behavior in vitro [195–198]. For example, we have demonstrated that highly metastatic K7M2 cells express and produce more bone morphogenetic protein-2 (BMP-2) and VEGF than less metastatic K12 cells. Additionally, we observed that the inhibition of these factors diminished the motility and viability of K7M2 cells [197, 198]. More recently, we have demonstrated important differences between K7M2 and K12 in terms of Notch1 expression and function [50].

To evaluate the role of Notch in regulating stemness behaviors, we first compared K7M2 and K12 cells with reverse transcription polymerase chain reaction (RT-PCR). We analyzed differences in the expression of Notch1, its downstream targets, and other important genes in OS biology. We observed a significant upregulation (nearly twofold) of Notch1, Notch2, and Notch4 expression [50]. We also observed the upregulation of the Notch1 target genes Hes1 and Stat3 in highly metastatic K7M2 cells compared with less metastatic K12 cells. Notch pathway inhibition using an inhibitor of γ-secretase (GSI) in K7M2 cells reduced expression of these genes down to levels similar to K12 and also reduced migration and invasiveness of K7M2. Activation of Notch in K12 using an exogenous ligand increased invasiveness and migration, confirming the vital role of the Notch pathway in regulating these processes in this model [50].

Aldehyde dehydrogenase (ALDH) is another putative cancer stem cell factor [199–202] that has been implicated in a variety of human cancers. ALDH is a tetrameric protein that oxidizes aldehydes to carboxylic acids and thus enables cells to withstand oxidative stress. Its activity has been associated with metastasis, drug resistance, and poor prognosis [199–203]. We have shown that K7M2 cells possess greater mean ALDH activity and a higher percentage of ALDH-positive cells than the less metastatic K12 cells [204]. GSI treatment of K7M2 cells reduced the expression of ALDH and rendered the cells less tolerant of oxidative stress (Fig. 4), while treatment of K12 cells with the Notch ligand jagged 1 increased ALDH expression and rendered cells more tolerant of oxidative stress (Fig. 5), confirming that Notch pathway signaling is upstream of ALDH expression [50].

Conclusions

These studies, taken together, support the concept that Notch pathway signaling plays a key role in maintaining a stem cell-like phenotype for osteosarcoma and highlights the importance of Notch in osteosarcoma growth and metastasis. It is

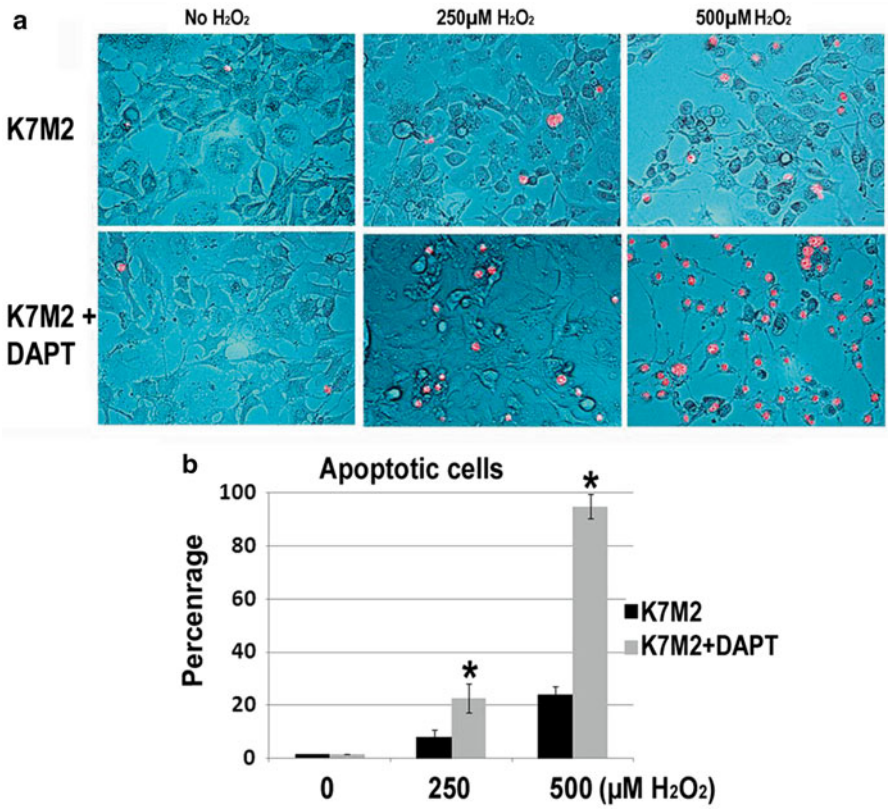


Fig. 4 K7M2 cells are resistant to H_2O_2 but become sensitive after treatment with DAPT. (**a**) K7M2 cells were treated with or without the γ-secretase inhibitor DAPT (10 μM) for 4 days and were then cultured with media containing H_2O_2 (0, 250, or 500 μM) for 6 h. Cell death was analyzed using PI exclusion assay. (**b**) The percentage of PI+ cells was determined for each group in (**a**). *Asterisk* indicates that the difference is significant comparing DAPT-treated or non-treated samples ($p < 0.05$). Figure taken from [50], used with permission

interesting to note, however, that the phenotype associated with Notch pathway expression could be induced by exposure to exogenous Notch ligand, calling into question the concept that tumor stem cells represent a discrete subpopulation in osteosarcoma. Given the importance of Notch signaling in tumor blood vessels and the high level of expression of Notch ligands in the vasculature, it is possible that the phenotype we associate with stemness in osteosarcoma really reflects proximity to tumor blood vessels and, therefore, exposure to Notch ligands. As therapies are developed to target Notch in cancer patients, the role of Notch in tumor vessel formation and expansion must also be considered.

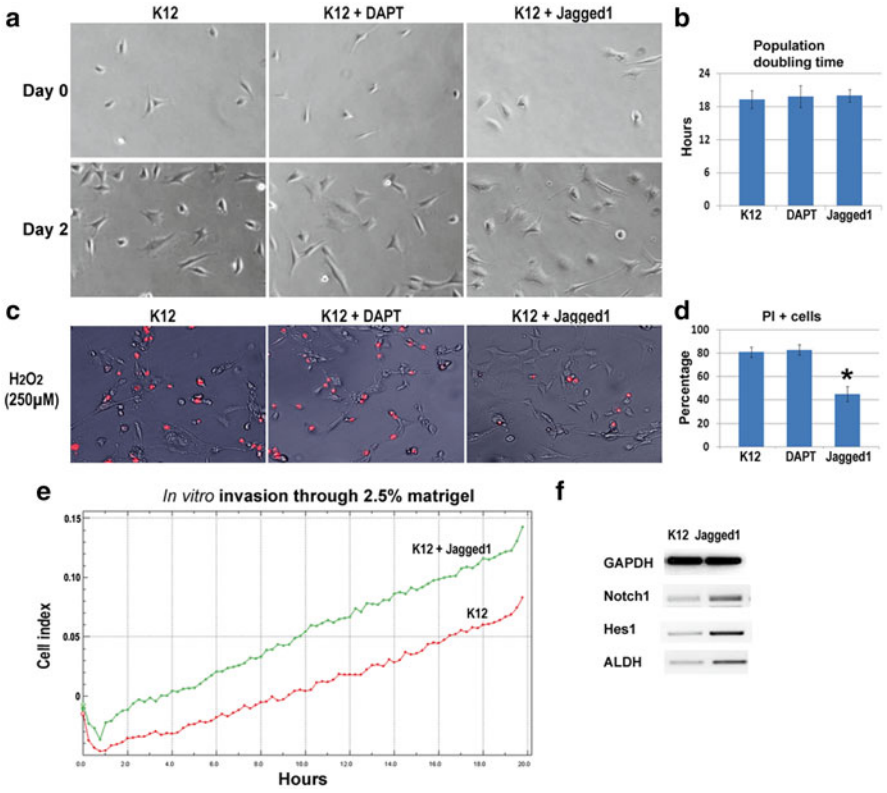


Fig. 5 Notch1 activation with jagged 1 increases K12 cells' resistance to oxidative stress. (**a**–**d**) K12 cells, which have low levels of Notch activation, were treated with soluble Jag1 to activate Notch pathway signaling. After Notch1 activation, K12 cells exhibited greater resistance to oxidative stress than untreated K12 cells. These images are presented as in Fig. 4. (**e**) K12 cell invasiveness increases with jagged 1 treatment. Jagged 1 treatment of K12 cells increased the in vitro invasion capacity of the cells through a semisolid matrigel matrix, suggesting that enhanced Notch expression contributes to metastatic potential in OS cells. Graphs depict the quantified mean invasion of tumor cells into matrigel over time. (**f**) Gene expression with Notch1 activation in less metastatic K12 cells. RT-PCR showed that the expression of Notch1, Hes1, and ALDH was upregulated in K12 cells with jagged 1 treatment, indicating a more aggressive phenotype. This figure is adapted from [50] and is used with permission

References

1. Iso T, Kedes L, Hamamori Y (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194(3):237–255
2. Kageyama R, Ohtsuka T (1999) The Notch-Hes pathway in mammalian neural development. Cell Res 9(3):179–188
3. Hilton M, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, Kronenberg H, Teitelbaum S, Ross F, Kopan R, Long F (2008) Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. Nat Med 14(3):306–314

4. Greenwald I, Kovall R (2013) Notch signaling: genetics and structure. WormBook : the online review of C elegans biology, pp 1–28
5. Kopan R (2012) Notch signaling. Cold Spring Harb Perspect Biol 4(10):a008904
6. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147
7. Crigler L, Kazhanie A, Yoon T-J, Zakhari J, Anders J, Taylor B, Virador V (2007) Isolation of a mesenchymal cell population from murine dermis that contains progenitors of multiple cell lineages. FASEB J 21(9):2050–2063
8. Tang N, Song W-X, Luo J, Haydon R, He T-C (2008) Osteosarcoma development and stem cell differentiation. Clin Orthopaed Relat Res 466(9):2114–2130
9. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G (1999) A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Gen Dev 13(8):1025–1036
10. Haydon R, Luu H, He T-C (2007) Osteosarcoma and osteoblastic differentiation: a new perspective on oncogenesis. Clin Orthopaed Relat Res 454:237–246
11. Hong J-H, Hwang E, McManus M, Amsterdam A, Tian Y, Kalmukova R, Mueller E, Benjamin T, Spiegelman B, Sharp P, Hopkins N, Yaffe M (2005) TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. Science 309(5737):1074–1078
12. Lian J, Stein G, Javed A, van Wijnen A, Stein J, Montecino M, Hassan M, Gaur T, Lengner C, Young D (2006) Networks and hubs for the transcriptional control of osteoblastogenesis. Rev Endoc Metab Disord 7(1–2):1–16
13. Luu H, Song W-X, Luo X, Manning D, Luo J, Deng Z-L, Sharff K, Montag A, Haydon R, He T-C (2007) Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthopaed Res 25(5):665–677
14. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng J, Behringer R, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108(1):17–29
15. Yamaguchi A, Komori T, Suda T (2000) Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endoc Rev 21(4):393–411
16. Deng Z-L, Sharff K, Tang N, Song W-X, Luo J, Luo X, Chen J, Bennett E, Reid R, Manning D, Xue A, Montag A, Luu H, Haydon R, He T-C (2008) Regulation of osteogenic differentiation during skeletal development. Front Biosci 13:2001–2021
17. Olsen B, Reginato A, Wang W (2000) Bone development. Annu Rev Cell Dev Biol 16:191–220
18. Harada S-i, Rodan G (2003) Control of osteoblast function and regulation of bone mass. Nature 423(6937):349–355
19. Ralston S, de Crombrugghe B (2006) Genetic regulation of bone mass and susceptibility to osteoporosis. Gen Dev 20(18):2492–2506
20. Gazzerro E, Canalis E (2006) Bone morphogenetic proteins and their antagonists. Rev Endoc Metab Disord 7(1–2):51–65
21. Krishnan V, Bryant H, Macdougald O (2006) Regulation of bone mass by Wnt signaling. J Clin Investig 116(5):1202–1209
22. Deregowski V, Gazzerro E, Priest L, Rydziel S, Canalis E (2006) Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not bone morphogenetic protein signaling. J Biol Chem 281(10):6203–6210
23. Banerjee C, McCabe L, Choi J, Hiebert S, Stein J, Stein G, Lian J (1997) Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 66(1):1–8
24. Ducy P, Zhang R, Geoffroy V, Ridall A, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89(5):747–754
25. Motohiko S, Natsuo Y, Takanobu N, Hirohisa K, Mizuo S, Seiichi H, Yukihiko K, Shintaro N, Takahiro O (1998) Expression of bone matrix proteins mRNA during distraction osteogenesis. J Bone Miner Res 13(8):1221–1231

26. Xiao Z, Hinson T, Quarles L (1999) Cbfa1 isoform overexpression upregulates osteocalcin gene expression in non-osteoblastic and pre-osteoblastic cells. J Cell Biochem 74(4):596–605
27. Prince M, Banerjee C, Javed A, Green J, Lian J, Stein G, Bodine P, Komm B (2001) Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. J Cell Biochem 80(3):424–440
28. Pregizer S, Barski A, Gersbach C, García A, Frenkel B (2007) Identification of novel Runx2 targets in osteoblasts: cell type-specific BMP-dependent regulation of Tram2. J Cell Biochem 102(6):1458–1471
29. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson R, Gao Y, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89(5):755–764
30. Otto F, Thornell A, Crompton T, Denzel A, Gilmour K, Rosewell I, Stamp G, Beddington R, Mundlos S, Olsen B, Selby P, Owen M (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89(5):765–771
31. Engin F, Yao Z, Yang T, Zhou G, Bertin T, Jiang M, Chen Y, Wang L, Zheng H, Sutton R, Boyce B, Lee B (2008) Dimorphic effects of Notch signaling in bone homeostasis. Nat Med 14(3):299–305
32. Sciaudone M, Gazzerro E, Priest L, Delany A, Canalis E (2003) Notch 1 impairs osteoblastic cell differentiation. Endocrinology 144(12):5631–5639
33. Tezuka K-I, Yasuda M, Watanabe N, Morimura N, Kuroda K, Miyatani S, Hozumi N (2002) Stimulation of osteoblastic cell differentiation by Notch. J Bone Miner Res 17(2):231–239
34. Zamurovic N, Cappellen D, Rohner D, Susa M (2004) Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. J Biol Chem 279(36):37704–37715
35. Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A (2003) Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. J Biol Chem 278(34):32227–32235
36. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, Takayanagi H (2005) NFAT and Osterix cooperatively regulate bone formation. Nat Med 11(8):880–885
37. Winslow M, Pan M, Starbuck M, Gallo E, Deng L, Karsenty G, Crabtree G (2006) Calcineurin/NFAT signaling in osteoblasts regulates bone mass. Dev Cell 10(6):771–782
38. Ikeda F, Nishimura R, Matsubara T, Hata K, Reddy S, Yoneda T (2006) Activation of NFAT signal in vivo leads to osteopenia associated with increased osteoclastogenesis and bone-resorbing activity. J Immunol 177(4):2384–2390
39. Dallas D, Genever P, Patton A, Millichip M, McKie N, Skerry T (1999) Localization of ADAM10 and Notch receptors in bone. Bone 25(1):9–15
40. Qi H, Rand M, Wu X, Sestan N, Wang W, Rakic P, Xu T, Artavanis-Tsakonas S (1999) Processing of the notch ligand delta by the metalloprotease Kuzbanian. Science 283(5398):91–94
41. Luo X, Chen J, Song W-X, Tang N, Luo J, Deng Z-L, Sharff K, He G, Bi Y, He B-C, Bennett E, Huang J, Kang Q, Jiang W, Su Y, Zhu G-H, Yin H, He Y, Wang Y, Souris J, Chen L, Zuo G-W, Montag A, Reid R, Haydon R, Luu H, He T-C (2008) Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects. Lab Investig 88(12):1264–1277
42. Reya T, Morrison S, Clarke M, Weissman I (2001) Stem cells, cancer, and cancer stem cells. Nature 414(6859):105–111
43. Thomas D, Kansara M (2006) Epigenetic modifications in osteogenic differentiation and transformation. Journal of cellular biochemistry 98(4):757–769
44. Wagner E, He B-C, Chen L, Zuo G-W, Zhang W, Shi Q, Luo Q, Luo X, Liu B, Luo J, Rastegar F, He C, Hu Y, Boody B, Luu H, He T-C, Deng Z-L, Haydon R (2010) Therapeutic Implications of PPARgamma in Human Osteosarcoma. PPAR Res 2010:956427

45. Thomas D, Johnson S, Sims N, Trivett M, Slavin J, Rubin B, Waring P, McArthur G, Walkley C, Holloway A, Diyagama D, Grim J, Clurman B, Bowtell D, Lee J-S, Gutierrez G, Piscopo D, Carty S, Hinds P (2004) Terminal osteoblast differentiation, mediated by runx2 and p27KIP1, is disrupted in osteosarcoma. J Cell Biol 167(5):925–934
46. Dailey D, Anfinsen K, Pfaff L, Ehrhart E, Charles J, Bønsdorff T, Thamm D, Powers B, Jonasdottir T, Duval D (2013) HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors. BMC Vet Res 9(1):130
47. Engin F, Bertin T, Ma O, Jiang MM, Wang L, Sutton RE, Donehower LA, Lee B (2009) Notch signaling contributes to the pathogenesis of human osteosarcomas. Hum Mol Genet 18(8):1464–1470
48. Hughes DPM (2009) How the NOTCH pathway contributes to the ability of osteosarcoma cells to metastasize. In: Jaffe N, Bruland OS, Bielack S (eds) Pediatric and adolescent osteosarcoma. Cancer treatment and research, 152nd edn. Springer, New York, NY, pp 479–496
49. Li Y, Zhang J, Ma D, Zhang L, Si M, Yin H, Li J (2012) Curcumin inhibits proliferation and invasion of osteosarcoma cells through inactivation of Notch-1 signaling. FEBS J 279(12):2247–2259
50. Mu X, Isaac C, Greco N, Huard J, Weiss K (2013) Notch signaling is associated with ALDH activity and an aggressive metastatic phenotype in murine osteosarcoma cells. Front Oncol 3:143
51. Tanaka M, Setoguchi T, Hirotsu M, Gao H, Sasaki H, Matsunoshita Y, Komiya S (2009) Inhibition of Notch pathway prevents osteosarcoma growth by cell cycle regulation. Br J Cancer 100(12):1957–1965
52. Zhang P, Yang Y, Nolo R, Zweidler-McKay PA, Hughes DP (2010) Regulation of NOTCH signaling by reciprocal inhibition of HES1 and Deltex 1 and its role in osteosarcoma invasiveness. Oncogene 29(20):2916–2926. doi:10.1038/onc.2010.62
53. Artavanis-Tsakonas S, Rand M, Lake R (1999) Notch signaling: cell fate control and signal integration in development. Science 284(5415):770–776
54. Shawber C, Kitajewski J (2004) Notch function in the vasculature: insights from zebrafish, mouse and man. Bioessays 26(3):225–234
55. Kume T (2009) Novel insights into the differential functions of Notch ligands in vascular formation. J Angiogen Res 1:8
56. Limbourg F, Takeshita K, Radtke F, Bronson R, Chin M, Liao J (2005) Essential role of endothelial Notch1 in angiogenesis. Circulation 111(14):1826–1832
57. Krebs L, Xue Y, Norton C, Shutter J, Maguire M, Sundberg J, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith G, Stark K, Gridley T (2000) Notch signaling is essential for vascular morphogenesis in mice. Gen Dev 14(11):1343–1352
58. Swiatek P, Lindsell C, del Amo F, Weinmaster G, Gridley T (1994) Notch1 is essential for postimplantation development in mice. Gen Dev 8(6):707–719
59. Hamada Y, Kadokawa Y, Okabe M, Ikawa M, Coleman J, Tsujimoto Y (1999) Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. Development 126(15):3415–3424
60. Xue Y, Gao X, Lindsell C, Norton C, Chang B, Hicks C, Gendron-Maguire M, Rand E, Weinmaster G, Gridley T (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum Mol Genet 8(5):723–730
61. Sörensen I, Adams R, Gossler A (2009) DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. Blood 113(22):5680–5688
62. HrabÄ de Angelis M, McIntyre 2nd J, Gossler A (1997) Maintenance of somite borders in mice requires the Delta homologue DII1. Nature 386(6626):717–721
63. Gale N, Dominguez M, Noguera I, Pan L, Hughes V, Valenzuela D, Murphy A, Adams N, Lin H, Holash J, Thurston G, Yancopoulos G (2004) Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A 101(45):15949–15954
64. Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J (2004) Dosage-sensitive requirement for mouse DLL4 in artery development. Gen Dev 18(20):2474–2478

65. Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M (2004) The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Gen Dev 18(8): 901–911
66. Krebs L, Shutter J, Tanigaki K, Honjo T, Stark K, Gridley T (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Gen Dev 18(20):2469–2473
67. Herreman A, Hartmann D, Annaert W, Saftig P, Craessaerts K, Serneels L, Umans L, Schrijvers V, Checler F, Vanderstichele H, Baekelandt V, Dressel R, Cupers P, Huylebroeck D, Zwijsen A, Van Leuven F, De Strooper B (1999) Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc Natl Acad Sci U S A 96(21):11872–11877
68. Nakajima M, Yuasa S, Ueno M, Takakura N, Koseki H, Shirasawa T (2003) Abnormal blood vessel development in mice lacking presenilin-1. Mech Dev 120(6):657–667
69. Uyttendaele H, Ho J, Rossant J, Kitajewski J (2001) Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proc Natl Acad Sci U S A 98(10):5643–5648
70. Krebs L, Starling C, Chervonsky A, Gridley T (2010) Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinB2 and EphB4 mutants. Genesis 48(3): 146–150
71. Shawber C, Das I, Francisco E, Kitajewski J (2003) Notch signaling in primary endothelial cells. Ann N Y Acad Sci 995:162–170
72. Kofler N, Shawber C, Kangsamaksin T, Reed H, Galatioto J, Kitajewski J (2011) Notch signaling in developmental and tumor angiogenesis. Gen Cancer 2(12):1106–1116
73. Koch S, Claesson-Welsh L (2012) Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med 2(7):a006502
74. Ferrara N, Gerber H-P, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9(6):669–676
75. Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck T, Pelletier N, Ferrara N (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem 276(5):3222–3230
76. Shalaby F, Rossant J, Yamaguchi T, Gertsenstein M, Wu X, Breitman M, Schuh A (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376(6535):62–66
77. Kappas N, Zeng G, Chappell J, Kearney J, Hazarika S, Kallianos K, Patterson C, Annex B, Bautch V (2008) The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. J Cell Biol 181(5):847–858
78. Shibuya M (2006) Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. J Biochem Mol Biol 39(5):469
79. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea K, Powell-Braxton L, Hillan K, Moore M (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380(6573):439–442
80. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380(6573):435–439
81. Suchting S, Freitas C, le Noble F, Benedito R, Bréant C, Duarte A, Eichmann A (2007) The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci U S A 104(9):3225–3230
82. Hellström M, Phng L-K, Hofmann J, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson A-K, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe M, Kalén M, Gerhardt H, Betsholtz C (2007) DLL4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445(7129):776–780

83. Lobov I, Renard R, Papadopoulos N, Gale N, Thurston G, Yancopoulos G, Wiegand S (2007) Delta-like ligand 4 (DLL4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci U S A 104(9):3219–3224
84. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161(6):1163–1177
85. del Toro R, Prahst C, Mathivet T, Siegfried G, Kaminker J, Larrivee B, Breant C, Duarte A, Takakura N, Fukamizu A, Penninger J, Eichmann A (2010) Identification and functional analysis of endothelial tip cell-enriched genes. Blood 116(19):4025–4033
86. Mats H, Li-Kun P, Holger G (2007) VEGF and notch signaling: the yin and yang of angiogenic sprouting. Cell Adh Migr 1(3):133–136
87. Benedito R, Hellström M (2013) Notch as a hub for signaling in angiogenesis. Exp Cell Res 319(9):1281–1288
88. Eilken H, Adams R (2010) Turning on the angiogenic microswitch. Nat Med 16(8):853–854
89. Harrington L, Sainson R, Williams C, Taylor J, Shi W, Li J-L, Harris A (2008) Regulation of multiple angiogenic pathways by DLL4 and Notch in human umbilical vein endothelial cells. Microvasc Res 75(2):144–154
90. Eilken H, Adams R (2010) Dynamics of endothelial cell behavior in sprouting angiogenesis. Curr Opin Cell Biol 22(5):617–625
91. Iruela-Arispe M, Davis G (2009) Cellular and molecular mechanisms of vascular lumen formation. Dev Cell 16(2):222–231
92. Dejana E, Tournier-Lasserve E, Weinstein B (2009) The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Dev Cell 16(2):209–221
93. Tammela T, Zarkada G, Wallgard E, Murtomäki A, Suchting S, Wirzenius M, Waltari M, Hellström M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Ylä-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K (2008) Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 454(7204):656–660
94. Benedito R, Rocha S, Woeste M, Zamykal M, Radtke F, Casanovas O, Duarte A, Pytowski B, Adams R (2012) Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. Nature 484(7392):110–114
95. Tammela T, Zarkada G, Nurmi H, Jakobsson L, Heinolainen K, Tvorogov D, Zheng W, Franco C, Murtomäki A, Aranda E, Miura N, Ylä-Herttuala S, Fruttiger M, Mäkinen T, Eichmann A, Pollard J, Gerhardt H, Alitalo K (2011) VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. Nat Cell Biol 13(10):1202–1213
96. Hisaki H, Tsutomu K (2009) Foxc2 transcription factor as a regulator of angiogenesis via induction of integrin β3 expression. Cell Adh Migr 3(1):24–26
97. Chappell J, Taylor S, Ferrara N, Bautch V (2009) Local guidance of emerging vessel sprouts requires soluble Flt-1. Dev Cell 17(3):377–386
98. Benedito R, Roca C, Sörensen I, Adams S, Gossler A, Fruttiger M, Adams R (2009) The notch ligands DLL4 and Jagged1 have opposing effects on angiogenesis. Cell 137(6):1124–1135
99. Thomas J-L, Baker K, Han J, Calvo C, Nurmi H, Eichmann A, Alitalo K (2013) Interactions between VEGFR and Notch signaling pathways in endothelial and neural cells. Cell Mol Life Sci 70(10):1779–1792
100. Phng L-K, Potente M, Leslie J, Babbage J, Nyqvist D, Lobov I, Ondr J, Rao S, Lang R, Thurston G, Gerhardt H (2009) Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. Dev Cell 16(1):70–82
101. Sainson R, Aoto J, Nakatsu M, Holderfield M, Conn E, Koller E, Hughes C (2005) Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. FASEB J 19(8):1027–1029
102. Kageyama R, Masamizu Y, Niwa Y (2007) Oscillator mechanism of Notch pathway in the segmentation clock. Dev Dyn 236(6):1403–1409
103. Jakobsson L, Franco C, Bentley K, Collins R, Ponsioen B, Aspalter I, Rosewell I, Busse M, Thurston G, Medvinsky A, Schulte-Merker S, Gerhardt H (2010) Endothelial cells dynami-

cally compete for the tip cell position during angiogenic sprouting. Nat Cell Biol 12(10):943–953
104. Leslie J, Ariza-McNaughton L, Bermange A, McAdow R, Johnson S, Lewis J (2007) Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. Development 134(5):839–844
105. Noguera-Troise I, Daly C, Papadopoulos N, Coetzee S, Boland P, Gale N, Lin H, Yancopoulos G, Thurston G (2006) Blockade of DLL4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 444(7122):1032–1037
106. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang W-C, Chanthery Y, Kowalski J, Watts R, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo J-AS, de Sauvage F, Plowman G, Yan M (2006) Inhibition of DLL4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 444(7122):1083–1087
107. Siekmann A, Lawson N (2007) Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. Nature 445(7129):781–784
108. Phng LK, Gerhardt H (2009) Angiogenesis: a team effort coordinated by notch. Dev Cell 16(2):196–208
109. Siekmann A, Affolter M, Belting H-G (2013) The tip cell concept 10 years after: new players tune in for a common theme. Exp Cell Res 319(9):1255–1263
110. Benjamin L, Hemo I, Keshet E (1998) A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125(9):1591–1598
111. Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. Circ Res 97(6):512–523
112. Villa N, Walker L, Lindsell C, Gasson J, Iruela-Arispe M, Weinmaster G (2001) Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech Dev 108(1–2):161–164
113. Liu H, Kennard S, Lilly B (2009) NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. Circ Res 104(4):466–475
114. Schadler KL, Zweidler-McKay PA, Guan H, Kleinerman ES (2010) Delta-like ligand 4 plays a critical role in pericyte/vascular smooth muscle cell formation during vasculogenesis and tumor vessel expansion in Ewing's sarcoma. Clin Cancer Res 16(3):848–856
115. Stewart KS, Zhou Z, Zweidler-McKay P, Kleinerman ES (2011) Delta-like ligand 4-Notch signaling regulates bone marrow-derived pericyte/vascular smooth muscle cell formation. Blood 117(2):719–726
116. Crowther M, Brown N, Bishop E, Lewis C (2001) Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. J Leukoc Biol 70(4):478–490
117. Schmidt T, Carmeliet P (2010) Blood-vessel formation: bridges that guide and unite. Nature 465(7299):697–699
118. Checchin D, Sennlaub F, Levavasseur E, Leduc M, Chemtob S (2006) Potential role of microglia in retinal blood vessel formation. Investig Ophthalmol Vis Sci 47(8):3595–3602
119. Fantin A, Vieira J, Gestri G, Denti L, Schwarz Q, Prykhozhij S, Peri F, Wilson S, Ruhrberg C (2010) Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. Blood 116(5):829–840
120. Bigas A, Martin D, Milner L (1998) Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol Cell Biol 18(4):2324–2333
121. Monsalve E, Pérez M, Rubio A, Ruiz-Hidalgo M, Baladrón V, García-Ramírez J, Gómez J, Laborda J, Díaz-Guerra M (2006) Notch-1 up-regulation and signaling following macrophage activation modulates gene expression patterns known to affect antigen-presenting capacity and cytotoxic activity. J Immunol 176(9):5362–5373
122. Singh N, Phillips R, Iscove N, Egan S (2000) Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. Exp Hematol 28(5):527–534
123. Murakami M, Zheng Y, Hirashima M, Suda T, Morita Y, Ooehara J, Ema H, Fong G-H, Shibuya M (2008) VEGFR1 tyrosine kinase signaling promotes lymphangiogenesis as well

as angiogenesis indirectly via macrophage recruitment. Arterioscler Thromb Vasc Biol 28(4):658–664
124. Outtz H, Wu J, Wang X, Kitajewski J (2010) Notch1 deficiency results in decreased inflammation during wound healing and regulates vascular endothelial growth factor receptor-1 and inflammatory cytokine expression in macrophages. J Immunol 185(7):4363–4373
125. Outtz H, Tattersall I, Kofler N, Steinbach N, Kitajewski J (2011) Notch1 controls macrophage recruitment and Notch signaling is activated at sites of endothelial cell anastomosis during retinal angiogenesis in mice. Blood 118(12):3436–3439
126. Leung D, Cachianes G, Kuang W, Goeddel D, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246(4935):1306–1309
127. Hu P, Liu W, Wang L, Yang M, Du J (2013) High circulating VEGF level predicts poor overall survival in lung cancer. J Cancer Res Clin Oncol 139(7):1157–1167
128. Claesson-Welsh L, Welsh M (2013) VEGFA and tumour angiogenesis. J Intern Med 273(2):114–127
129. Yu X-W, Wu T-Y, Yi X, Ren W-P, Zhou Z-B, Sun Y-Q, Zhang C-Q (2013) Prognostic significance of VEGF expression in osteosarcoma: a meta-analysis. Tumour Biol 35(1):155–160
130. Chen D, Zhang Y-J, K-w Z, Wang W-C (2013) A systematic review of vascular endothelial growth factor expression as a biomarker of prognosis in patients with osteosarcoma. Tumour Biol 34(3):1895–1899
131. Lammli J, Fan M, Rosenthal H, Patni M, Rinehart E, Vergara G, Ablah E, Wooley P, Lucas G, Yang S-Y (2012) Expression of vascular endothelial growth factor correlates with the advance of clinical osteosarcoma. Int Orthopaed 36(11):2307–2313
132. Ranganathan P, Weaver K, Capobianco A (2011) Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 11(5):338–351
133. Patel N, Li J-L, Generali D, Poulsom R, Cranston D, Harris A (2005) Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. Cancer Res 65(19):8690–8697
134. Rehman A, Wang C-Y (2006) Notch signaling in the regulation of tumor angiogenesis. Trend Cell Biol 16(6):293–300
135. Dufraine J, Funahashi Y, Kitajewski J (2008) Notch signaling regulates tumor angiogenesis by diverse mechanisms. Oncogene 27(38):5132–5137
136. Chi Sabins N, Taylor J, Fabian K, Appleman L, Maranchie J, Stolz D, Storkus W (2013) DLK1: a novel target for immunotherapeutic remodeling of the tumor blood vasculature. Mol Ther 21(10):1958–1968
137. Li J-L, Sainson R, Shi W, Leek R, Harrington L, Preusser M, Biswas S, Turley H, Heikamp E, Hainfellner J, Harris A (2007) Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. Cancer Res 67(23):11244–11253
138. Segarra M, Williams C, Sierra MDLL, Bernardo M, McCormick P, Maric D, Regino C, Choyke P, Tosato G (2008) DLL4 activation of Notch signaling reduces tumor vascularity and inhibits tumor growth. Blood 112(5):1904–1911
139. Trindade A, Kumar S, Scehnet J, Lopes-da-Costa L, Becker J, Jiang W, Liu R, Gill P, Duarte A (2008) Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. Blood 112(5):1720–1729
140. Williams C, Li J-L, Murga M, Harris A, Tosato G (2006) Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. Blood 107(3):931–939
141. Zhang J-P, Qin H-Y, Wang L, Liang L, Zhao X-C, Cai W-X, Wei Y-N, Wang C-M, Han H (2011) Overexpression of Notch ligand DLL1 in B16 melanoma cells leads to reduced tumor growth due to attenuated vascularization. Cancer Lett 309(2):220–227
142. Zhao X-C, Dou G-R, Wang L, Liang L, Tian D-M, Cao X-L, Qin H-Y, Wang C-M, Zhang P, Han H (2013) Inhibition of tumor angiogenesis and tumor growth by the DSL domain of human delta-like 1 targeted to vascular endothelial cells. Neoplasia 15(7):815–825
143. Scehnet J, Jiang W, Kumar S, Krasnoperov V, Trindade A, Benedito R, Djokovic D, Borges C, Ley E, Duarte A, Gill P (2007) Inhibition of DLL4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. Blood 109(11):4753–4760

144. Yan M, Callahan C, Beyer J, Allamneni K, Zhang G, Ridgway J, Niessen K, Plowman G (2010) Chronic DLL4 blockade induces vascular neoplasms. Nature 463(7282):7
145. Folkman J (2002) Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29(6, Supplement 16):15–18
146. Indraccolo S, Minuzzo S, Masiero M, Pusceddu I, Persano L, Moserle L, Reboldi A, Favaro E, Mecarozzi M, Di Mario G, Screpanti I, Ponzoni M, Doglioni C, Amadori A (2009) Cross-talk between tumor and endothelial cells involving the Notch3-DLL4 interaction marks escape from tumor dormancy. Cancer Res 69(4):1314–1323
147. Indraccolo S, Favaro E, Amadori A (2006) Dormant tumors awaken by a short-term angiogenic burst: the spike hypothesis. Cell Cycle 5(16):1751–1755
148. Almog N, Ma L, Raychowdhury R, Schwager C, Erber R, Short S, Hlatky L, Vajkoczy P, Huber PE, Folkman J, Abdollahi A (2009) Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype. Cancer Res 69(3):836–844
149. Indraccolo S (2013) Insights into the regulation of tumor dormancy by angiogenesis in experimental tumors. Adv Exp Med Biol 734:37–52
150. Lai EC (2004) Notch signaling: control of cell communication and cell fate. Development 131(5):965–973
151. Bray SJ (2006) Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7(9):678–689
152. Zweidler-McKay P, Pear W (2004) Notch and T cell malignancy. Semin Cancer Biol 14(5):329–340
153. Hu YY, Zheng MH, Zhang R, Liang YM, Han H (2012) Notch signaling pathway and cancer metastasis. Adv Exp Med Biol 727:186–198
154. Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM, Leach SD (2003) Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell 3(6):565–576
155. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H (2005) Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435(7044):959–963
156. Wang Z, Li Y, Banerjee S, Kong D, Ahmad A, Nogueira V, Hay N, Sarkar FH (2010) Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-kappaB signaling pathways. J Cell Biochem 109(4):726–736
157. Weng AP, Ferrando AA, Lee W, Morris JPT, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306(5694):269–271
158. Bai F, Tagen M, Colotta C, Miller L, Fouladi M, Stewart CF (2010) Determination of the gamma-secretase inhibitor MK-0752 in human plasma by online extraction and electrospray tandem mass spectrometry (HTLC-ESI-MS/MS). J Chromatogr B Analyt Technol Biomed Life Sci 878(25):2348–2352
159. Cheng X, O'Neill HC (2009) Oncogenesis and cancer stem cells: current opinions and future directions. J Cell Mol Med 13(11–12):4377–4384
160. Fouladi M, Stewart CF, Olson J, Wagner LM, Onar-Thomas A, Kocak M, Packer RJ, Goldman S, Gururangan S, Gajjar A, Demuth T, Kun LE, Boyett JM, Gilbertson RJ (2011) Phase I trial of MK-0752 in children with refractory CNS malignancies: a pediatric brain tumor consortium study. J Clin Oncol 29(26):3529–3534
161. Mazumdar J, Dondeti V, Simon MC (2009) Hypoxia-inducible factors in stem cells and cancer. J Cell Mol Med 13(11–12):4319–4328
162. Shih Ie M, Wang TL (2007) Notch signaling, gamma-secretase inhibitors, and cancer therapy. Cancer Res 67(5):1879–1882
163. Wang Z, Li Y, Banerjee S, Sarkar FH (2009) Emerging role of Notch in stem cells and cancer. Cancer Lett 279(1):8–12

164. Zweidler-McKay PA, He Y, Xu L, Rodriguez CG, Karnell FG, Carpenter AC, Aster JC, Allman D, Pear WS (2005) Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. Blood 106(12):3898–3906
165. Kannan S, Sutphin RM, Hall MG, Golfman LS, Fang W, Nolo RM, Akers LJ, Hammitt RA, McMurray JS, Kornblau SM, Melnick AM, Figueroa ME, Zweidler-McKay PA (2013) Notch activation inhibits AML growth and survival: a potential therapeutic approach. J Exp Med 210(2):321–337
166. Proweller A, Tu L, Lepore JJ, Cheng L, Lu MM, Seykora J, Millar SE, Pear WS, Parmacek MS (2006) Impaired Notch signaling promotes de novo squamous cell carcinoma formation. Cancer Res 66(15):7438–7444
167. Zage P, Nolo R, Fang W, Stewart J, Garcia-Manero G, Zweidler-McKay P (2012) Notch pathway activation induces neuroblastoma tumor cell growth arrest. Pediatr Blood Cancer 58(5):682–689
168. Kunnimalaiyaan M, Chen H (2007) Tumor suppressor role of Notch-1 signaling in neuroendocrine tumors. Oncologist 12(5):535–542
169. Dumont AG, Yang Y, Reynoso D, Katz D, Trent JC, Hughes DP (2012) Anti-tumor effects of the Notch pathway in gastrointestinal stromal tumors. Carcinogenesis 33(9):1674–1683
170. Hughes DP (2009) How the NOTCH pathway contributes to the ability of osteosarcoma cells to metastasize. Cancer Treat Res 152:479–496
171. Capaccione KM, Pine SR (2013) The Notch signaling pathway as a mediator of tumor survival. Carcinogenesis 34(7):1420–1430
172. Fan X, Matsui W, Khaki L, Stearns D, Chun J, Li Y-M, Eberhart CG (2006) Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. Cancer Res 66(15):7445–7452
173. Farnie G, Clarke R (2007) Mammary stem cells and breast cancer – role of Notch signalling. Stem Cell Rev 3(2):169–175
174. Hassan KA, Wang L, Korkaya H, Chen G, Maillard I, Beer DG, Kalemkerian GP, Wicha MS (2013) Notch pathway activity identifies cells with cancer stem cell-like properties and correlates with worse survival in lung adenocarcinoma. Clin Cancer Res 19(8): 1972–1980
175. Korkaya H, Wicha MS (2009) HER-2, Notch, and breast cancer stem cells: targeting an axis of evil. Clin Cancer Res 15(6):1845–1847
176. Sullivan JP, Spinola M, Dodge M, Raso MG, Behrens C, Gao B, Schuster K, Shao C, Larsen JE, Sullivan LA, Honorio S, Xie Y, Scaglioni PP, DiMaio JM, Gazdar AF, Shay JW, Wistuba II, Minna JD (2010) Aldehyde dehydrogenase activity selects for lung adenocarcinoma stem cells dependent on notch signaling. Cancer Res 70(23):9937–9948
177. Wang J, Sullenger B, Rich J (2012) Notch signaling in cancer stem cells. Adv Exp Med Biol 727:174–185
178. Adhikari AS, Agarwal N, Wood BM, Porretta C, Ruiz B, Pochampally RR, Iwakuma T (2010) CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. Cancer Res 70(11):4602–4612
179. Williams S, Maecker H, French D, Liu J, Gregg A, Silverstein L, Cao T, Carano R, Dixit V (2011) USP1 deubiquitinates ID proteins to preserve a mesenchymal stem cell program in osteosarcoma. Cell 146(6):918–930
180. Wang L, Park P, Lin C (2009) Characterization of stem cell attributes in human osteosarcoma cell lines. Cancer Biol Ther 8(6):543–552
181. Siclari V, Qin L (2010) Targeting the osteosarcoma cancer stem cell. J Orthop Surg Res 5:78
182. Gibbs C, Levings P, Ghivizzani S (2011) Evidence for the osteosarcoma stem cell. Curr Orthop Pract 22(4):322–326
183. Di Fiore R, Fanale D, Drago-Ferrante R, Chiaradonna F, Giuliano M, De Blasio A, Amodeo V, Corsini L, Bazan V, Tesoriere G, Vento R, Russo A (2012) Genetic and molecular characterization of the human osteosarcoma 3AB-OS cancer stem cell line: a possible model for studying osteosarcoma origin and stemness. J Cell Physiol 228(6):1189–1201

184. Di Fiore R, Santulli A, Ferrante R, Giuliano M, De Blasio A, Messina C, Pirozzi G, Tirino V, Tesoriere G, Vento R (2009) Identification and expansion of human osteosarcoma-cancer-stem cells by long-term 3-aminobenzamide treatment. J Cell Physiol 219(2):301–313
185. Tang Q, Zhao Z, Li J, Liang Y, Yin J, Zou C, Xie X, Zeng Y, Shen J, Kang T, Wang J (2011) Salinomycin inhibits osteosarcoma by targeting its tumor stem cells. Cancer Lett 311(1):113–121
186. Wilson H, Huelsmeyer M, Chun R, Young K, Friedrichs K, Argyle D (2008) Isolation and characterisation of cancer stem cells from canine osteosarcoma. Vet J 175(1):69–75
187. Martins-Neves S, Lopes Á, do Carmo A, Paiva A, Simoes P, Abrunhosa A, Gomes C (2012) Therapeutic implications of an enriched cancer stem-like cell population in a human osteosarcoma cell line. BMC Cancer 12:139
188. Liu B, Ma W, Jha R, Gurung K (2011) Cancer stem cells in osteosarcoma: recent progress and perspective. Acta Oncol 50(8):1142–1150
189. Basu-Roy U, Basilico C, Mansukhani A (2012) Perspectives on cancer stem cells in osteosarcoma. Cancer Lett 338(1):158–167
190. Wang L, Park P, Zhang H, La Marca F, Lin C (2011) Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity. Int J Cancer 128(2):294–303
191. Wang L, Park P, Zhang H, La Marca F, Claeson A, Valdivia J, Lin C (2011) BMP-2 inhibits the tumorigenicity of cancer stem cells in human osteosarcoma OS99-1 cell line. Cancer Biol Ther 11(5):457–463
192. Rainusso N, Man T, Lau C, Hicks J, Shen J, Yu A, Wang L, Rosen J (2011) Identification and gene expression profiling of tumor-initiating cells isolated from human osteosarcoma cell lines in an orthotopic mouse model. Cancer Biol Ther 12(4):278–287
193. Yang M, Yan M, Zhang R, Li J, Luo Z (2011) Side population cells isolated from human osteosarcoma are enriched with tumor-initiating cells. Cancer Sci 102(10):1774–1781
194. Huang Y, Dai H, Guo Q (2012) TSSC3 overexpression reduces stemness and induces apoptosis of osteosarcoma tumor-initiating cells. Apoptosis 17(8):749–761
195. Khanna C, Prehn J, Yeung C, Caylor J, Tsokos M, Helman L (2000) An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. Clin Exp Meta 18(3):261–271
196. Khanna C, Khan J, Nguyen P, Prehn J, Caylor J, Yeung C, Trepel J, Meltzer P, Helman L (2001) Metastasis-associated differences in gene expression in a murine model of osteosarcoma. Cancer Res 61(9):3750–3759
197. Weiss KR (2010) Inhibition of osteosarcoma metastatic potential with noggin and s-Flt. In: 2010 Meeting of the Musculoskeletal Tumor Society, Philadelphia, PA, 2010
198. Weiss KR, Cooper GM, Jadlowiec JA, McGough RL 3rd, Huard J (2006) VEGF and BMP expression in mouse osteosarcoma cells. Clin Orthop Relat Res 450:111–117
199. Charafe-Jauffret E, Ginestier C, Iovino F, Tarpin C, Diebel M, Esterni B, Houvenaeghel G, Extra JM, Bertucci F, Jacquemier J, Xerri L, Dontu G, Stassi G, Xiao Y, Barsky SH, Birnbaum D, Viens P, Wicha MS (2010) Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer. Clin Cancer Res 16(1):45–55
200. Cheung AM, Wan TS, Leung JC, Chan LY, Huang H, Kwong YL, Liang R, Leung AY (2007) Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. Leukemia 21(7):1423–1430
201. Cho HJ, Lee TS, Park JB, Park KK, Choe JY, Sin DI, Park YY, Moon YS, Lee KG, Yeo JH, Han SM, Cho YS, Choi MR, Park NG, Lee YS, Chang YC (2007) Disulfiram suppresses invasive ability of osteosarcoma cells via the inhibition of MMP-2 and MMP-9 expression. J Biochem Mol Biol 40(6):1069–1076
202. Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM (2009) Aldehyde dehydrogenase 1 is a marker for normal and malignant human

colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res 69(8):3382–3389
203. Honoki K, Fujii H, Kubo A, Kido A, Mori T, Tanaka Y, Tsujiuchi T (2010) Possible involvement of stem-like populations with elevated ALDH1 in sarcomas for chemotherapeutic drug resistance. Oncol Rep 24(2):501–505
204. Mu X, Isaac C, Schott T, Huard J, Weiss K (2013) Rapamycin Inhibits ALDH Activity, Resistance to Oxidative Stress, and Metastatic Potential in Murine Osteosarcoma Cells. Sarcoma 2013:11
205. Hughes DP (2009) Strategies for the targeted delivery of therapeutics for osteosarcoma. Expert Opin Drug Deliv 6(12):1311–1321
206. Blake JA, Bult CJ, Eppig JT, Kadin JA, Richardson JE, The Mouse Genome Database Group (2014) The Mouse Genome Database: integration of and access to knowledge about the laboratory mouse. Nucleic Acids Res 42(D1):D810–D817
207. Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze DV, Weinmaster G, Gridley T (1998) Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. Genes Dev 12(7):1046–1057
208. Sorensen I, Adams RH, Gossler A (2009) DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. Blood 113(22):5680–5688. doi:10.1182/blood-2008-08-174508
209. Dunwoodie SL, Clements M, Sparrow DB, Sa X, Conlon RA, Beddington RS (2002) Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm. Development 129(7):1795–1806
210. Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, Klonjkowski B, Berrou E, Mericskay M, Li Z, Tournier-Lasserve E, Gridley T, Joutel A (2004) Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev 18(22):2730–2735
211. Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. Cell 89(4):629–639

Developmental Pathways Hijacked by Osteosarcoma

Jared R. Mortus, Yi Zhang, and Dennis P.M. Hughes

Abstract Cancer of any type often can be described by an arrest, alteration or disruption in the normal development of a tissue or organ, and understanding of the normal counterpart's development can aid in understanding the malignant state. This is certainly true for osteosarcoma and the normal developmental pathways that guide osteoblast development that are changed in the genesis of osteogenic sarcoma. A carefully regulated crescendo–decrescendo expression of RUNX2 accompanies the transition from mesenchymal stem cell to immature osteoblast to mature osteoblast. This pivotal role is controlled by several pathways, including bone morphogenic protein (BMP), Wnt/β-catenin, fibroblast growth factor (FGF), and protein kinase C (PKC). The HIPPO pathway and its downstream target YAP help to regulate proliferation of immature osteoblasts and their maturation into non-proliferating mature osteoblasts. This pathway also helps regulate expression of the mature osteoblast protein osteocalcin. YAP also regulates expression of MT1-MMP, a membrane-bound matrix metalloprotease responsible for remodeling the extracellular matrix surrounding the osteoblasts. YAP, in turn, can be regulated by the ERBB family protein Her-4. Osteosarcoma may be thought of as a cell held at the immature osteoblast stage, retaining some of the characteristics of that developmental stage. Disruptions of several of these pathways have been described in osteosarcoma, including BMP, Wnt/b-catenin, RUNX2, HIPPO/YAP, and Her-4. Further, PKC can be activated by several receptor tyrosine kinases implicated in osteosarcoma, including

J.R. Mortus
The College of Natural Sciences, The University of Texas at Austin, Austin, TX, USA

Y. Zhang, M.D. • D.P.M. Hughes, M.D., Ph.D. (✉)
The Children's Cancer Hospital, University of Texas at MD Anderson Cancer Center, Houston, TX, USA
e-mail: dphughes@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_5,

the ERBB family (EGFR, Her-2 and Her-4 in osteosarcoma), IGF1R, FGF, and others. Understanding these functions may aid in the understanding the mechanisms underpinning clinical observations in osteosarcoma, including both the lytic and blastic phenotypes of tumors, the invasiveness of the disease, and the tendency for treated tumors to ossify rather than shrink. Through a better understanding of the relationship between normal osteoblast development and osteosarcoma, we may gain insights into novel therapeutic avenues and improved outcomes.

Keywords Osteosarcoma • Organogenesis • Osteoblast • Development • Cancer signaling • ERBB4 • RUNX2 • Wnt/β-catenin • FGF • IGF1R • MMP • BMP • TWIST • YAP

Introduction

To better understand the biology of osteosarcoma, it can be helpful to think of this disease as a cancer arising from the malignant transformation of osteoblasts, the cells responsible for normal bone formation. While the cell of origin for osteosarcoma typically is defined as the mesenchymal stem cell, mesenchymal stem cells also are the progenitor cell for not only osteoblasts [1] but also adipocytes and chondrocytes [2]. The term "osteosarcoma" itself is, in fact, a contraction of the older term, "osteogenic sarcoma," meaning "the sarcoma that gives birth to bone," based on the disease-defining characteristic of malignant bone formation within tumors, first identified 80 years ago [3, 4]. Further, transgenic mice with driver mutations (i.e., p53 and Rb) limited exclusively to the osteoblast lineage do develop osteosarcoma [5], indicating the link between the malignancy and its normal counterpart. Because of the phenotypic and functional similarity between immature osteoblasts and osteosarcoma cells, it is useful to examine osteosarcoma from the perspective of arrested development of osteoblasts, evaluating those pathways defined as important in normal bone development. With that in mind, we focus here on those pathways that regulate normal osteoblast development, weighing the evidence for each pathway's involvement in osteosarcoma pathobiology.

Runx2

RUNX2 is a major transcriptional factor in bone formation, acting as the master regulator for osteoblast differentiation [6]. RUNX2 is a DNA binding protein that comes from a family of very well described Runt-related transcription factors, with homologs RUNX1 and RUNX3 [7]. These proteins in this family are heterodimeric proteins, which typically not only involve the cofactor CBF-β [8] but also include a

variety of co-activators, including, but not limited to, HES1 [9] and YAP [10]. RUNX2 induces bone formation and mineralization in vivo [11] through activation of bone matrix proteins such as osteocalcin [12] and osteopontin [13]. RUNX2$^{-/-}$ transgenic mice have normal skeletal development, but lack mineralization and die just after birth [14]. In humans, defective RUNX2 results in cleidocranial dysplasia [15].

In addition to its major function as the master regulator of bone formation and mineralization, RUNX2 also regulates cell cycle exit and apoptosis. In proliferating osteoblasts, RUNX2 is present only in the G1 phase of cell cycle, being suppressed in S, G2, and M [16]. RUNX2 has been shown in normal osteoblasts to upregulate p27^{KIP1} [17], a known regulator of growth arrest and cell cycle control. The effects of RUNX2 on cell cycle also result from its interactions with the hypophosphorylated retinoblastoma protein (Rb), which has been shown to be mediated by HES1 [18]. This interaction between Rb and RUNX2 facilitates cell cycle exit into G_0, inducing cellular senescence. Additionally, HES1 and pRB interact physically to cause transcriptional activation of RUNX2 [18]. The physical interaction of the C-termini of RUNX2 and HES1 has been implied to prevent TLE/Groucho mediated silencing of target genes. Further, the retinoblastoma binding protein-1 (RBP1) serves as a co-activator of RUNX2, promoting target gene expression and subsequent differentiation [19].

Another function of RUNX2 is its ability to suppress p53 mediated apoptosis in response to DNA damage, through physical interaction with and use of HDAC6 [20]. Although many osteosarcoma cell lines lack endogenous or functional p53 [21], those which still possess p53 are subject to suppression by RUNX2 and HDAC6. In osteosarcoma, RUNX2 gain of function models indicate that RUNX2 levels remain high throughout the cell cycle in osteosarcoma, in contrast to its role in normal osteoblasts [16, 22]. It has been suggested that the effects of RUNX2 on p27^{KIP1} are abrogated in osteosarcoma. This loss of the RUNX2-p^{27KIP1} signaling axis prevents osteosarcoma cells from terminally differentiating and exiting the cell cycle.

Expression of several osteoblast-specific proteins is controlled in part by RUNX2. RUNX2 interacts with the Mastermind-like 1 protein (MAML1), a component of the CSL complex that is activated by Notch signaling, to activate osteocalcin expression through binding to a promoter element called osteoblast-specific element 2 [23]. Overexpression of MAML1 in murine derived mesenchymal stem cells resulted in enhanced alkaline phosphatase (ALP) expression, which is an early marker of osteoblast differentiation. Osteoblast differentiation also is heightened by p300/CBF-associated factor (PCAF) which directly binds to and acetylates RUNX2, resulting in an increase in transcriptional activity of RUNX2 target genes [24]. ALP activity and mineralization were both attenuated with PCAF siRNA mediated knockdown, suggesting that PCAF is important in normal osteoblast differentiation.

RUNX2 also is subject to control by a variety of extracellular and intracellular signals. TNFα is a pro-apoptotic and antiproliferative protein in osteoblasts [25, 26]; however, RUNX2 activation in SaOS2 cells results in protection from these effects, accompanied by an increase in anti-apoptotic factor Bcl-1 and sequestering of

pro-apoptotic factor Bax [27]. Interestingly enough, EWS-FLI, the major fusion protein responsible for Ewing's Sarcomas, directly binds to and inhibits RUNX2, giving rise to a loss of RUNX2 activation, which translates to a loss of function of RUNX2 and prevention of lineage commitment in these cells carrying the EWS-FLI t(11;22) translocation [28]. A variety of other factors can influence osteoblast differentiation in RUNX2 dependent manners, such as epidermal growth factor receptor (EGFR) [29], insulin-growth factor receptor (IFGR), fibroblast growth factor receptor (FGFR), and the bone morphogenetic protein receptor (BMPR), as well as canonical Wnt/β-catenin signaling axis and Hippo signaling. In addition, RUNX2 regulates several genes responsible for cell motility and adhesion [30]. Clearly then, RUNX2 plays multiple roles in osteoblast differentiation and aberrations in these signals play a vital role in the malignant phenotype observed in osteosarcoma.

Wnt/β-Catenin

The Wnt family of proteins includes soluble peptide ligands which bind to FRIZZLED receptors, as well as LRP5/6 coreceptors, which affect cells through both canonical and noncanonical signaling pathways. Canonical Wnt signaling includes the stabilization, accumulation, and nuclear translocation of β-catenin, allowing it to cooperate with the Lef/Tcf transcriptional factor family of proteins, resulting in activation of target genes [31]. β-catenin is critical for the proliferation of osteoblasts during early development [32]. The specific roles of Wnt5a in osteoblast differentiation have been reviewed recently [33]. Wnt5b also has been shown to associate closely with Nestin, a marker for bone marrow mesenchymal stem cells, which implies that Wnt5b aids in maintaining the stem cell population [34].

Some of the noncanonical functions of Wnt signaling in osteoblast differentiation are mediated by interleukin-1β (IL-1β). This important cytokine signals through Wnt5a and receptor tyrosine kinase-like orphan receptor 2 (Ror2) to induce differentiation [35]. Addition of IL-1β to mesenchymal stem cells in vitro alone demonstrated marked increase in mineralization (as determined by Alizarin Red Stain) and increase in alkaline phosphatase levels, both indicative of advanced differentiation. These effects were mediated through Wnt5 and Ror2.

Although there are several activators and enhancers of the Wnt signaling pathways present in bone cells, there are also a variety of molecules which inhibit Wnt/β-catenin signaling. Sclerostin, for example, is a molecule produced by osteocytes which inhibits osteoblast activity by suppressing Wnt signaling. Typically associated with osteoporosis, sclerostin not only serves to prevent further bone development by suppressing osteoblast function but also acts as a negative regulator of osteoblast proliferation [36]. Additionally, sclerostin is induced by BMP in the osteosarcoma line SaOS2 [37]. Sclerostin has been shown to be downstream of osterix (discussed below). The role of sclerostin in osteosarcoma has not been determined.

Several important negative regulators of Wnt signaling are included in the phosphorylation complex responsible for degradation of β-catenin, including glycogen

synthase kinase 3β, Axin, casein kinase 1α, and adenomatous polyposis coli 1 (AP1). This complex hyperphosphorylates β-catenin, allowing for F-box/WD repeat-containing protein 1A (FBXW1A) to target β-catenin for degradation, supposedly through its action as part of an E3 ubiquitin ligase [38].

Another inhibitor of Wnt signaling is the family of proteins known as the Dickkopf (Dkk) proteins, which have been implied to be significant regulators of osteoblast activity and osteosarcoma behavior. In a recent study, Dkk-3 was shown to decrease tumor growth and reduce pulmonary metastasis by 88.7 % in vivo, while reducing cell motility, viability, and anchorage independent growth in vitro. In the same study, Dkk-3 was shown to downregulate MMP2 and MMP9, both of which are key in invasion, migration, and metastasis [39]. Consistent with this function, Dkk-3 in osteosarcoma cell lines is downregulated in comparison to normal human osteoblasts, suggesting that loss of Dkk-3 may be important for osteosarcoma pathogenesis.

Taken together, Wnt appears to be a very important pathway in osteoblasts and osteosarcoma and manipulation of Wnt seems to have very potent effects both on normal osteoblasts and on osteosarcoma cells. Novel therapies targeting Wnt signaling are being developed for use in a variety of bone diseases, and possibly osteosarcoma [34, 40–42].

FGF

The fibroblast growth factor (FGF) proteins are a family of molecules which signal through four distinct fibroblast growth factor receptor (FGFR) proteins [43]. The mechanism of FGF signaling involves the binding of soluble ligand by the immunoglobulin-like (Ig-like) regions of the receptor, dimerization, and subsequent activation through its intracellular tyrosine kinase domain. Additionally, alternatively spliced mRNA of the FGFR genes determines which isoform, IIIb or IIIc, is produced based on the C-terminal half of the third Ig-like domain [44]. FGFR3, when missing exons 8–10, becomes soluble in SaOS2 cells [45]. Full length FGFR3, without the missing C-terminus Ig-like domain, only binds FGF-1 with high specificity, but loss of this domain resulted in binding of both FGF-1 and FGF-2, which reduces the specificity of the receptor and allows for increased nonspecific activation by FGF2 in SaOS2.

The biological effects of soluble FGF receptors have been shown in other cell types, such as breast cancer [46]. Soluble FGFR4 resulted in a decrease in the response of breast cancer line MCF7 to FGF-1, with a marked decrease in mitogen activated protein kinase activity. Additionally, soluble FGFR transcripts lacking the transmembrane domain have been shown to both compete for ligand and heterodimerize with surface FGFRs in vivo, both decreasing the response of the cell to FGF [47, 48]. Thus, soluble FGFRs have the potential to exert significant biological effects.

Fibroblast growth factors play a vital role in normal human osteoblasts. FGF-8, for example, promotes bone formation in vivo by upregulating osteocalcin,

allowing for greater deposition of hydroxyapatite [49]. FGF-8b was shown to increase alkaline phosphatase staining and activity in vitro after short exposure. However, prolonged exposure of FGF-8 led to inhibition of bone nodule formation. On the contrary, FGF-2 inhibits osteoblast differentiation of mesenchymal stem cells in vitro through transcriptional suppression of BMP ligand and receptor, mediated by activation of ERK and JNK [50]. Drug mediated inhibition of ERK and JNK rescued cells from the effects of FGF-2, thus giving strong support to the hypothesis that FGF-2 signals through ERK and JNK to suppress BMP and BMPR upregulation during osteoblast differentiation.

FGF2 signaling in osteosarcoma cells, then, may mediate two of the hallmark behaviors of cancer: blockade of differentiation and sustained proliferation. Since specific receptors can bind FGF-2, and the previously reported soluble FGFR-3 gain of function mutation results in nonspecific binding to FGF-2 in SaOS2, it appears as though these receptors may be good therapeutic targets. As discussed later in this review, FGF-2 also has been shown to signal downstream of protein kinase c delta (PKCδ), which causes phosphorylation of RUNX2, resulting in an increase in transcriptional activity of RUNX2 target genes [51]. Basic fibroblast growth factor (bFGF) and basic growth factor receptors (bFGFR) have been shown to be overexpressed in osteosarcoma line MG-63 [52]. This overexpression is correlated with the differentiation state of the cells, which has been correlated with prognosis.

MMP

The matrix metalloproteinase (MMP) proteins are part of a group of endopeptidases which are responsible for degradation of most extracellular matrix molecules [53]. Although MMPs are necessary for normal osteoblast function, expression of these proteins is associated with metastasis, invasiveness, increased migration, and angiogenesis in osteosarcoma [54]. Of all MMPs, MMP-2 and MMP-9 seem to be the major proteases functioning in osteosarcoma pathogenesis. Gallic acid has been shown to decrease invasiveness and migration in osteosarcoma cell line U2OS mainly through downregulation of MMP-2 and MMP-9 [55]. These effects were mediated through known upstream regulators of MMP-2 and MMP-9, mainly protein kinase c (PKC), PI3K/AKT, and NF-κB. This decrease in expression and activity of MMP-2/-9 led both to decreased invasiveness through Matrigel and o decreased metastatic capability of the cells. In addition, the use of *Phyllanthus urinaria* extracts (PUE), a known anti-inflammatory, antiviral, and antibacterial agent, in osteosarcoma cell line SaOS2 resulted in decreased migration and invasion [56]. The effects of PUE on SaOS2 cells were traced to the effects on MMP-2, mainly in the decreased expression of MMP-2. Once again, these effects were mediated through suppression of the ERK and PI3K/AKT signaling pathways. MMP-9 expression correlated with metastatic disease in several cancers [57] and is important in the context of osteoblasts and osteosarcoma, since MMP-9 is an integral

proteinase involved in bone remodeling [58]. This gave rise to the theory that aberrant expression of MMP-9 in osteosarcoma may be responsible for metastatic behavior. In osteosarcoma, MMP-9 is strongly expressed in pulmonary metastases, suggesting that MMP-9 may be necessary for the metastatic phenotype. Although MMP-9 has not yet been correlated with outcome, studies suggest that this may warrant investigation. ERK5 has also been implied to be upstream of MMP-9 [59]. Short hairpin RNA targeting ERK5 resulted in suppression of MMP-9 without having significant effects on MMP-2, TIMP, or matrix tethered MMP14 (MT1-MMP). Therefore, it was concluded that ERK5 expression may be correlated with metastatic disease and could pose as a therapeutic target for metastatic osteosarcoma tumors expressing MMP-9. MT1-MMP modulates the fibroblast signaling axis by proteolytically inhibiting ADAM9, which has been shown to mediate ectodomain shedding of FGFR2 [60]. MMP-14$^{-/-}$ mice experience a greater accumulation of the truncated FGFR2 receptor, which results in defective FGFR signaling. Removal of ADAM9 rescues cells from this effect. MT1-MMP also has implications in the β1-integrin/YAP/TAZ signaling axis by controlling β1-integrin activation in skeletal stem cells by regulating extracellular matrix remodeling [61]. Loss of MT1-MMP results in loss of osteoblastic cell lineage commitment in skeletal stem cells and allows for increased commitment in the adipogenic and chondrogenic pathways. Additionally, since YAP/TAZ signaling is dependent on cell–cell contact, through Hippo signaling (see elsewhere in this chapter), the effects of ECM remodeling by MT1-MMP clearly play a vital role in regulation of downstream activation of YAP/TAZ target genes. Since YAP/TAZ signaling has been tied to osteogenesis, upregulation of MT1-MMP would result in an increased movement towards the osteoblast cell fate, while downregulation would result in higher rates of adipogenesis and chondrogenesis. Thus, MT1-MMP plays a vital role in regulating osteoblast differentiation. MMP-1 also promotes tumorigenesis and pulmonary metastases in vivo [62]. Knockdown of MMP-1 in vivo resulted in significantly fewer pulmonary metastases, while adenoviral mediated expression of MMP-1 in osteosarcoma line HOS, which lacks endogenous MMP-1 expression, results in greater lung metastases. Additionally, MMP-1 expression promotes anchorage independent growth in soft agar, with overexpression resulting in more numerous and larger colonies and knockdown resulting in smaller and less numerous colonies. MMP-1 also correlated with tumor burden in mouse xenograph models, with significant increases in tumor burden correlating with increased MMP-1 expression. Thus, MMPs have a vital role in osteosarcoma pathogenesis and metastasis.

IGFR

The insulin growth factor (IGF) signaling axis is a pivotal component of the osteoblast phenotype [63]. IGFR regulates proliferation, differentiation, apoptosis, and metabolism in cell through activation of Akt and MAPK [64]. In osteosarcoma, IGFs and IGFRs have been linked to tumor progression, chemoresistance, and tumorigenesis.

Due to its involvement in these phenotypes, the IGFR signaling axis is an attractive therapeutic target. Additionally, IGF1R has been shown to be correlated with prognosis in osteosarcoma patients [65]. Analysis of primary lesions for IGF1R expression was linked with overall survival rate, with high IGF1R leading to poor outcome. The small molecule inhibitor tyrphostin AG1024 targets IGF1R by preventing phosphorylation of its intracellular domain, resulting in inhibition of IGF1R downstream signaling [66]. This inhibition was linked with increased sensitivity to doxorubicin, a chemotherapy agent. Dual treatment with AG1024 and doxorubicin resulted in increased growth inhibition, onset of cell cycle arrest, and increased apoptosis than treatment with either drug alone. Additionally, IGF1R has been implicated in angiogenesis, driving production of VEGF in sarcomas [67]. Suppression of VEGF could be achieved by addition of an antibody targeting IGF1R, CP-751,871. Unfortunately, clinical trials using anti-IGF1R antibodies for osteosarcoma rarely showed more than a brief, transient benefit, underscoring both the complexity and plasticity of signaling in osteosarcoma.

BMP

Canonical bone morphogenic protein (BMP) signaling is initiated when BMP ligand induces dimerization of BMP receptor (BMPR). which activates downstream phosphorylation of the SMAD family of proteins. This phosphorylation causes nuclear trans-localization of SMAD 1/5/8 by chaperone protein SMAD 4, activating transcription of target genes [68]. BMP-2 is a potent inducer of osteoblast differentiation, through induction of osteocalcin, which results in bone nodule formation [69]. BMP-7 has been shown to have similar effects in vitro by increasing alkaline phosphatase activity, a marker of osteoblastic differentiation, and increased mineralization [70]. Synergistic effects are seen between BMP and FGF, as well as BMP and Wnt signaling pathways. In calvarial osteoblasts, FGF signaling results in upregulation of BMP-2 [71]. During bone fracture healing, FGF-2 and BMP-2 have synergistic effects, with FGF-2 promoting proliferation of osteoblasts during early stages and BMP-2 serving as an inducer of differentiation during later stages [72]. BMP and Wnt signaling also seem to have synergistic effects through the cooperative effects of Wnt3A and BMP-9, resulting in induction of alkaline phosphatase in mesenchymal stem cells [1]. Wnt/β-catenin signaling has been shown to regulate BMP-2 expression in osteoblasts. Forced overexpression of β-catenin results in upregulation of BMP-2 while knockdown results in decreased expression [73]. BMP-2 is also unique in its ability to serve as a chemotactic migration agent for osteosarcoma cells [74]. Although BMP-4 and BMP-6 function elsewhere in osteoblast differentiation, only BMP-2 was shown to increase in vitro migration of U2OS cells. Thus, the BMP signaling axis is critical for normal osteoblast differentiation, but may be altered or subverted as a part of osteosarcoma pathology.

Osterix

Osterix (OSX), also called Sp7 Transcription Factor, is a zinc finger transcription factor that serves as a regulator of osteoblast differentiation, promoting transcription of osteocalcin, osteopontin, and bone sialoprotein [75]. OSX has been described as an antitumor agent, serving as a potent inducer of the commitment step from immature osteoblast to mature osteoblast. There is a clear lack of OSX expression in osteosarcoma [76]. Transfection with an OSX expressing vector caused a clear reduction in tumorigenesis, decreased osteolysis, decreased migration, and decreased pulmonary metastases in vivo. Although upregulation of OSX in vivo results in decreased metastasis, a panel of patient samples demonstrated that there were not significant differences in the expression of OSX in metastatic osteosarcomas vs. nonmetastatic osteosarcomas [77]. Neither the primary tumors nor the metastases had significant differences in OSX expression, which could imply that loss of OSX is not necessary for osteosarcoma pathogenesis. Although RUNX2 and OSX classically work in synergy to promote differentiation in osteoblasts, it appears as though heightened expression of RUNX2, together with loss of OSX expression, results in the osteosarcoma phenotype, keeping the cells in an immature, undifferentiated state [78]. Further investigation is warranted to fully establish the role of signaling through the RUNX2-OSX axis and its implications in osteosarcoma pathogenesis.

TWIST

The TWIST family of basis helix loop helix transcriptional factors [79] have been linked to a variety of cancers, including breast cancer [80], neuroblastoma [81], and other epithelial cancers [82]. TWIST1 is a potent regulator of the osteoblast lineage due to its ability to regulate RUNX2. In mesenchymal stem cells, enforced expression of TWIST1 results in an increase in life span and reduction of osteogenic potential as measured by calcium deposition [83]. There are several mechanisms proposed for this effect. One includes TWIST1 mediated inhibition of BMP signaling through recruitment of HDAC1 to SMAD4, which would effectively silence BMP signaling as well as TGF-β signaling [84]. Although this is the case in mesenchymal stem cells, TWIST has other implications in osteoblast cells. TWIST has been shown to promote RUNX2 expression in osteoblast cells [85]. Reduced expression of TWIST results in decreased RUNX2 target gene transcription, including bone sialoprotein, osteopontin, and osteocalcin, all of which are markers of mature osteoblasts. TWIST also has been implicated in the β-catenin signaling pathway, where it may induce chemoresistance [86]. TWIST was shown to regulate β-catenin target genes, suggesting that TWIST is upstream of β-catenin. (Alternatively, TWIST may act directly on these genes in a b-catenin-independent fashion.)

Twist also appears important in the biology of osteosarcoma. SaOS2 cells which overexpress TWIST have marked reduction of alkaline phosphatase, reduced proliferation, and lose their response to basic fibroblast growth factor, while cells which have decreased TWIST expression have an upregulation of alkaline phosphatase and type I collagen, induced osteopontin expression, and reduced proliferation [87]. Overexpression of TWIST resulted in increased apoptosis by cisplatin and knockdown of β-catenin furthered this effect in SaOS2 cells. Knockdown of TWIST in MG-63 cells resulted in decreases apoptosis mediated by cisplatin and knockdown of β-catenin completely abolished this effect. These results offer evidence to the idea that TWIST expression decreases osteosarcoma cell resistance to cisplatin, mediated by a decrease in soluble β-catenin through the PI3K pathway. Additionally, shTWIST1 in mesenchymal stem cells resulted in increased proliferation, but had no effect on cell survival in serum free media [88]. shTWIST1 resulted in increased alkaline phosphatase expression and increased mineral deposition, both indicative of osteoblast differentiation. In the same study, silencing TWIST1 resulted in FGFR2 upregulation. These effects were mediated by RUNX2. TWIST1 is downstream of parathyroid hormone in osteoblasts [89]. Expression of parathyroid hormone results in decreased TWIST1 expression. The effects of parathyroid hormone may be meditated in part by this decrease in TWIST1 since TWIST1 was shown to inhibit bone-specific ATF4 transcriptional activity. Physical interactions between the C-terminus of TWIST1 and N-terminus of ATF4 allow for suppression of osteocalcin mRNA transcription. Exogenous expression of TWIST1 in osteosarcoma cells lacking endogenous TWIST1 resulted in suppression of the differentiation inductive effects of parathyroid hormone. Studies of archival osteosarcoma tumor samples pointed toward a role for TWIST in osteosarcoma pathogenesis. Metastatic osteosarcoma cases showed higher rates of TWIST abnormalities and amplification [90]. Relapsed tumors also had statistically significant increases in TWIST abnormalities. Hypoxia has also been shown to induce TWIST in mesenchymal stem cells [91]. Overexpression of TWIST resulted in decreased RUNX2 and osteocalcin, while siTWIST resulted in increased RUNX2 and osteocalcin. Thus, hypoxia was shown to inhibit osteogenesis through HIF-1α and TWIST. Thus, there is an important role for TWIST both in normal osteoblast development and in osteosarcoma pathogenesis, and there may be therapeutic benefit to manipulating this pathway in osteosarcoma patients. Further study is needed.

Hippo/YAP Pathway in Osteoblast Differentiation

First discovered in Drosophila, the Hippo signaling pathway plays an important role in cell development and organ size control [92]. The discovery of the Hippo signaling pathway in mammals provides us a novel important entry point to the control of organ size and the mechanisms which regulate this process [93–95]. At least 35 proteins or complexes have so far been identified in the Hippo signaling pathway, which consist of a large network of proteins [96, 97].

The core part of the Hippo pathway includes mammalian STE20-like protein kinase1 (MST1) and MST2, large tumor suppressor 1(LATS1) and LATS2 together with the adaptor proteins Salvador (SAV1), Mob kinase activator 1A (MOB1A) and MOB1B [98, 99]. MST1/2, activated by caspase cleavage under apoptotic stress, can phosphorylate SAV1, LATS1/2, and MOB1-A/B [100, 101]. The interaction between MST1/2 and SAV1 regulates MST1/2 nuclear translocation, which is mediated by the SARAH (Sav/RASSF/Hpo) domains within both MST1/2 and SAV1. MST1/2 directly phosphorylates LATS1/2 at the hydrophobic motif and also is required for LATS1/2 expression [102]. Moreover, it is also reported that SAV1 provides a bridge to bring MST1/2 and LATS1/2 together [103]. After being phosphorylated by MST1/2, MOB can bind to the auto-inhibitory motif in LATS1/2 which leads to the phosphorylation of the Lats activation loop [103, 104].

These proteins, which constitute the core part of the Hippo pathway, inhibit cell growth by facilitating LATS1- and LATS2-dependent phosphorylation of the homologous oncoproteins Yes-associated protein (YAP) and TAZ (also known as WWTR1), the transcriptional co-activator with PDZ-binding motif [105]. This interaction is mediated by the interaction of PPxY motifs on LATS-1/2 and WW domains on the YAP/TAZ [106]. YAP can be phosphorylated at various serine residues by its two upstream kinases, MST1/2 and LATS1/2 [106]. After YAP is phosphorylated in the cytoplasm, it will be retained in the cytosol so that YAP cannot combine with transcriptional enhancer factor domain family members (TEADs) as a transcriptional complex to drive growth-promoting and anti-apoptotic gene expression [107]. Both YAP and TEAD transcription factors are enriched in multiple types of stem cells [108]. YAP phosphorylation is activated at high cell density, while YAP will translocate to nucleus to facilitate cell proliferation at low cell density [106, 109, 110]. Three potential steps are recognized in regulation of the YAP/TAZ complex: (1) phosphorylated YAP/TAZ interact with 14-3-3, which leads to cytoplasmic retention; (2) YAP/TAZ phosphorylation results in protein degradation; (3) translocate to nucleus to interact with DNA-binding transcription factors [111].

While YAP/TAZ does not contain intrinsic DNA-binding domains, it binds to the promoters of its target genes through interaction with TEAD family members (TEADs) [112]. Besides TEADs, YAP/TAZ also can interact with other transcription factors, such as SMAD1-3, RUNX1/2, p63/73, and ERBB4 [113–116]. These interactions can mediate expression of diverse genes involved in proliferation, differentiation, development and other biological processes. Since many of these transcription factors are known to affect osteoblast development (see elsewhere in this chapter), it is reasonable to assume that the Hippo signaling pathway can regulate osteoblast differentiation.

Another way in which HIPPO signaling can affect cell death and organ size is through interaction with RASSF (Ras-association domain family) protein family (which has 10 members, RASSF1–10), which induce cell-cycle arrest and apoptosis. RASSF 2 binds to and stabilizes STK3/MST2 via the SARAH (Sav/RASSF/Hpo) domain, and knockout of RASSF2 causes bone remodeling defects. Since the RASSFs are KRAS-specific effectors, this interaction may help explain the

differential effects of geranylgeranylated KRAS vs farnesylated KRAS in promoting growth arrest or apoptosis [117]. Moreover, in vitro cell differentiation experiments show that RASSF2 promotes osteoblast differentiation and inhibits osteoclast differentiation. This effect might be mediated by nuclear factor (NF)-κB hyperactivation, as transduction of RASSF2 into RASSF2$^{-/-}$ osteoclast or osteoblast precursors inhibited NF-κB and normalized osteoclast and osteoblast differentiation [118, 119].

The SoxC group, which includes Sox4, Sox11, and Sox12, also facilitates differentiation of osteoblasts, T or B lymphocytes, and pancreatic β-cells. In mice with one copy of Sox4 inactivated (heterozygotes), osteoblast cell development and bone formation is impaired [120]. Sox4 and Sox11 are up-regulated in several cancer types, and may serve as prognosis indicators in some cancers. SoxC genes are upstream of the TEAD family, facilitating their association with YAP and TAZ coactivators to mediate cell survival and proliferation downstream of the Hippo signaling pathway [121].

Protein kinase A and Rho GTPases facilitate LATS1/2 kinase activity and YAP phosphorylation by cyclic adenosine monophosphate (cAMP), which is a second messenger downstream from $G\alpha_s$-coupled receptors. YAP phosphorylation is strongly decreased when PKA expression is inhibited by shRNA, while overexpression of the catalytic subunit PKAα stimulates YAP phosphorylation. Further, YAP/TAZ dephosphorylation is required for the PKA inhibitor to suppress differentiation, whereas YAP/TAZ phosphorylation is crucial to inducing differentiation. Thus, YAP/TAZ activity is required for PKA signaling to regulate adipocyte differentiation. Rho GTPases mediate the effect of PKA on Hippo-YAP regulation, implying that the Rho family helps mediate the effect of PKA on LATS1/2. PKA also inhibits osteoblast differentiation, so it suggests that the effects of YAP on osteogenesis and adipogenesis are mediated by the Gαs-cAMP-PKA-Rho signaling pathway [122, 123].

Another protein that regulates YAP function is Angiomotin (AMOT). This protein inhibits the mobility of endothelial cells in angiogenesis and wound healing, and is required in embryogenesis to prevent differentiation of the inner cell mass (ICM). Depletion of AMOT and Amot-like 2 (AMOTl2) can promote ICM differentiation and compromise embryonic development. Two PPxY motifs and several other motifs mediate interaction between YAP and AMOTl [124]. Further, AMOT can regulate YAP localization, and, consequently, the expression of transcription factor Cdx2, a key regulator of vitamin D receptor (VDR) expression. This function is independent of classical Hippo pathway signaling, as AMOT function can compensate for the absence of LATS1/2. In the absence of the Hippo pathway, high levels of AMOT are sufficient to regulate YAP translocation to the nucleus [125].

So, YAP localization and its impact on the expression of transcriptional targets such as Cdx2 is regulated not only through Hippo signaling but also through a Hippo pathway-independent mechanism [126]. Both types of regulation should be considered in evaluating the impact of YAP expression in tumor cell biology.

Hippo/YAP Pathway in Osteosarcoma

Since the Hippo/YAP pathway plays a vital role in regulating organ size, it is not surprising to find that this pathway is frequently "hijacked" during tumorigenesis. In recent years, numerous studies identified Hippo pathway gene mutations involved in cancer development and metastasis [127–129].

Neurofibromin 2 (NF2, also known as Merlin) is a cytosolic protein typically found near the membrane in filipodia and ruffled membranes, which can help mediate activation of the Sav1/MST1/2 complex. As the apical upstream component of the Hippo pathway, NF2 often is involved in sarcoma pathogenesis. In a murine model of NF2 inactivation, heterozygous mice developed a variety of high grade, frequently metastatic cancers, including osteosarcoma or fibrosarcoma. Thus, NF2 might act as a tumor suppressor in osteosarcoma [130].

Hypermethylation of MST1/2, one of the core parts of the Hippo pathway, is frequently detected in soft tissue sarcoma and MST1 hypermethylation is a prognostic marker for soft tissue sarcoma patients. LATS1, another core protein in the Hippo pathway, is associated with organogenesis; LATS1$^{-/-}$ mice develop soft tissue sarcomas and ovarian stromal cell tumors, especially after treatment with carcinogens. Those data indicate that LATS1/2 functions as a tumor suppressor [131–133]. MOBKlA/B and the TEAD family, the other two core components of the Hippo pathway, have been associated with several types of cancer, but the role of these proteins in osteosarcoma need further investigation.

Other Hippo pathway modulators such as the RASSF family members also are associated with sarcoma, among other cancers. Both RASSF1A and RASSF2 genes frequently are methylated in Ewing sarcoma (either tumors or cell lines); the overexpression of these two genes can reduce Ewing sarcoma cell colony formation ability. RASSF2 methylation correlates with poor overall survival, especially in younger patients (under 18y) [134]. Moreover, RASSF10 promoter hypermethylation also is involved in osteosarcoma (and other several cancer types); its expression level is elevated by cell contact and regulated by protein kinase A (PKA) and activator Protein 1 (AP-1), linking the RASSF10 to the cAMP signaling pathway [135].

YAP activity also is affected by Hippo-independent pathways that are altered in sarcoma. BMI-1, which is one of the polycomb group family proteins, suppresses cell contact inhibition and promotes the tumorigenicity of Ewing sarcoma through CDKN2A repression. Moreover, BMI-1 stabilized YAP expression and nuclear localization [136]. In osteosarcoma, overexpression of BMI1 promotes cell cycle and confers cisplatin resistance [137]. In various cancers, nuclear localization of YAP correlates with poor prognosis [92]. Hippo signaling regulates YAP expression and translocation into nucleus vs. sequestration in the cytosol. YAP overexpression promotes resistance to chemotherapy-induced apoptosis and to anoikis in cancer cells [138]. YAP/TAZ hyperactivity also promoted tumorigenic ability by increasing stem-cell-like properties [61]. Several key junctional proteins such as α-cadherin and E-cadherin can complex with and regulate YAP/TAZ activity to control cancer cell proliferation and metastasis [139, 140].

The Hippo/YAP pathway also plays an important role in cancer metastasis. Cancer cells must resist anoikis and survive transit through the circulation before they can recolonize in a secondary site. Hippo pathway deregulation is a potential promoter of metastasis, as YAP overexpression can both inhibit anoikis and promote epithelial–mesenchymal transition (EMT), which is an important process in carcinoma prior to cancer cell dissociation from the primary tumor [141, 142]. YAP/TAZ activity was significantly elevated in high-grade metastatic breast cancer compared with low-grade non-metastatic ones; further, the expression of LATS1/2, which could inhibit YAP/TAZ activity, was lower in metastatic prostate cancers [143, 144].

Multiple cancer-associated signaling networks engage in regulatory crosstalk with the Hippo pathway, often at the level of YAP/TAZ. Several pathways including WNT, transforming growth factor-β (TGF-β) -bone morphogenetic protein (BMP), Notch, insulin, and mTOR all have been reported to interact functionally with the Hippo pathway [145]. Mutations of these pathways frequently are associated with lung, colon, and other cancers [146–148]. For example, β-catenin activated by WNT signaling pathway in colorectal cancer can activate YAP to promote its nuclear accumulation [149]. The current data for the involvement of many of these pathways in osteosarcoma pathogenesis is discussed elsewhere in this chapter.

Since YAP is an important protein downstream of the Hippo pathway and at the convergence of several pathways, chemical and biological agents that affect YAP localization or expression may provide novel methods for cancer treatment [150]. The efficiency of this therapeutic strategy functionally depends on the expression and activity of pathway components in different cancer types. As the core parts of the Hippo pathway, MST1/2, SAV1, and LATS1/2 will effect Hippo signaling directly, so they are potential targets to design Hippo-specific drugs [150]. Most Hippo pathway components are tumor suppressor genes, so the conventional approach of designing small-molecule kinase inhibitors might be unlikely to work for the Hippo pathway. By contrast, the YAP/TAZ-TEAD complex might be a more promising target. Interestingly, dobutamine, which is a β-adrenergic receptor agonist, compromises YAP-dependent transcription by inhibiting its nuclear translocation. There are three major sites within TEAD to bind with YAP/TAZ [151]. However, further investigation on the interaction between TAZ and TEAD are still needed to design new inhibitors.

ERBB4 Signaling in Osteoblast Differentiation

As an important co-activator of YAP, the receptor tyrosine-protein kinase ERBB4 also is highly associated with cell differentiation. ERBB4 regulates many key cellular responses in normal organogenesis and maintenance of mature tissues, such as control of cell division, migration, differentiation, and apoptosis [152, 153]. Characterizing the contribution of ERBB4 to osteoblast development is challenging, as the receptor has 4 juxtamembranous isoforms (JM-A, JM-B, JM-C, and JM-D), which

arise from alternative use of two in-frame exons, 15b and 16. While all four isoforms encode a functioning receptor tyrosine kinase, exon 16 encodes a recognition sequence for cleavage by ADAMS17 protease, which then renders the residual protein susceptible to γ-secretase cleavage. Thus, the JM-A and JM-D isoforms have the potential, after activation, of being cleaved to form a soluble intracellular fragment [115, 154] that can function as a transcription modifier [155]. There also is alternative use of exon 26, a 48-base pair region that encodes an AKT binding site, generating the CYT1 isoform when exon 26 is included, and CYT2 when it is not [156]. Several proteins, including YAP, WWOX, p63, and p73, are capable of binding with the ERBB4 intracellular domain (4ICD) as molecular chaperones to facilitate 4ICD translocation to the nucleus, where it can affect the transcription of target genes [157]. Not all studies exploring the impact of ERBB expression in osteoblast development or osteosarcoma biology have appreciated this complexity.

The cleavable JM-A isoform is capable of suppressing cell differentiation, while the two soluble intracellular isoforms of ERBB4 (CYT-1 and CYT-2) exert opposite effects on epithelial differentiation [158, 159]. The ligand of ERBB4, neuregulin (NRG) can induce mesenchymal and neuronal cell differentiation. Heparin binding-epidermal growth factor (HB-EGF), which binds to both EGFR and ERBB4, contributes to differentiation via ERBB4; downregulation of EGFR or ERBB4 during embryonic lung development prevented stretch-induced type II cell differentiation via ERK signaling [160]. The expression and activation of ERBB4 induces expression of the differentiation marker, GAP-43, to promote neuronal differentiation [161]. The role of the ERBB family in bone biology has been reviewed recently [162]. While the full story is too complex to explain here, ERBB signaling generally serves to promote proliferation of immature osteoblasts while blocking differentiation.

ERBB4 in Osteosarcoma

Abnormal expression and activity of ERBB pathway proteins can lead to tumorigenesis [115, 163], as it can activate and maintain several pivotal transcription factors to mediate cell proliferation, cell cycle, apoptosis, and metastasis [164]. Polymorphisms in the ERBB4 promoter region increase the risk for breast and colorectal cancers [165]. The protease-cleavable ERBB4 isoforms are also found to promote Estrogen Receptor (ER)-positive breast cancer growth and enhances ER-mediated gene transcription [166]. Nuclear expression of ERBB4 confers a worse prognosis in esophageal cancer [167].

We have found that nuclear localization of 4ICD, as well as the cell surface expression of ErbB-2 (Her-2) and EGFR, contributes to osteosarcoma pathogenesis [168]. Osteosarcoma cell lines demonstrate constitutive phosphorylation of ERBB4, as well as EGFR and ERBB2 [169]. Further, ERBB4, especially the 4ICD fragment, is a protective factor when the cancer cells are exposed to multiple exogenous apoptotic stimuli, including anoikis, nutrient deficiency, and cytotoxic chemotherapy [170]. These effects are likely to lead to osteosarcoma metastasis and recurrence.

E-cadherin dependent ERBB4 activation can mediate anoikis resistance and increase resistance to chemotherapeutic agents in Ewing sarcoma [171]. In one small series, expression of ERBB4 was associated with worse outcome for adult patients with osteosarcoma [172].

While it may be presumed that the interactions with YAP and other WW-containing signaling proteins provide the basis for the adverse impact of nuclear ERBB4 expression on multiple cancers, including osteosarcoma, the precise mechanisms remain to be defined. It also remains to be seen is pan-ERBB kinase inhibitors will serve to block all of the pro-survival effects of ERBB4 expression in these cells.

Conclusions

Osteoblast development is a complex and tightly regulated process, involving multiple signaling pathways that regulate cellular proliferation, lineage commitment, differentiation and eventual terminal differentiation and growth arrest. Among the key pathways and molecules are Runx2, Wnt/β-catenin, BMP, Osterix, Twist, HIPPO/YAP, and the ERBB family, especially ERBB4. For each of these pathways and molecules, there are examples of osteosarcomas in which the process has become dysregulated, or "hijacked" by the malignant process. Since osteosarcomas are genetically diverse and lack a common molecular alteration that drives their behavior, it is unlikely that any of these alterations would be found in all osteosarcomas. Rather, by understanding normal osteoblast development, we may find frequent alterations that explain the diversity of tumor behaviors and patient outcomes seen. Further, once it is clear which of these pathways are most essential for malignant transformation or the persistence of malignant cells, these pathways and molecules may provide a new set of therapeutic targets for a disease that desperately needs novel therapies.

References

1. Tang N, Song W-X, Luo J, Luo X, Chen J, Sharff K, Bi Y, He B-C, Huang J-Y, Zhu G-H, Su Y-X, Jiang W, Tang M, He Y, Wang Y, Chen L, Zuo G-W, Shen J, Pan X, Reid R, Luu H, Haydon R, He T-C (2009) BMP-9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical Wnt/beta-catenin signalling. J Cell Mol Med 13(8B):2448–2464. doi:10.1111/j.1582-4934.2008.00569.x
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147. doi:10.1126/science.284.5411.143
3. Harding WG, Courville CB (1934) Bone formation in metastases of osteogenic sarcoma: report of case with metastases to the brain. Am J Cancer 21(4):787–794. doi:10.1158/ajc.1934.787
4. Phillips C (1935) Osteogenic sarcoma: its pathologic characteristics. Radiology 24(6):728–734. doi:10.1148/24.6.728

5. Berman SD, Calo E, Landman AS, Danielian PS, Miller ES, West JC, Fonhoue BD, Caron A, Bronson R, Bouxsein ML, Mukherjee S, Lees JA (2008) Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. Proc Natl Acad Sci 105(33):11851–11856. doi:10.1073/pnas.0805462105
6. Komori T, Kishimoto T (1998) Cbfa1 in bone development. Curr Opin Genet Dev 8(4):494–499
7. Blyth K, Cameron E, Neil J (2005) The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer 5(5):376–387
8. Kundu M, Javed A, Jeon J, Horner A, Shum L, Eckhaus M, Muenke M, Lian J, Yang Y, Nuckolls G, Stein G, Liu P (2002) Cbfbeta interacts with Runx2 and has a critical role in bone development. Nat Genet 32(4):639–644
9. McLarren KW, Theriault FM, Stifani S (2001) Association with the nuclear matrix and interaction with Groucho and RUNX proteins regulate the transcription repression activity of the basic helix loop helix factor Hes1. J Biol Chem 276(2):1578–1584. doi:10.1074/jbc.M007629200
10. Lian J, Stein J, Stein G, van Wijnen A, Montecino M, Javed A, Gutierrez S, Shen J, Zaidi S, Drissi H (2003) Runx2/Cbfa1 functions: diverse regulation of gene transcription by chromatin remodeling and co-regulatory protein interactions. Connect Tissue Res 44(Suppl 1):141–148
11. Otto F, Lubbert M, Stock M (2003) Upstream and downstream targets of RUNX proteins. J Cell Biochem 89(1):9–18
12. Thiede M, Smock S, Petersen D, Grasser W, Thompson D, Nishimoto S (1994) Presence of messenger ribonucleic acid encoding osteocalcin, a marker of bone turnover, in bone marrow megakaryocytes and peripheral blood platelets. Endocrinology 135(3):929–937. doi:10.1210/en.135.3.929
13. Morrison N, Shine J, Fragonas J, Verkest V, McMenemy M, Eisman J (1989) 1,25-dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. Science 246(4934):1158–1161. doi:10.1126/science.2588000
14. Kamekura S, Kawasaki Y, Hoshi K, Shimoaka T, Chikuda H, Maruyama Z, Komori T, Sato S, Takeda S, Karsenty G, Nakamura K, Chung U, Kawaguchi H (2006) Contribution of runt-related transcription factor 2 to the pathogenesis of osteoarthritis in mice after induction of knee joint instability. Arthritis Rheum 54(8):2462–2470
15. Bergwitz C, Prochnau A, Mayr B, Kramer F, Rittierodt M, Berten H, Hausamen J, Brabant G (2001) Identification of novel CBFA1/RUNX2 mutations causing cleidocranial dysplasia. J Inherit Metab Dis 24(6):648–656
16. Galindo M, Pratap J, Young DW, Hovhannisyan H, Im H-J, Choi J-Y, Lian JB, Stein JL, Stein GS, van Wijnen AJ (2005) The bone-specific expression of Runx2 OSCILLATES DURING THE CELL CYCLE TO SUPPORT a G1-related antiproliferative function in osteoblasts. J Biol Chem 280(21):20274–20285. doi:10.1074/jbc.M413665200
17. Thomas DM, Johnson SA, Sims NA, Trivett MK, Slavin JL, Rubin BP, Waring P, McArthur GA, Walkley CR, Holloway AJ, Diyagama D, Grim JE, Clurman BE, Bowtell DDL, Lee J-S, Gutierrez GM, Piscopo DM, Carty SA, Hinds PW (2004) Terminal osteoblast differentiation, mediated by runx2 and p27KIP1, is disrupted in osteosarcoma. J Cell Biol 167(5):925–934. doi:10.1083/jcb.200409187
18. Lee J, Thomas D, Gutierrez G, Carty S, Yanagawa S, Hinds P (2006) HES1 cooperates with pRb to activate RUNX2-dependent transcription. J Bone Miner Res 21(6):921–933
19. Monroe D, Hawse J, Subramaniam M, Spelsberg T (2010) Retinoblastoma binding protein-1 (RBP1) is a Runx2 coactivator and promotes osteoblastic differentiation. BMC Musculoskelet Disord 11:104
20. Ozaki T, Wu D, Sugimoto H, Nagase H, Nakagawara A (2013) Runt-related transcription factor 2 (RUNX2) inhibits p53-dependent apoptosis through the collaboration with HDAC6 in response to DNA damage. Cell Death Dis 4:e610. doi:10.1038/cddis.2013.127
21. Park HR, Won Jung W, Bertoni F et al (2004) Molecular analysis of p53, MDM2 and H-ras genes in low-grade central osteosarcoma. Pathology Research and Practice 200(6):439–445

22. Martin J, Zielenska M, Stein G, van Wijnen A, Squire J (2011) The role of RUNX2 in osteosarcoma oncogenesis. Sarcoma 2011:282745
23. Watanabe T, Oyama T, Asada M, Harada D, Ito Y, Inagawa M, Suzuki Y, Sugano S, K-i K, Karsenty G, Komori T, Kitagawa M, Asahara H (2013) MAML1 enhances the transcriptional activity of Runx2 and plays a role in bone development. PLoS Genet 9(1):e1003132. doi:10.1371/journal.pgen.1003132
24. Wang CY, Yang SF, Wang Z, Tan JM, Xing SM, Chen DC, Xu SM, Yuan W (2013) PCAF acetylates Runx2 and promotes osteoblast differentiation. J Bone Miner Metab 31(4):381–389. doi:10.1007/s00774-013-0428-y
25. Gilbert L, He X, Farmer P, Boden S, Kozlowski M, Rubin J, Nanes MS (2000) Inhibition of osteoblast differentiation by tumor necrosis factor-{alpha}. Endocrinology 141(11):3956–3964. doi:10.1210/en.141.11.3956
26. Li Y, Li A, Strait K, Zhang H, Nanes M, Weitzmann M (2007) Endogenous TNFalpha lowers maximum peak bone mass and inhibits osteoblastic Smad activation through NF-kappaB. J Bone Miner Res 22(5):646–655
27. Olfa G, Christophe C, Philippe L, Romain S, Khaled H, Pierre H, Odile B, Jean-Christophe D (2010) RUNX2 regulates the effects of TNFalpha on proliferation and apoptosis in SaOs-2 cells. Bone 46(4):901–910
28. Li X, McGee-Lawrence M, Decker M, Westendorf J (2010) The Ewing's sarcoma fusion protein, EWS-FLI, binds Runx2 and blocks osteoblast differentiation. J Cell Biochem 111(4):933–943
29. Zhu J, Shimizu E, Zhang X, Partridge N, Qin L (2011) EGFR signaling suppresses osteoblast differentiation and inhibits expression of master osteoblastic transcription factors Runx2 and Osterix. J Cell Biochem 112(7):1749–1760. doi:10.1002/jcb.23094
30. van der Deen M, Akech J, Lapointe D, Gupta S, Young D, Montecino M, Galindo M, Lian J, Stein J, Stein G, van Wijnen A (2012) Genomic promoter occupancy of runt-related transcription factor RUNX2 in osteosarcoma cells identifies genes involved in cell adhesion and motility. J Biol Chem 287(7):4503–4517. doi:10.1074/jbc.M111.287771
31. Rao T, Kühl M (2010) An updated overview on Wnt signaling pathways: a prelude for more. Circ Res 106(12):1798–1806. doi:10.1161/CIRCRESAHA.110.219840
32. Rodda S, McMahon A (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 133(16):3231–3244. doi:10.1242/dev.02480
33. Maeda K, Takahashi N, Kobayashi Y (2013) Roles of Wnt signals in bone resorption during physiological and pathological states. J Mol Med (Berlin, Germany) 91(1):15–23. doi:10.1007/s00109-012-0974-0
34. Wan Y, Lu C, Cao J, Zhou R, Yao Y, Yu J, Zhang L, Zhao H, Li H, Zhao J, Zhu X, He L, Liu Y, Yao Z, Yang X, Guo X (2013) Osteoblastic Wnts differentially regulate bone remodeling and the maintenance of bone marrow mesenchymal stem cells. Bone 55(1):258–267. doi:10.1016/j.bone.2012.12.052
35. Sonomoto K, Yamaoka K, Oshita K, Fukuyo S, Zhang X, Nakano K, Okada Y, Tanaka Y (2012) Interleukin-1β induces differentiation of human mesenchymal stem cells into osteoblasts via the Wnt-5a/receptor tyrosine kinase-like orphan receptor 2 pathway. Arthritis Rheum 64(10):3355–3363. doi:10.1002/art.34555
36. Włodarski K, Galus R, Brodzikowska A, Włodarski P (2013) Sclerostin, an osteocytes-derived bone-forming inhibitor. Pol Orthop Traumatol 78:151–154
37. Yu L, van der Valk M, Cao J, Han C-YE, Juan T, Bass M, Deshpande C, Damore M, Stanton R, Babij P (2011) Sclerostin expression is induced by BMPs in human Saos-2 osteosarcoma cells but not via direct effects on the sclerostin gene promoter or ECR5 element. Bone 49(6):1131–1140. doi:10.1016/j.bone.2011.08.016
38. Hart M, Concordet J, Lassot I, Albert I, del los Santos R, Durand H, Perret C, Rubinfeld B, Margottin F, Benarous R, Polakis P (1999) The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr Biol 9(4):207–210. doi:10.1016/S0960-9822(99)80091-8

39. Lin C, Guo Y, Ghaffar S, McQueen P, Pourmorady J, Christ A, Rooney K, Ji T, Eskander R, Zi X, Hoang B (2013) Dkk-3, a secreted wnt antagonist, suppresses tumorigenic potential and pulmonary metastasis in osteosarcoma. Sarcoma 2013:147541. doi:10.1155/2013/147541
40. Loeser R (2013) Osteoarthritis year in review 2013: biology. Osteoarthritis Cartilage. doi:10.1016/j.joca.2013.05.020
41. Ruan J, Trotter T, Nan L, Luo R, Javed A, Sanderson R, Suva L, Yang Y (2013) Heparanase inhibits osteoblastogenesis and shifts bone marrow progenitor cell fate in myeloma bone disease. Bone. doi:10.1016/j.bone.2013.07.024
42. Steensma M, Tyler W, Shaber A, Goldring S, Ross F, Williams B, Healey J, Purdue P (2013) Targeting the giant cell tumor stromal cell: functional characterization and a novel therapeutic strategy. PLoS One 8(7):e69101. doi:10.1371/journal.pone.0069101
43. Ornitz D, Marie P (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev 16(12):1446–1465. doi:10.1101/gad.990702
44. Hanneken A (2001) Structural characterization of the circulating soluble FGF receptors reveals multiple isoforms generated by secretion and ectodomain shedding. FEBS Lett 489(2–3):176–181. doi:10.1016/S0014-5793(00)02409-1
45. Jang J-H (2002) Identification and characterization of soluble isoform of fibroblast growth factor receptor 3 in human SaOS-2 osteosarcoma cells. Biochem Biophys Res Commun 292(2):378–382. doi:10.1006/bbrc.2002.6668
46. Ezzat S, Zheng L, Yu S, Asa S (2001) A soluble dominant negative fibroblast growth factor receptor 4 isoform in human MCF-7 breast cancer cells. Biochem Biophys Res Commun 287(1):60–65. doi:10.1006/bbrc.2001.5546
47. Celli G, LaRochelle W, Mackem S, Sharp R, Merlino G (1998) Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. EMBO J 17(6):1642–1655. doi:10.1093/emboj/17.6.1642
48. Peters K, Werner S, Liao X, Wert S, Whitsett J, Williams L (1994) Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. EMBO J 13(14):3296–3301
49. Valta M, Hentunen T, Qu Q, Valve E, Harjula A, Seppänen J, Väänänen H, Härkönen P (2006) Regulation of osteoblast differentiation: a novel function for fibroblast growth factor 8. Endocrinology 147(5):2171–2182. doi:10.1210/en.2005-1502
50. Biver E, Soubrier A-S, Thouverey C, Cortet B, Broux O, Caverzasio J, Hardouin P (2012) Fibroblast growth factor 2 inhibits up-regulation of bone morphogenic proteins and their receptors during osteoblastic differentiation of human mesenchymal stem cells. Biochem Biophys Res Commun 427(4):737–742. doi:10.1016/j.bbrc.2012.09.129
51. Choi K-Y, Kim H-J, Lee M-H, Kwon T-G, Nah H-D, Furuichi T, Komori T, Nam S-H, Kim Y-J, Kim H-J, Ryoo H-M (2005) Runx2 regulates FGF2-induced Bmp2 expression during cranial bone development. Dev Dyn 233(1):115–121. doi:10.1002/dvdy.20323
52. Bodo M, Lilli C, Bellucci C, Carinci P, Calvitti M, Pezzetti F, Stabellini G, Bellocchio S, Balducci C, Carinci F, Baroni T (2002) Basic fibroblast growth factor autocrine loop controls human osteosarcoma phenotyping and differentiation. Mol Med 8(7):393–404
53. Birkedal-Hansen H, Moore W, Bodden M, Windsor L, Birkedal-Hansen B, DeCarlo A, Engler J (1993) Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 4(2):197–250. doi:10.1177/10454411930040020401
54. Bjørnland K, Flatmark K, Pettersen S, Aaasen A, Fodstad O, Maelandsmo G (2005) Matrix metalloproteinases participate in osteosarcoma invasion. J Surg Res 127(2):151–156. doi:10.1016/j.jss.2004.12.016
55. Liao C-L, Lai K-C, Huang A-C, Yang J-S, Lin J-J, Wu S-H, Gibson Wood W, Lin J-G, Chung J-G (2012) Gallic acid inhibits migration and invasion in human osteosarcoma U-2 OS cells through suppressing the matrix metalloproteinase-2/-9, protein kinase B (PKB) and PKC signaling pathways. Food Chem Toxicol 50(5):1734–1740. doi:10.1016/j.fct.2012.02.033
56. Lu K-H, Yang H-W, Su C-W, Lue K-H, Yang S-F, Hsieh Y-S (2013) Phyllanthus urinaria suppresses human osteosarcoma cell invasion and migration by transcriptionally inhibiting u-PA via ERK and Akt signaling pathways. Food Chem Toxicol 52:193–199. doi:10.1016/j.fct.2012.11.019

57. Himelstein B, Asada N, Carlton M, Collins M (1998) Matrix metalloproteinase-9 (MMP-9) expression in childhood osseous osteosarcoma. Med Pediatr Oncol 31(6):471–474. doi:10.1002/(SICI)1096-911X(199812)31:6<471::AID-MPO2>3.0.CO;2-M
58. Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H, Seiki M (1995) Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. Lab Invest 72(3):311–322
59. Kim S-M, Lee H, Park Y-S, Lee Y, Seo S (2012) ERK5 regulates invasiveness of osteosarcoma by inducing MMP-9. J Orthop Res 30(7):1040–1044. doi:10.1002/jor.22025
60. Chan K, Wong H, Jin G, Liu B, Cao R, Cao Y, Lehti K, Tryggvason K, Zhou Z (2012) MT1-MMP inactivates ADAM9 to regulate FGFR2 signaling and calvarial osteogenesis. Dev Cell 22(6):1176–1190. doi:10.1016/j.devcel.2012.04.014
61. Tang Y, Rowe R, Botvinick E, Kurup A, Putnam A, Seiki M, Weaver V, Keller E, Goldstein S, Dai J, Begun D, Saunders T, Weiss S (2013) MT1-MMP-dependent control of skeletal stem cell commitment via a β1-integrin/YAP/TAZ signaling axis. Dev Cell 25(4):402–416. doi:10.1016/j.devcel.2013.04.011
62. Husmann K, Arlt M, Muff R, Langsam B, Bertz J, Born W, Fuchs B (2013) Matrix Metalloproteinase 1 promotes tumor formation and lung metastasis in an intratibial injection osteosarcoma mouse model. Biochim Biophys Acta 1832(2):347–354. doi:10.1016/j.bbadis.2012.11.006
63. Zhang W, Shen X, Wan C, Zhao Q, Zhang L, Zhou Q, Deng L (2012) Effects of insulin and insulin-like growth factor 1 on osteoblast proliferation and differentiation: differential signalling via Akt and ERK. Cell Biochem Funct 30(4):297–302. doi:10.1002/cbf.2801
64. Taniguchi C, Emanuelli B, Kahn C (2006) Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 7(2):85–96. doi:10.1038/nrm1837
65. Wang Y-H, Han X-D, Qiu Y, Xiong J, Yu Y, Wang B, Zhu Z-Z, Qian B-P, Chen Y-X, Wang S-F, Shi H-F, Sun X (2012) Increased expression of insulin-like growth factor-1 receptor is correlated with tumor metastasis and prognosis in patients with osteosarcoma. J Surg Oncol 105(3):235–243. doi:10.1002/jso.22077
66. Luk F, Yu Y, Walsh W, Yang J-L (2011) IGF1R-targeted therapy and its enhancement of doxorubicin chemosensitivity in human osteosarcoma cell lines. Cancer Invest 29(8):521–532. doi:10.3109/07357907.2011.606252
67. Kurmasheva R, Dudkin L, Billups C, Debelenko L, Morton C, Houghton P (2009) The insulin-like growth factor-1 receptor-targeting antibody, CP-751,871, suppresses tumor-derived VEGF and synergizes with rapamycin in models of childhood sarcoma. Cancer Res 69(19):7662–7671. doi:10.1158/0008-5472.CAN-09-1693
68. Miyazono K, Maeda S, Imamura T (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16(3):251–263. doi:10.1016/j.cytogfr.2005.01.009
69. Noël D, Gazit D, Bouquet C, Apparailly F, Bony C, Plence P, Millet V, Turgeman G, Perricaudet M, Sany J, Jorgensen C (2004) Short-term BMP-2 expression is sufficient for in vivo osteochondral differentiation of mesenchymal stem cells. Stem Cells 22(1):74–85. doi:10.1634/stemcells.22-1-74
70. Gu K, Zhang L, Jin T, Rutherford R (2004) Identification of potential modifiers of Runx2/Cbfa1 activity in C2C12 cells in response to bone morphogenetic protein-7. Cells Tissues Organs 176(1–3):28–40. doi:10.1159/000075025
71. Fakhry A, Ratisoontorn C, Vedhachalam C, Salhab I, Koyama E, Leboy P, Pacifici M, Kirschner R, Nah H-D (2005) Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. Bone 36(2):254–266. doi:10.1016/j.bone.2004.10.003
72. Hughes-Fulford M, Li C-F (2011) The role of FGF-2 and BMP-2 in regulation of gene induction, cell proliferation and mineralization. J Orthop Surg Res 6:8. doi:10.1186/1749-799X-6-8
73. Zhang R, Oyajobi B, Harris S, Chen D, Tsao C, Deng H-W, Zhao M (2013) Wnt/β-catenin signaling activates bone morphogenetic protein 2 expression in osteoblasts. Bone 52(1):145–156. doi:10.1016/j.bone.2012.09.029

74. Lind M, Eriksen E, Bünger C (1996) Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. Bone 18(1):53–57
75. Nakashima K, de Crombrugghe B (2003) Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet 19(8):458–466. doi:10.1016/S0168-9525(03)00176-8
76. Cao Y, Zhou Z, de Crombrugghe B, Nakashima K, Guan H, Duan X, Jia S-F, Kleinerman E (2005) Osterix, a transcription factor for osteoblast differentiation, mediates antitumor activity in murine osteosarcoma. Cancer Res 65(4):1124–1128. doi:10.1158/0008-5472.CAN-04-2128
77. Salinas-Souza C, De Oliveira R, Alves M, Garcia Filho R, Petrilli A, Toledo S (2013) The metastatic behavior of osteosarcoma by gene expression and cytogenetic analyses. Hum Pathol. doi:10.1016/j.humpath.2013.04.013
78. Horvai A, Roy R, Borys D, O'Donnell R (2012) Regulators of skeletal development: a cluster analysis of 206 bone tumors reveals diagnostically useful markers. Mod Pathol 25(11):1452–1461. doi:10.1038/modpathol.2012.110
79. Bourgeois P, Stoetzel C, Bolcato-Bellemin A, Mattei M, Perrin-Schmitt F (1996) The human H-twist gene is located at 7p21 and encodes a B-HLH protein that is 96 % similar to its murine M-twist counterpart. Mamm Genome 7(12):915–917. doi:10.1007/s003359900269
80. Martin T, Goyal A, Watkins G, Jiang W (2005) Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer. Ann Surg Oncol 12(6):488–496. doi:10.1245/ASO.2005.04.010
81. Puisieux A, Valsesia-Wittmann S, Ansieau S (2006) A twist for survival and cancer progression. Br J Cancer 94(1):13–17. doi:10.1038/sj.bjc.6602876
82. Wu K-J, Yang M-H (2011) Epithelial-mesenchymal transition and cancer stemness: the Twist1-Bmi1 connection. Biosci Rep 31(6):449–455. doi:10.1042/BSR20100114
83. Isenmann S, Arthur A, Zannettino A, Turner J, Shi S, Glackin C, Gronthos S (2009) TWIST family of basic helix-loop-helix transcription factors mediate human mesenchymal stem cell growth and commitment. Stem Cells (Dayton, Ohio) 27(10):2457–2468. doi:10.1002/stem.181
84. Hayashi M, Nimura K, Kashiwagi K, Harada T, Takaoka K, Kato H, Tamai K, Kaneda Y (2007) Comparative roles of Twist-1 and Id1 in transcriptional regulation by BMP signaling. J Cell Sci 120(Pt 8):1350–1357. doi:10.1242/jcs.000067
85. Yousfi M, Lasmoles F, Marie P (2002) TWIST inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalcin promoter in osteoblasts. Biochem Biophys Res Commun 297(3):641–644. doi:10.1016/S0006-291X(02)02260-X
86. Wu J, Liao Q, He H, Zhong D, Yin K (2012) TWIST interacts with β-catenin signaling on osteosarcoma cell survival against cisplatin. Mol Carcinog. doi:10.1002/mc.21991
87. Lee M, Lowe G, Strong D, Wergedal J, Glackin C (1999) TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. J Cell Biochem 75(4):566–577. doi:10.1002/(SICI)1097-4644(19991215)75:4<566::AID-JCB3>3.0.CO;2-0
88. Miraoui H, Severe N, Vaudin P, Pagès J-C, Marie P (2010) Molecular silencing of Twist1 enhances osteogenic differentiation of murine mesenchymal stem cells: implication of FGFR2 signaling. J Cell Biochem 110(5):1147–1154. doi:10.1002/jcb.22628
89. Danciu T, Li Y, Koh A, Xiao G, McCauley L, Franceschi R (2012) The basic helix loop helix transcription factor Twist1 is a novel regulator of ATF4 in osteoblasts. J Cell Biochem 113(1):70–79. doi:10.1002/jcb.23329
90. Entz-Werle N, Lavaux T, Metzger N, Stoetzel C, Lasthaus C, Marec P, Kalifa C, Brugieres L, Pacquement H, Schmitt C, Tabone M-D, Gentet J-C, Lutz P, Babin A, Oudet P, Gaub M, Perrin-Schmitt F (2007) Involvement of MET/TWIST/APC combination or the potential role of ossification factors in pediatric high-grade osteosarcoma oncogenesis. Neoplasia (New York, NY) 9(8):678–688

91. Yang D-C, Yang M-H, Tsai C-C, Huang T-F, Chen Y-H, Hung S-C (2011) Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. PLoS One 6(9):e23965. doi:10.1371/journal.pone.0023965
92. Pan D (2010) The hippo signaling pathway in development and cancer. Dev Cell 19(4):491–505. doi:10.1016/j.devcel.2010.09.011
93. Stanger BZ (2008) Organ size determination and the limits of regulation. Cell Cycle 7(3):318–324
94. Halder G, Johnson RL (2011) Hippo signaling: growth control and beyond. Development 138(1):9–22. doi:10.1242/dev.045500
95. Lian I, Kim J, Okazawa H, Zhao J, Zhao B, Yu J, Chinnaiyan A, Israel MA, Goldstein LS, Abujarour R, Ding S, Guan KL (2010) The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. Genes Dev 24(11):1106–1118. doi:10.1101/gad.1903310
96. Grusche FA, Degoutin JL, Richardson HE, Harvey KF (2011) The Salvador/Warts/Hippo pathway controls regenerative tissue growth in *Drosophila melanogaster*. Dev Biol 350(2):255–266. doi:10.1016/j.ydbio.2010.11.020
97. Cao X, Pfaff SL, Gage FH (2008) YAP regulates neural progenitor cell number via the TEA domain transcription factor. Genes Dev 22(23):3320–3334. doi:10.1101/gad.1726608
98. Wu S, Huang J, Dong J, Pan D (2003) hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell 114(4):445–456
99. Udan RS, Kango-Singh M, Nolo R, Tao C, Halder G (2003) Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat Cell Biol 5(10):914–920. doi:10.1038/ncb1050
100. Harvey KF, Pfleger CM, Hariharan IK (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell 114(4):457–467
101. Pantalacci S, Tapon N, Leopold P (2003) The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat Cell Biol 5(10):921–927. doi:10.1038/ncb1051
102. Tapon N, Harvey KF, Bell DW, Wahrer DC, Schiripo TA, Haber D, Hariharan IK (2002) salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell 110(4):467–478
103. Praskova M, Xia F, Avruch J (2008) MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Curr Biol 18(5):311–321. doi:10.1016/j.cub.2008.02.006
104. Chan EH, Nousiainen M, Chalamalasetty RB, Schafer A, Nigg EA, Sillje HH (2005) The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. Oncogene 24(12):2076–2086. doi:10.1038/sj.onc.1208445
105. Huang J, Wu S, Barrera J, Matthews K, Pan D (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell 122(3):421–434. doi:10.1016/j.cell.2005.06.007
106. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai ZC, Guan KL (2007) Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev 21(21):2747–2761. doi:10.1101/gad.1602907
107. Zhao B, Tumaneng K, Guan KL (2011) The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. Nat Cell Biol 13(8):877–883. doi:10.1038/ncb2303
108. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298(5593):597–600. doi:10.1126/science.1072530
109. Lallemand D, Curto M, Saotome I, Giovannini M, McClatchey AI (2003) NF2 deficiency promotes tumorigenesis and metastasis by destabilizing adherens junctions. Genes Dev 17(9):1090–1100. doi:10.1101/gad.1054603
110. Okada T, Lopez-Lago M, Giancotti FG (2005) Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. J Cell Biol 171(2):361–371. doi:10.1083/jcb.200503165

111. Yu FX, Guan KL (2013) The Hippo pathway: regulators and regulations. Genes Dev 27(4):355–371. doi:10.1101/gad.210773.112
112. Zhang L, Ren F, Zhang Q, Chen Y, Wang B, Jiang J (2008) The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev Cell 14(3):377–387. doi:10.1016/j.devcel.2008.01.006
113. Ferrigno O, Lallemand F, Verrecchia F, L'Hoste S, Camonis J, Atfi A, Mauviel A (2002) Yes-associated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-beta/Smad signaling. Oncogene 21(32):4879–4884. doi:10.1038/sj.onc.1205623
114. Ge L, Smail M, Meng W, Shyr Y, Ye F, Fan KH, Li X, Zhou HM, Bhowmick NA (2011) Yes-associated protein expression in head and neck squamous cell carcinoma nodal metastasis. PLoS One 6(11):e27529. doi:10.1371/journal.pone.0027529
115. Komuro A, Nagai M, Navin NE, Sudol M (2003) WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. J Biol Chem 278(35):33334–33341
116. Stein GS, Lian JB, Stein JL, van Wijnen AJ, Choi JY, Pratap J, Zaidi SK (2003) Temporal and spatial parameters of skeletal gene expression: targeting RUNX factors and their coregulatory proteins to subnuclear domains. Connect Tissue Res 44(Suppl 1):149–153
117. Geryk-Hall M, Yang Y, Hughes DPM (2010) Driven to death: inhibition of farnesylation increases Ras activity in osteosarcoma and promotes growth arrest and cell death. Mol Cancer Ther 9(5):1111–1119. doi:10.1158/1535-7163.mct-09-0833
118. Zhao B, Lei QY, Guan KL (2008) The Hippo-YAP pathway: new connections between regulation of organ size and cancer. Curr Opin Cell Biol 20(6):638–646. doi:10.1016/j.ceb.2008.10.001
119. Song H, Kim H, Lee K, Lee DH, Kim TS, Song JY, Lee D, Choi D, Ko CY, Kim HS, Shin HI, Choi J, Park H, Park C, Jeong D, Lim DS (2012) Ablation of Rassf2 induces bone defects and subsequent haematopoietic anomalies in mice. EMBO J 31(5):1147–1159. doi:10.1038/emboj.2011.480
120. Nissen-Meyer LS, Jemtland R, Gautvik VT, Pedersen ME, Paro R, Fortunati D, Pierroz DD, Stadelmann VA, Reppe S, Reinholt FP, Del Fattore A, Rucci N, Teti A, Ferrari S, Gautvik KM (2007) Osteopenia, decreased bone formation and impaired osteoblast development in Sox4 heterozygous mice. J Cell Sci 120(Pt 16):2785–2795. doi:10.1242/jcs.003855
121. Bhattaram P, Penzo-Mendez A, Sock E, Colmenares C, Kaneko KJ, Vassilev A, Depamphilis ML, Wegner M, Lefebvre V (2010) Organogenesis relies on SoxC transcription factors for the survival of neural and mesenchymal progenitors. Nat Commun 1:9. doi:10.1038/ncomms1008
122. Yu FX, Zhang Y, Park HW, Jewell JL, Chen Q, Deng Y, Pan D, Taylor SS, Lai ZC, Guan KL (2013) Protein kinase A activates the Hippo pathway to modulate cell proliferation and differentiation. Genes Dev 27(11):1223–1232. doi:10.1101/gad.219402.113
123. Kim M, Kim M, Lee S, Kuninaka S, Saya H, Lee H, Lee S, Lim DS (2013) cAMP/PKA signalling reinforces the LATS-YAP pathway to fully suppress YAP in response to actin cytoskeletal changes. EMBO J 32(11):1543–1555. doi:10.1038/emboj.2013.102
124. Wang W, Huang J, Chen J (2011) Angiomotin-like proteins associate with and negatively regulate YAP1. J Biol Chem 286(6):4364–4370. doi:10.1074/jbc.C110.205401
125. Nishioka N, Inoue K, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N, Makita R, Kurihara H, Morin-Kensicki EM, Nojima H, Rossant J, Nakao K, Niwa H, Sasaki H (2009) The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell 16(3):398–410. doi:10.1016/j.devcel.2009.02.003
126. Leung CY, Zernicka-Goetz M (2013) Angiomotin prevents pluripotent lineage differentiation in mouse embryos via Hippo pathway-dependent and -independent mechanisms. Nat Commun 4:2251. doi:10.1038/ncomms3251
127. Tschop K, Conery AR, Litovchick L, Decaprio JA, Settleman J, Harlow E, Dyson N (2011) A kinase shRNA screen links LATS2 and the pRB tumor suppressor. Genes Dev 25(8):814–830. doi:10.1101/gad.2000211

128. Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M (2006) A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. Genes Dev 20(19):2687–2700. doi:10.1101/gad.1447006
129. Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS, Haber DA (2006) Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc Natl Acad Sci U S A 103(33):12405–12410. doi:10.1073/pnas.0605579103
130. McClatchey AI, Saotome I, Mercer K, Crowley D, Gusella JF, Bronson RT, Jacks T (1998) Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors. Genes Dev 12(8):1121–1133
131. St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, Parlow AF, McGrath J, Xu T (1999) Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. Nat Genet 21(2):182–186. doi:10.1038/5965
132. Bothos J, Tuttle RL, Ottey M, Luca FC, Halazonetis TD (2005) Human LATS1 is a mitotic exit network kinase. Cancer Res 65(15):6568–6575. doi:10.1158/0008-5472.CAN-05-0862
133. Genevet A, Wehr MC, Brain R, Thompson BJ, Tapon N (2010) Kibra is a regulator of the Salvador/Warts/Hippo signaling network. Dev Cell 18(2):300–308. doi:10.1016/j.devcel.2009.12.011
134. Gharanei S, Brini AT, Vaiyapuri S, Alholle A, Dallol A, Arrigoni E, Kishida T, Hiruma T, Avigad S, Grimer R, Maher ER, Latif F (2013) RASSF2 methylation is a strong prognostic marker in younger age patients with Ewing sarcoma. Epigenetics 8(9)
135. Richter AM, Walesch SK, Wurl P, Taubert H, Dammann RH (2012) The tumor suppressor RASSF10 is upregulated upon contact inhibition and frequently epigenetically silenced in cancer. Oncogenesis 1:e18. doi:10.1038/oncsis.2012.18
136. Hsu JH, Lawlor ER (2011) BMI-1 suppresses contact inhibition and stabilizes YAP in Ewing sarcoma. Oncogene 30(17):2077–2085. doi:10.1038/onc.2010.571
137. Wu Z, Min L, Chen D, Hao D, Duan Y, Qiu G, Wang Y (2011) Overexpression of BMI-1 promotes cell growth and resistance to cisplatin treatment in osteosarcoma. PLoS One 6(2):e14648
138. Zhao B, Li L, Wang L, Wang CY, Yu J, Guan KL (2012) Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes Dev 26(1):54–68. doi:10.1101/gad.173435.111
139. Keely PJ (2013) Proteolytic remodeling of the ECM and the geometric control of stem cell fate. Dev Cell 25(4):325–326. doi:10.1016/j.devcel.2013.05.012
140. Kim JE, Finlay GJ, Baguley BC (2013) The role of the hippo pathway in melanocytes and melanoma. Front Oncol 3:123. doi:10.3389/fonc.2013.00123
141. De Craene B, Berx G (2013) Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 13(2):97–110. doi:10.1038/nrc3447
142. Wang Y, Shang Y (2013) Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. Exp Cell Res 319(2):160–169. doi:10.1016/j.yexcr.2012.07.019
143. Yang N, Morrison CD, Liu P, Miecznikowski J, Bshara W, Han S, Zhu Q, Omilian AR, Li X, Zhang J (2012) TAZ induces growth factor-independent proliferation through activation of EGFR ligand amphiregulin. Cell Cycle 11(15):2922–2930. doi:10.4161/cc.21386
144. Hergovich A (2012) YAP-Hippo signalling downstream of leukemia inhibitory factor receptor: implications for breast cancer. Breast Cancer Res 14(6):326. doi:10.1186/bcr3349
145. Irvine KD (2012) Integration of intercellular signaling through the Hippo pathway. Semin Cell Dev Biol 23(7):812–817. doi:10.1016/j.semcdb.2012.04.006
146. Polakis P (2012) Wnt signaling in cancer. Cold Spring Harb Perspect Biol 4(5). doi:10.1101/cshperspect.a008052
147. Louvi A, Artavanis-Tsakonas S (2012) Notch and disease: a growing field. Semin Cell Dev Biol 23(4):473–480. doi:10.1016/j.semcdb.2012.02.005
148. Tumaneng K, Schlegelmilch K, Russell RC, Yimlamai D, Basnet H, Mahadevan N, Fitamant J, Bardeesy N, Camargo FD, Guan KL (2012) YAP mediates crosstalk between

the Hippo and PI(3)K-TOR pathways by suppressing PTEN via miR-29. Nat Cell Biol 14(12):1322–1329. doi:10.1038/ncb2615
149. Konsavage WM Jr, Kyler SL, Rennoll SA, Jin G, Yochum GS (2012) Wnt/beta-catenin signaling regulates Yes-associated protein (YAP) gene expression in colorectal carcinoma cells. J Biol Chem 287(15):11730–11739. doi:10.1074/jbc.M111.327767
150. Fujii M (2012) Exploration of a new drug that targets YAP. J Biochem 152(3):209–211. doi:10.1093/jb/mvs072
151. Bao Y, Nakagawa K, Yang Z, Ikeda M, Withanage K, Ishigami-Yuasa M, Okuno Y, Hata S, Nishina H, Hata Y (2011) A cell-based assay to screen stimulators of the Hippo pathway reveals the inhibitory effect of dobutamine on the YAP-dependent gene transcription. J Biochem 150(2):199–208. doi:10.1093/jb/mvr063
152. Casalini P, Iorio MV, Galmozzi E, Menard S (2004) Role of HER receptors family in development and differentiation. J Cell Physiol 200(3):343–350. doi:10.1002/jcp.20007
153. Muraoka-Cook RS, Sandahl M, Husted C, Hunter D, Miraglia L, Feng SM, Elenius K, Earp HS 3rd (2006) The intracellular domain of ErbB4 induces differentiation of mammary epithelial cells. Mol Biol Cell 17(9):4118–4129. doi:10.1091/mbc.E06-02-0101
154. Aqeilan RI, Donati V, Palamarchuk A, Trapasso F, Kaou M, Pekarsky Y, Sudol M, Croce CM (2005) WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res 65(15):6764–6772. doi:10.1158/0008-5472.CAN-05-1150
155. Junttila T, Sundvall M, Maatta J, Elenius K (2000) Erbb4 and its isoforms: selective regulation of growth factor responses by naturally occurring receptor variants. Trends Cardiovasc Med 10(7):304–310
156. Veikkolainen V, Vaparanta K, Halkilahti K, Iljin K, Sundvall M, Elenius K (2011) Function of ERBB4 is determined by alternative splicing. Cell Cycle 10(16):2647–2657
157. Citri A, Yarden Y (2006) EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol 7(7):505–516. doi:10.1038/nrm1962
158. Sardi SP, Murtie J, Koirala S, Patten BA, Corfas G (2006) Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. Cell 127(1):185–197. doi:10.1016/j.cell.2006.07.037
159. Muraoka-Cook RS, Sandahl MA, Strunk KE, Miraglia LC, Husted C, Hunter DM, Elenius K, Chodosh LA, Earp HS 3rd (2009) ErbB4 splice variants Cyt1 and Cyt2 differ by 16 amino acids and exert opposing effects on the mammary epithelium in vivo. Mol Cell Biol 29(18):4935–4948. doi:10.1128/MCB.01705-08
160. Huang Z, Wang Y, Nayak PS, Dammann CE, Sanchez-Esteban J (2012) Stretch-induced fetal type II cell differentiation is mediated via ErbB1-ErbB4 interactions. J Biol Chem 287(22):18091–18102. doi:10.1074/jbc.M111.313163
161. Vaskovsky A, Lupowitz Z, Erlich S, Pinkas-Kramarski R (2000) ErbB-4 activation promotes neurite outgrowth in PC12 cells. J Neurochem 74(3):979–987
162. Schneider MR, Sibilia M, Erben RG (2009) The EGFR network in bone biology and pathology. Trends Endocrinol Metabol 20(10):517–524, http://dx.doi.org/10.1016/j.tem.2009.06.008
163. Hong W, Guan KL (2012) The YAP and TAZ transcription co-activators: key downstream effectors of the mammalian Hippo pathway. Semin Cell Dev Biol 23(7):785–793. doi:10.1016/j.semcdb.2012.05.004
164. Hollmen M, Elenius K (2010) Potential of ErbB4 antibodies for cancer therapy. Future Oncol 6(1):37–53. doi:10.2217/fon.09.144
165. Rokavec M, Justenhoven C, Schroth W, Istrate MA, Haas S, Fischer HP, Vollmert C, Illig T, Hamann U, Ko YD, Glavac D, Brauch H (2007) A novel polymorphism in the promoter region of ERBB4 is associated with breast and colorectal cancer risk. Clin Cancer Res 13(24):7506–7514. doi:10.1158/1078-0432.CCR-07-0457

166. Junttila TT, Sundvall M, Lundin M, Lundin J, Tanner M, Harkonen P, Joensuu H, Isola J, Elenius K (2005) Cleavable ErbB4 isoform in estrogen receptor-regulated growth of breast cancer cells. Cancer Res 65(4):1384–1393
167. Xu S, Kitayama J, Yamashita H, Souma D, Nagawa H (2008) Nuclear translocation of HER-4/c-erbB-4 is significantly correlated with prognosis of esophageal squamous cell carcinoma. J Surg Oncol 97(1):44–50
168. Hughes DP, Thomas DG, Giordano TJ, Baker LH, McDonagh KT (2004) Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. Cancer Res 64(6):2047–2053
169. Hughes DPM, Thomas DG, Giordano TJ, McDonagh KT, Baker LH (2006) Essential erbB family phosphorylation in osteosarcoma as a target for CI-1033 inhibition. Pediatr Blood Cancer 46(5):614–623
170. Hua Y, Gorshkov K, Yang Y, Wang W, Zhang N, Hughes D (2012) Slow down to stay alive: HER4 protects against cellular stress and confers chemoresistance in neuroblastoma. Cancer 118(20):5140–5154
171. Kang HG, Jenabi JM, Zhang J, Keshelava N, Shimada H, May WA, Ng T, Reynolds CP, Triche TJ, Sorensen PH (2007) E-cadherin cell-cell adhesion in ewing tumor cells mediates suppression of anoikis through activation of the ErbB4 tyrosine kinase. Cancer Res 67(7):3094–3105. doi:10.1158/0008-5472.CAN-06-3259
172. Merimsky O, Kollender Y, Issakov J, Inbar M, Flusser G, Benayahu D, Meller I, Bickels J (2003) Induction chemotherapy for bone sarcoma in adults: correlation of results with erbB-4 expression. Oncol Rep 10(5):1593–1599

MicroRNAs in Osteosarcomagenesis

Lisa A. Kafchinski and Kevin B. Jones

Abstract The etiology of osteosarcoma (OS) remains enigmatic. Particular clinical and molecular patterns, observed with high frequency in OS, suggest that it results from some yet-to-be-discovered central driver. How else can biology generate such an aggressive, metastatic, genetically and chromosomally unstable malignancy with virtually no apparent precursor neoplasms that are partway along a disease path toward OS? With this conundrum as a backdrop, the discovery of every new native molecule with power to impact a cell's biology is usually quickly followed by a search to see if this type of molecule contains the key to unlock OS biology.

Keywords MircoRNA • miRNA • Prognosis • Apoptosis • Chemoresponsiveness

This pattern was followed closely as the appreciation of microRNAs (miRs) dawned on the biology of cancer and development over the last decade. MiRs are short noncoding RNAs, typically 20–22 nucleotides in length, with profound impact on the posttranscriptional control of gene expression [1]. Typically, miRs bind to the 3′ untranslated regions of target genes, limiting the level of translation. MiRs destabilize some gene transcripts, limiting the duration for which the messenger RNAs are available for translation. Other transcripts are not degraded any faster because of the bound miR, but are blocked from the ribosomal translational machinery. A single miR can impact a variety of genes. Families of miRs, sometimes grouped by sequence similarities and sometimes by co-expression from a single genomic locus, have proven to be master regulators of broad transcriptional profiles in development and

L.A. Kafchinski • K.B. Jones (✉)
Department of Orthopaedics and Center for Children's Cancer Research, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA
e-mail: kevin.jones@hci.utah.edu

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_6,

disease. The modulating role of miRs in oncogenesis has been demonstrated in numerous cancers. In a few malignancies, dominant, cancer-initiating roles have been elucidated for specific miRs. In most cancers, they have been found to play a role.

Many investigators have sought a potential role for miRs in OS. Most have given up the initially tantalizing thought that a miR or miR family might be the driving force behind OS. However, even without some core discovery explaining OS biology, patterns of miR expression in OS and individual miRs function in OS cell lines has led to an understanding that miRs comprise an important part of the molecular landscape of OS.

A role for miRs in the management of OS has been sought by the profiling of snap frozen specimens, paraffin embedded specimens [2], and even patient serum samples [3–5]. Most of these have sought prognostic markers of survival, metastasis, or chemotherapeutic response. A variety of methods have been utilized to profile miRs, including locked nucleic acid (LNA) microarray, beads array, and TaqMan quantitative real-time PCR low density array (TLDA). Each array type has demonstrated high intra-platform reliability, but poor inter-platform reliability [6]. The wide variations in miR collection and profiling likely contribute to the difficulty of parsing the different profiles reported, but some themes are discernible.

Additional work has attempted to characterize the expression levels of individual miRs, for prognostication, deciphering of biological pathways, identification of therapeutic targets, or identification of potentially therapeutic miRs.

Profiles and Patterns of MicroRNAs in Osteosarcoma

In an early miR profiling paper, the miR-181 family (miR-181a, b, c) were overexpressed in OS tumor samples and higher expression of miR-181c was associated with development of metastasis. Multiple members of the miR-16 family were decreased in OS tumor samples, and lower levels of one family member associated with chemoresistance [7]. These two groups of oncogenic and tumor suppressive miRs have been identified by other groups as well [8]. For example, the well-known MG-63 OS cell line was found to overexpress miR-181a [9]. Others have also shown miR-181 overexpression [10]. MG-63 cells also overexpress miR-195, a miR-16 family (generally tumor suppressing) member.

A possibly explanation for the prominent role of miR-16 family members in various profiles is their participation in osteoblast differentiation [11]. Other osteoblast differentiation associated miRs, such as miR-29a/miR-29b, the let-7 family, and the miR-34 family also figure prominently in a variety of reported profiles of usually downregulated miRs [11–18]. Levels of the master osteoblast regulator, RUNX2, are related directly with miR-34 [17].

Another generally oncogenic miR group identified in OS cells is the miR-17-92 cluster. The miR-17-92 cluster and its two paralogous clusters miR-106a-92 and miR-106b-25, all associated with stemness and poor outcome in a variety of cancers, were found to be upregulated in multiple OS cell lines [19, 20].

MiR-126/126*, a well-established tumor suppressing miR in colon cancer and other carcinomas, was also found to be downregulated in osteosarcoma by a few different investigative teams [12, 20, 21]. This may be due to its recently established role antagonizing the SDF-1α cytokine, which recruits inflammatory monocytes and mesenchymal stem cells to tumors, prompting metastasis [22], or to its suppression of Sirt1 [21].

The remainder of the data available for miRs in OS either focuses on individual miRs or profiling that has not been reproduced in multiple series.

Pronostic MicroRNAs in Osteosarcoma

General Aggressiveness

Some miRs have been identified as individually prognostic of survival in OS. Others have been shown in cell lines to increase invasiveness or aggressiveness. For example, silencing of the 14q32 locus has an important role in OS progression. A group of miRs expressed from this locus, including miR-382, miR-134, and miR-544, have prognostic value in OS [2, 3]. In contrast, decreased levels of miR-206 [23] and miR-145 [24] are associated with more advanced clinical stages of OS and histologic de-differentiation. Other miRs have been shown to be dysregulated in OS, but when manipulated in OS cell lines have impacted their aggressiveness. These would include miR-16, as noted above, but also miR-210 [25] and miR-21 [26], which are expressed more in OS cells than osteoblasts and modulate tumor aggressiveness in cell lines. Invasiveness due to pathologic angiogenesis is related to a loss of miR-132 expression [27].

Metastasis

A number of individual miRs have been associated with metastasis by different study groups in OS patient cohorts. These include miR-27a and miR-181c [12], miR-206 [23], miR-145 [24], and miR-93 [28]. The modulation of expression of other miRs has been demonstrated to impact metastatic phenotypes of cell lines. This second, partly overlapping group includes miR-27a [12], miR-340 [29], miR-183 [30, 31], miR-424 [32], miR-195 [33], and miR-20a [34]. Specific gene transcript targets of some of these have also been validated. MiRs-424, -195, and -20a all target fatty acid synthase (FASN), which has previously established function in OS metastasis. The last of these, miR-20a, is part of the miR-17-92 cluster that figures prominently in more than one general profile of OS miRs. MiR-340 targets Rho-associated protein kinase 1 (ROCK1), awareness of which as a general driver of metastasis in cancer is growing rapidly [29]. MiR-183 targets ezrin, a gene central to the metastatic program for OS [30, 31].

Chemoresistance

In addition to miR-15b, as noted above, dysregulation of other miRs in OS tumor samples have correlated with resistance to chemotherapy. Chemoresistance was noted in OS cells that had increased levels of miR-21 [4]. Decreased responsiveness to the specific chemotherapeutic agent cisplatin was found in OS cells that had increased expression of miR-221 [35]. Five miRs were identified as being prognostic for ifosfamide response, miR-92a, miR-99b, miR-132, miR-193a-5p and miR-422a, impacting the TGF-β, Wnt, and MAP kinase signaling pathways [36]. Resistance to both methotrexate and raltitrexed (Tomudex®) was found to correlate with increased levels of miR-215 [37]. Overexpression of miR-140 in OS cells caused resistance to methotrexate and 5-fluorouracil [38].

Pathways of Influence for MicroRNAs

Although the appreciation of miRs has elevated to our awareness the critical impact of noncoding RNA molecules, we still interpret most of the biological influence of miRs through the language of the genes whose translation they ultimately impact. Naturally, most investigators have looked for target genes in the major developmental and oncogenic pathways. As most of these pathways have been implicated one at a time and by single miRs, it is difficult to summarize these data without apparent lists.

Proliferation

The group of miRs expressed from the 14q32 locus, typically lost in OS, impact a number of important pathways in proliferation, including Notch, RAS/p21, MAPK, Wnt, and the Jun/FOS [39]. Other miRs also impact Notch signaling, including miR34c, a tumor suppressor miR downregulated in OS [7]. In contradistinction, Notch signaling activity is increased by miR-199b-5p, which is often overexpressed in OS and has proven to be a potential therapeutic target. Transfection with a miR-199b-5p inhibitor decreased Notch signaling and proliferation. A downstream effect was a reduction in HES1 expression, which diminished cell invasiveness [40]. Other members of the miR-199 family, such as miR-199a-3p, reduce cell proliferation by affecting the G1/S cell cycle checkpoint of the cell cycle [41].

Signal transducer and activator of transcription 3 (STAT3), another central driver of proliferation, is a downstream target of miR-125b, which is often decreased in OS [42]. Transforming growth factor alpha (TGF-α), a ligand for the epidermal growth factor receptor, is often overexpressed in OS, functioning in autocrine fashion. MiR-376c decreases levels of TGF-α and is noted to be downregulated itself in OS [43].

Insulin-like growth factor 1 receptor (IGF-R1) is involved in the proliferation of many cancers. In osteosarcoma, it is a target of miR-16, which represses cell proliferation. When miR-16 is underexpressed, then cell proliferation increases via IGF-R1 and the Raf1-MEK1/2-ERK1/2 pathway [8]. MiR-15a and miR-16-1 also impact proliferation partly by targeting cyclin D1 [44].

Another regulator of increased OS cell proliferation is lysophosphatidic acid acyltransferase β (LPAATβ), a target of miR24. In many OS cell lines, miR-24 is downregulated, leading to increased LPAATβ activity and OS cell proliferation [45].

MiR-34a levels are low in OS cells leading to upregulation of ether à go-go 1 (Eag1) pathway activity and dependent proliferation [15].

Apoptosis

MiR-133a is downregulated in OS, resulting in increased cell proliferation. When levels of miR-133a are reestablished, it functions as a tumor suppressor via inhibition of Bcl-xL and Mcl-1 expression [46]. Similarly, pro-differentiation, tumor-suppressing miR-29a silenced Bcl-2 and Mcl-1 and is typically downregulated in OS [14]. Bcl-2 alone is targeted by miR-143, which is low in OS specimens [47].

Transcript levels of the regulatory gene, c-MYC, can be affected by a group of miRs at the chromosome 14q32 locus, including miR-382, miR-369-3p, miR 544, and miR-134, downregulated in OS cells. Reinstating functional levels of these miRs causes decreased c-MYC activity, triggering induction of apoptosis [48].

In contrast, suppression of the oncogenic miR-181a leads to increased apoptosis in OS [10]. Heat shock protein 90 (Hsp90) is a target of miR-223, which produces increased apoptosis in OS cells as well as G0/G1 arrest when the miR is antagonized [49].

DNA Damage Repair

DNA repair is aided by a phosphorylated histone H2AX. MicroRNA-138 inhibits formation of this important histone complex, thereby improving the responsiveness to both radiation therapy and chemotherapy [50].

Invasion

Matrix metalloproteinases (MMPs), integral in cell migration contribute to the metastatic phenotype. OS cells with decreased levels of miR-143 demonstrate resultant upregulation of its target MMP-13 and therefore metastasis [51].

Angiogenesis

Angiogenesis in previously dormant osteosarcoma cells was associated with decreased expression of miR-190 [52]. Vascular endothelial growth factor is down-regulated when miR-145 is over-expressed, therefore also limiting invasion of OS cells [53].

Driving MicroRNA Dysregulation

While so much research has focused on how miRs manage the expression levels of a variety of coding genes, much less is known about what factors directly influence the expression levels of miRs. A few associations have been identified.

For example, miR34a, a member of the illustrious miR 34 family, with its varied and sundry effects in OS, is a target of the key tumor suppressor gene p53 [13, 16]. p53 also targets miR-211, indirectly. Noncoding RNA loc 285194, a p53 regulated tumor suppressor, leads to decreased levels of miR211 as well as decreased proliferation [54].

Changes in the expression of 13 different miRs were identified by microarray and qRT-PCR when cells were transfected with apurinic/apyrimidinic endonuclease1 (APE1). This enzyme functions in both cellular DNA repair and redox regulation. Downstream pathways that were affected include p53 signaling, Wnt, TGF- β, and MAPK. Therefore, multiple cellular processes including differentiation and signaling can be regulated by APE1 through alteration of gene expression by miRs [55].

Some therapeutic chemicals applied to OS also have been shown to impact miR expression profiles. Exposure of OS cells to epirubicin increased levels of miR-302b, which inhibits OS cell proliferation via promotion of apoptosis [56].

Diallyl trisulfide (DATS) decreases angiogenesis, cell survival, and invasion of OS cells. Mechanistically, it causes a drop in the expression of Notch-1 signaling pathway and its downstream genes, such as matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF). However, expression of a group of miRs, normally decreased in OS, is increased. This group of miRs acts as tumor suppressors and include miR-34a, miR-143, miR-145, and miR200b/c [18].

Future Directions

Doubtless, we have only begun to understand the breadth and depth of impact miRs have on osteosarcomagenesis, progression, metastasis, and chemoresistance. As technology and our understanding of miRs continue to improve, additional utilization of miRs in diagnostic, prognostic, and hopefully therapeutic purposes will be made.

References

1. Croce CM (2009) Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10(10):704–714
2. Kelly AD et al (2013) MicroRNA paraffin-based studies in osteosarcoma reveal reproducible independent prognostic profiles at 14q32. Genome Med 5(1):2
3. Sarver AL et al (2013) MicroRNAs at the human 14q32 locus have prognostic significance in osteosarcoma. Orphanet J Rare Dis 8:7
4. Yuan J et al (2012) Identification of serum microRNA-21 as a biomarker for chemosensitivity and prognosis in human osteosarcoma. J Int Med Res 40(6):2090–2097
5. Ouyang L et al (2013) A three-plasma miRNA signature serves as novel biomarkers for osteosarcoma. Med Oncol 30(1):340
6. Liu S, Gibb BC (2011) Solvent denaturation of supramolecular capsules assembled via the hydrophobic effect. Chem Commun (Camb) 47(12):3574–3576
7. Bae Y et al (2012) miRNA-34c regulates Notch signaling during bone development. Hum Mol Genet 21(13):2991–3000
8. Chen L et al (2013) miR-16 inhibits cell proliferation by targeting IGF1R and the Raf1-MEK1/2-ERK1/2 pathway in osteosarcoma. FEBS Lett 587(9):1366–1372
9. Hu H et al (2012) Changes in microRNA expression in the MG-63 osteosarcoma cell line compared with osteoblasts. Oncol Lett 4(5):1037–1042
10. Jianwei Z et al (2013) MicroRNA 181a improves proliferation and invasion, suppresses apoptosis of osteosarcoma cell. Tumour Biol 34(6):3331–3337
11. Eguchi T et al (2013) OstemiR: a novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesencymal stem cells. PLoS One 8(3):e58796
12. Jones KB et al (2012) miRNA signatures associate with pathogenesis and progression of osteosarcoma. Cancer Res 72(7):1865–1877
13. Yan K et al (2012) MicroRNA-34a inhibits the proliferation and metastasis of osteosarcoma cells both in vitro and in vivo. PLoS One 7(3):e33778
14. Zhang W et al (2012) The microRNA-29 plays a central role in osteosarcoma pathogenesis and progression. Mol Biol (Mosk) 46(4):622–627
15. Wu X et al (2013) MicroRNA-34a inhibits human osteosarcoma proliferation by downregulating ether a go-go 1 expression. Int J Med Sci 10(6):676–682
16. He C et al (2009) Functional elucidation of MiR-34 in osteosarcoma cells and primary tumor samples. Biochem Biophys Res Commun 388(1):35–40
17. van der Deen M et al (2013) MicroRNA-34c inversely couples the biological functions of the Runt-related transcription factor RUNX2 and the tumor suppressor p53 in osteosarcoma. J Biol Chem 288(29):21307–21319
18. Li Y et al (2013) Diallyl trisulfide inhibits proliferation, invasion and angiogenesis of osteosarcoma cells by switching on suppressor microRNAs and inactivating of Notch-1 signaling. Carcinogenesis 34(7):1601–1610
19. Baumhoer D et al (2012) MicroRNA profiling with correlation to gene expression revealed the oncogenic miR-17-92 cluster to be up-regulated in osteosarcoma. Cancer Genet 205(5):212–219
20. Namlos HM et al (2012) Modulation of the osteosarcoma expression phenotype by microRNAs. PLoS One 7(10):e48086
21. Xu JQ et al (2013) MicroRNA-126 inhibits osteosarcoma cells proliferation by targeting Sirt1. Tumour Biol 34(6):3871–3877
22. Ren G, Kang Y (2013) A one-two punch of miR-126/126* against metastasis. Nat Cell Biol 15(3):231–233
23. Bao YP et al (2013) Roles of microRNA-206 in osteosarcoma pathogenesis and progression. Asian Pac J Cancer Prev 14(6):3751–3755
24. Tang M et al (2013) MicroRNA-145 downregulation associates with advanced tumor progression and poor prognosis in patients suffering osteosarcoma. Onco Targets Ther 6:833–838

25. Cai H et al (2013) Prognostic evaluation of microRNA-210 expression in pediatric osteosarcoma. Med Oncol 30(2):499
26. Ziyan W et al (2011) MicroRNA-21 is involved in osteosarcoma cell invasion and migration. Med Oncol 28(4):1469–1474
27. Yang J et al (2013) Loss of microRNA-132 predicts poor prognosis in patients with primary osteosarcoma. Mol Cell Biochem 381(1–2):9–15
28. Montanini L et al (2012) MicroRNA cloning and sequencing in osteosarcoma cell lines: differential role of miR-93. Cell Oncol (Dordr) 35(1):29–41
29. Zhou X, Wei M, Wang W (2013) MicroRNA-340 suppresses osteosarcoma tumor growth and metastasis by directly targeting ROCK1. Biochem Biophys Res Commun 437(4):653–658
30. Zhao H et al (2012) miR-183 inhibits the metastasis of osteosarcoma via downregulation of the expression of Ezrin in F5M2 cells. Int J Mol Med 30(5):1013–1020
31. Zhu J et al (2012) Down-regulation of miR-183 promotes migration and invasion of osteosarcoma by targeting Ezrin. Am J Pathol 180(6):2440–2451
32. Long XH et al (2013) Tumor suppressive microRNA-424 inhibits osteosarcoma cell migration and invasion via targeting fatty acid synthase. Exp Ther Med 5(4):1048–1052
33. Mao JH et al (2012) microRNA-195 suppresses osteosarcoma cell invasion and migration in vitro by targeting FASN. Oncol Lett 4(5):1125–1129
34. Huang G et al (2012) miR-20a encoded by the miR-17-92 cluster increases the metastatic potential of osteosarcoma cells by regulating Fas expression. Cancer Res 72(4):908–916
35. Zhao G et al (2013) MicroRNA-221 induces cell survival and cisplatin resistance through PI3K/Akt pathway in human osteosarcoma. PLoS One 8(1):e53906
36. Gougelet A et al (2011) Micro-RNA profiles in osteosarcoma as a predictive tool for ifosfamide response. Int J Cancer 129(3):680–690
37. Song B et al (2010) Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. Mol Cancer 9:96
38. Song B et al (2009) Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. Oncogene 28(46):4065–4074
39. Maire G et al (2011) Analysis of miRNA-gene expression-genomic profiles reveals complex mechanisms of microRNA deregulation in osteosarcoma. Cancer Genet 204(3):138–146
40. Won KY et al (2013) MicroRNA-199b-5p is involved in the Notch signaling pathway in osteosarcoma. Hum Pathol 44(8):1648–1655
41. Duan Z et al (2011) MicroRNA-199a-3p is downregulated in human osteosarcoma and regulates cell proliferation and migration. Mol Cancer Ther 10(8):1337–1345
42. Liu LH et al (2011) miR-125b suppresses the proliferation and migration of osteosarcoma cells through down-regulation of STAT3. Biochem Biophys Res Commun 416(1–2):31–38
43. Jin Y et al (2013) MicroRNA-376c inhibits cell proliferation and invasion in osteosarcoma by targeting to transforming growth factor-alpha. DNA Cell Biol 32(6):302–309
44. Cai CK et al (2012) miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma. Oncol Rep 28(5):1764–1770
45. Lipton A et al (2013) Reply to Araki et al. Eur J Cancer. doi:10.1016/j.ejca.2013.02.037
46. Ji F et al (2013) MicroRNA-133a, downregulated in osteosarcoma, suppresses proliferation and promotes apoptosis by targeting Bcl-xL and Mcl-1. Bone 56(1):220–226
47. Zhang H et al (2010) microRNA-143, down-regulated in osteosarcoma, promotes apoptosis and suppresses tumorigenicity by targeting Bcl-2. Oncol Rep 24(5):1363–1369
48. Thayanithy V et al (2012) Perturbation of 14q32 miRNAs-cMYC gene network in osteosarcoma. Bone 50(1):171–181
49. Li G et al (2012) Heat shock protein 90B1 plays an oncogenic role and is a target of microRNA-223 in human osteosarcoma. Cell Physiol Biochem 30(6):1481–1490
50. Wang Y et al (2011) MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. Mol Cancer Res 9(8):1100–1111
51. Osaki M et al (2011) MicroRNA-143 regulates human osteosarcoma metastasis by regulating matrix metalloprotease-13 expression. Mol Ther 19(6):1123–1130

52. Gordin FM et al (1990) Prevalence of human immunodeficiency virus and hepatitis B virus in unselected hospital admissions: implications for mandatory testing and universal precautions. J Infect Dis 161(1):14–17
53. Fan L et al (2012) MicroRNA-145 targets vascular endothelial growth factor and inhibits invasion and metastasis of osteosarcoma cells. Acta Biochim Biophys Sin (Shanghai) 44(5):407–414
54. Liu Q et al (2013) LncRNA loc285194 is a p53-regulated tumor suppressor. Nucleic Acids Res 41(9):4976–4987
55. Dai N et al (2013) Alteration of the microRNA expression profile in human osteosarcoma cells transfected with APE1 siRNA. Neoplasma 60(4):384–394
56. Zhang Y et al (2013) Epirubicin-mediated expression of miR-302b is involved in osteosarcoma apoptosis and cell cycle regulation. Toxicol Lett 222(1):1–9

RECQ DNA Helicases and Osteosarcoma

Linchao Lu, Weidong Jin, Hao Liu, and Lisa L. Wang

Abstract The RECQ family of DNA helicases is a conserved group of enzymes that are important for maintaining genomic integrity. In humans, there are five RECQ helicase genes, and mutations in three of them—*BLM*, *WRN*, and *RECQL4*—are associated with the genetic disorders Bloom syndrome, Werner syndrome, and Rothmund–Thomson syndrome (RTS), respectively. Importantly all three diseases are cancer predisposition syndromes. Patients with RTS are highly and uniquely susceptible to developing osteosarcoma; thus, RTS provides a good model to study the pathogenesis of osteosarcoma. The "tumor suppressor" role of RECQL4 and the other RECQ helicases is an area of active investigation. This chapter reviews what is currently known about the cellular functions of RECQL4 and how these may relate to tumorigenesis, as well as ongoing efforts to understand RECQL4's functions in vivo using animal models. Understanding the RECQ pathways may provide insight into avenues for novel cancer therapies in the future.

Keywords RECQ • RECQL4 • DNA helicase • Rothmund–Thomson syndrome • RTS • Bloom syndrome • Werner syndrome • Osteosarcoma

L. Lu, Ph.D. • W. Jin, M.S. • L.L. Wang, M.D. (✉)
Section of Hematology/Oncology, Department of Pediatrics, Texas Children's Cancer Center, Baylor College of Medicine, 1102 Bates Avenue, Suite 1200, Houston, TX 77030, USA
e-mail: linchaol@bcm.edu; weidong.jin@bcm.edu; llwang@bcm.edu

H. Liu, Ph.D.
Section of Hematology/Oncology, Department of Medicine, Division of Biostatistics, Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA
e-mail: haol@bcm.edu

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_7,

Introduction

The role of the RECQ helicases in cancer and specifically the role of RECQL4 in osteosarcoma (OS) are active areas of investigation. While it is known that constitutional mutations in the *RECQ* genes predispose patients to developing cancer, the exact mechanisms of tumorigenesis remain to be discovered. As basic science research continues to reveal the normal cellular functions of the RECQ helicases, application of this knowledge to OS pathogenesis will provide avenues for future investigation into targeted therapies for this disease. This chapter focuses on what is currently known about the *RECQL4* DNA helicase gene, which is mutated in the OS predisposition disorder Rothmund–Thomson syndrome (RTS).

RECQ Family of DNA Helicases and Cancer Predisposition

The RECQ DNA helicases are a family of proteins that are important in maintaining genomic integrity. DNA helicases are ubiquitous molecular motor proteins which harness the chemical-free energy of ATP hydrolysis to catalyze the unwinding of duplex DNA, and as such play important roles in nearly all aspects of nucleic acid metabolism, including replication, repair, recombination, and transcription [83]. The RECQ helicases belong to the SF2 superfamily of DNA helicases that unwind DNA in a $3' \rightarrow 5'$ direction in an ATP- and Mg^{2+}-dependent fashion [3, 5]. As such, they contain a conserved region that includes the seven characteristic helicase motifs (I, Ia, II, III, IV, V, and VI) that define this family of helicases and that are important for coupling ATP hydrolysis to the separation of DNA strands. The first RECQ helicase was discovered in *Escherichia coli* (*E. coli*) in a screen for resistance to thymineless death [57]. Subsequently, RECQ proteins have been identified in multiple species. These evolutionarily conserved proteins are defined by their common central helicase motif, a highly conserved region of approximately 400 amino acids (Fig. 1) [5, 38]. The number of RECQ helicases increases from lower to higher organisms. Bacteria such as *E. coli* have one (RecQ), as do yeast (*Sgs1* in *Saccharomyces cerevisiae* and *Rqh1* in *Schizosaccharomyces pombe*), while *Caenorhabditis elegans* has two and *Arabidopsis thaliana* has seven [40].

In humans, there are five RECQ helicases (Fig. 1). Three of them, WRN, BLM, and RECQL4, are associated with human diseases [54]. Mutations in the *WRN* gene [99] cause Werner syndrome [51], and mutations in the *BLM* gene [21] are responsible for Bloom syndrome [24]. Mutations in *RECQL4* are associated with three overlapping disorders: RTS, RAPADILINO syndrome, and Baller–Gerold syndrome (BGS) [39, 71, 85]. *RECQL1* and *RECQL5* have not thus far been associated with any human diseases.

All of the human RECQ disorders are cancer predisposition syndromes, but their cancer profiles are different (Table 1). Patients with Werner syndrome display features of premature aging, such as diabetes, coronary artery disease, cataracts, and osteoporosis. They are susceptible primarily to thyroid cancer, melanoma, meningioma,

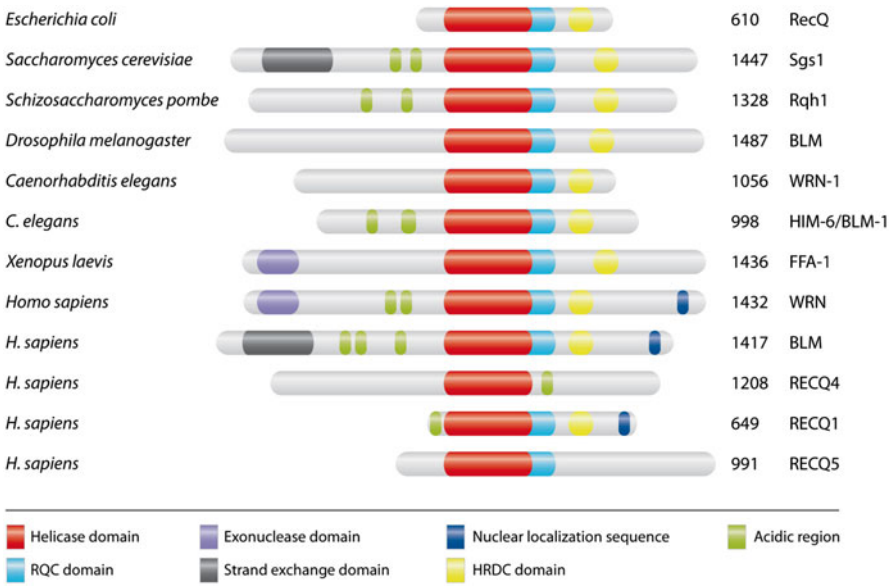


Fig. 1 Structural features of RecQ helicases. The RecQ proteins have several structural domains that are conserved from bacteria through humans. All RecQ proteins have a core helicase domain. Most RecQ proteins also contain conserved helicase and RNase D C-terminal (HRDC) and RecQ C-terminal (RQC) domains that are thought to mediate interactions with nucleic acid and other proteins, respectively. Many RecQ proteins have acidic regions that enable protein–protein interactions, and some of the RecQ proteins have nuclear localization sequences. WRN and FFA-1 protein are unique in that they also contain an exonuclease domain. Sgs1 and Blm are the first characterized members of this family of proteins containing a functional strand exchange domain in their N-terminus. The number of amino acids in each protein is indicated on the right. (Reprinted with permission from Bernstein KA, Gangloff S, Rothstein R. The RecQ DNA helicases in DNA repair. Annu Rev Genet 2010; 44:393–417)

Table 1 Human RECQ helicase syndromes

Disease	Main clinical features	Cancer predisposition	Gene location
Bloom syndrome	Small stature, photosensitive rash, immunodeficiency	Multiple tumor types, including leukemia, lymphoma, solid tumors	*BLM* 15q26.1
Werner syndrome	Premature aging, cataracts, diabetes, atherosclerosis	Soft tissue sarcomas, skin (melanoma), thyroid cancer, osteosarcoma	*WRN* 8p11
Rothmund–Thomson syndrome	Poikiloderma, radial ray and other skeletal defects, alopecia	*Osteosarcoma*, skin cancer (squamous and basal cell carcinomas)	*RECQL4* 8q24.3
RAPADILINO syndrome	Small stature, radial ray and limb deformities, palatal defects, absent patella	Lymphoma and osteosarcoma	*RECQL4* 8q24.3
Baller–Gerold syndrome	Craniosynostosis, radial ray defects, poikiloderma	Possibly lymphoma	*RECQL4* 8q24.3

soft tissue sarcomas, and OS. In a study of the spectrum of cancers in Werner syndrome patients, OS was found to comprise 7.7 % of all neoplasms [43]. In contrast, patients with Bloom syndrome are susceptible to all types of cancers seen in the general population but at a much higher frequency and at an earlier age. These include leukemias and lymphomas, epithelial cancers of the colon, breast, head and neck, cervix, as well as OS, which accounted for 2 % of the first 100 cases of cancers reported in the Bloom Registry [25]. Among the *RECQL4*-associated disorders, patients with RTS have a very high and *specific* risk for OS, in addition to non-melanoma skin cancers (squamous and basal cell carcinomas). In one clinical cohort study of 41 RTS patients, 30 % had a diagnosis OS [90]. Patients with RAPADILINO syndrome and *RECQL4* mutations are also at risk for cancer, most commonly lymphomas as well as OS [72]. These patients share many of the same phenotypes as RTS patients, including small stature, limb deformities, radial ray defects, and absent patellae. Interestingly, these patients do not display poikiloderma, which is a defining feature of RTS. BGS is the least well-characterized of the *RECQL4* disorders. These patients are characterized by craniosynostosis and radial ray defects, as well as poikiloderma in some patients. So far only a few cases have been described to have *RECQL4* mutations, and cancer has only been described in one patient who developed a midline NK cell lymphoma [17]. Overall there have been over 60 *RECQL4* mutations identified among these three disorders [84]. Exact genotype–phenotype correlations with respect to specific mutations and resultant phenotypes, including cancer, remain to be elucidated.

As a group, the RECQ helicase are felt to be "caretakers" of the genome and as such do not necessarily directly regulate tumorigenesis, but prevent genomic instability that results in accumulation of structural changes in oncogenes or tumor suppressors that could then lead to cancer [11]. This protection of genome stability is achieved through their various roles in DNA replication, repair, and telomere maintenance. It is also possible that the RECQ helicases could play a more direct role in affecting tumorigenesis. For example, it has been shown that the MYC oncoprotein directly stimulates transcription of *WRN*, which may promote MYC-driven tumorigenesis through prevention of cellular senescence normally mediated by the WRN protein [28]. While the exact molecular mechanisms of tumor suppression have yet to be worked out fully, it is clear that deficiency of the WRN, BLM, and RECQL4 proteins in humans predisposes to the development of cancer.

Functions of the RECQL4 DNA Helicase

RECQL4 is unusual among the other human RECQ helicases because it lacks several of the common conserved domains, such as the RecQ C-terminal (RQC) domain which is thought to be important for mediating protein–protein interactions, as well as the Helicase-and-RNase D C-terminal (HRDC) domain which is felt to be important for interactions with nucleic acids (Fig. 1) [5, 55]. However, unlike the other RECQ members, RECQL4 has an N-terminal domain (amino acids 1–200)

with homology to the yeast replication initiation protein Sld2 in *S. cerevisiae* and DRC1 in *S. pombe* [11, 52, 67], which are important for establishing replication forks during the process of DNA replication. The N-terminus also contains several localization regions, including two nuclear localization domains [6], a region of acetylation by p300 which regulates nuclear to cytoplasmic localization [18], and a predicted mitochondrial localization signal in amino acids 1–84 [16]. For several years, researchers were unable to demonstrate actual DNA unwinding activity by RECQL4 using a variety of DNA substrates [48, 98]. Finally after many attempts, in 2009, helicase activity was demonstrated for RECQL4 by several groups [9, 64, 77, 95], which was likely masked in previous assays by the strong annealing activity of the enzyme. In vitro biochemical data suggest that RECQL4 possesses another N-terminal region contributing to DNA unwinding besides the well-known conserved helicase domain [95], although known helicase motifs and nucleotide binding sites are not found to be present in that region. The in vivo function of this extra helicase domain is not clear. Furthermore, the first 54 amino acids of N-terminus of human RECQL4 forms a homeodomain which has DNA-binding activity without any specific DNA-binding sequences [61].

The role of RECQL4 in DNA replication has been extensively studied, and it appears that while RECQL4 may participate in many cellular functions, its primary role is in the initiation of DNA replication [33, 52, 67, 81, 93, 96, 97]. The N-terminal domain of RECQL4 shares homology to the yeast replication factor Sld2 that, after phosphorylation by cyclin-dependent kinases, binds Dpb11, a key mediator of the formation of the active replicative helicase complex on replication origins and a crucial factor in the initiation of DNA replication [36, 78, 87]. In Xenopus, it has been shown that xRECQL4 belongs to the replication initiation complex and helps to promote loading of replication factors at the origins, after pre-replication complex formation [67]. The N-terminal amino acid region 1–596 has been demonstrated to interact directly with xCut5 (frog ortholog of Dpb11), which is responsible for recruiting DNA polymerases to the sites of replication [52]. RECQL4 has been shown by mass spectrometry in human cells to interact with multiple DNA replication factors, such as MCM10, MCM2-7, CDC45, GINS, and SLD5 which are essential for initiation of DNA replication [33, 96] as well as TopBP1, the vertebrate ortholog of Dpb11 [61]. The function of the C-terminus of RECQL4 in replication is not as well understood, but studies by Kohzaki et al. demonstrated that the helicase domain and C-terminus region, while not critical for unperturbed replication, are important in the replication elongation process in cells exposed to ionizing radiation, perhaps by allowing replication forks to negotiate the radiation-damaged DNA templates [41].

RECQL4 has also been implicated to function in various aspects of DNA repair, including DSB repair [42, 63, 73], nucleotide excision repair (NER) [13, 22], and base excision repair (BER) [68]. Many of these studies have shown that RECQL4 localizes to sites of DNA damage or that it colocalizes or interacts directly with proteins well known to be involved in the various forms of DNA damage. For example, RECQL4 has been shown to interact physically by co-immunoprecipitation with RAD51, a key protein involved in the homologous recombination pathway of DSB

repair, and to associate with RAD51 by immunofluorescence in DNA damage foci [42, 63, 73]. RECQL4 has also been shown to colocalize with XPA, a key protein involved in NER, and to interact with this protein directly by GST-pulldown assay [22]. The NER pathway is a major mediator of repair of UV damage. RECQL4 was also found to colocalize and functionally interact with key proteins involved in BER, including APE1, FEN1, and DNA polymerase β, after treatment with H_2O_2 [68]. The BER pathway is the main mechanism for repair of oxidative DNA lesions. Werner et al. showed that after H_2O_2 treatment RECQL4 translocates from the cytoplasm to the nucleus and forms nuclear foci in normal human fibroblasts. After recovery from oxidant damage, viable RTS patient fibroblasts underwent irreversible growth arrest and had significantly decreased DNA synthesis [91]. Woo et al. also showed that in response to oxidative stress, RECQL4 had altered cellular localization to the nucleolus, and using a T7 phage display screen showed that RECQL4 C-terminus interacts with the single-strand break repair protein, poly(ADP-ribose) polymerase-1 (PARP-1) [92]. PARP-1 is activated in response to a wide variety of DNA damaging agents and modulates the cellular sensitivity to γ-irradiation [47]. RECQL4 has also been shown to interact with BLM helicase, which like RECQL4 probably has many functions in the cell, the most important of which is its role in homologous recombination. This interaction was strengthened in S-phase and after ionizing radiation treatment in human cells, indicating that RECQL4 coordinates with BLM to function in DNA replication and DNA damage repair [74].

The responses to different genotoxic agents in *RECQL4* mutant cells have been investigated by several groups; these have included UV and ionizing radiation, hydrogen peroxide, topoisomerases inhibitors, and chemotherapy agents such as doxorubicin and cisplatin [7, 13, 22, 35, 41, 73, 91]. However, the results have been somewhat inconsistent between studies, likely reflecting the use of different primary cells or cell lines (transformed cells vs. untransformed cells, RTS patient cells vs. *RECQL4* knockdown cells), different assays to determine sensitivity, and different *RECQL4* mutations present in the cells. For example, some studies have demonstrated increased sensitivity to UV radiation [62, 70, 75], while others have not [35, 41]. Taken together, the above studies suggest that RECQL4 is involved in DNA repair and that mutations in *RECQL4* can lead to defects in DNA damage repair, which can then result in accumulation of genome instability and increased cancer susceptibility.

In addition to its function in DNA replication and DNA damage repair, RECQL4 has also been shown to play a role in telomere maintenance [26]. RTS patient cells and human cells with *RECQL4* knockdown have been shown to exhibit increased fragile telomeric ends. In addition, human RECQL4 localizes to telomeres and interacts with shelterin protein telomeric repeat-binding factor 2 (TRF2) which maintains telomere integrity [26]. RECQL4 also interacts with the WRN protein and stimulates WRN's activity on telomeric D-loops. Similar to WRN and BLM, RECQL4 also appears to be able resolve these D-loops, which is necessary for replication to take place at the telomeres, and this resolving activity is stimulated by TRF1 and TRF2 as well as the shelterin protein POT1 [26]. Also similar to WRN and BLM, RECQL4 seems to be more active on telomeric D-loops that contain

8-oxoguanine base lesions, indicative of oxidative damage. Unlike WRN, however, RECQL4 also has a clear preference for unwinding D-loops that contain thymine glycol (Tg) lesions, which are the most common oxidation product of the thymine base, and this activity is stimulated by TRF2 [23]. Thus, mutations in *RECQL4* could result in dysfunctional telomeres, which are well known to play a role in both tumor suppression and tumor progression, depending on the cellular milieu, particularly with respect to the checkpoint status of the cells [94].

In addition to these nuclear functions, RECQL4 has also been shown to localize in the cytosol [18, 98] and more recently in the mitochondria [10, 14, 16]. Yin et al. showed that RECQL4 interacts with cytosolic ubiquitin ligases UBR1 and UBR2 which function in the N-end rule pathway by ubiquitination and degradation of proteins [98]. Dietschy et al. demonstrated that RECQL4 can be acetylated by histone acetyltransferase p300 resulting in the cytosolic translocation of RECQL4 from the nucleus [18], providing a mechanism to modulate RECQL4 nuclear activities. In the mitochondria, RECQL4 has recently been shown to be important for maintenance of mitochondrial DNA (mtDNA) integrity [14] and in mtDNA oxidative damage repair [10]. Furthermore, De et al. demonstrated that RECQL4 interacts with and directs p53 localization in the mitochondria of human cells under unstressed conditions, and that DNA damage and *RECQL4* mutations lead to p53 nuclear stabilization and activation [16]. This provides a novel regulation of p53 activity by RECQL4. Interestingly, RECQL4 was also previously shown to be transcriptionally repressed by p53 [69]. The interaction between RECQL4 and p53 is particular interesting and warrants further investigation since constitutional mutations in either gene gives rise to cancer predisposition syndromes.

There have been a few studies analyzing the biochemical functional consequences resulting from specific mutations in *RECQL4* [15, 34]. For example, Croteau et al. demonstrated that mutant RECQL4 protein lacking exon 7 and caused by the c.1390 + 2delT splice-site mutation frequently found in RAPADILINO patients was deficient in DNA helicase activity when expressed in *E. coli* [15]. Furthermore, using similar biochemical assays, RECQL4 missense mutations in the DNA helicase domain were demonstrated to dramatically weaken the function of RECQL4 on DNA unwinding and ATP hydrolysis [34]. These findings are invaluable in dissecting the function of RECQL4 in multiple aspects of DNA metabolism and provide the basis for ongoing genotype–phenotype analyses.

Rothmund–Thomson Syndrome: Nature's Model of Osteosarcoma

RTS was first described in 1868 by Dr. Auguste Rothmund, who was a German ophthalmologist. He described poikiloderma, the classic skin finding in RTS, along with rapidly developing bilateral juvenile cataracts in several families in an isolated region in the Bavarian Alps [65]. In 1921, Dr. Sydney Thomson, a British dermatologist, described a similar rash in two sisters, but instead of juvenile cataracts,

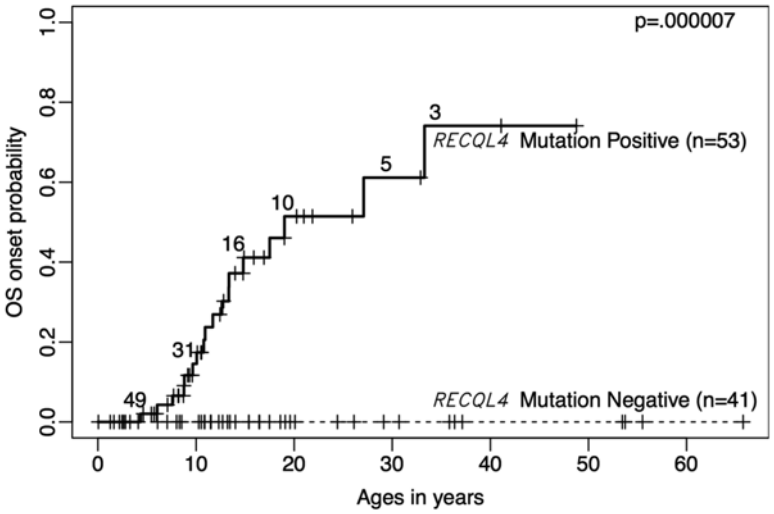


Fig. 2 Estimated probability of osteosarcoma onset in Rothmund–Thomson syndrome, classified by *RECQL4* mutation status. The time to OS onset was defined from the date of birth to the first diagnosis of OS. Event-time data were analyzed by Kaplan–Meier method, and the difference between the RECQL4 mutation positive and negative was compared by the log-rank method

they had bone abnormalities (radial ray defects) [82]. Later, Dr. William Taylor in the USA proposed that the two disorders described by Rothmund and Thomson were the same, and he proposed the eponym Rothmund–Thomson syndrome [80]. Mutations in the *RECQL4* gene in RTS was not discovered until 1999 [38, 39], 131 years after the original description by Rothmund. It is now known that approximately two-thirds of patients with RTS have mutations in the *RECQL4* gene (designated Type 2 RTS). The gene defect(s) in the other one-third (Type 1 RTS) has not yet been identified. Studies have shown that the presence of deleterious mutations in *RECQL4* correlates significantly with risk of developing OS (Fig. 2) [89]. None of the patients with Type 1 RTS have developed OS thus far, while every RTS patient with OS has *RECQL4* mutations. These deleterious mutations included nonsense, frameshift, splice site, and intronic deletions. Unlike other hereditary cancer syndromes known to predispose patients to OS, such as Li-Fraumeni syndrome and hereditary retinoblastoma, where the causative genes, p53 and RB, respectively, are commonly mutated in sporadic OS [88], mutations in *RECQL4* have not been detected in sporadic OS tumors [60]. Thus, *RECQL4* does not appear to be a direct target for somatic mutations in sporadic OS. However, the extremely high and specific risk for OS in Type 2 RTS patients suggests that the RECQL4 helicase plays a clear role in OS tumor suppression, making RTS a relevant model for the study of OS pathogenesis.

In addition to *cancer* of the bone, patients with RTS also have prominent bone *developmental* defects. In a study of 28 RTS patients who underwent skeletal

surveys, 75 % were found to have major skeletal abnormalities, including radial, ulnar or thumb agenesis/hypoplasia, radio-ulnar and radio-humeral synostoses, abnormal metaphyseal trabeculation, brachymesophalangy, and osteopenia [53]. This risk correlated with the presence of *RECQL4* mutations. Understanding the role that RECQL4 plays in normal skeletal development will provide additional insight into the specific risk for OS, since many developmental pathways, such as the Wnt, Hedgehog, and Notch signaling pathways, are not only critical for normal skeletal development [27, 31, 79] but also play important roles in tumorigenesis [4, 12, 37, 86, 100].

Early case reports suggested that OS arising in RTS patients may be different from sporadic OS, i.e., arising in unusual or multiple (multifocal) sites [19]. In addition, because of the implicated role of *RECQL4* in DNA damage repair, clinicians have often considered decreasing chemotherapy doses up-front for RTS patients diagnosed with OS. However, a study of 12 RTS patients with OS showed that their tumors had features that mirrored OS in the general population with regard to location of primary tumor (distal long bones), histology (conventional OS), histologic response to neoadjuvant chemotherapy, and overall outcomes [29]. The major difference was that the age of onset was younger in the RTS cohort compared to sporadic OS, which is not surprising given the genetic predisposition of RTS patients to OS. Some patients developed mucositis requiring dose modifications, particularly to doxorubicin (no more than 25 % decrease), but there is no current method to determine a priori who will experience increased toxicities. Therefore, current recommendations are to treat with standard doses of chemotherapy and to adjust according to the patient's individual course. The similarities between OS in RTS and sporadic OS support the further study of the contribution of the RECQL4 pathways in the pathogenesis of OS.

Understanding the Role of RECQL4 in Osteosarcoma Development

Previous Recql4 Mouse Models

In order to understand the function of RECQL4 in OS tumorigenesis in vivo, three mouse models of global *Recql4* disruption have been generated. In the first mouse model, exons 5–8 of *Recql4* upstream of the conserved helicase domain (exons 9–15) were replaced with *PGKneo* and *LacZ* cassettes [32]. The homozygous mutants died during early embryonic stage E3.5–6.5. Blastocyst cultures of E3.5 mutants showed severely reduced growth of inner cell mass and trophoblasts. Heterozygous mutants were phenotypically similar to wild type littermates. Although there was no information about transcripts and protein levels of *Recql4* in the paper, presumably this targeting strategy generated a null mutation as a result of nonsense-mediated decay.

The second mouse model by Hoki et al. targeted exon 13 of the helicase domain of the *Recql4* gene with a neomycin cassette [30]. The mutant *Recql4* transcripts without exon 13 could be detected by RT-PCR in the primary mouse embryonic fibroblasts (MEFs) and testes of these mutant mice, but with significantly reduced expression levels indicating that the stability of the mutant transcripts could be affected by the neomycin cassette. These homozygous mutants were viable at birth, but 95 % of them died within 2 weeks. The remaining 5 % exhibited growth retardation, skin atrophy, hair abnormalities, and tissue hypoplasia, such as severely reduced bone trabeculae and fewer and smaller villi of the small intestine. The MEFs from these mutants showed reduced proliferation. However, there was no malignancy reported in these mice. It is worth noting that the authors also showed that a number of short transcripts spanning exon 1–12 of *Recql4* could be detected, indicating that the relatively milder phenotypes in this mouse model compared with the first model could be due to these potentially translated truncated Recql4 proteins.

The third mouse model was generated by replacing exons 9–13 in the conserved helicase domain of *Recql4* with a *PGK-HPRT* cassette [50]. Homozygous mutants were born alive with normal Mendelian ratio, but 16 % of them died within 24 h of birth. The remaining mutants exhibited tail pigmentation defects by 12 months, and palatal patterning defects were seen in all examined animals. Furthermore, 5.7 % of these mutants developed limb defects at birth, ranging from preaxial polydactyly of hindlimbs to forelimb aplasia. Transcript analysis showed weak and truncated products spanning exon 1–8 of *Recql4*. Chromosome analysis showed that these mutants had significantly increased aneuploidy in MEFs as well as in bone marrow cells and lymphocytes. Premature centromere separation was found to be markedly increased in the mutant cells. Interestingly, 5 % of these mutants developed OS or lymphoma by 20 months, while heterozygous and WT mice had no tumor formation, although this difference was not found to be statistically significant. Mutants were crossed with Apc^{min} mice which are highly susceptible to spontaneous intestinal adenoma formation and die by 120 days of age [56]. The double mutants had larger and increased incidence of macroadenomas in the gastrointestinal tract. However, there was no difference in tumor histological grade between double mutants and Apc^{min} mice.

These three mouse models were generated by targeting different regions of the mouse *Recql4* gene which could explain the different phenotypes between mutant mice. Viable mice in the latter two models exhibited phenotypes mimicking some of the clinical findings in *RECQL4* associated diseases, e.g., pigmentation defects, hair and skin abnormalities, and limb defects. However, these mutant mice failed to recapitulate the dramatic OS susceptibility observed in patients carrying *RECQL4* mutations. The first model gave the strongest "null" phenotype, indicating that exons 5–8 may be an ideal targeting region for studying the function of this gene. However, due to early embryonic lethality, it was not possible to analyze the function of Recql4 in development and tumorigenesis in these mice. Generation of a conditional tissue-specific knockout mouse model would be an alternative strategy to circumvent this issue.

Generation of a Conditional (Bone-Specific) Mouse Model of RTS

In an attempt to further investigate the function of RECQL4 in both skeletal development and OS pathogenesis in vivo, our laboratory has generated a conditional allele of mouse *Recql4* by gene targeting. Based on the first mouse model by Ichikawa et al. in which global targeting of exons 5–8 resulted in embryonic lethality, we flanked exons 5–8 by *loxP* sites. Utilizing the *Cre/loxP* recombination system, a conditional knockout of *Recql4* specifically in the mouse skeletal system can then be generated using a variety of transgenic mouse lines expressing Cre recombinase controlled by promoters expressed at different stages of the skeletal lineage [20]. These include the paired-related homeobox gene-1 (Prx1, primarily expressed in mesenchymal progenitor cells) [46], osterix (Osx, primarily expressed in osteoprogenitor cells), collagen type 1 alpha (Col1a1, primarily expressed in osteoblasts), and collagen type 2 alpha (Col2a1, primarily expressed in chondrocytes). Early results show that conditional deletion of *Recql4* using Prx1-Cre (*Recql4*$^{fl/fl}$; *Prx1-Cre*$^{+}$) leads to limb developmental defects (deformed forelimbs, aplasia/hypoplasia of digits, missing patellae, and low bone mass), growth retardation, and bilateral synostoses of cranial coronal and squamosal sutures, which faithfully recapitulate the major skeletal findings in RECQL4-related human disorders. *Recql4*$^{fl/fl}$; *Prx1-Cre*$^{+}$ mutant embryos develop smaller forelimbs at embryonic day E12.5 and exhibit markedly increased apoptosis in the forelimb tissues. Real-time RT-PCR analysis of mutant E13.5 forelimbs display dramatically increased transcripts of the pro-apoptotic factor *Bax* and cyclin-dependent kinase inhibitor 1A (*CDKN1A/p21*) which induces cell cycle arrest, indicating activation of the p53 signaling pathway. Western blot analysis shows that the level of phosphorylated p53 at residue serine 15 is strongly increased, while the transcripts and protein levels of unmodified p53 are unchanged in the forelimbs of *Recql4*$^{fl/fl}$; *Prx1-Cre*$^{+}$ mutants (data not shown). Our in vivo data indicate that inactivation of *Recql4* in mouse skeletal progenitor cells leads to increased p53 activation which could be caused by replication defects and/or DNA damage or by increased p53 nuclear stabilization [16]. The skeletal findings in RTS patients carrying *RECQL4* mutations may be a result of elevated genome instability leading to p53 activation which results in increased cell death and cell cycle arrest in skeletal progenitor cells during development. For OS tumorigenesis in RTS patients, a small proportion of cells may somehow escape the increased p53 activation and acquire additional mutations or oncogenic events that lead to the development of malignant tumor clones. Interestingly, those *Recql4*$^{fl/fl}$; *Prx1-Cre*$^{+}$ mutant mice that survived the weaning period had no increased OS susceptibility at 18 months although they had severely deformed limbs. This could be partially accounted for by differences between humans and mice, but other underlying mechanisms of osteosarcomagenesis in the setting of Recql4 deficiency are currently actively being investigated. For example, crossing the floxed *Recql4* mice with a mouse model whereby floxed p53 is inactivated using the Prx1-Cre transgene and which gives rise to OS [8, 44] may provide further information which will allow us to dissect the exact function of RECQL4 in OS pathogenesis.

Implications for Understanding and Potentially Targeting RECQ-Related Pathways for Cancer Therapy

Based on the roles of the RECQ proteins in normal cellular proliferation, DNA damage response, DNA repair, and telomere maintenance, there is growing interest in exploring inhibition of these functions in susceptible cancer cell types. Recent work has identified small molecule inhibitors of the WRN protein [1] and BLM protein [59] as potential anti-proliferative cancer therapies. Both of these molecules were identified through in vitro helicase activity screens. The WRN inhibitor, a small molecule inhibitor identified from the National Cancer Institute Diversity Set, designated NSC 19630 [2], was shown to inhibit cell proliferation and to induce apoptosis in a WRN-dependent manner. It also caused increase in double-strand breaks and accumulation of blocked replication forks in human tumor cells grown in culture. NSC 19630 also had a synergistic effect on inhibiting cell proliferation when cells were co-treated along with telomestatin, a small molecule that binds G4 structures and causes disruption of telomere associated proteins, as well as a PARP inhibitor KU0058948. It also acted synergistically with the topoisomerases inhibitor topotecan in inducing double-strand breaks. Investigators later characterized a structurally related compound, NSC 617145, which they demonstrated was able to sensitize cancer cells to mitomycin C, resulting in decreased cell proliferation, increased DNA damage and chromosomal abnormalities [1]. Taken together, small molecule inhibitors to the WRN protein may be useful to enhance existing or developing DNA-damaging anticancer therapies, particularly in tumor cell types with DNA repair deficiencies.

The small molecule inhibitor of BLM, ML216 [59], was found to exert its action by preventing BLM from binding to DNA. Cells treated with ML216 showed decreased proliferation as well as an increase in sister chromatid exchanges, a hallmark of Bloom syndrome. One of the proposed future uses of this BLM-specific inhibitor would be to test its efficacy to treat tumor cells that depend on the ALT (alternative lengthening of telomeres) mechanism for maintenance of telomeres, since previous work showed that the BLM ortholog Sgs1 is required for telomere maintenance in the absence of telomerase. Approximately 5–10 % of tumors depend on the ALT pathway for continued proliferation, including osteosarcomas; therefore, further exploration of this BLM-specific inhibitor could open up a new therapeutic strategy for targeting susceptible tumors.

Expression of *RECQL4* has been found to be upregulated in a variety of cancer types in addition to sporadic OS [49, 66], including soft tissue sarcomas [45], prostate cancer [76], and cervical cancer [58], suggesting that inactivation of RECQL4, and thus inhibition of its functions in cellular replication/viability, genome stability, DNA repair, and telomere maintenance may be attractive as a potential adjunct to cancer therapy in susceptible tumor cells. Ongoing basic science research is needed to fully understand the cellular context and molecular mechanisms by which RECQL4 exerts its actions in tumorigenesis.

References

1. Aggarwal M, Banerjee T, Sommers JA et al (2013) Targeting an Achilles' heel of cancer with a WRN helicase inhibitor. Cell Cycle 12(20):3329–3335
2. Aggarwal M, Sommers JA, Shoemaker RH et al (2011) Inhibition of helicase activity by a small molecule impairs Werner syndrome helicase (WRN) function in the cellular response to DNA damage or replication stress. Proc Natl Acad Sci U S A 108(4):1525–1530
3. Bachrati CZ, Hickson ID (2003) RecQ helicases: suppressors of tumorigenesis and premature aging. Biochem J 374(Pt 3):577–606
4. Belyea B, Kephart JG, Blum J et al (2012) Embryonic signaling pathways and rhabdomyosarcoma: contributions to cancer development and opportunities for therapeutic targeting. Sarcoma 2012:406239
5. Bernstein KA, Gangloff S, Rothstein R (2010) The RecQ DNA helicases in DNA repair. Annu Rev Genet 44:393–417
6. Burks LM, Yin J, Plon SE (2007) Nuclear import and retention domains in the amino terminus of RECQL4. Gene 391(1–2):26–38
7. Cabral RE, Queille S, Bodemer C et al (2008) Identification of new RECQL4 mutations in Caucasian Rothmund-Thomson patients and analysis of sensitivity to a wide range of genotoxic agents. Mutat Res 643(1–2):41–47
8. Calo E, Quintero-Estades JA, Danielian PS et al (2010) Rb regulates fate choice and lineage commitment in vivo. Nature 466(7310):1110–1114
9. Capp C, Wu J, Hsieh TS (2009) Drosophila RecQ4 has a 3′-5′ DNA helicase activity that is essential for viability. J Biol Chem 284(45):30845–30852
10. Chi Z, Nie L, Peng Z et al (2012) RecQL4 cytoplasmic localization: implications in mitochondrial DNA oxidative damage repair. Int J Biochem Cell Biol 44(11):1942–1951
11. Chu WK, Hickson ID (2009) RecQ helicases: multifunctional genome caretakers. Nat Rev Cancer 9(9):644–654
12. Cleton-Jansen AM, Anninga JK, Briaire-de Bruijin IH et al (2009) Profiling of high-grade central osteosarcoma and its putative progenitor cells identifies tumourigenic pathways. Br J Cancer 101(11):1909–1918
13. Crevel G, Vo N, Crevel I et al (2012) Drosophila RecQ4 is directly involved in both DNA replication and the response to UV damage in S2 cells. PLoS One 7(11):e49505
14. Croteau DL, Rossi ML, Canugovi C et al (2012) RECQL4 localizes to mitochondria and preserves mitochondrial DNA integrity. Aging Cell 11(3):456–466
15. Croteau DL, Rossi ML, Ross J et al (2012) RAPADILINO RECQL4 mutant protein lacks helicase and ATPase activity. Biochim Biophys Acta 1822(11):1727–1734
16. De S, Kumari J, Mudgal R et al (2012) RECQL4 is essential for the transport of p53 to mitochondria in normal human cells in the absence of exogenous stress. J Cell Sci 125(Pt 10):2509–2522
17. Debeljak M, Zver A, Jazbec J (2009) A patient with Baller-Gerold syndrome and midline NK/T lymphoma. Am J Med Genet A 149A(4):755–759
18. Dietschy T, Shevelev I, Pena-Diaz J et al (2009) p300-mediated acetylation of the Rothmund-Thomson-syndrome gene product RECQL4 regulates its subcellular localization. J Cell Sci 122(Pt 8):1258–1267
19. el-Khoury JM, Haddad SN, Atallah NG (1997) Osteosarcomatosis with Rothmund-Thomson syndrome. Br J Radiol 70:215–218
20. Elefteriou F, Yang X (2011) Genetic mouse models for bone studies strengths and limitations. Bone 49(6):1242–1254
21. Ellis NA, Groden J, Ye TZ et al (1995) The Bloom's syndrome gene product is homologous to RecQ helicases. Cell 83(4):655–666
22. Fan W, Luo J (2008) RecQ4 facilitates UV light-induced DNA damage repair through interaction with nucleotide excision repair factor xeroderma pigmentosum group A (XPA). J Biol Chem 283(43):29037–29044

23. Ferrarelli LK, Popuri V, Ghosh AK et al (2013) The RECQL4 protein, deficient in Rothmund-Thomson syndrome is active on telomeric D-loops containing DNA metabolism blocking lesions. DNA Repair (Amst) 12(7):518–528
24. German J (1995) Bloom's syndrome. Dermatol Clin 13(1):7–18
25. German J (1997) Bloom's syndrome. XX. The first 100 cancers. Cancer Genet Cytogenet 93(1):100–106
26. Ghosh AK, Rossi ML, Singh DK et al (2012) RECQL4, the protein mutated in Rothmund-Thomson syndrome, functions in telomere maintenance. J Biol Chem 287(1):196–209
27. Glass DA, Karsenty G (2007) In vivo analysis of Wnt signaling in bone. Endocrinology 148(6):2630–2634
28. Grandori C, Wu KJ, Fernandez P et al (2003) Werner syndrome protein limits MYC-induced cellular senescence. Genes Dev 17(13):1569–1574
29. Hicks MJ, Roth JR, Kozinetz CA et al (2007) Clinicopathologic features of osteosarcoma in patients with Rothmund-Thomson syndrome. J Clin Oncol 25(4):370–375
30. Hoki Y, Araki R, Fujimori A et al (2003) Growth retardation and skin abnormalities of the Recql4-deficient mouse. Hum Mol Genet 12(18):2293–2299
31. Hu H, Hilton MJ, Tu X et al (2005) Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development 132(1):49–60
32. Ichikawa K, Noda T, Furuichi Y (2002) Preparation of the gene targeted knockout mice for human premature aging diseases, Werner syndrome, and Rothmund-Thomson syndrome caused by the mutation of DNA helicases. Nippon Yakurigaku Zasshi 119(4):219–226
33. Im JS, Ki SH, Farina A et al (2009) Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. Proc Natl Acad Sci U S A 106(37):15628–15632
34. Jensen MB, Dunn CA, Keijzers G et al (2012) The helicase and ATPase activities of RECQL4 are compromised by mutations reported in three human patients. Aging (Albany NY) 4(11):790–802
35. Jin W, Liu H, Zhang Y et al (2008) Sensitivity of RECQL4-deficient fibroblasts from Rothmund-Thomson syndrome patients to genotoxic agents. Hum Genet 123(6):643–653
36. Kamimura Y, Masumoto H, Sugino A et al (1998) Sld2, which interacts with Dpb11 in *Saccharomyces cerevisiae*, is required for chromosomal DNA replication. Mol Cell Biol 18(10):6102–6109
37. Kansara M, Tsang M, Kodjabachian L et al (2009) Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcomagenesis in mice. J Clin Invest 119(4):837–851
38. Kitao S, Ohsugi I, Ichikawa K et al (1998) Cloning of two new human helicase genes of the RecQ family: biological significance of multiple species in higher eukaryotes. Genomics 54(3):443–452
39. Kitao S, Shimamoto A, Goto M et al (1999) Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. Nat Genet 22(1):82–84
40. Kobbe D, Focke M, Puchta H (2010) Purification and characterization of RecQ helicases of plants. Methods Mol Biol 587:195–209
41. Kohzaki M, Chiourea M, Versini G et al (2012) The helicase domain and C-terminus of human RecQL4 facilitate replication elongation on DNA templates damaged by ionizing radiation. Carcinogenesis 33(6):1203–1210
42. Kumata Y, Tada S, Yamanada Y et al (2007) Possible involvement of RecQL4 in the repair of double-strand DNA breaks in Xenopus egg extracts. Biochim Biophys Acta 1773(4):556–564
43. Lauper JM, Krause A, Vaughan TL et al (2013) Spectrum and risk of neoplasia in Werner syndrome: a systematic review. PLoS One 8(4):e59709
44. Lin PP, Pandey MK, Jin F et al (2009) Targeted mutation of p53 and Rb in mesenchymal cells of the limb bud produces sarcomas in mice. Carcinogenesis 30(10):1789–1795
45. Linton KM, Hey Y, Saunders E et al (2008) Acquisition of biologically relevant gene expression data by Affymetrix microarray analysis of archival formalin-fixed paraffin-embedded tumours. Br J Cancer 98(8):1403–1414

46. Logan M, Martin JF, Nagy A et al (2002) Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis 33(2):77–80
47. Luo X, Kraus WL (2012) On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1. Genes Dev 26(5):417–432
48. Macris MA, Krejci L, Bussen W et al (2006) Biochemical characterization of the RECQ4 protein, mutated in Rothmund-Thomson syndrome. DNA Repair (Amst) 5(2):172–180
49. Maire G, Yoshimoto M, Chilton-Macneill S et al (2009) Recurrent RECQL4 imbalance and increased gene expression levels are associated with structural chromosomal instability in sporadic osteosarcoma. Neoplasia 11(3):260–268, 3p
50. Mann MB, Hodges CA, Barnes E et al (2005) Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund-Thomson syndrome. Hum Mol Genet 14(6):813–825
51. Martin GM, Oshima J (2000) Lessons from human progeroid syndromes. Nature 408(6809):263–266
52. Matsuno K, Kumano M, Kubota Y et al (2006) The N-terminal noncatalytic region of Xenopus RecQ4 is required for chromatin binding of DNA polymerase alpha in the initiation of DNA replication. Mol Cell Biol 26(13):4843–4852
53. Mehollin-Ray AR, Kozinetz CA, Schlesinger AE et al (2008) Radiographic abnormalities in Rothmund-Thomson syndrome and genotype-phenotype correlation with RECQL4 mutation status. AJR Am J Roentgenol 191(2):W62–W66
54. Monnat RJ Jr (2010) Human RECQ helicases: roles in DNA metabolism, mutagenesis and cancer biology. Semin Cancer Biol 20(5):329–339
55. Morozov V, Mushegian AR, Koonin EV et al (1997) A putative nucleic acid-binding domain in Bloom's and Werner's syndrome helicases. Trends Biochem Sci 22(11):417–418
56. Moser AR, Pitot HC, Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247(4940):322–324
57. Nakayama K, Irino N, Nakayama H (1985) The recQ gene of *Escherichia coli* K12: molecular cloning and isolation of insertion mutants. Mol Gen Genet 200(2):266–271
58. Narayan G, Bourdon V, Chaganti S et al (2007) Gene dosage alterations revealed by cDNA microarray analysis in cervical cancer: identification of candidate amplified and overexpressed genes. Genes Chromosomes Cancer 46(4):373–384
59. Nguyen GH, Dexheimer TS, Rosenthal AS et al (2013) A small molecule inhibitor of the BLM helicase modulates chromosome stability in human cells. Chem Biol 20(1):55–62
60. Nishijo K, Nakayama T, Aoyama T et al (2004) Mutation analysis of the RECQL4 gene in sporadic osteosarcomas. Int J Cancer 111(3):367–372
61. Ohlenschlager O, Kuhnert A, Schneider A et al (2012) The N-terminus of the human RecQL4 helicase is a homeodomain-like DNA interaction motif. Nucleic Acids Res 40(17):8309–8324
62. Park SJ, Lee YJ, Beck BD et al (2006) A positive involvement of RecQL4 in UV-induced S-phase arrest. DNA Cell Biol 25(12):696–703
63. Petkovic M, Dietschy T, Freire R et al (2005) The human Rothmund-Thomson syndrome gene product, RECQL4, localizes to distinct nuclear foci that coincide with proteins involved in the maintenance of genome stability. J Cell Sci 118(Pt 18):4261–4269
64. Rossi ML, Ghosh AK, Kulikowicz T et al (2010) Conserved helicase domain of human RecQ4 is required for strand annealing-independent DNA unwinding. DNA Repair (Amst) 9(7):796–804
65. Rothmund A (1868) Ueber cataracten in verbindung mit einer eigenthumlichen hautdegeneration. Arch Ophthamol 14:159–182
66. Sadikovic B, Thorner P, Chilton-Macneill S et al (2010) Expression analysis of genes associated with human osteosarcoma tumors shows correlation of RUNX2 overexpression with poor response to chemotherapy. BMC Cancer 10:202
67. Sangrithi MN, Bernal JA, Madine M et al (2005) Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome. Cell 121(6):887–898
68. Schurman SH, Hedayati M, Wang Z et al (2009) Direct and indirect roles of RECQL4 in modulating base excision repair capacity. Hum Mol Genet 18(18):3470–3483

69. Sengupta S, Shimamoto A, Koshiji M et al (2005) Tumor suppressor p53 represses transcription of RECQ4 helicase. Oncogene 24(10):1738–1748
70. Shinya A, Nishigori C, Moriwaki S et al (1993) A case of Rothmund-Thomson syndrome with reduced DNA repair capacity. Arch Dermatol 129(3):332–336
71. Siitonen HA, Kopra O, Kaariainen H et al (2003) Molecular defect of RAPADILINO syndrome expands the phenotype spectrum of RECQL diseases. Hum Mol Genet 12(21):2837–2844
72. Siitonen HA, Sotkasiira J, Biervliet M et al (2008) The mutation spectrum in RECQL4 diseases. Eur J Hum Genet 17(2):151–158
73. Singh DK, Karmakar P, Aamann M et al (2010) The involvement of human RECQL4 in DNA double-strand break repair. Aging Cell 9(3):358–371
74. Singh DK, Popuri V, Kulikowicz T et al (2012) The human RecQ helicases BLM and RECQL4 cooperate to preserve genome stability. Nucleic Acids Res 40(14):6632–6648
75. Smith PJ, Paterson MC (1982) Enhanced radiosensitivity and defective DNA repair in cultured fibroblasts derived from Rothmund Thomson syndrome patients. Mutat Res 94(1):213–228
76. Su Y, Meador JA, Calaf GM et al (2010) Human RecQL4 helicase plays critical roles in prostate carcinogenesis. Cancer Res 70(22):9207–9217
77. Suzuki T, Kohno T, Ishimi Y (2009) DNA helicase activity in purified human RECQL4 protein. J Biochem 146(3):327–335
78. Tanaka S, Komeda Y, Umemori T et al (2013) Efficient initiation of DNA replication in eukaryotes requires Dpb11/TopBP1-GINS interaction. Mol Cell Biol 33(13):2614–2622
79. Tao J, Chen S, Lee B (2010) Alteration of Notch signaling in skeletal development and disease. Ann N Y Acad Sci 1192:257–268
80. Taylor WB (1957) Rothmund's syndrome-Thomson's syndrome. Arch Dermatol 75:236–244
81. Thangavel S, Mendoza-Maldonado R, Tissino E et al (2010) Human RECQ1 and RECQ4 helicases play distinct roles in DNA replication initiation. Mol Cell Biol 30(6): 1382–1396
82. Thomson MS (1923) An hitherto undescribed familial disease. Br J Dermatol 35:455–462
83. Tuteja N, Tuteja R (2004) Prokaryotic and eukaryotic DNA helicases. Essential molecular motor proteins for cellular machinery. Eur J Biochem 271(10):1835–1848
84. Van Maldergem L, Piard J, Larizza L, Wang LL (2013) RECQL4-related recessive conditions. In: Epstein CJ, Erickson RP, Winshaw-Boris A (eds) Inborn errors of development, 3rd edn. Oxford University Press, Oxford
85. Van Maldergem L, Siitonen HA, Jalkh N et al (2006) Revisiting the craniosynostosis-radial ray hypoplasia association: Baller-Gerold syndrome caused by mutations in the RECQL4 gene. J Med Genet 43(2):148–152
86. Vijayakumar S, Liu G, Rus IA et al (2011) High-frequency canonical Wnt activation in multiple sarcoma subtypes drives proliferation through a TCF/beta-catenin target gene, CDC25A. Cancer Cell 19(5):601–612
87. Wang H, Elledge SJ (1999) DRC1, DNA replication and checkpoint protein 1, functions with DPB11 to control DNA replication and the S-phase checkpoint in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 96(7):3824–3829
88. Wang LL (2005) Biology of osteogenic sarcoma. Cancer J 11(4):294–305
89. Wang LL, Gannavarapu A, Kozinetz CA et al (2003) Association between osteosarcoma and deleterious mutations in the RECQL4 gene in Rothmund-Thomson syndrome. J Natl Cancer Inst 95(9):669–674
90. Wang LL, Levy ML, Lewis RA et al (2001) Clinical manifestations in a cohort of 41 Rothmund-Thomson syndrome patients. Am J Med Genet 102(1):11–17
91. Werner SR, Prahalad AK, Yang J et al (2006) RECQL4-deficient cells are hypersensitive to oxidative stress/damage: insights for osteosarcoma prevalence and heterogeneity in Rothmund-Thomson syndrome. Biochem Biophys Res Commun 345(1):403–409
92. Woo LL, Futami K, Shimamoto A et al (2006) The Rothmund-Thomson gene product RECQL4 localizes to the nucleolus in response to oxidative stress. Exp Cell Res 312(17):3443–3457

93. Wu J, Capp C, Feng L et al (2008) Drosophila homologue of the Rothmund-Thomson syndrome gene: essential function in DNA replication during development. Dev Biol 323(1):130–142
94. Xu L, Li S, Stohr BA (2013) The role of telomere biology in cancer. Annu Rev Pathol 8:49–78
95. Xu X, Liu Y (2009) Dual DNA unwinding activities of the Rothmund-Thomson syndrome protein, RECQ4. EMBO J 28(5):568–577
96. Xu X, Rochette PJ, Feyissa EA et al (2009) MCM10 mediates RECQ4 association with MCM2-7 helicase complex during DNA replication. EMBO J 28(19):3005–3014
97. Xu Y, Lei Z, Huang H et al (2009) dRecQ4 is required for DNA synthesis and essential for cell proliferation in Drosophila. PLoS One 4(7):e6107
98. Yin J, Kwon YT, Varshavsky A et al (2004) RECQL4, mutated in the Rothmund-Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. Hum Mol Genet 13(20):2421–2430
99. Yu CE, Oshima J, Fu YH et al (1996) Positional cloning of the Werner's syndrome gene. Science 272(5259):258–262
100. Zhang P, Yang Y, Zweidler-McKay PA et al (2008) Critical role of notch signaling in osteosarcoma invasion and metastasis. Clin Cancer Res 14(10):2962–2969

Autophagy in Osteosarcoma

Janice Santiago O'Farrill and Nancy Gordon

Abstract Osteosarcoma (OS) metastatic disease is resistant to conventional chemotherapy. Tumor resistance to chemotherapy has been one of the major areas of concern to clinicians and the topic of many laboratory investigators. Evaluation of mechanisms implicated in OS lung metastasis resistance to chemotherapy has been the focus of some of our most recent work. We have previously demonstrated the therapeutic efficacy of aerosol gemcitabine (GCB) in OS lung metastases. However, a subset of cells fails to respond to GCB treatment and persists as isolated lung metastases in vivo. Autophagy, a physiological mechanism that supports nutritional deprivation under stressful conditions, has been implicated in tumor resistance to chemotherapy. We demonstrated the induction of autophagy by GCB in LM7 metastatic human OS cells and K7M3 metastatic murine OS cells. Inhibition of autophagy resulted in increased sensitivity to GCB in LM7 cells. By contrast, inhibiting autophagy in K7M3 cells decreased GCB sensitivity. Defining the role autophagy plays in chemotherapy response in different tumor types has become of greater importance in order to identify the best suitable therapeutic approach. In this chapter, we summarize some of the most recent work related to autophagy in OS, identify some of the known mechanisms, and address the different roles autophagy plays in chemotherapy response.

Keywords Osteosarcoma • Autophagy • Pro-tumorigenic • Tumor suppressor • Clinical trials • Hydroxychloroquine

J.S. O'Farrill • N. Gordon (✉)
Department of Pediatrics-Research,
The Children's Cancer Hospital, University of Texas M.D. Anderson Cancer Center,
Houston, TX 77030, USA
e-mail: ngordon@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_8,

Introduction

Osteosarcoma (OS) overall survival has remained the same in the last 15 years. Pulmonary metastasis continues to be a major concern in patients with OS. New therapeutic strategies are being sought. Using a human OS mouse model, we have previously demonstrated that aerosol Gemcitabine (GCB) has a significant therapeutic effect in OS lung metastases [1]. Nevertheless, a subset of cells fails to respond to GCB treatment and persist as small isolated lung metastases in vivo (Fig. 1). Resistance to GCB is also evidenced by survival of OS cells after treatment in vitro. One of the main concerns and limitations of cancer treatment is acquired resistance by tumor cells. Scientists have focused on trying to understand the underlying molecular mechanisms implicated in tumor resistance to chemotherapy to identify targets that will enhance therapeutic efficacy and further improve patient survival. For this purpose, autophagy constitutes one of the mechanisms most recently studied.

Autophagy, a Greek term that refers to "self-eating," is a catabolic process with a housekeeping role in protein turnover that allows cells to eliminate unwanted proteins or damaged organelles. Under stressful conditions such as starvation, hypoxia, and cytotoxicity, it serves as a source of energy, providing the cells amino acids to sustain cell survival [2, 3]. It exerts a critical role in different biological functions that implicates not only cell survival but also cell metabolism and development. It has also been related to different physiological and pathophysiological settings such as aging, muscular diseases, neurodegenerative disorders, and cancer [4]. Even though the process of autophagy has been mostly defined as a protective mechanism to enhance cell survival under adverse environmental conditions, recent evidence suggests that excessive autophagy can lead to cell death. To this end, autophagy has been recognized as programmed cell death (PCD) type II. PCD type I is apoptotic cell death and type III programmed necrosis [5, 6]. These processes are not mutually exclusive. They can occur simultaneously or sequentially depending on the cell context and the stimulus. Additionally, autophagy can work as an independent

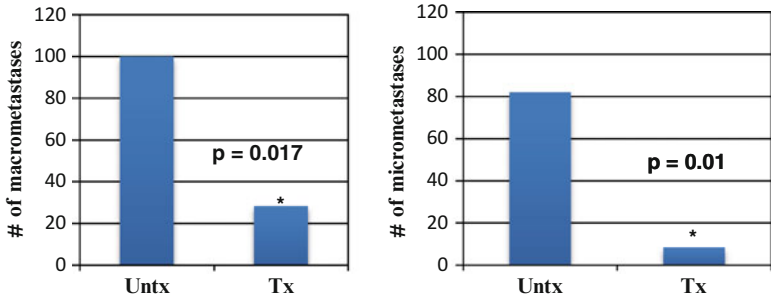


Fig. 1 Therapeutic effect of aerosol Gemcitabine (GCB) in K7M3 murine osteosarcoma (OS) lung metastases. Balb/c mice were injected i.v. with K7M3 OS cells. Aerosol GCB was started 10 days after i.v. injection for 3 weeks. Mice were sacrificed and macro- and micrometastases were quantified

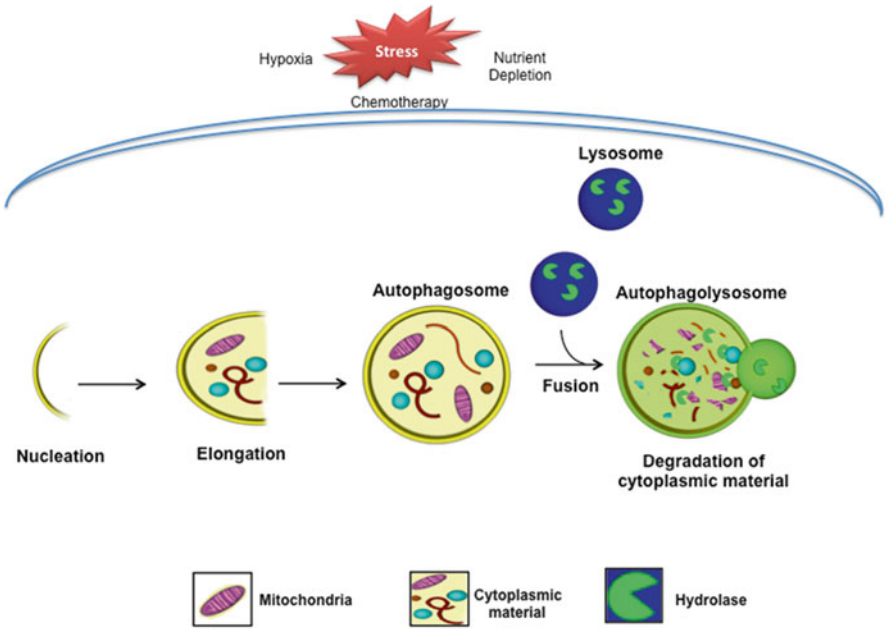


Fig. 2 The process of autophagy

process capable of antagonizing or delaying apoptosis. The cross talk between these processes in tumorigenesis is not clear, but it is thought to be determined by the tumor type, context, and disease stage [3, 7].

Three different types of autophagy have been described: (1) Chaperone mediated-autophagy, (2) microautophagy, and (3) macroautophagy. They all differ in the delivery of the substrates to the lysosome [5]. This chapter focuses on macroautophagy (hereafter referred as autophagy), which is the most studied type [5].

The process of autophagy has been divided into several steps that comprise induction, autophagosome formation-nucleation, elongation, completion, lysosome fusion, degradation, and recycling (Fig. 2). All steps are regulated by more than 30 autophagy-related (*Atg*) genes. Under situations of nutrient deprivation, hypoxic conditions, and/or chemotherapeutic treatment, activation of Atg 1 complex occurs leading to formation of a flat membrane cistern, phagophore, or isolation membrane and vesicle nucleation via activation of a complex formed by Vps34, a phosphatidyl inositol 3 kinase (PI3K) and Beclin1 one of the first characterized autophagy proteins. This last complex is involved in vesicle trafficking. Interaction of these complexes and other factors help to recluse proteins and lipids necessary for the autophagosome formation [2]. The next step is elongation, where the autophagosome formation is completed. This step is mediated by two ubiquitin-like systems. The first system is involved in the formation of Atg12, Atg5, and Atg16 complex which is mediated by the E1-like enzyme, Atg7. The second system regulates the conjugation of the microtubule-associated protein 1 light chain 3 (LC3-I/Atg8) with

phosphatidylethanolamine (PE). LC3 is first synthesized as an unprocessed form, proLC3 which is converted to a proteolytically processed form, LC3-I. LC3-I is cleaved by the protease Atg4, modified into the PE-conjugated form, LC3-II and translocated from the cytoplasm to the autophagosome membrane [3, 8]. LC3 is the only known marker for autophagosome. At this stage, the lysosome fuses to the autophagosome, forming the autolysosome. As a final step, proteins are degraded in the autolysosome and amino acids are released into the cytoplasm. These final products can be used for protein synthesis or can be oxidized by mitochondria electron transport chain to produce adenosine triphosphate (ATP) to use for cell survival.

Regulation of Autophagy in OS

Different mechanisms have been described to regulate the process of autophagy. The most studied mechanism involves the PI3K/AKT/mTOR pathway. The mammalian target of rapamycin (mTOR) kinase is downstream of the nutrient sensor PI3K and negatively regulates autophagy in response to nutrient deprivation [2, 9]. This process is accomplished through phosphorylation of many different proteins necessary to cell metabolism. Under normal nutrient conditions, PI3K/AKT/mTOR is activated, autophagy is suppressed and cells undergo normal proliferation [8, 10]. However, when cells are deprived from nutrients or under stressful conditions, mTOR is inhibited and autophagy is activated [9] (Fig. 3).

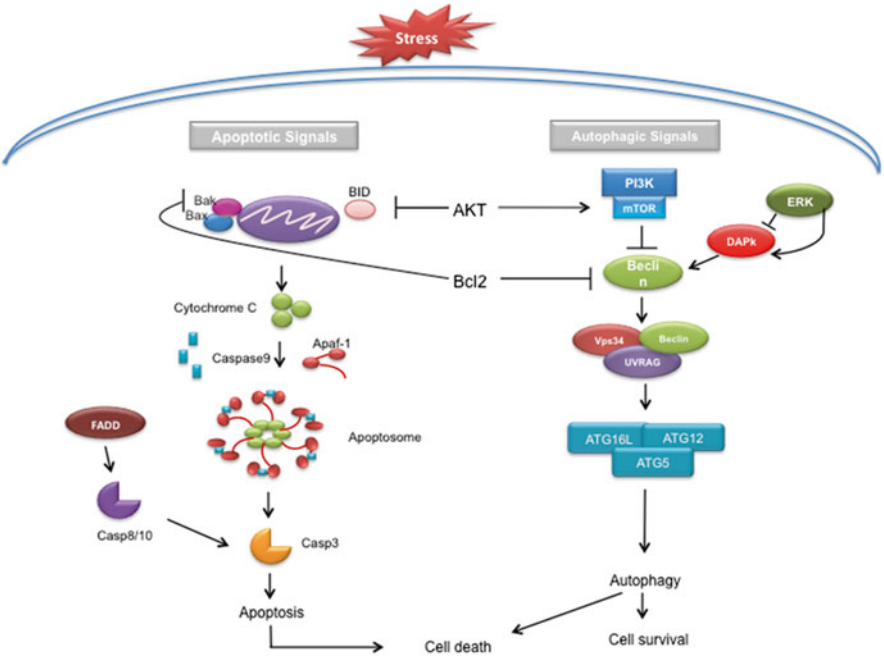


Fig. 3 Regulation of autophagy

Table 1 Mechanisms implicated in the induction of autophagy in osteosarcoma (OS)

Mechanism	Cell line	Reference
Barkor–UVRAG	U2OS	Sun et al. [16]
↑DRAM — (+)JNK	SaOS	Lorin et al. [17]
Hypoxia→p62 degradation	Solid tumors	Pursiheimo et al. [18, 19]
Rb —| E2F1→↓Bcl-2	SaOS	Jiang et al. [20]
RIP3	U2OS	Ciupienne et al. [21]
HMGB1→Beclin/Vps34	Mg63, U2OS, SaOS	Huang et al. [22, 23]
STAT3 —| PKR —| sIF2α	U2OS	Wong et al. [24]
↓PI3K/AKT/mTOR	K7M3/LM7	Santiago-O'Farril [55]

An additional mechanism found to participate in autophagy regulation and tumorigenesis involves Beclin-1, originally recognized as a Bcl-2 binding protein. It is part of a multi-protein complex formed by Vps34/class III PI3K. Beclin-1/Vps34 interaction can be modulated by anti-apoptotic molecules such as B cell lymphoma 2 (Bcl-2) and B cell lymphoma extralarge (Bcl-xL). Interaction of these molecules with the Beclin1 complex inactivates autophagy [7, 11–15].

In OS, activation of the Beclin1 complex by additional upstream mediators appears as the most common mechanism involved in induction of autophagy. Table 1 summarizes the most recent mechanisms implicated in the induction of autophagy in OS. Beclin1-associated autophagy-related key regulator (Barkor), a cloned autophagy specific-protein was shown to play a critical role in autophagy induction in U2OS human OS cells. Under starvation conditions, Barkor knock-down U2OS cells showed decrease autophagy as evidenced by decrease in LC3 II and autophagosome formation. Barkor re-expression increased autophagy. In this case, Barkor was shown to interact directly with Beclin1 and compete with UV radiation resistant gene product (UVRAG), an additional gene bound to the Vps34 complex, to induce autophagy [16]. In addition, human SaOS OS cells treated with an anti-tumoral compound 2-methoxyestradiol, upregulate damaged regulated autophagy modulator (DRAM), a p53 target gene, and enhance activation of c-Jun NH2 terminal kinase (JNK) which in turn activates autophagy by the induction of Bcl-2 phosphorylation and activation of Beclin 1/Vps34 complex [17]. Hypoxia, a common feature of solid tumors was also shown to induce autophagy by enhancing degradation of a multifunctional signal adaptor protein sequestosome 1 (SQSTM11/p62) by a mechanism independent of the hypoxic signal mediators, hypoxic inducible factor (HIF) [18, 19]. Furthermore, the well-known retinoblastoma (Rb) gene has been shown to activate autophagy in OS by repressing E2 transcription factor 1 (E2F1) which in turn down-modulates Bcl-2 allowing activation of the Beclin1/Vps34 complex [20]. Jiang et al. demonstrated that introduction of Rb into SaOS-2 human OS cells, induced autophagy confirmed by an increase in Beclin-1 expression. No changes were observed in mTOR phosphorylation suggesting that Rb-induced autophagy is more relevant to E2F1-mediated Bcl-2 expression than the mTOR pathway [20]. Moreover, the receptor-interacting protein 3 (RIP3), a key necrosis mediator has also been shown to play a role in activation of autophagy as a

protective mechanism in U2OS human OS cells [21]. High mobility group box 1 (HMGB1), a chromatin binding nuclear protein induces autophagy by controlling the formation of the Beclin1/Vps34 complex and decreasing OS sensitivity to chemotherapy. In accordance with this, three human OS cells MG63, SaOS, and U2OS transfected with HMGB1 cDNA showed decrease sensitivity to chemotherapy [22, 23].

Most recently an additional mechanism that involves the cytoplasmic form of the signal transducer and activator of transcription 3 (STAT3) was found to inhibit autophagy in U2OS human OS cells by a mechanism that involves inhibition of protein kinase R (PKR) which in turn inhibits phosphorylation of eukaryotic initiation factor 2α (eIF2α) a step required for the activation of autophagy [24]. Lastly, we have recent evidence to suggest that the activation of autophagy in human LM7 and murine K7M3 OS cells involves the PI3K/Akt/mTOR pathway. Gemcitabine, a nucleoside analog previously shown to induce autophagy in pancreatic tumor cells [25–27], induces autophagy in OS by decreasing Akt and mTOR phosphorylation and Beclin 1/Vps34 complex activation.

Overall, there is enough evidence to suggest that autophagy is induced in OS. Many intermediates are involved in the process. Nevertheless, the end result is determined by the trigger, the type, and context of the tumor cell and the tumor development stage.

Autophagy and Tumorigenesis: Cell Survival vs. Cell Death

Several studies have reported autophagy to exert a dual role in tumorigenesis. Under certain conditions, autophagy appears to contribute to either cell survival or cell death by either increasing or decreasing tumor sensitivity to chemotherapy [5, 28].

Autophagy as a Cell Death Mechanism

Currently, the role of autophagy in cancer has received increased interest within the scientific community since many chemotherapeutic agents induce autophagy in cancer cells [6, 7, 29]. Overall, chemotherapy constitutes the most effective treatment for many cancers and has been part of the standard of care for more than 50 years. This type of treatment targets cancer cells and induces cell death. Apoptosis and necrosis were thought to be the only mechanisms of drug-induced cell death [30]. Autophagy is now considered an additional mechanism to induce cell death.

The term "autophagic cell death" is used to describe an important form of cell death that results from excessive levels of cellular autophagy (cellular consumption), causing massive degradation of cellular content [31, 32]. This type of cell death is distinguished by the excessive presence of autophagic markers and morphological features [4]. Table 2 summarizes the major differences between apoptosis and autophagy. In general, cells undergoing autophagy are

Table 2 Difference between autophagy and apoptosis

	Apoptosis	Autophagy
Morphological characteristics	Nuclear fragmentation, DNA fragmentation, membrane blebbing, apoptotic bodies formation	Vacuolization, organelles, and proteins degradation
Biochemical features	Caspase activation, PARP cleavage, phagocytosis	LC3I/LC3II lipidation, p62/SQSTM1 degradation, lysosome activity
Stimulus	Oxidative stress	Starvation, hypoxia, chemotherapy
	Death receptor ligands	Growth factor deprivation
	Chemotherapy	PI3-kinase, mTOR inhibition
	Extrinsic and intrinsic signals	

morphologically characterized by the absence of chromatin condensation and by cytoplasmic vacuolation [33]. By contrary, apoptosis is characterized by chromatin condensation, cytoplasmic membrane blebbing, and formation of apoptotic bodies. In addition, autophagy is a caspase *independent* process hallmarked by the cleavage and lipidation of LC3I to LC3II and the degradation of another important protein called p62/SQSTM1 [34–37]. By contrast, apoptosis is a caspase *dependent* process that involves DNA degradation and cellular fragmentation [3]. Finally, in autophagy, the autophagic vesicles are destroyed and removed by the lysosomal system of the same cell [2, 33], whereas in apoptosis, apoptotic bodies are recognized by macrophages and removed by phagocytosis.

Autophagy as a Cell Survival Mechanism

Chemotherapy-induced autophagy has recently been described as a mechanism of tumor cell resistance. In an attempt to understand resistance to cancer chemotherapy, studies have focused on mechanisms thought to be specific to one drug class or another.

Most recently, different resistance patterns associated with the majority of nonspecific cytotoxic agents have been described. These patterns include alterations in drug transport, tumor-cell apoptosis, the DNA damage response, tumor microenvironment, and the function of cancer stem cells [38–41] (Fig. 4).

Within the *alterations in drug transport* are specific factors that influence drug uptake, metabolism, and sequestration. Factors specifically related to *drug uptake* include decrease expression or mutation of solute carriers responsible for cellular uptake of different chemotherapies. Decrease expression or mutation of these carriers impairs drug and plasma membrane interactions resulting in decrease intracellular accumulation of the drug and decrease therapeutic effect. Some of these solute carriers include SLC29A1, A2, and SLC28A1, described as solute carriers that mediate the cellular uptake of nucleoside analogs such as Gemcitabine.

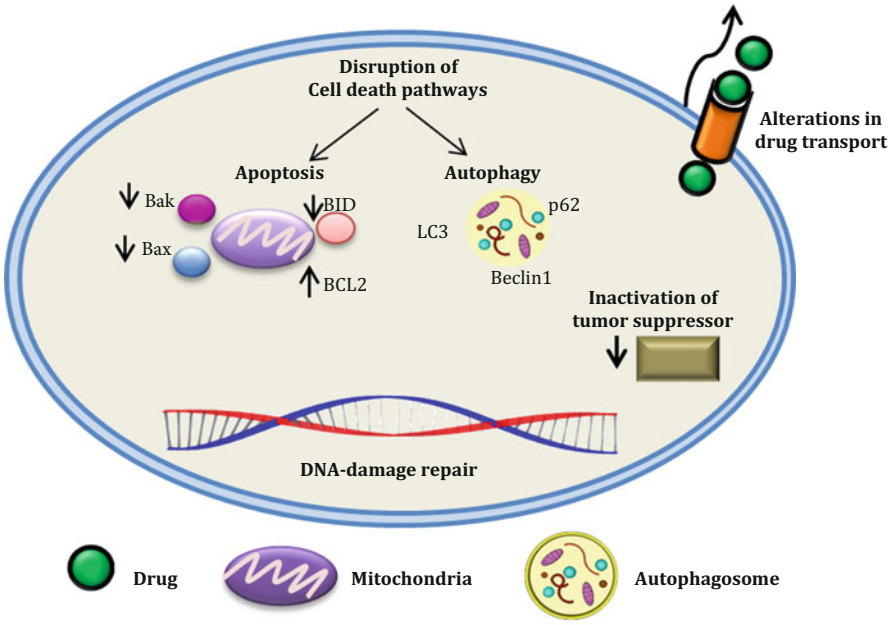


Fig. 4 Mechanisms implicated in chemotherapy resistance

In addition, proteins that belong to the ATP-binding cassette (ABC) transporter family, also consider drug efflux pumps, can affect drug uptake by maintaining a low intracellular drug concentration. 15 of these proteins have been characterized to confer resistance to most of the currently used anticancer drugs. The most frequently associated to multidrug resistance are p-glycoprotein (MDR, Pgp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1), and ABCG2. These proteins can be constitutively highly expressed in cells from which tumors originate or can be up-regulated by exposure of the tumor cells to the drugs. In regards to *drug metabolism*, alterations in the metabolic enzymes such as mutations can significantly decrease the success of chemotherapy treatment. Lastly, *drug sequestration* in secretory vesicles such as lysosome and Golgi can influence drug therapeutic effect and confer resistance [41].

The *DNA-damage response network* is another way by which cancer cells become resistant to chemotherapy. Many chemotherapeutic drugs induce cell death by causing DNA lesions. However, cancer cells have the ability to repair these lesions through a complex network of repair systems. This network involves the mismatch repair (MMR) pathway, the nucleotide excision repair (NER), the base excision repair (BER), homologous recombination (HR), and the non-homologous recombination pathway (NHR). Deficiencies in some of these DNA damage signaling pathways lead to chemoresistance since cell cycle arrest is not triggered upon DNA damage and the cells go through the cell cycle unrepaired.

The *tumor microenvironment* can also contribute to cancer cells resistance to chemotherapy. Enough evidence indicates the presence of hypoxic areas in most human solid tumors. Oxygen deficiency triggers activation of hypoxia-specific factors, the HIFs (Hypoxia-inducing factors) responsible for a lot of the cellular reprogramming that compromises the effectiveness of chemotherapy. In addition, tumor stromal cells have also been implicated in chemotherapy resistance in some instances due to adaptive signaling dialog between tumor cells and their surrounding environment or cell adhesion-mediated resistance.

Cancer stem cells can also influence chemotherapy response by their inherent insensitivity to chemotherapy. If tumors develop from CSCs, these are the cells that need to be killed upon treatment to eradicate the tumor. Any left intact will be responsible for tumor relapse, mainly because these cells are equipped with specialized defenses against anticancer drugs.

Evasion of drug induced cell death or tumor cell apoptosis also constitutes another mechanism of tumor cells resistance to chemotherapy. Apoptotic-cell death is the most conventional end result expected from chemotherapy treatments. However, tumor cells have developed smart ways to circumvent apoptotic signals. Some of them include overexpression of anti-apoptotic molecules such as Bcl-2, Mcl-1, Bcl-xL, FLIP, and IAPs or inactivation of pro-apoptotic molecules such as Bax and Bid and mutations in genes encoding caspases or alterations in the p53 pathway. Most recently, additional non-apoptotic mechanisms have been identified as cell death mechanisms. Disruption of these pathways has been a major limitation in the success of cancer treatment. Within the non-apoptotic mechanisms are necroptosis, senescence, and autophagy. Conventionally, *autophagy* has been described as a cell survival mechanism induced by cancer cells under stressful conditions. As such, autophagy has a cytoprotective role that enables cancer cells to cope with stresses. Autophagy, when triggered by chemotherapeutic drugs has shown to mostly participate in tumor resistance rather than cell death [38, 42].

In general, once autophagy is found to occur, identification of the role of autophagy in the specific tumor context is necessary to determine therapeutic interventions using autophagy inhibitors or activators as a way to improve cell killing efficacy of chemotherapeutic agents.

Implications of Autophagy in OS

In OS, several agents have shown to trigger autophagy and either increase drug sensitivity or induce drug resistance (Table 3). More specifically, natural products such as *curcumin*, *pancratistatin*, *and voacamine* (*VOA*) were shown to enhance the sensitivity of OS cells to the cytotoxic action of chemotherapy [43–49]. VOA induces autophagy in multidrug resistant (MDR) U2OS human OS cells as confirmed by electron microscopy and LC3 conversion. Treatment of MDR U2OS with doxorubicin in the presence of VOA increased cell death. Modulation of autophagy by pre-treatment with autophagy inhibitors or by transfection of OS cells with

Table 3 Dual role of autophagy in Osteosarcoma (OS)

Agents	Effect	References
Natural products (curcumin, pancratistatin, voacamine)	Tumor suppressor	[43–49]
BH3-mimetic GX 15-070	Tumor suppressor	[50]
Delta-24-RGD (conditionally replicating adenovirus)	Tumor suppressor	[51]
Cannabinoids	Unknown	[49]
Cisplatin	Pro-tumorigenic	[50]
Doxorubicin, cisplatin, methotrexate-mediated HMGB1 upregulation	Pro-tumorigenic	[22, 23]
Biphosphinic palladacycle complex (BPC)	Pro-tumorigenic	[48]
Natural product (Dihydroptychantol-DHA)	Pro-tumorigenic	[46]
Gemcitabine	Pro-tumorigenic and/or tumor suppressor	

siRNA against Atg genes reduced the ability of VOA to augment doxorubicin-mediated cytotoxicity [44]. The *BH3-mimetic agent, GX 15-070*, was also shown to have a tumor cell death promoter effect on U2OS human OS cells. Treatment of these cells with 3-methyladenine and chloroquine, inhibitors of autophagy through two different mechanisms, potentiated the GX15-070 cytotoxicity effect [50, 51]. Interestingly, *Cisplatin*, *Doxorubicin*, *and Methotrexate*, three of the agents included in the standard treatment of OS, were shown in at least three different studies, to induce autophagy in OS cells and promote tumor cell survival by inducing tumor cell resistance to chemotherapy [43, 50, 52, 53].

Using an OS mouse model, we have previously demonstrated that aerosol *Gemcitabine* (*GCB*) has a significant therapeutic effect in OS lung metastases as evidenced by a significant increase in the mean survival of the untreated vs. treated group (35 days vs. >155 days, respectively) [54]. However, OS cells resistance to GCB is evidenced by survival of cells after treatment in vitro and persistence of small isolated lung metastases in vivo. Most recently, our preliminary data suggests that GCB induces autophagy in different human (LM7, CCH-OS-D) and mouse (K7M3) OS cells and either increases sensitivity to chemotherapy contributing to cell death or promotes resistance and increase tumor cell survival. The difference in the autophagy effects induced by the treatment of the OS cell lines with GCB could not be attributed to difference in species since opposing effects were found in the two human OS cells tested (LM7 and CCH-OS-D) confirming once again that the different effects of autophagy induced by different chemotherapeutic agents is cell and context dependent.

In conclusion, the role of autophagy in the tumor cell's sensitivity or resistance to chemotherapy is complex. In OS, as shown in other tumors, autophagy plays a dual role either by promoting cell survival and tumor cell resistance to chemotherapy or by acting as one of the mechanisms responsible for chemotherapy-induced cell death. Better understanding of the molecular pathways that govern the process of autophagy will allow identification of a mode to modulate these pathways in order to enhance the activity of chemotherapy.

Modulation of Autophagy

Experimental studies have defined different stages where autophagy can be modulated to increase chemotherapy efficacy. Early- and late-stage autophagy inhibitors have been identified. The *early-stage inhibitors* target Vps34 directly and impede its recruitment to the membrane. The *late-stage inhibitors* act at different levels, however only two members of this group have been evaluated in humans. They include two known anti-malarial drugs chloroquine and hydroxychloroquine. Both drugs work by preventing acidification of lysosomes whose digestive hydrolases depend on low pH. Hydroxychloroquine is the most widely used due to its minor side effects. However, since excessive autophagy has the potential to induce cell death, *autophagy inducers* have been considered as potential cancer treatments. Since mTOR is one of the major autophagy down-regulators, the most used autophagy inducers are the mTOR inhibitors Rapamycin and its analogs Temsirolimus (CC-779), Everolimus (RAD-001), and Deforolimus (A3-23573). However, with the exception of certain types of cancers, these autophagy inducers have had limited activity in clinical trials.

In conclusion, different strategies have been identified to modulate the process of autophagy. However, more research is necessary to delineate the complex functions of the autophagy process. Specifically, there is a need to determine how autophagy fits into the picture of drug resistance or drug sensitivity. Currently, several studies are evaluating the effectiveness of these autophagy modulators in different types of cancers. So far, none of the studies have a particular focus in OS.

Summary

Eradication of OS pulmonary metastases remains the main challenge in OS. Here, we present *autophagy* as one of the mechanisms involved in OS resistance to chemotherapy. We summarized the most recent data to identify known mediators of autophagy in OS and address some of the different roles autophagy plays in OS. Understanding the role of autophagy, its regulatory mechanisms and how it affects chemotherapy-induced cell death may allow identification of potential agents to use in combination with conventional chemotherapy to improve disease-response and long-term survival in these patients. The dual role of autophagy underlines the necessity to carefully identify and define its role in tumor cells before applying autophagy-based therapies. It is of paramount importance to determine which cancer cell undergoes autophagy in response to therapy, and whether increased autophagy leads to chemotherapy resistance or is an important part of the drug-induced cell death process. Moreover, because only a subpopulation of cells undergo autophagy, it is unlikely for autophagy inhibitors to be used as single agents. As a consequence, the main strategy of ongoing studies is to incorporate anti-autophagic therapy into the existing anti-cancer regimens.

Acknowledgements We are grateful to Amy A. Sisson from the UT MD Anderson Research Medical library for her clerical assistance.

References

1. Gordon N, Kleinerman ES (2009) The role of Fas/FasL in the metastatic potential of osteosarcoma and targeting this pathway for the treatment of osteosarcoma lung metastases. Cancer Treat Res 152:497–508. doi:10.1007/978-1-4419-0284-9_29
2. Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P (2009) Major cell death pathways at a glance. Microbes Infect 11(13):1050–1062. doi:10.1016/j.micinf.2009.08.013
3. Gozuacik D, Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23(16):2891–2906. doi:10.1038/sj.onc.1207521
4. Shen HM, Codogno P (2011) Autophagic cell death: Loch Ness monster or endangered species? Autophagy 7(5):457–465
5. Aredia FGL, Giansanti V, Scovassi AI (2012) Autophagy and cancer. Cells 1(3):520–534
6. Sun Y, Peng ZL (2009) Programmed cell death and cancer. Postgrad Med J 85(1001):134–140. doi:10.1136/pgmj.2008.072629
7. Yang ZJ, Chee CE, Huang S, Sinicrope FA (2011) The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther 10(9):1533–1541. doi:10.1158/1535-7163.MCT-11-0047
8. Liu EY, Ryan KM (2012) Autophagy and cancer—issues we need to digest. J Cell Sci 125(Pt 10):2349–2358. doi:10.1242/jcs.093708
9. Huber A, Rubio A, Loewith RJ (2009) Mammalian target of rapamycin signaling and cancer treatment. Cancer Chemother Rev 4(4):210–223
10. Liu Q, Thoreen C, Wang J, Sabatini D, Gray NS (2009) MTOR mediated anti-cancer drug discovery. Drug Discov Today Ther Strat 6(2):47–55, http://dx.doi.org/10.1016/j.ddstr.2009.12.001
11. Kang R, Zeh HJ, Lotze MT, Tang D (2011) The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 18(4):571–580. doi:10.1038/cdd.2010.191
12. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122(6):927–939. doi:10.1016/j.cell.2005.07.002
13. Furuya N, Yu J, Byfield M, Pattingre S, Levine B (2005) The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. Autophagy 1(1):46–52
14. Ciechomska IA, Goemans GC, Skepper JN, Tolkovsky AM (2009) Bcl-2 complexed with Beclin-1 maintains full anti-apoptotic function. Oncogene 28(21):2128–2141. doi:10.1038/onc.2009.60
15. Boya P, Kroemer G (2009) Beclin 1: a BH3-only protein that fails to induce apoptosis. Oncogene 28(21):2125–2127. doi:10.1038/onc.2009.83
16. Sun Q, Fan W, Chen K, Ding X, Chen S, Zhong Q (2008) Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A 105(49):19211–19216. doi:10.1073/pnas.0810452105
17. Lorin S, Borges A, Dos Santos LR, Souquere S, Pierron G, Ryan KM, Codogno P, Djavaheri-Mergny M (2009) c-Jun NH2-terminal kinase activation is essential for DRAM-dependent induction of autophagy and apoptosis in 2-methoxyestradiol-treated ewing sarcoma cells. Cancer Res 69(17):6924–6931, http://dx.doi.org/10.1158/0008-5472.CAN-09-1270
18. Pursiheimo JP, Rantanen K, Heikkinen PT, Johansen T, Jaakkola PM (2009) Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. Oncogene 28(3):334–344. doi:10.1038/onc.2008.392
19. Noman MZ, Janji B, Berchem G, Mami-Chouaib F, Chouaib S (2012) Hypoxia-induced autophagy: a new player in cancer immunotherapy? Autophagy 8(4):704–706. doi:10.4161/auto.19572

20. Jiang H, Martin V, Gomez-Manzano C, Johnson DG, Alonso M, White E, Xu J, McDonnell TJ, Shinojima N, Fueyo J (2010) The RB-E2F1 pathway regulates autophagy. Cancer Res 70(20):7882–7893. doi:10.1158/0008-5472.can-10-1604
21. Coupienne I, Fettweis G, Piette J (2011) RIP3 expression induces a death profile change in U2OS osteosarcoma cells after 5-ALA-PDT. Lasers Surg Med 43(7):557–564. doi:10.1002/lsm.21088
22. Huang J, Liu K, Yu Y, Xie M, Kang R, Vernon P, Cao L, Tang D, Ni J (2012) Targeting HMGB1-mediated autophagy as a novel therapeutic strategy for osteosarcoma. Autophagy 8(2):275–277. doi:10.4161/auto.8.2.18940
23. Huang J, Ni J, Liu K, Yu Y, Xie M, Kang R, Vernon P, Cao L, Tang D (2012) HMGB1 promotes drug resistance in osteosarcoma. Cancer Res 72(1):230–238. doi:10.1158/0008-5472.can-11-2001
24. Wong W (2013) Inhibit autophagy, STAT! Sci Signal 6(256):ec4, http://dx.doi.org/10.1126/scisignal.2003913
25. Roy S, Debnath J (2010) Autophagy and tumorigenesis. Semin Immunopathol 32(4):383–396. doi:10.1007/s00281-010-0213-0
26. Chen N, Debnath J (2010) Autophagy and tumorigenesis. FEBS Lett 584(7):1427–1435. doi:10.1016/j.febslet.2009.12.034
27. Pardo R, Lo Re A, Archange C, Ropolo A, Papademetrio DL, Gonzalez CD, Alvarez EM, Iovanna JL, Vaccaro MI (2010) Gemcitabine induces the VMP1-mediated autophagy pathway to promote apoptotic death in human pancreatic cancer cells. Pancreatology 10(1):19–26. doi:10.1159/000264680
28. Eskelinen EL (2011) The dual role of autophagy in cancer. Curr Opin Pharmacol 11(4):294–300. doi:10.1016/j.coph.2011.03.009
29. Akin D, Law B, Progulske-fox A, Dunn WA (2009) Identification of compounds that enhance and suppress autophagy. Autophagy 5(6):910–911
30. Notte A, Leclere L, Michiels C (2011) Autophagy as a mediator of chemotherapy-induced cell death in cancer. Biochem Pharmacol 82(5):427–434. doi:10.1016/j.bcp.2011.06.015
31. Shao Y, Gao Z, Marks PA, Jiang X (2004) Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci U S A 101(52):18030–18035. doi:10.1073/pnas.0408345102
32. Periyasamy-Thandavan S, Jiang M, Schoenlein P, Dong Z (2009) Autophagy: molecular machinery, regulation, and implications for renal pathophysiology. Am J Physiol Renal Physiol 297(2):F244–F256. doi:10.1152/ajprenal.00033.2009
33. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16(1):3–11. doi:10.1038/cdd.2008.150
34. Le XF, Mao W, Lu Z, Carter BZ, Bast RC Jr (2010) Dasatinib induces autophagic cell death in human ovarian cancer. Cancer 116(21):4980–4990. doi:10.1002/cncr.25426
35. Seibenhener ML, Geetha T, Wooten MW (2007) Sequestosome 1/p62—more than just a scaffold. FEBS Lett 581(2):175–179. doi:10.1016/j.febslet.2006.12.027
36. Komatsu M, Ichimura Y (2010) Physiological significance of selective degradation of p62 by autophagy. FEBS Lett 584(7):1374–1378. doi:10.1016/j.febslet.2010.02.017
37. Ichimura Y, Komatsu M (2010) Selective degradation of p62 by autophagy. Semin Immunopathol 32(4):431–436. doi:10.1007/s00281-010-0220-1
38. Rebucci M, Michiels C (2013) Molecular aspects of cancer cell resistance to chemotherapy. Biochem Pharmacol 85(9):1219–1226. doi:10.1016/j.bcp.2013.02.017
39. Doroshow JH (2013) Overcoming resistance to targeted anticancer drugs. N Engl J Med 369(19):1852–1853. doi:10.1056/NEJMe1311325
40. Luqmani YA (2005) Mechanisms of drug resistance in cancer chemotherapy. Med Princ Pract 14(Suppl 1):35–48. doi:10.1159/000086183

41. Gillet JP, Gottesman MM (2010) Mechanisms of multidrug resistance in cancer. Methods Mol Biol 596:47–76. doi:10.1007/978-1-60761-416-6_4
42. Rufini A, Melino G (2011) Cell death pathology: the war against cancer. Biochem Biophys Res Commun 414(3):445–450, http://dx.doi.org/10.1016/j.bbrc.2011.09.110
43. Meschini S, Condello M, Marra M, Formisano G, Federici E, Arancia G (2007) Autophagy-mediated chemosensitizing effect of the plant alkaloid voacamine on multidrug resistant cells. Toxicol In Vitro 21(2):197–203. doi:10.1016/j.tiv.2006.09.007
44. Meschini S, Condello M, Calcabrini A, Marra M, Formisano G, Lista P, De Milito A, Federici E, Arancia G (2008) The plant alkaloid voacamine induces apoptosis-independent autophagic cell death on both sensitive and multidrug resistant human osteosarcoma cells. Autophagy 4(8):1020–1033
45. Dhule SS, Penfornis P, Frazier T, Walker R, Tan G, He J, John V, Pochampally R (2011) Curcumin in cyclodextrin in liposome as a delivery vehicle against osteosarcoma. Cancer Res http://dx.doi.org/10.1158/1538-7445.AM2011-4227
46. Li X, Wu WK, Sun B, Cui M, Liu S, Gao J, Lou H (2011) Dihydroptychantol A, a macrocyclic bisbibenzyl derivative, induces autophagy and following apoptosis associated with p53 pathway in human osteosarcoma U2OS cells. Toxicol Appl Pharmacol 251(2):146–154. doi:10.1016/j.taap.2010.12.007
47. Ma D, Tremblay P, Mahngar K, Collins J, Hudlicky T, Pandey S (2011) Selective cytotoxicity against human osteosarcoma cells by a novel synthetic C-1 analogue of 7-deoxypancratistatin is potentiated by curcumin. PLoS One 6(12). http://dx.doi.org/10.1371/journal.pone.0028780
48. Bechara A, Pereira GJS, Paredes-Gamero EJ, Barbosa CMV, Nascimento FD, Hirata H, Caires ACF, Smaili SS, Bincoletto C (2011) Autophagy and apoptosis modulation in osteosarcoma cells exposed to a new palladacycle compound. Basic Clin Pharmacol Toxicol 109:59, http://dx.doi.org/10.1111/j.1742-7843.2011.00722.x
49. Calvaruso G, Pellerito O, Notaro A, Giuliano M (2012) Cannabinoid-associated cell death mechanisms in tumor models (review). Int J Oncol 41(2):407–413, http://dx.doi.org/10.3892/ijo.2012.1476
50. Pan J, Cheng C, Verstovsek S, Chen Q, Jin Y, Cao Q (2010) The BH3-mimetic GX15-070 induces autophagy, potentiates the cytotoxicity of carboplatin and 5-fluorouracil in esophageal carcinoma cells. Cancer Lett 293(2):167–174. doi:10.1016/j.canlet.2010.01.006
51. Urquiza L, Zalacain M, Marrodan L, Odero MD, Lecanda F, Gomez-Manzano C, Fueyo J, Sierrasesumaga L, Patino-Garcia A, Alonso MM (2011) Enhancing autophagy as a novel approach to target osteosarcoma: combination of oncolytic adenovirus and chemotherapy. Cancer Res. http://dx.doi.org/10.1158/1538-7445.AM2011-5402
52. Lambert LA, Qiao N, Hunt KK, Lambert DH, Mills GB, Meijer L, Keyomarsi K (2008) Autophagy: a novel mechanism of synergistic cytotoxicity between doxorubicin and roscovitine in a sarcoma model. Cancer Res 68(19):7966–7974, http://dx.doi.org/10.1158/0008-5472.CAN-08-1333
53. Zhang Z, Shao Z, Xiong L, Che B, Deng C, Xu W (2009) Expression of Beclin1 in osteosarcoma and the effects of down-regulation of autophagy on the chemotherapeutic sensitivity. J Huazhong Univ Sci Technolog Med Sci 29(6):737–740. doi:10.1007/s11596-009-0613-3
54. Gordon N, Kleinerman ES (2010) Aerosol therapy for the treatment of osteosarcoma lung metastases: targeting the Fas/FasL pathway and rationale for the use of gemcitabine. J Aerosol Med Pulm Drug Deliv 23(4):189–196. doi:10.1089/jamp.2009.0812
55. Santiago-O'Farril (2013) in progress

HER-2 Involvement in Osteosarcoma

Jonathan Gill, David Geller, and Richard Gorlick

Abstract The major goals of translational research in osteosarcoma entail the identification of prognostic factors and therapeutic targets. Given the relevance of epidermal growth factor receptor pathway to breast cancer and the finding that HER-2 was expressed in a proportion of osteosarcoma, it was reasonable to investigate this pathway further. Investigations of HER-2 in osteosarcoma have led to the publication of numerous conflicting reports with regard to the level and prognostic value of HER-2 expression, which are reviewed and discussed. Numerous lessons provided by this research experience are described. This pathway has also been explored as a therapeutic target with at least one study of trastuzumab for the treatment of osteosarcoma completed. Other studies utilizing alternative approaches to target the HER-2 receptor for the treatment of osteosarcoma have been considered.

Keywords Osteosarcoma • Human epidermal growth factor receptor (HER-2) • Trastuzumab • Immunohistochemistry • Targeted therapy

J. Gill, M.D. • R. Gorlick, M.D. (✉)
Department of Pediatrics, Montefiore Medical Center, The Children's Hospital at Montefiore, 3415 Bainbridge Avenue, Rosenthal 3rd Floor, Bronx, NY, USA

The Albert Einstein College of Medicine, Bronx, NY, USA
e-mail: rgorlick@montefiore.org

D. Geller, M.D.
Department of Orthopedic Surgery, Montefiore Medical Center and The Children's Hospital at Montefiore, Bronx, NY, USA

The Albert Einstein College of Medicine, Bronx, NY, USA

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_9,

Introduction

The major goals of translational research in osteosarcoma entail the identification of prognostic factors and therapeutic targets. The human epidermal growth factor receptor (HER-2) pathway has been demonstrated to have biological relevance in breast cancer, and its inhibition has been shown to have clinically significant results. A portion of osteosarcomas express HER-2. The level of HER-2 expression and its prognostic relevance in osteosarcoma remains controversial. This chapter reviews and discusses the germane literature and clinical implications.

HER-2 Biology

HER-2 was first described by multiple groups in the 1980s, which has led to its multiple names in the literature. The term *neu* was derived from studies in rats which developed neuroglioblastomas following in utero exposure to ethylnitrosourea. The *neu* gene transformed NIH3T3 cells. The protein product was shown to have homology with the epidermal growth factor receptor (EGFR) encoded by the *erb-B* gene [1]. Using the avian erythroblastosis virus transforming gene, v-*erbB*, which has similarity to human EGFR, as a probe, the human homologue of the rat *neu* gene was isolated. Due to it similarity to EGFR, it was named HER-2 [2]. Using the same probe, another group isolated two genes: *c-erB-1*, encoding EGFR, and *c-erB-2* [3]. In a mammary carcinoma cell line, another group also using the v-*erB* probe described MAC117 [4]. Subsequently, *neu*, *c-erB-2*, MAC117, and HER-2 were all shown to be the same gene by having sequence homology and the same chromosome locus.

Like its homologue, EGFR, HER-2 is a transmembrane tyrosine kinase receptor [5]. While EGFR is localized to chromosome 7, HER-2 is found on chromosome 17q21. During fetal development, HER-2 is widely expressed in tissues including placenta, liver, kidney, lung, and brain. Lower levels of expression are also seen in adult tissues: kidney, liver, skin, lung, jejunum, uterus, stomach, and colon. The HER-2 null mouse is embryonic lethal due to complete absence of cardiac trabeculae [6]. Conditional knockout of HER-2 in mouse ventricular cardiomyocytes leads to the development of severe dilated cardiomyopathy [7]. HER-2 is expressed throughout mammary duct development from the nulliparous mouse to lactation [8]. The dominant negative truncated HER-2 receptor expressed under the control of a mouse mammary cell specific promoter leads to a failure to form lactationally active lobuloaveoli [9]. In addition, HER-2 expression has been implicated in the development of the sympathetic nervous system, peripheral nerves, as well as spinal cord oligodendrocytes [10–12].

There are four members of the family of epidermal growth factor receptor tyrosine kinases: ErbB-1 (EGFR), ErbB-2 (HER-2), ErbB-3 (HER-3), and ErbB-4 (HER-4). All of these receptors need to dimerize to initiate the signaling cascade and frequently form heterodimers. HER-2 is unique in that it is the only member of this family for which there is no known ligand. However, it has been shown to be the

preferred partner for the other members to form heterodimers. Heterodimers with HER-2 as a partner have enhancement and prolongation of intracellular signaling [13]. HER2 heterodimerization and activation has been implicated in multiple downstream signaling cascades including mitogen-activated protein kinase (MAPK), PI3K/Akt, mTOR, Src kinase, and Signal Transducer and Activator of Transcription (STAT). The complexity of these downstream effects is mediated by the differing ligand affinity between the different members of the HER family and the specific heterodimer at the time of activation [14, 15].

HER-2 overexpression has been shown to be tumorigenic. Transfection of NIH3T3 cells with HER-2 transforms the cells and leads to tumor formation in mice. The tumorigenicity is associated with level of expression of HER-2 within the transformed cells [16, 17]. Transgenic mice expressing HER-2 under the control of a mouse mammary cell specific promoter form mammary tumors consistent with adenocarcinomas at 4 months of age. Ultimately most of the mice develop lung metastases as well [18].

HER-2 in Breast Cancer

HER2 has been shown to be overexpressed in many human adenocarcinomas including breast, ovaries, lung, stomach, and salivary gland [19]. It has been the most thoroughly evaluated in breast cancer. Shortly after HER-2 was described as a possible oncogene, it was demonstrated to be amplified in greater than 30 % of breast cancers. This initial evaluation also noted a trend for the increased number of copies being associated with increased number of involved lymph nodes at diagnosis. In addition, when the authors evaluated a cohort of node positive patients, HER2 amplification was significantly associated with the number of involved nodes as well as shorter time to relapse and shorter overall survival [20]. Amplification of HER-2 was also demonstrated to correlate with overexpression by Northern, Western, and immunohistochemistry. Western blot analysis was most discordant because of excessive stromal elements in the tumor tissue. In 10 % of the cases, while there was no evidence of amplification, there was clear overexpression at the level of RNA and protein [21]. This suggests that gene amplification may not be the only mechanism leading to HER-2 overexpression in breast cancer.

Trastuzumab is a humanized mouse monoclonal antibody directed at the extracellular domain of HER-2. In xenograft models of human breast cancer cell lines overexpressing HER-2, trastuzumab was shown to have a dose-dependent antitumor activity that was additive with paclitaxel or doxorubicin [22]. In a phase II study of single-agent trastuzumab in women with relapsed, metastatic breast cancer overexpressing HER-2, 5 of 43 evaluable patients (11.6 %) exhibited response to treatment; 37 % had some response or stable disease. One patient exhibited compete response. HER-2 status was defined by immunohistochemistry demonstrating membrane staining in greater than 25 % of cells [23]. When trastuzumab was added to cisplatin in women with relapsed, metastatic breast cancer overexpressing HER-2 the overall response

rate was 23 %. HER-2 status was determined by immunohistochemistry grading 0–3: 2+ and 3+ were considered eligible for study participation [24]. In a phase III study of women with metastatic breast cancer overexpressing HER-2, many of whom had received prior chemotherapy, women were randomized to chemotherapy with or without trastuzumab. The addition of trastuzumab prolonged median time to progression from 4.6 months to 7.4 months ($p<0.001$). The eligibility criterion for HER-2 overexpression was immunohistochemical membrane staining of 2+ or 3+ on 10 % of the tumor cells [25]. Trastuzumab exhibited survival advantage when combined with adjuvant chemotherapy in women with operable HER-2 positive breast cancer. In a report of two randomized trials, the patients in the trastuzumab group had an event free survival of 87.1 % compared to 75.4 % in the control arm. The overall survival rates for the trastuzumab and control group at 3 years were 94.3 % and 91.7 %, respectively. For participation on this study, the tumors required to have immunohistochemical staining of 3+ on greater than 10 % of the tumor cells or demonstration of amplification of HER-2 by fluorescence in situ hybridization [26]. The different criteria for HER-2 positivity led to significant confusion about the relevance of HER-2 in breast cancer. Contradictory reports with varying percentages of HER-2 positive tumors using different antibodies added to the controversy [27].

During the initial pivotal trials of trastuzumab in breast cancer, cardiac dysfunction became readily apparent as a major toxicity of treatment. This led to the establishment of an independent Cardiac Review and Evaluation Committee (CERC). The results of the review of the CERC revealed that 3–7 % of patients treated with trastuzumab alone experienced cardiac dysfunction. This compares to 1 % of patients treated with paclitaxel alone and 8 % of patients treated with anthracyclines. When trastuzumab is combined with paclitaxel or anthracyclines, the rates of cardiac toxicity increased to 13 % and 27 %, respectively. If trastuzumab was combined with other chemotherapy, the rates of cardiac dysfunction remained between 3 and 6 %. The majority of patients with cardiac dysfunction required post-treatment medical therapy. Functional impairment was most pronounced in the patients receiving trastuzumab in combination with anthracyclines, occurring in 16 % versus no greater than 4 % in all the other regimens. However, given the improvement in time to treatment failure associated with trastuzumab, the authors conclude that the risk of cardiac dysfunction is justified. The addition of trastuzumab to chemotherapy other than anthracyclines led to similar outcomes observed with the anthracyclines based regimens [28].

HER-2 in Osteosarcoma Cell Lines

Unlike in breast cancer cells, in osteosarcoma cell lines HER-2 displays primarily cytoplasmic or mixed membranous and cytoplasmic staining. Hughes, et al examined the expression pattern of all the members of the family of epidermal growth factor receptors in primary as well as established osteosarcoma cell lines. They evaluated the expression patterns by immunohistochemistry, western blot, polymerase chain reaction (PCR), and flow cytometry. They demonstrated that HER-3

was not expressed in osteosarcoma. EGFR expression was detectable in a primarily membranous pattern by immunohistochemistry in most of the cell lines studied. The expression of EGFR was confirmed by PCR as well as western blot. However, flow cytometry revealed minimal surface EGFR expression, which the authors suggest may be secondary to internalization in endocytosed vesicles. Supporting this assertion, the authors found that EGFR immunohistochemistry staining in archival specimens displayed a diffuse pattern consistent with localization of the activated receptor within the cytoplasm. HER-4 demonstrated diffuse and nuclear patterns of staining by immunohistochemistry in the primary tumor samples, and primarily nuclear localization in the archival tissue. The protein levels by western blot were consistent with the levels of expression detected by immunohistochemistry. HER-2 demonstrated primarily a diffuse pattern of staining consistent with cytoplasmic localization by immunohistochemistry in both the primary cell lines as well as the archival samples of osteosarcoma. The expression of HER-2 by immunohistochemistry was less intense than that seen by EGFR. The expression levels by immunohistochemistry were consistent with the levels of messenger RNA detected by PCR and protein by western blots. Unexpectedly, despite the lack of detection of HER-2 on the membrane by immunohistochemistry, flow cytometry revealed higher quantities of HER-2 than EGFR on the surface of the primary osteosarcoma cell lines [29].

The detection by flow cytometry of HER-2 on the cell surface of osteosarcoma cell lines has been corroborated by two other studies. Hassan et al. demonstrated in primary as well as established osteosarcoma cell lines that HER-2 is detectable in greater quantities than EGFR [30]. Scotlandi et al. found that 62 % of the primary and established osteosarcoma cell lines tested demonstrated HER-2 expression by flow cytometry, albeit at lower levels than the breast and ovarian cancer cell lines used as positive controls. None of the osteosarcoma cell lines demonstrated amplification of the *HER2* gene by fluorescence in situ hybridization. When treated with trastuzumab, the primary cell lines demonstrated only modest growth inhibition. In contrast, the established osteosarcoma cell line, SaoS-2, showed similar growth inhibition to the positive control breast cancer cell line. However, since the insulin-like growth factor receptor (IGF-1R) is known to play a role in resistance to treatment with trastuzumab and because IGF-1R has been implicated in the pathogenesis of osteosarcoma, the combination of trastuzumab with an antibody targeting IGF-1R was found to have significant growth inhibitory effects, greater than with either antibody alone [31]. Unlike the data in cell lines, the studies in patient samples have described conflicting results regarding whether HER-2 is expressed in osteosarcoma and its role in defining prognosis.

HER-2 Is a Negative Prognostic Indicator in Osteosarcoma

Six studies have demonstrated that HER-2 expression in osteosarcoma portends a poor outcome. Onda et al. in 1996 first described HER-2 expression in osteosarcoma. Using frozen and paraffin embedded tissue from 26 patients, they evaluated HER-2 expression by immunoblotting, immunohistochemical staining, and Southern

blotting, which they correlated with known clinical outcomes. They found that 42 % of tissues demonstrated various levels of expression by immunoblotting, which was scored from 0 to 3+ (no staining, weak, moderate, and high, respectively). This was corroborated by immunohistochemistry, revealing a primarily membranous pattern of staining. Southern blot analysis did not reveal any amplification of the *HER-2* gene. Patients whose tumors expressed HER-2 (1 to 3+) had significantly worse response to preoperative chemotherapy and survival as measured by Kaplan-Meier curves. In this series, patients who had no HER-2 expression demonstrated a 1-year survival rate of 100 % and 3-year survival rate of 84 %. In contrast those with weak to high expression of HER-2 had significantly worse outcomes with 1- and 3-year survival rates of 61 % and 14 %, respectively [32].

In another, single-institution, retrospective analysis, Gorlick et al. reviewed 53 patients treated on the T12 protocol. This randomized trial found no survival benefit to dose intensification of the preoperative chemotherapy, allowing all the samples to be treated as a single cohort [33]. HER-2 expression levels were evaluated by immunohistochemistry and scored according to according to the percentage of cells staining positive: 0 (no staining), 1+ (1–25 %), 2+ (26–50 %), 3+ (51–75 %), and 4+ (76–100 %). HER-2 staining localized primarily to the cell membrane. Overexpression was defined as greater than 2+ staining. HER-2 was overexpressed in 45.3 % of the patients' tumors, which was similar to the 42.6 % detected from the initial biopsy specimens. Overexpression of HER-2 was found to be correlated with decreased response to preoperative chemotherapy and a worse event-free survival. At 5-years, patients whose tumors overexpressed HER-2 had a 40 % event-free survival compared to 78 % for patients with low or undetectable levels of HER-2 expression. The difference in event-free survival remained significant even when the 13 % of patients who presented with metastatic disease were excluded from the analysis (47 % versus 79 %) [34].

Zhou et al. reviewed HER-2 expression from 25 patients treated at their institution from 1981 to 1996. They included in their analysis 25 primary tumor samples and 12 specimens from metastatic lung lesions. They evaluated the samples using immunohistochemistry for levels of HER-2 expression and FISH for amplification. Immunohistochemistry was defined as positive if greater than 25 % of tumor cells demonstrated immunoreactivity. Amplification was defined as positive if greater than 10 % of the cells demonstrated more than two signals or if more than three cells showed a large number of signals by FISH probe for the *HER-2* gene. They found focal to diffuse cytoplasmic staining in the majority of the tumor cells staining positive for HER-2. HER-2 expression was detectable in 44 % of the primary tumor samples and 58 % of the pulmonary metastases. HER-2 expression was not found to be correlated with response to chemotherapy. However, patients whose tumors stained positive for HER-2 were found to have a significantly worse metastasis-free survival. In this cohort, 19 patients presented with localized disease at diagnosis. Of those 19 patients, 7 had tumors staining positive for HER-2, and 5 went on to develop recurrences. To evaluate for amplification of the *HER*-2 gene FISH was performed on 12 samples. Increased signal consistent with amplification was observed in 6 of 7 immunostain-positive samples and 2 of 5 immunostain-negative samples. In

the two immunostain-negative samples which were found to have amplification of *HER-2*, the immunohistochemistry revealed focal HER-2 staining which did not meet the criteria for positive [35]. As discussed above, the cytoplasmic staining of HER-2 has uncertain biologic significance because of the protein's known function as a transmembrane receptor. In colon cancer, cytoplasmic staining for HER-2 has been demonstrated to correlate with a worse overall survival. Western blots were performed to corroborate protein expression on tumors that had both cytoplasmic and membranous staining and those that demonstrated only cytoplasmic staining. In the tumors that demonstrated both cytoplasmic and membranous pattern of staining, they found two bands: one at 185-kDa (corresponding to the expected size of the HER-2 protein) and one at 155-kDa. In the tumors that stained positive for HER-2 solely in the cytoplasm, only the 155-kDa band was detected by western blot [36]. The biological significance of this truncated version of HER-2 has not been examined. Given the discrepancy in size, it also raises the concern that cytoplasmic staining for HER-2 may indicate false positive staining and possible cross-reactivity with another protein expressed by these tumors.

In 2004, Fellenberg et al. attempted to address some of these issues with immunohistochemistry by assessing HER-2 expression at the level of mRNA by real-time reverse-transcription PCR (RT-PCR). To enrich the samples, they used laser microdissection to isolate osteosarcoma cells for analysis. They evaluated 17 pretreatment biopsies from a single institution using histologic response as their primary clinical endpoint. They found that HER-2 mRNA could be detected in all the samples tested. HER-2 expression was significantly elevated in patients who demonstrated a poor histologic response to preoperative chemotherapy. For internal validation, they corroborated their findings by testing two different areas of the tumors to ensure reproducibility. When they analyzed the samples for protein expression by immunohistochemistry, they found strong cytoplasmic staining in all the samples. There was no correlation between mRNA levels and protein expression of HER-2 [37]. This study serves as a proof of concept, that they were able to enrich tumor cells and perform RT-PCR on paraffin-embedded tissue. Using histologic response as the primary clinical endpoint, did not provide data on the significance of HER-2 overexpression on survival. Secondarily, the lack of correlation between mRNA and protein levels may have implications for the clinical significance of cytoplasmic staining for HER-2 by immunohistochemistry.

In the same year, Ferrari et al. published a report on a cohort of 19 patients who presented with localized disease who subsequently experienced a pulmonary relapse. They evaluated differences in the expression pattern between the primary tumor and the subsequent pulmonary metastasis. They examined HER-2 expression by immunohistochemistry according to the percentage of cells staining positive on the membrane, 0 to 4+. The tumor was considered to be positive if it exhibited 2+ or greater staining. They found HER-2 to be expressed in 32 % of the primary tumors and 53 % of the patients had at least one nodule expressing HER-2. The accordance rate, defined as the presence of the same expression pattern in the primary and metastatic samples, was 42 %. Patients with HER-2 positive primary tumors had a shorter recurrence-free interval of 17.2 months versus 31.8 months for patients with HER-2

negative primary tumors. Likewise, patients with HER-2 positive primary tumors were more likely to recur with multiple pulmonary metastases [38].

A large, single-institution, retrospective analysis of HER-2 expression in osteosarcoma in 84 patients treated on two similar protocols was published by Scotlandi et al. in 2005. They examined pretreatment biopsy specimens, using two different antibodies, and for half of the specimens three different antibodies. They defined expression as having greater than 25 % of the cells stain positive. They detected HER-2 expression in 32 % of the samples with a pattern of focal to diffuse cytoplasmic staining. Between the two antibodies tested they found a concordance rate of 78 %. For the samples tested with the third antibody similar results were obtained with 28 % of the samples positive for HER-2 expression. Patients whose tumors expressed HER-2 were found to have a higher rate of relapse and a worse event-free survival. Patients with HER-2 negative tumors exhibited an event-free survival of greater than 60 % compared to approximately 40 % for those expressing HER-2 [31]. This analysis demonstrated cytoplasmic staining for HER-2 in osteosarcoma using multiple antibodies. The high-rate of concordance in the pattern of staining between these three antibodies suggests that the cytoplasmic staining is less likely to be due to cross-reactivity with another protein. However, the clinical significance of HER-2 in osteosarcoma remains controversial as several studies have found contradictory results in HER-2 expression.

HER-2 Is Not Prognostic in Osteosarcoma

At the same time as the retrospective analyses discussed previously demonstrated the correlation with poor prognosis in patients whose tumors expressed HER-2, eight studies also reported that HER-2 expression is not prognostic in osteosarcoma. Maitra et al., in 2001, using immunohistochemistry and FISH examined 21 diagnostic biopsy specimens from a single institution. For immunohistochemistry analysis, they defined as positive only cell membrane staining, excluding cytoplasmic and nuclear staining, and graded according to a four-tier grading scheme: negative, low, medium, and high. They did not find HER-2 overexpression by immunohistochemistry in any of the samples. Likewise, they did not detect any amplification of the *HER-2* gene by FISH [39].

Kilpatrick et al., in the same year, reported on a retrospective analysis from two centers between 1985 and 2000. They examined HER-2 expression by immunohistochemistry comparing two different antibodies as well as decalcified versus nondecalcified specimens. They delineated between membranous and cytoplasmic staining, but did not exclude cytoplasmic staining. Cytoplasmic staining was scored from 0 to 3+. Positive was defined as 2+ or 3+: weak to moderate staining in more than 10 % of cells; or moderate to strong staining in more than 10 % of cells. None of the osteosarcoma specimens demonstrated staining for HER-2 on the cell membrane. Focal cytoplasmic staining in more than 10 % of the cells was found in

83 and 98 % of the samples, using the different antibodies. There was poor agreement between the antibodies in the extent of cytoplasmic staining, even when they were collapsed to positive and negative. Neither antibody demonstrated correlation with response to preoperative chemotherapy, metastasis, or survival [40].

Thomas et al. performed a retrospective analysis of osteosarcomas in a single-institution from 33 patients that included 25 primary biopsies, 29 resections after chemotherapy, 9 pulmonary metastatic lesions, and 6 other lesions. The samples were analyzed by immunohistochemistry and RT-PCR. They graded the immunohistochemical staining according to a five-tier system: negative, cytoplasmic, low-positive membranous, medium-positive membranous, and high-positive membranous. None of the samples demonstrated staining for HER-2 on the cell membrane, but 47 % of the specimens did demonstrate diffuse cytoplasmic staining. None of the samples had HER-2 mRNA amplifiable by RT-PCR. mRNAs from housekeeping genes were amplifiable suggesting that this was not due to issues with technique or failure of RNA extraction. Likewise they were able to RT-PCR HER-2 from a breast cancer specimen, suggesting that this negative result is not secondary to failure of the primers [41]. Since they were unable to detect the corresponding mRNA in the samples, the authors concluded that the cytoplasmic staining of HER-2 should be discounted as a positive finding.

Another single-institution, retrospective analysis was performed by Anninga et al. They included in their analysis 15 pretreatment biopsy specimens as well as 12 specimens including post-chemotherapy resections or pulmonary, distant bone, or local relapse specimens. They evaluated the samples by quantitative real-time RT-PCR (qPCR) and by immunohistochemistry. Tumor samples were scored 0 to 3+ according to the level of membrane staining. Cytoplasmic staining was not considered positive. Of the 27 evaluable specimens, only one sample (from a pretreatment biopsy) displayed membranous staining, which was scored as moderate. Focal cytoplasmic staining was detected in two other samples. None of the samples had overexpression of HER-2 mRNA when compared to a HER-2 overexpressing cell line. The levels of HER-2 expression detected by qPCR were described as within the range of normal breast tissue. In the one sample with HER-2 membranous staining, FISH did not reveal *HER-2* amplification [42]. The authors likewise concluded that HER-2 does not play a significant role in osteosarcoma.

A collaborative project, involving four institutions, evaluated HER-2 expression in 22 samples from 20 patients. They were all reviewed at one institution by immunohistochemistry and fluorescence in situ hybridization. Immunohistochemistry was graded from 0 to 3+ according to level (>10 % of cells) and intensity (mild, moderate, strong) of membranous staining. Scores of 0 and 1+ were considered to be negative. Four of the samples (18 %) showed focal positivity for HER-2 (1+ grading). None of the samples revealed amplification of HER-2 by fluorescence in situ hybridization. When the authors interpreted 1+ staining as positive, univariate analysis did not reveal a statistically significant difference in survival in the two groups [43]. The authors note that major concerns of this study include the small sample size and the limited follow-up, as median survival had not been reached at the time of publication.

Somers et al reviewed 34 samples from 18 patients in a single-institution. They examined tumor samples on tissue microarrays for HER-2 expression by immunohistochemistry and amplification by chromogenic in situ hybridization (CISH). They graded the immunostaining from 0 to 3+ according to intensity of membrane staining. Cytoplasmic staining was graded as 0. They found that four osteosarcoma specimens from two patients displayed HER-2 immunostaining. Two revealed cytoplasmic staining (0), and two cytoplasmic and membranous staining (1+). None of the samples were evaluated as having overexpression of HER-2 by immunohistochemistry. None of the samples demonstrated *HER2* gene amplification by CISH. In 39 % of the tumors, aneuploidy (having multiple signals to the CISH probe) was detected in less than 10 % of the cells. They also noted that four samples exhibited three nuclear signals in greater than 50 % of the cells, which they state is suggestive for trisomy 17. None of the tumors with increased signal by CISH probe displayed expression for HER-2 [44]. Since there was no concordance between the increased chromogenic signal and immunohistochemistry, the authors concluded that the increased signal should not be interpreted as amplification of the gene. These findings if interpreted by the criteria used by Zhou et al. would have been described as positive for amplification.

HER-2 gene amplification was evaluated by Willmore-Payne et al using FISH as well as multiplex and monoplex PCR. They also performed immunohistochemistry on the samples, grading from 0 to 3+. Cytoplasmic staining was graded as 0. In the initial 21 cases evaluated by multiplex PCR and FISH, there was no evidence of *HER-2* gene amplification. Of these cases, 11 demonstrated cytoplasmic staining for HER-2 by immunohistochemistry, which were all graded as 0. No samples demonstrated membranous staining. Given the negative findings, they obtained an additional 35 paraffin blocks from 26 patients from another institution to perform monoplex PCR and FISH. Again, they were not able to detect any *HER-2* gene amplification. In these 26 patients, they detected two samples with cytoplasmic staining for HER-2 by immunohistochemistry, and one sample with 1+ membranous staining [45]. The authors concluded that HER-2 is not amplified or expressed in osteosarcoma.

Bakhshi et al. evaluated HER-2 expression by immunohistochemistry in 63 patients. They delineated the pattern of staining as cytoplasmic versus membranous. They graded the samples according to the percentage of cells stained: 0, 0–10 %; 1+, 11–30 %; 2+, 31–50 %; 3+, 51–100 %. They observed HER-2 staining (1+ and greater) in 47.6 % of samples. All of the samples demonstrated cytoplasmic staining, and four samples demonstrated both cytoplasmic and membranous staining. Positive staining for HER-2 was not correlated with metastatic disease at presentation [46]. The authors did not evaluate HER-2 for any clinical outcomes. Also confounding the results is that almost half of the patients presented with metastatic disease. The high proportion of patients presenting with advance disease which is the most powerful predictor of outcome in osteosarcoma may obscure the relevance of a biological marker.

HER-2 Is a Positive Prognostic Indicator in Osteosarcoma

Adding to the controversy over the relevance of HER-2 in osteosarcoma, Akatsuka et al. published a report of 81 patients with localized disease from two centers. They evaluated initial biopsy specimens for HER-2 expression by immunohistochemistry. The samples were graded from 0 to 3+ based on the percentage of cells staining positive: 0, negative; 1+, 1–30 %; 2+, 31–75 %; 3+, 76–100 %. The section with the highest degree of staining was used as representative, and overexpression was defined as tumors with 2+ or 3+ staining. They found that 63 % of the tumors had overexpression of HER-2. HER-2 expression did not correlate with response to chemotherapy. Overexpression of HER-2 was significantly correlated with event-free survival. At 5 years, the event-free survival of patients with overexpression of HER-2 was 72 % compared to 46 % for patients without HER-2 overexpression [47]. In a separate report, these authors also demonstrate that the rate of HER-2 expression is lower in metachronous pulmonary metastases as compared to initial biopsy specimens. They suggest that HER-2 does not play a role in the development of lung metastasis [48].

Summary of HER-2 Studies

All of these studies (summed in Table 1) provide limited clarity of the role of HER-2 in osteosarcoma. The confounding features include issues with immunohistochemistry staining and gene amplification. In the studies examining the levels of expressions of HER-2 by immunohistochemistry, there are differences in the antibodies being used, differences in the interpretations of positive staining, and differences in the grading systems used to define overexpression; recapitulating the experience in breast cancer during the incipient years following the identification of HER-2. In the studies revealing that HER-2 is not prognostic in osteosarcoma, the point of contention lies in whether HER-2 is truly overexpressed in osteosarcoma. Two studies which identified defined positive HER-2 staining in osteosarcoma demonstrated that it was not associated with a worse prognosis. The study by Bakhshi et al. was complicated by the increased numbers of patients presenting with advance disease. In contrast, Akatsuka et al. demonstrated positive HER-2 staining, but showed that it improved survival.

In regards to the second confounding feature, in breast cancer, the basis of overexpression of HER-2 is gene amplification in the majority of tumors. In osteosarcoma, there is evidence that gene amplification of *HER-2* is not involved in the pathogenesis. Again, there is disagreement in the literature in terms of the definition of positive criteria for gene amplification.

A meta-analysis published in 2010 evaluated the association of HER-2 overexpression with prognosis in osteosarcoma. Of the 28 evaluable reports, 23 were excluded.

Table 1 Studies evaluating HER-2 as a prognostic indicator in osteosarcoma

Study	Assay	*n*	Positive (%)	Outcome
Onda	Immunoblotting IHC Southern	26	Membranous: 42 0	Survival: 1-yr, 3-yr Neg: 100 %, 84 % Pos: 61 %, 14 %
Gorlick	IHC	53	Membranous: 42.6	Event-free survival: 5-yr Neg: 78 % Pos: 40 %
Zhou	IHC FISH	25 primary 12 metastases 7 IHC pos 5 IHC neg	Cytoplasmic: 44 Cytoplasmic: 58 85.7 40	Metastasis-free survival Worse
Fellenberg	RT-PCR IHC	10 good response 7 poor response NR	0 85 Cytoplasmic: 100	Histologic response: mRNA levels 94 % predictive of histologic response
Ferrari	IHC	17	Primary: 32 Metastases: 53	Recurrence-free interval: Neg: 31.8 months Pos: 17.2 months
Scotlandi	IHC	84	28–32	Event-free survival: Worse
Maitra	IHC FISH	21	0 0	NR
Kilpatrick	IHC	41	Membranous: 0 Cytoplasmic: 83–98	Response to chemotherapy, metastasis, survival: No association
Thomas	IHC RT-PCR	66	Membranous: 0 Cytoplasmic: 47 0	NR
Anninga	RT-PCR IHC FISH	27 27 1	0 Membranous: 3.7 Cytoplasmic: 7.4 0	NR
Tsai	IHC FISH	22 22	Focal: 18 0	No association (limited follow-up)
Somers	IHC microarray CISH microarray	34 34	Membranous and cytoplasmic: 5.8 Cytoplasmic: 5.8 0	NR
Willmore-Payne	FISH PCR IHC	47 46	0 0 Membranous: 0 Cytoplasmic: 4.3	NR
Bakhshi	IHC	63	Cytoplasmic: 41.2 Membranous and cytoplasmic: 6.3	Expression in patients with metastatic disease and grade: No difference
Akatsuka	IHC	81	63 %	Event-free survival: 5-yr Neg: 46 Pos: 72

IHC immunohistochemistry, *NR* not reported, *yr* year, *RT-PCR* reverse-transcription polymerase chain reaction, *FISH* fluorescent in situ hybridization, *CISH* chromogenic in situ hybridization

In the remaining five reports, the authors had difficulty with standardization of the cohorts as the reports as described above used different modalities to evaluate HER-2 overexpression, different antibodies, and different criteria for the evaluation of immunohistochemistry staining. The authors conclude that HER-2 positivity revealed a trend for a 1.26-fold higher risk of death, which was not statistically significant [49]. Another major confounder of the meta-analysis was the lack of standardization of the populations and the treatments across the studies.

More recently, results from the Children's Oncology Group have been presented. They evaluated 191 samples from 149 patients for whom there were confirmed histologic diagnosis of osteosarcoma, adequate staining, and survival information. Most of the patients were enrolled on clinical trial and had standardized treatment. HER-2 overexpression was evaluated by immunohistochemistry and graded according to the percentage of cells staining positive: negative (no staining), 1+ (0–25 %), 2+ (26–50 %), 3+ (51–75 %), and 4 (>75 %). Positive for HER-2 overexpression was defined by a grade of 3+ or 4+. According to these criteria, the investigators found that HER-2 was overexpressed in 13.4 % of the samples evaluated. HER-2 overexpression did not correlate with survival [50].

Trastuzumab in Osteosarcoma

Given the promising clinical benefit of trastuzumab in breast cancer and the early retrospective analyses in osteosarcoma, the Children's Oncology Group initiated a phase II trial of trastuzumab in patients with metastatic osteosarcoma. Eligible patients were required to have newly diagnosed metastatic disease, defined as bone, bone and lung, bilateral lung, or greater than four unilateral lung metastases. Immunohistochemistry staining was used to evaluate the HER-2 status of the patients' biopsy specimen performed and graded by an independent, centralized facility. The specimens were graded according the percentage of cells staining positive for HER-2 (membranous, cytoplasmic, and nuclear staining all considered positive): 0, <10 %; 1+, 10–50 %; 2+, >50 %. Patients with 2+ staining received trastuzumab in addition to the five-drug regimen of methotrexate, doxorubicin, cisplatin, ifosfamide, and etoposide. Patients treated with trastuzumab initiated therapy prior to week 6 and continued weekly until they had completed the course of 34 doses. The primary outcome of event-free survival was compared to patients without HER-2 expression receiving five-drug chemotherapy without trastuzumab. Between July 2001 and November 2005, 96 evaluable were enrolled on the study: 41 HER-2 positive and 55 HER-2 negative. Of the patient samples submitted for review, 33–35 % demonstrated HER-2 positive expression. The results of the trial were disappointing. There was no difference in the event-free and overall survival in the two treatment arms. The 30-month event-free survival was 32 % in both the trastuzumab arm and the non-HER-2 expressing arm. The 30-month overall survival was 59 % in the trastuzumab cohort and 50 % in the non-HER-2 expressing cohort. Despite the high-doses of anthracyclines, there was no increase in cardiac toxicity in the trastuzumab treated arm [51]. The addition of trastuzumab to

cytotoxic chemotherapy was well tolerated in this group of patients. The lack of clinical benefit noted in the trial may have been due to trastuzumab overcoming the negative prognostic effects associated with the overexpression of HER-2.

Lessons Learned

There is limited value of institutional retrospective analyses in defining possible targeted therapy. The sample sizes available are too small to detect differences in subpopulations. Secondarily, inconsistencies in techniques make reproducibility and validation very challenging. Target validation requires proving *in vivo*, using available rodent and canine models, the tumor localization and efficacy of the therapeutic agent. In conjunction, understanding the biologic basis for the targets and the mechanisms of cellular dependencies will lend confidence to the applicability of the therapeutic agent for targeted therapy. These requirements need to be standardized in the foundation of a coherent drug development plan in osteosarcoma.

Conclusion

HER-2 expression, like P-glycoprotein, is too controversial and cannot be used as a prognostic factor or in the treatment of osteosarcoma.

References

1. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA (1984) The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature 312(5994):513–516
2. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U et al (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230(4730):1132–1139
3. Semba K, Kamata N, Toyoshima K, Yamamoto T (1985) A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci U S A 82(19):6497–6501
4. King CR, Kraus MH, Aaronson SA (1985) Amplification of a novel v-erbB-related gene in a human mammary carcinoma. Science 229(4717):974–976
5. Bargmann CI, Hung MC, Weinberg RA (1986) The neu oncogene encodes an epidermal growth factor receptor-related protein. Nature 319(6050):226–230. doi:10.1038/319226a0
6. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378(6555):394–398. doi:10.1038/378394a0
7. Ozcelik C, Erdmann B, Pilz B, Wettschureck N, Britsch S, Hubner N, Chien KR, Birchmeier C, Garratt AN (2002) Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes

leads to dilated cardiomyopathy. Proc Natl Acad Sci U S A 99(13):8880–8885. doi:10.1073/pnas.122249299
8. Schroeder JA, Lee DC (1998) Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. Cell Growth Differ 9(6):451–464
9. Jones FE, Stern DF (1999) Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. Oncogene 18(23):3481–3490. doi:10.1038/sj.onc.1202698
10. Britsch S, Li L, Kirchhoff S, Theuring F, Brinkmann V, Birchmeier C, Riethmacher D (1998) The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev 12(12):1825–1836
11. Morris JK, Lin W, Hauser C, Marchuk Y, Getman D, Lee KF (1999) Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron 23(2):273–283
12. Park SK, Miller R, Krane I, Vartanian T (2001) The erbB2 gene is required for the development of terminally differentiated spinal cord oligodendrocytes. J Cell Biol 154(6):1245–1258. doi:10.1083/jcb.200104025
13. Graus-Porta D, Beerli RR, Daly JM, Hynes NE (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 16(7):1647–1655. doi:10.1093/emboj/16.7.1647
14. Hynes NE, MacDonald G (2009) ErbB receptors and signaling pathways in cancer. Curr Opin Cell Biol 21(2):177–184. doi:10.1016/j.ceb.2008.12.010
15. Olayioye MA, Neve RM, Lane HA, Hynes NE (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 19(13):3159–3167. doi:10.1093/emboj/19.13.3159
16. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA (1987) erbB-2 is a potent oncogene when overexpressed in NIH/3 T3 cells. Science 237(4811):178–182
17. Hudziak RM, Schlessinger J, Ullrich A (1987) Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3 T3 cells. Proc Natl Acad Sci U S A 84(20):7159–7163
18. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 89(22):10578–10582
19. Hynes NE (1993) Amplification and overexpression of the erbB-2 gene in human tumors: its involvement in tumor development, significance as a prognostic factor, and potential as a target for cancer therapy. Semin Cancer Biol 4(1):19–26
20. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235(4785):177–182
21. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A et al (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244(4905):707–712
22. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J (1998) Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res 58(13):2825–2831
23. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L (1996) Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol 14(3):737–744
24. Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D, Baly D, Baughman SA, Twaddell T, Glaspy JA, Slamon DJ (1998) Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol 16(8):2659–2671

25. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344(11):783–792. doi:10.1056/NEJM200103153441101
26. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr, Davidson NE, Tan-Chiu E, Martino S, Paik S, Kaufman PA, Swain SM, Pisansky TM, Fehrenbacher L, Kutteh LA, Vogel VG, Visscher DW, Yothers G, Jenkins RB, Brown AM, Dakhil SR, Mamounas EP, Lingle WL, Klein PM, Ingle JN, Wolmark N (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 353(16):1673–1684. doi:10.1056/NEJMoa052122
27. Press MF, Hung G, Godolphin W, Slamon DJ (1994) Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res 54(10):2771–2777
28. Seidman A, Hudis C, Pierri MK, Shak S, Paton V, Ashby M, Murphy M, Stewart SJ, Keefe D (2002) Cardiac dysfunction in the trastuzumab clinical trials experience. J Clin Oncol 20(5):1215–1221
29. Hughes DP, Thomas DG, Giordano TJ, Baker LH, McDonagh KT (2004) Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. Cancer Res 64(6):2047–2053
30. Hassan SE, Bekarev M, Kim MY, Lin J, Piperdi S, Gorlick R, Geller DS (2012) Cell surface receptor expression patterns in osteosarcoma. Cancer 118(3):740–749. doi:10.1002/cncr.26339
31. Scotlandi K, Manara MC, Hattinger CM, Benini S, Perdichizzi S, Pasello M, Bacci G, Zanella L, Bertoni F, Picci P, Serra M (2005) Prognostic and therapeutic relevance of HER2 expression in osteosarcoma and Ewing's sarcoma. Eur J Cancer 41(9):1349–1361. doi:10.1016/j.ejca.2005.03.015
32. Onda M, Matsuda S, Higaki S, Iijima T, Fukushima J, Yokokura A, Kojima T, Horiuchi H, Kurokawa T, Yamamoto T (1996) ErbB-2 expression is correlated with poor prognosis for patients with osteosarcoma. Cancer 77(1):71–78. doi:10.1002/(SICI)1097-0142(19960101)77:1<71::AID-CNCR13>3.0.CO;2-5
33. Meyers PA, Gorlick R, Heller G, Casper E, Lane J, Huvos AG, Healey JH (1998) Intensification of preoperative chemotherapy for osteogenic sarcoma: results of the Memorial Sloan-Kettering (T12) protocol. J Clin Oncol 16(7):2452–2458
34. Gorlick R, Huvos AG, Heller G, Aledo A, Beardsley GP, Healey JH, Meyers PA (1999) Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 17(9):2781–2788
35. Zhou H, Randall RL, Brothman AR, Maxwell T, Coffin CM, Goldsby RE (2003) Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. J Pediatr Hematol Oncol 25(1):27–32
36. Osako T, Miyahara M, Uchino S, Inomata M, Kitano S, Kobayashi M (1998) Immunohistochemical study of c-erbB-2 protein in colorectal cancer and the correlation with patient survival. Oncology 55(6):548–555
37. Fellenberg J, Krauthoff A, Pollandt K, Delling G, Parsch D (2004) Evaluation of the predictive value of Her-2/neu gene expression on osteosarcoma therapy in laser-microdissected paraffin-embedded tissue. Lab Invest 84(1):113–121. doi:10.1038/sj.labinvest.3700006
38. Ferrari S, Bertoni F, Zanella L, Setola E, Bacchini P, Alberghini M, Versari M, Bacci G (2004) Evaluation of P-glycoprotein, HER-2/ErbB-2, p53, and Bcl-2 in primary tumor and metachronous lung metastases in patients with high-grade osteosarcoma. Cancer 100(9):1936–1942. doi:10.1002/cncr.20151
39. Maitra A, Wanzer D, Weinberg AG, Ashfaq R (2001) Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. Cancer 92(3):677–683
40. Kilpatrick SE, Geisinger KR, King TS, Sciarrotta J, Ward WG, Gold SH, Bos GD (2001) Clinicopathologic analysis of HER-2/neu immunoexpression among various histologic subtypes and grades of osteosarcoma. Mod Pathol 14(12):1277–1283. doi:10.1038/modpathol.3880474
41. Thomas DG, Giordano TJ, Sanders D, Biermann JS, Baker L (2002) Absence of HER2/neu gene expression in osteosarcoma and skeletal Ewing's sarcoma. Clin Cancer Res 8(3):788–793

42. Anninga JK, van de Vijver MJ, Cleton-Jansen AM, Kristel PM, Taminiau AH, Nooij M, Egeler RM, Hogendoorn PC (2004) Overexpression of the HER-2 oncogene does not play a role in high-grade osteosarcomas. Eur J Cancer 40(7):963–970. doi:10.1016/j.ejca.2003.10.025
43. Tsai JY, Aviv H, Benevenia J, Chang VT, Patterson F, Aisner S, Hameed M (2004) HER-2/neu and p53 in osteosarcoma: an immunohistochemical and fluorescence in situ hybridization analysis. Cancer Invest 22(1):16–24
44. Somers GR, Ho M, Zielenska M, Squire JA, Thorner PS (2005) HER2 amplification and overexpression is not present in pediatric osteosarcoma: a tissue microarray study. Pediatr Dev Pathol 8(5):525–532. doi:10.1007/s10024-005-0044-5
45. Willmore-Payne C, Holden JA, Zhou H, Gupta D, Hirschowitz S, Wittwer CT, Layfield LJ (2006) Evaluation of Her-2/neu gene status in osteosarcoma by fluorescence in situ hybridization and multiplex and monoplex polymerase chain reactions. Arch Pathol Lab Med 130(5):691–698. doi:10.1043/1543-2165(2006)130[691:EONGSI]2.0.CO;2
46. Bakhshi S, Gupta A, Sharma MC, Khan SA, Rastogi S (2009) Her-2/neu, p-53, and their coexpression in osteosarcoma. J Pediatr Hematol Oncol 31(4):245–251. doi:10.1097/MPH.0b013e318197947e
47. Akatsuka T, Wada T, Kokai Y, Kawaguchi S, Isu K, Yamashiro K, Yamashita T, Sawada N, Yamawaki S, Ishii S (2002) ErbB2 expression is correlated with increased survival of patients with osteosarcoma. Cancer 94(5):1397–1404
48. Akatsuka T, Wada T, Kokai Y, Sawada N, Yamawaki S, Ishii S (2001) Loss of ErbB2 expression in pulmonary metastatic lesions in osteosarcoma. Oncology 60(4):361–366, doi:58533
49. Li YG, Geng X (2010) A meta-analysis on the association of HER-2 overexpression with prognosis in human osteosarcoma. Eur J Cancer Care 19(3):313–316. doi:10.1111/j.1365-2354.2008.00970.x
50. Gorlick R, Barkauskas DA, Krailo M, Piperdi S, Sowers R, Gorlick S, Gill J, Geller D, Randall RL, Janeway KA, Schwartz CL, Grier H, Meyers P, Bernstein M, Marina N (2012) HER-2 expression is not prognostic in osteosarcoma: A report of a Children's Oncology Group Prospective Biology Study. Connective Tissue Oncology Society, 2012 meeing abstract
51. Ebb D, Meyers P, Grier H, Bernstein M, Gorlick R, Lipshultz SE, Krailo M, Devidas M, Barkauskas DA, Siegal GP, Ferguson WS, Letson GD, Marcus K, Goorin A, Beardsley P, Marina N (2012) Phase II trial of trastuzumab in combination with cytotoxic chemotherapy for treatment of metastatic osteosarcoma with human epidermal growth factor receptor 2 overexpression: a report from the children's oncology group. J Clin Oncol 30(20):2545–2551. doi:10.1200/JCO.2011.37.4546

Part II
Tumor Microenvironment

Role of Ezrin in Osteosarcoma Metastasis

Ling Ren and Chand Khanna

Abstract The cause of death for the vast majority of cancer patients is the development of metastases at sites distant from that of the primary tumor. For most pediatric sarcoma patients such as those with osteosarcoma (OS), despite successful management of the primary tumor through multimodality approaches, the development of metastases, commonly to the lungs, is the cause of death. Significant improvements in long-term outcome for these patients have not been seen in more than 30 years. Furthermore, the long-term outcome for patients who present with metastatic disease is grave [1–5]. New treatment options are needed.

Opportunities to improve outcomes for patients who present with metastases and those at-risk for progression and metastasis require an improved understanding of cancer progression and metastasis. With this goal in mind we and others have identified ezrin as a metastasis-associated protein that associated with OS and other cancers. Ezrin is the prototypical ERM (Ezrin/Radixin/Moesin) protein family member. ERMs function as linker proteins connecting the actin cytoskeleton and the plasma membrane. Since our initial identification of ezrin in pediatric sarcoma, an increasing

L. Ren, Ph.D.
Molecular Oncology Section - Metastasis Biology Group, Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 37 Convent Dr., Rm 2144, Bethesda, MD 20892, USA
e-mail: renl@mail.nih.gov

C. Khanna, D.V.M., Ph.D., Dipl. A.C.V.I.M. (Oncology) (✉)
Molecular Oncology Section - Metastasis Biology Group, Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 37 Convent Dr., Rm 2144, Bethesda, MD 20892, USA

Comparative Oncology Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 37 Convent Dr., Rm 2144, Bethesda, MD 20892, USA
e-mail: khannac@mail.nih.gov

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_10,

understanding the role of ezrin in metastasis has emerged. Briefly, ezrin appears to allow metastatic cells to overcome a number of stresses experienced during the metastatic cascade, most notably the stress experienced as cells interact with the microenvironment of the secondary site. Cells must rapidly adapt to this environment in order to survive. Evidence now suggests a connection between ezrin expression and a variety of mechanisms linked to this important cellular adaptation including the ability of metastatic cells to initiate the translation of new proteins and to allow the efficient generation of ATP through a variety of sources. This understanding of the role of ezrin in the biology of metastasis is now sufficient to consider ezrin as an important therapeutic target in osteosarcoma patients. This chapter reviews our understanding of ezrin and the related ERM proteins in normal tissues and physiology, summarizes the expression of ezrin in human cancers and associations with clinical parameters of disease progression, reviews reports that detail a biological understanding of ezrin's role in metastatic progression, and concludes with a rationale that may be considered to target ezrin and ezrin biology in osteosarcoma.

Keywords Ezrin • ERM Proteins • Osteosarcoma • Metastasis • Sarcoma

Ezrin, Radixin, and Moesin Proteins

Functional Relationship and Structure Organization in Normal Tissues

Structure and Domain Organization

Ezrin, radixin, and moesin are widely expressed proteins known as ERMs that link the actin cytoskeleton to membrane and membrane-associated proteins. ERM proteins share homology in sequence structure and function. They are composed of three domains, an N-terminal globular domain (N-terminal ERM association domain, N-ERMAD); an extended alpha-helical domain; and a charged C-terminal domain (C-terminal ERM association domain, C-ERMAD). The N-terminal domain of ERM proteins, called the FERM (*f*our-point-one protein, *e*zrin, *r*edixin, *m*oesin) domain is highly conserved, and is also found in the protein merlin, band 4.1 proteins and members of the band 4.1 superfamily. The crystal structures of ERM proteins have revealed the FERM domain to be composed of three modules that together form a compact clover-shaped structure. In contrast to the globular FERM domain, the carboxy-terminal domain adopts an extended structure and is composed of one β-strand and six helical regions that bind to and cover an extensive area on the FERM-domain surface, potentially masking recognition sites of other proteins. Ezrin and radixin also contain a polyproline region between the helical and C-terminal domains (Fig. 1). ERM proteins can undergo intramolecular and/or intermolecular head-to-tail interactions: both monomers and oligomers exist in cells, but it is not clear whether all forms are biologically active.

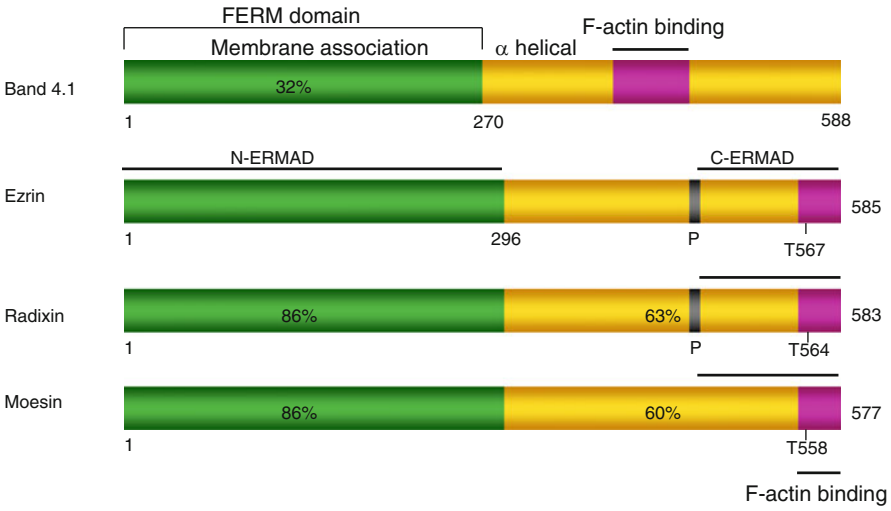


Fig. 1 Band 4.1 protein and ERM (ezrin, radixin, and moesin) family members. ERM proteins consist of three domains: a globular N-terminal membrane binding domain (FERM domain or N-ERMAD), following by an extended **α**-helical domain and a positively charged C-terminal actin-binding domain (C-ERMAD). The percentage sequence identity with ezrin in N-terminal domain is indicated at amino acid sequence level. *P* polyproline region

Tissue Localization and Functional Relationship

Although ezrin, radixin, and moesin are co-expressed in most cultured cells, they exhibit somewhat distinctive tissue-specific expression patterns. For example, ezrin is highly concentrated in intestine, stomach, lung and kidney. Moesin is predominantly expressed in lung and spleen, and radixin is primarily found in the liver and intestine. Ezrin is normally expressed in epithelial and mesothelial cells while moesin is expressed in lymphoid and endothelial cells. The brush border of intestinal epithelial cells expresses only ezrin, and hepatocytes express only radixin.

The functional redundancy that exists between ERM proteins is supported by the phenotype of moesin, radixin, and ezrin gene knockout mice. *Moesin*$^{-/-}$ mice do not carry any observable phenotype in any of the tissue examined [6]. Not surprisingly, the expression and subcellular distribution of ezrin and radixin in the tissues from the moesin$^{-/-}$ mice are not changed. Similarly, *Rdx* (encoding radixin)$^{-/-}$ animals are viable and only exhibit deafness and hepatic abnormalities [7]. Deafness in these mice results from defective stereocilia in the inner and outer hair cells, which exclusively express radixin and no ezrin or other ERM proteins [8]. The ezrin knockout mouse is viable at birth suggesting the ability of radixin and moesin to compensate for ezrin during development. Interestingly the fatal phenotype of this mouse is characterized by intestinal villous malformations seen at day 13 post partum. As may be predicted, normal intestinal epithelial cells nearly exclusively express ezrin [9]. Genetic studies using *Drosophila* and *C. elegans*

extend further to support the functional redundancy of ERM proteins [10, 11]. In *Drosophila* only a single ERM protein/orthologue (Dmoesin) is present. The functional loss of Dmoesin, in *Drosophila*, leads to a wide range of developmental and morphologenic defects which can be largely attributed to abnormal epithelial morphogenesis [11–15].

Conformational Regulation: Physiology

ERM proteins are conformationally regulated. ERM proteins exist in proposed dormant forms in which the C-terminal tail binds to and masks the N-terminal FERM domain. The activation of ERM protein is mediated by both exposure to PIP2 and phosphorylation of the C-terminal threonine (T567 in ezrin, T564 in radixin, T558 in moesin). It is likely that phosphorylation at other residues in ERM proteins are needed to maintain an open activated conformation for ezrin and to direct ezrin specific effects in cells [16]. The studies of ezrin using atomic force microscopy (AFM) demonstrated that ezrin activation, induced by T567 phosphorylation, is a 2-step process [17]. In the resting stage, ezrin is initially folded, with an association of C-ERMAD with the α-helical region followed by a second association of N-FERM/C-ERMAD, which forms a completely closed ezrin molecule. During the process of ezrin activation, modification of the N-terminus following PIP2 binding disrupts the N-FERM/C-ERMAD contact to allow Thr567 to be exposed for subsequent phosphorylation [18]. This phosphorylation subsequently releases the extreme carboxyl terminal region for F-actin binding [19]. Several protein kinases have been found to phosphorylate the C-terminal threonine residue of the ERM proteins. Examples include protein kinase C (PKC)α [20], PKCθ [21], PKCι [22], Rho kinases/ROCK [23], G protein-coupled receptor kinase 2 (GRK2) [24], Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) [25], and the ste20-related kinase Nck-interacting kinase (NIK) [26] (Fig. 2). Although we have focused on the role of ERM proteins in their proposed active and open conformation, it is reasonable that ERM proteins have some biological activity in their closed conformations.

The deactivation of ERM proteins is also important for physiologic functions including the dynamics of actin-rich membrane projections. This process might be triggered by ERM protein dephosphorylation. Moesin T558 dephosphorylation has been suggested to be a crucial step for lymphocyte adhesion and transendothelial migration. The disassembly of microvilli on lymphocyte cell surfaces caused by dephosphorylation of moesin facilitates the cell–cell (lymphocyte–endothelium) contact. Protein phosphatase 2C is involved in the dephosphorylation of moesin through the activation of Rac1 small GTPase [27]. A recent study in the transgenic mice whose T-lymphocytes express low levels of ezrin phosphomimetic protein (T567E) showed a decreased lymphocyte migration across the endothelium [28]. In vivo, dephosphorylation of ERM proteins correlates with microvilli breakdown induced by anoxia and apoptosis [29, 30].

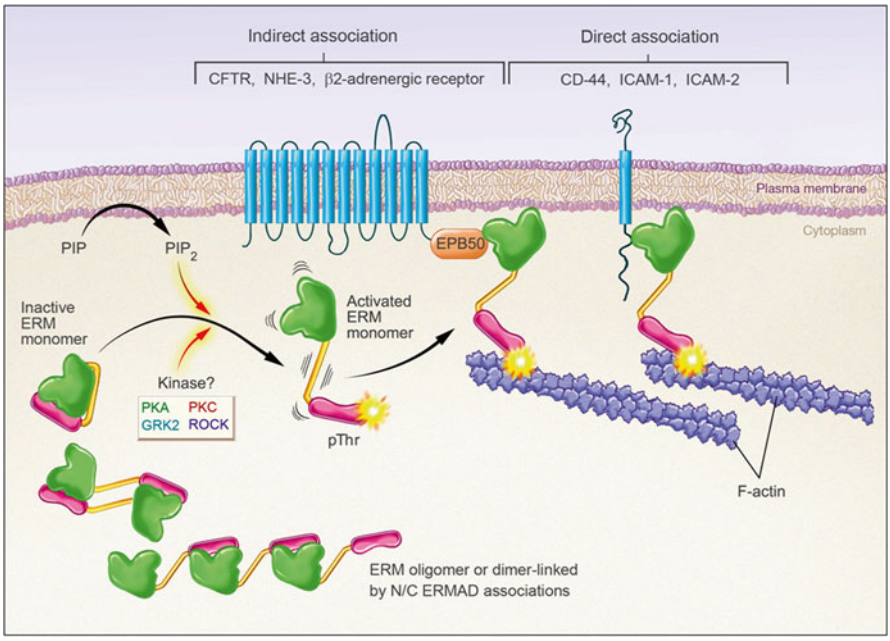


Fig. 2 Activation of ERM proteins. The dormant ERM proteins exist as monomers, dimers, and oligomers with a closed conformation. The activation of ERM proteins is mediated by both exposure to PIP2 and phosphorylation of the C-terminal threonine. The C-terminal of activated ERM proteins bind to F-actin filaments. The N-terminal domains of activated ERM proteins are associated directly with the molecules such as CD44 and ICAM-1, -2 and -3 or indirectly with other transmembrane proteins such as NHE3 through EPB50 [31]

Cellular Functions

Membrane and Cytoskeleton Linker

ERM proteins either directly associate with the cytoplasmic domains of some trans membrane proteins, such as CD44 [32–34], CD43 [35, 36], ICAM-1, -2 and -3 [37–39], Fas [40], p-glycoprotein (Pgp) [41] or indirectly associate with membrane proteins via PDZ-containing adaptors EBP50 and E3KAP [42–44]. The role of ERM proteins is to not only recruit the membrane proteins in the correct localization on the cell membrane but also regulate their physiological functions [44]. ERM proteins can localize signaling molecules that regulate the activity of these membrane proteins [45–49]. ERMs may also contribute to the transport/delivery of membrane proteins for their recycling and vesicular trafficking [50–52]. Regulated attachment of membrane proteins to F-actin is essential for many fundamental cellular processes, including the determination of cell shape, polarity and surface structure, cell adhesion, motility, cytokinesis, phagocytosis, apoptosis, drug resistance. and integration of membrane transport with signaling pathways.

Intracellular Signaling

ERM proteins lie both downstream and upstream in signal-transduction pathways in epithelial and mesenchymal tissues. As described earlier, ERM protein conformation (activity) is regulated by a combination of phospholipid binding and phosphorylation. Both events have been proposed to lie downstream of signals mediated by Rho [23, 53–56]. Recent evidence also places ERM proteins upstream of Rho-pathway activation through direct association with proteins that regulate Rho functions. In vitro studies indicate that ERM binding to RhoGDI, a potent sequestering factor, releases inactive Rho from the GDI, thereby allowing activation through the exchange of GDP to GTP [57].

In a similar fashion, ERM proteins seem to act as effectors in PKCα and PKCθ signaling events [21, 34, 58]. Both kinases have been shown to phosphorylate the conserved c-terminal threonine and thereby facilitate conformational activation of ERM proteins. In addition, PKC mediated phosphorylation has been proposed to regulate interactions between CD44 and ezrin by altering phosphorylation of the intracellular domain of CD44 [34].

Several lines of evidence link ezrin to Fas-mediated apoptosis in human T lymphocytes. Ezrin has been found to directly interact with Fas (CD95) in the lipid raft on the uropod and facilitate the susceptibility of Fas-mediated apoptosis [40], which is important in the control of cell homeostasis and may have a role in some human diseases in which programmed cell death seems to be a central pathogenetic mechanism, such as AIDS [49].

Ezrin has been found to bind to Pgp in the plasma membrane and exerts a pivotal role in the establishment of Pgp-mediated MDR in human cancer cells [41, 59, 60]. Pgp membrane localization is maintained by Pgp-to-actin anchorage through ezrin, allowing a stable localization in membrane lipid rafts.

Nuclear Localization

To further complicate our understanding of ERM protein biology, Batchelor et al. found ERM proteins to localize in nucleus [61]. The nuclear localization of endogenous ezrin and moesin is regulated by cell density and is resistant to detergent extraction, suggesting a tight association with nuclear structures. Phosphorylation in the C-terminal actin-binding domain of ERM proteins is not a prerequisite for nuclear localization. A specific nuclear localization sequence has been identified and is conserved and functional in all ERM family members. The nuclear localization of ezrin was also found in breast cancer tissues [62, 63] and OS patient samples [64]. Although the precise nuclear function of ERM proteins is not clear, these data provide further evidence that an increasing number of cytoskeletal components directly link the plasma membrane with nuclear events.

Ezrin Expression in Cancer

Ezrin has been recently shown to be expressed in most human cancers and linked to progression in several cancers including carcinomas of endometrium, breast, colon, ovary, nasopharynx, and in uveal and cutaneous melanoma, brain tumors, non-small-cell lung cancer, soft tissue sarcoma, and osteosarcoma (Table 1). A brief summary of the associations between ezrin and some of these human cancers is presented below.

Table 1 Summary of clinical associations between human cancers and ezrin expression

Cancer type	Correlation of ezrin expression and cellular localization with tumor grade and patient outcomes	References
Breast cancer	Cytoplasmic ezrin expression increases from benign to malignant breast tumor. Ezrin and CD44 co-expression was associated with a poorer prognosis in patient subsets.	[65, 66]
Ovarian cancer	A study performed on tissue microarrays revealed a favorable outcome for patients with ezrin-positive invasive ovarian carcinomas. The other study using whole tissue sections showed that ezrin expression correlates with reduced overall survival.	[67, 68]
Melanoma	Ezrin expression is directly correlated with risk for metastasis in cutaneous and uveal melanoma. Ezrin was identified as an independent predictor of outcome in aggressive uveal melanoma.	[69, 70]
Head and neck cancer	There is a significant association of increased cytoplasmic ezrin with poor cancer survival in the head and neck squamous cell carcinoma. Patients with tumors expressing high ezrin had a shorter disease-free survival.	[71–73]
Lung cancer	In non-small-cell lung cancer, the up-regulation of ezrin expression at both mRNA and protein levels were significantly associated with increased tumor stage and lymph node metastasis. Ezrin is expressed diffusely in the cytoplasm. The invading tumor cells have stronger ezrin staining.	[74–76]
Brain cancer	Increased ezrin expression correlates significantly with increasing malignancy of astrocytic tumors. Ezrin is over expressed and inactivates tumor suppressor NF2/Merlin in glioblastoma.	[77, 78]
Colorectal cancer	Ezrin expression in the colorectal cells was typically cytoplasmic and is more intense in colon than in rectal carcinomas. Ezrin is a disease-specific survival predictor. Negative/weak ezrin expression correlates with favorable disease outcome, High ezrin expression in the tumors correlates with shorter time of local recurrence.	[79–83]
Gastric cancer	Positive expression of ezrin correlated with age, size of tumor, location of tumor, depth of invasion, vessel invasion, lymph node and distant metastasis, and TNM stage. Ezrin overexpression was correlated with a poor outcome. Ezrin is mainly localized in the cytoplasm, and nuclear localization can be detected in few primary cancer cells.	[84, 85]
Soft tissue sarcoma	Increased ezrin expression predicted development of metastasis and local recurrence. Ezrin expression correlates significantly with an infiltrative growth pattern.	[86, 87]
Osteosarcoma	High ezrin expression associates to the poor overall survival of OS patients and confers high risk of recurrence. Ezrin is localized on cell membrane, cytoplasm, and nucleus in OS tumors cells.	[88–91]

Melanoma

Several studies have attempted to find molecular signatures that correlate with the malignant phenotype (aggressive behavior and poor prognosis) of melanoma. Indeed, in these studies in melanoma, ezrin expression has been shown to be associated with metastatic potential. [69, 70]. Moreover, in aggressive uveal melanoma, following a multivariate study, ezrin was identified as an independent predictor of outcome in patients [92]. In metastatic melanoma, ezrin mediates aberrant linkage of the cytoskeleton to various proteins, including CD44 and LAMP-1, inducing marked changes in the general framework of cellular functions that are important to metastasis [69]. This leads to an aberrant engagement with the extracellular microenvironment that is directly involved in metastatic behavior of tumor cells. Intriguingly, ezrin has also been correlated with the capacity of metastatic melanoma cells to cannibalize other cells either dead or alive [70, 93], which is demonstrated by the fact that ezrin knockdown abolished tumor cell phagocytic activities [94, 95].

Head and Neck Cancer

Over-expression of ERMs has been linked to tumor progression in the head and neck squamous cell carcinoma (UADT-SCC) using DNA microarrays [96]. At the protein level high cytoplasmic ezrin expression was significantly associated with decreased survival in patients. Strong cytoplasmic moesin expression was associated with poorer survival, albeit not significantly. In contrast, membranous ezrin expression was associated with improved overall survival [71–73] in these patients.

Non Small Cell Lung Cancer (NSCLC)

Western blot analysis showed that the level of ezrin in the NSCLC tissues was significantly higher than that in the normal tissues. The expression of ezrin protein and mRNA was up-regulated in highly metastatic human NSCLC. In two independent studies, ezrin immunohistochemical staining was performed using patient tumor tissues, and the ezrin protein expression was detected by Western blot in freshly collected or frozen NSCLC tissues [74, 76]. The up-regulation of ezrin expression was significantly associated with increased tumor stage and lymph node (LN) metastasis but not correlated with age, sex, tumor size, histological type, clinical

TNM system or pathological grade. The 5-year survival rate of patients with no ezrin expression was significantly higher than that of patients with positive ezrin expression [74]. These results suggested that high-level ezrin expression contributed to NSCLC progression.

Brain Cancer

In glioblastoma both ezrin and neurofibromatosis type 2 (NF2/merlin), a cytoskeletal-related protein, have opposite yet interdependent activities. Ezrin overexpression was observed in glioblastoma, and interestingly ezrin enhanced cell proliferation and anchorage-independent growth only in cells expressing NF2/merlin. Ezrin interacted and delocalized NF2/merlin from the cortical compartment releasing its inhibition on Rac1. Ezrin expression may represent another mechanism for NF2/merlin inactivation in glioblastoma [77].

Ezrin immunoreactivity was examined in normal human brain tissues and tissues from human glial tumors including astrocytoma, ependymoma, oligodendroglioma, and glioblastoma [78]. The increase of ezrin expression correlated significantly with increasing malignancy of astrocytic tumors ($P<0.0001$). It was found that weak staining of peripheral processes in normal human brain astrocytes and in World Health Organization grade II benign astrocytoma. Staining was markedly increased in anaplastic astrocytoma (World Health Organization grade III) and clearly strongest in glioblastoma (World Health Organization grade IV). Ezrin immunoreactivity may provide a useful tool for the distinction of oligodendroglioma and astrocytoma and for the grading of astrocytic tumors.

Ezrin in Sarcomas/Osteosarcoma

Expression

Studies of ezrin expression in cancer and corresponding normal tissues have suggested uniquely aberrant expression of ezrin in tissues of mesenchymal origin. Normal human mesenchymal tissues express very little to no ezrin; whereas, mesenchymal human cancers (i.e., sarcomas) are amongst the highest expressers of ezrin [97]. This may suggest a distinct role for ezrin in sarcoma. In adult soft tissue sarcoma, a direct correlation has been made between histological grade and ezrin staining intensity using immunohistochemistry [86]. Furthermore, multivariate analysis has suggested that high ezrin staining intensity in primary tumors is inversely associated with metastasis-free interval. A strong correlation between ezrin expression and infiltrative growth pattern of soft tissue sarcoma was also observed [86, 87].

By utilizing microarray analysis to identify gene expression patterns that were specific to highly metastatic derivatives of murine tumor cell lines, we and others [98, 99] have found that ezrin was significantly overexpressed in highly metastatic osteosarcoma and murine rhabdomyosarcoma (RMS) and cell lines relative to their poorly metastatic counterparts. We determined that ezrin inhibition by stable expression of antisense constructs or short hairpin RNA directed at ezrin, or an through functional disruption of ezrin by a dominant negative ezrin mutant. In these studies a significantly reduced metastatic capability was seen in cells that had reduced ezrin expression or function in both cancer types. Conversely, overexpression of wild-type ezrin conferred higher metastatic capability to nonmetastatic RMS cells. Finally, we extended our findings demonstrating clear correlations between high ezrin levels and both increasing RMS grade in humans and with reduced disease free-interval in OS that naturally developed in dogs and in human OS patients. These data, together with the work of others, provide compelling evidence for a metastasis-promoting function of ezrin [98, 100, 101] in osteosarcoma and other sarcoma subtypes. In follow-up studies, ezrin was found to be a direct transcriptional target of Six1, a homeodomain containing transcription factor [99]. RNA interference (RNAi)-based knockdown of ezrin fully inhibited the ability of Six1 to promote metastasis in RMS cells [102].

High level expression of ezrin was also found in Ewing's sarcoma tissues. But unlike the other sarcomas, the alteration of ezrin activity in the Ewing's sarcoma cell lines had profound effects on cell proliferation in vitro and primary tumor growth in vivo. Molecular examination reveals that the action of ezrin in Ewing's sarcoma is dependent on the AKT/mTOR signal transduction cascade, but not MAP Kinase [103].

Ezrin Expression Predicts Outcome in OS Patients

Recently, a systematic review and meta-analysis of published studies on prognostic role of ezrin expression in OS were conducted by two research groups [90, 104]. Final analysis of 318 patients from five eligible studies was performed. Combined hazard ratio (HR) of ezrin immunohistochemical staining suggested that positive immunoexpression had an unfavorable impact on OS patients' overall survival ($n=223$ in four studies; HR = 4.79; 95 % CI: 1.50–15.30; $P=0.008$) but not on event-free survival ($n=202$ in three studies; HR = 1.59; 95 % CI: 0.61–4.15; $P=0.342$). Combined odds ratio (OR) of ezrin immunohistochemical staining indicated that positive immunoexpression was associated with recurrence ($n=134$ in two studies; OR = 3.79; 95 % CI: 1.49–9.64; $P=0.005$) but not with serum ALP level ($n=160$ in two studies; OR = 2.16; 95 % CI: 0.09–52.50; $P=0.637$) and histological response to neoadjuvant chemotherapy ($n=260$ in 4 studies; OR = 0.87; 95 % CI: 0.37–2.03; $P=0.740$). The results of this meta-analysis suggest that ezrin positive immunoexpression confers a higher risk of recurrence and a worse survival in OS patients. Large prospective studies are needed to confirm these results and understand the precise prognostic significance of ezrin.

A Biological Understanding of Ezrin in OS Metastasis

Increasing evidence highlights that ezrin may have a pleiotropic roles in the development of the metastatic process. Indeed, ezrin has been reported to exert multiple effects on the metastatic cascade [62, 69, 100, 101, 105, 106]. Ultimately, a better understanding of exactly how ezrin confers metastatic advantage will provide important insight into this key problem in cancer biology. Followings are some advances on the study of ezrin's role in OS metastasis:

Ezrin Increases Survival of OS Cells Early After Their Arrival in the Lung

The metastatic spread of cancer cells from a primary tumor to a distant secondary site involve a complex set of discrete steps and processes that are in many ways distinct from those associated with primary tumor growth. Most studies of this metastatic cascade suggest that cancer cells readily gain entry to the circulation from the primary tumor and that the majority of circulating cancer cells successfully arrive and extravasate at the distant secondary site; however, only a small minority of cells is able to survive at the distant and foreign microenvironment. Indeed, managing this critical stage of vulnerability is a defining feature of metastatic cells. Ezrin expression appears to provide an early survival advantage for OS cells that metastasize to the lung. Using an experimental tail vein injection model of metastasis, we found that reducing ezrin expression led to decreased survival of highly metastatic OS cells that were able to reach the lung. Reduced ezrin expression was accompanied by reduced levels of active MAPK and Akt, which can both promote cell survival. It is important to note that the inhibition or ezrin expression in these cells appeared to do little to influence primary tumor growth of these cancers [98].

Dynamic Regulation During Metastasis

The active phosphorylated form of ezrin does not appear to be constitutively expressed during metastasis, rather, it is dynamically regulated. By following the progression of metastasis in highly metastatic murine and human OS cells, high expression of phosphorylated ezrin was observed early after cells arrived in the lung. This is consistent with earlier findings suggesting that ezrin was actively involved in the survival of cancer cells that arrive at the secondary site. Surprisingly, at later points in the metastatic process there was a loss of phosphorylated ezrin most evident as metastatic lesions progressed in size, particularly within the central portions of large viable metastases. Re-expression of phosphorylated ezrin was then found at the invasive front of larger metastatic lesions. This re-expression of ezrin at

the invasive front was consistent with the hypothesis that ezrin was necessary to augment survival of cells experiencing the stress of interacting with the foreign microenvironment of the lung. This dynamic pattern of overexpression of phosphorylated ezrin early after cells arrive in the lung, followed by a subsequent decline was also found in mice xenografted with canine OS cells [107]. The kinase responsible for regulating this dynamic process of ezrin phosphorylation in OS metastasis was found to be the classical PKC family members [108]. The connection between PKC and ezrin expression was further observed in canine OS cells [109] and several other cancer cell lines [110]. Collectively these findings indicate that the regulation of ERM activation/phosphorylation, and not simply the expression of ERM proteins, may play an important role in tumor metastasis.

Early Metastatic Stress and PKC Inhibition

As outlined above, PKC was found to be responsible for the regulation of ezrin activation in OS [108] and its activation was significantly linked to survival of highly metastatic OS and breast cancer cells [111]. Using novel fluorescent-based imaging strategies (Single Cell Video Microscopy) that assess tumor cell interaction with the lung microenvironment, most high and low metastatic cells can be distinguished within 6 h of their arrival in the lung. Furthermore this difference is defined by the ability of high metastatic cells to resist apoptosis at the secondary site. These observations, in both murine and human OS, suggested that the metastatic phenotype is notably defined by the ability of metastatic cells to endure stresses early hours after their arrival in the lung. The stresses experienced by single cancer cells are likely related to differences in microenvironment at the secondary site compared to the primary site, including differences in tissue oxygen tension [112], reactive oxygen species [113], inflammation [114], nutrition sources [115], pH, and other metabolic features [116]. The cells that are able to resist these stresses will survive, progress, and yield metastases. The ezrin associated survival advantage for highly metastatic cells was found to be Akt independent but PKC and caspase 3 dependent. After exposure to the PKC inhibitors, a complete inhibition of metastatic progression of OS cells and marked and effective reduction metastatic burden ex vivo and in vivo were observed [111].

Alteration of Cellular Metabolism

To further evaluate the relative contributions and importance of phosphorylated (open) and dephosphorylated (closed) forms of ezrin, an open (ezrin phosphomimic; ezrinT567D), closed (ezrinT567A) or full-length forms of ezrin were expressed in OS cells. Surprisingly, OS cells expressing constitutively open ezrin (ezrinT567D) could neither form primary orthotopic tumors or lung metastases.

In contrast, ezrinT567A over-expression inhibited metastasis but had no effect on primary tumor growth. Single-cell imaging of cells progressing through the metastatic cascade in mice suggested that the transitions between ezrin conformational forms contributed to metastatic progression early after cells arrived in the secondary site (lung). Gene expression analysis of cells expressing ezrinT567A revealed a significant increase of genes that were functionally linked to carbohydrate and amino acid metabolism. Indeed, characterization of the metabolic competency of the ezrinT567A expressing cells or cells in which ezrin expression was suppressed revealed reduced lactate production (ECAR), basal oxygen consumption (OCR), and ATP dependent OCR [117]. Collectively, these results suggest that dynamic regulation of phosphorylation of ezrin at T567 is associated with alterations in cellular metabolism, and this ability to simultaneously undergo efficient oxidative phosphorylation and glycolysis may contribute to OS metastasis.

Regulation of Protein Translation

To better understand the role of ezrin in metastasis, two non-candidate analyses of ezrin function including a microarray subtraction of high and low ezrin expressing OS cells and a proteomic approach to identify proteins that bound the N-terminus of ezrin in tumor lysates. Functional analyses of these data led to a novel hypothesis that ezrin contributes to the efficiency of metastasis through regulation of protein translation. In support of this hypothesis, ezrin was found to be part of the ribonucleoprotein complex, to facilitate the expression of complex mRNAs in cells and to bind with poly A binding protein 1 (PABA1; *PABPC1*). These findings were further supported by the identification of ezrin and components of the translational machinery in pseudopodia of highly metastatic cells during the process of cell invasion [118]. In addition, two small molecule inhibitors recently shown to inhibit the ezrin metastatic phenotype disrupted the Ezrin/PABP1 association [119].

Linkage to mTOR Pathway

A mechanism of ezrin-related metastatic behavior is also believed to be linked to a mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (S6K1)/eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) pathway. Suppression of ezrin expression either by antisense transfection or by small interfering RNAs or disruption of ezrin function by transfection of a phospho-inactive ezrin mutant (ezrinT567A) led to decreased expression and decreased phosphorylation of both S6K1 and 4E-BP1. Blockade of the mTOR pathway with rapamycin or its analog, temsirolimus led to significant inhibition of experimental lung metastasis in vivo [120]. These results suggest that blocking the mTOR/S6K1/4E-BP1 pathway may be an appropriate target for strategies to reduce OS metastasis.

Inhibition of Ezrin Functions as Therapeutic Target in OS Metastasis

The collective evidence linking the expression and activation of ezrin in OS cells is sufficient to consider ezrin and the functional consequences of ezrin expression as a therapeutic target. It is important to note that based on the genetic knockdown of ezrin or dysregulation of ezrin function, the outcome of targeting ezrin therapeutically will be to prevent metastatic progression but not have significant effect against a primary or measurable tumor. Accordingly, novel clinical development paths for agents that uniquely target the metastatic phenotype will be needed. For example our biological understanding of ezrin expression and activity suggest a rationale for targeting ezrin itself, or targeting ezrin associated pathway components including the initiation of protein translation and the mTOR pathway, PKC, or the metabolic efficiency that is associated with highly metastatic and ezrin expressing cells. As alluded to earlier efforts to identify ezrin inhibitors through a small molecule library screen have been initiated. Specifically small molecules were screened for their abilities to directly interact with ezrin protein using surface plasmon resonance. Through a multilevel screen of ezrin binding compounds, two molecules, NSC305787 and NSC668394, which directly bind to ezrin with low micromolar affinity, have been identified. These compounds have been shown to inhibit ezrin phosphorylation, ezrin-actin interaction and ezrin-mediated motility of OS cells in culture. NSC305787 mimicked the ezrin morpholino phenotype, and NSC668394 caused a unique developmental defect consistent with reduced cell motility in developing zebrafish embryos. Following tail vein injection of OS cells into mice, both molecules inhibited lung metastasis of OS ezrin-sensitive cells, but not ezrin-resistant cells [119]. The small molecule inhibitors NSC305787 and NSC668394 demonstrate a novel targeted therapy that directly inhibits ezrin protein as an approach to prevent tumor metastasis.

In addition to targeting ezrin directly, studies of ezrin's role in OS metastasis provide a rationale for targeting mTOR and the initiation of protein translation that appears to be needed for the survival of metastatic cancer cells. Indeed, as predicted by studies with ezrin, the inhibition of mTOR signaling reduces growth and metastasis in OS models by blocking S6 kinase 1 and 4EBP-1 phosphorylation [121]. The rapalog inhibitors of mTOR, including rapamycin have been found to inhibit ezrin-mediated metastatic behavior [120, 122].

Similarly the connection between PKC signaling and cancer metastasis of OS provides a biologic rationale for the use of PKC inhibition in the prevention of metastatic progression. Classical PKC isoforms regulate the phosphorylation of ezrin in OS [108]. As predicted, UCN-01, a PKC inhibitor, can effectively reduce metastatic burden in OS mouse models [111]. The development of additional selective inhibitors of PKC are needed before this therapeutic rational in the setting of OS metastasis can be fully exploited.

Conclusion and Future Directions

Ezrin is an example of a metastasis-associated protein that appears to be important in OS and other cancers. The specific roles that ezrin plays in OS metastatic progression have been recently examined and have thus far focused on the early events following the arrival of metastatic cells at a secondary site. Additional studies are now needed to expand this understanding and clarify how ezrin may contribute to later steps in the metastatic cascade. Furthermore, ezrin appears to act at the nexus or as central node in the metastatic cascade. In this way ezrin appears to regulate a variety of important processes that may contribute to parts of the metastatic cascade. These processes include protein translation, cellular metabolism, and signal transduction. Studies are necessary to define where, in the metastatic cascade, that each of these ezrin-associated processes is important. The development of inhibitors of ezrin and ezrin-associated processes will rely on this biological understanding to allow the rapid translation of these agents to OS patients at risk for metastatic progression.

Acknowledgement This work is supported by the Intramural Research Program of NIH, National Cancer Institute, Center of Cancer Research.

References

1. Eccles SA, Welch DR (2007) Metastasis: recent discoveries and novel treatment strategies. Lancet 369(9574):1742–1757
2. Ferrari S, Briccoli A, Mercuri M, Bertoni F, Picci P, Tienghi A, Del Prever AB, Fagioli F, Comandone A, Bacci G (2003) Postrelapse survival in osteosarcoma of the extremities: prognostic factors for long-term survival. J Clin Oncol 21(4):710–715
3. Harris MB, Gieser P, Goorin AM, Ayala A, Shochat SJ, Ferguson WS, Holbrook T, Link MP (1998) Treatment of metastatic osteosarcoma at diagnosis: a Pediatric Oncology Group Study. J Clin Oncol 16(11):3641–3648
4. Hawkins DS, Arndt CA (2003) Pattern of disease recurrence and prognostic factors in patients with osteosarcoma treated with contemporary chemotherapy. Cancer 98(11):2447–2456
5. Hayden JB, Hoang BH (2006) Osteosarcoma: basic science and clinical implications. Orthop Clin N Am 37(1):1–7
6. Doi Y, Itoh M, Yonemura S, Ishihara S, Takano H, Noda T, Tsukita S (1999) Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout. J Biol Chem 274(4):2315–2321
7. Kikuchi S, Hata M, Fukumoto K, Yamane Y, Matsui T, Tamura A, Yonemura S, Yamagishi H, Keppler D, Tsukita S, Tsukita S (2002) Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. Nat Genet 31(3):320–325
8. Kitajiri S, Fukumoto K, Hata M, Sasaki H, Katsuno T, Nakagawa T, Ito J, Tsukita S, Tsukita S (2004) Radixin deficiency causes deafness associated with progressive degeneration of cochlear stereocilia. J Cell Biol 166(4):559–570
9. Saotome I, Curto M, McClatchey AI (2004) Ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine. Dev Cell 6(6):855–864
10. Polesello C, Payre F (2004) Small is beautiful: what flies tell us about ERM protein function in development. Trends Cell Biol 14(6):294–302

11. Gobel V, Barrett PL, Hall DH, Fleming JT (2004) Lumen morphogenesis in C. elegans requires the membrane-cytoskeleton linker erm-1. Dev Cell 6(6):865–873
12. Jankovics F, Sinka R, Lukacsovich T, Erdelyi M (2002) MOESIN crosslinks actin and cell membrane in Drosophila oocytes and is required for OSKAR anchoring. Curr Biol 12(23):2060–2065
13. Polesello C, Delon I, Valenti P, Ferrer P, Payre F (2002) Dmoesin controls actin-based cell shape and polarity during Drosophila melanogaster oogenesis. Nat Cell Biol 4(10):782–789
14. Hipfner DR, Keller N, Cohen SM (2004) Slik Sterile-20 kinase regulates Moesin activity to promote epithelial integrity during tissue growth. Genes Dev 18(18):2243–2248
15. Pilot F, Philippe JM, Lemmers C, Lecuit T (2006) Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. Nature 442(7102):580–584
16. Wu YX, Uezato T, Fujita M (2000) Tyrosine phosphorylation and cellular redistribution of ezrin in MDCK cells treated with pervanadate. J Cell Biochem 79(2):311–321
17. Liu D, Ge L, Wang F, Takahashi H, Wang D, Guo Z, Yoshimura SH, Ward T, Ding X, Takeyasu K, Yao X (2007) Single-molecule detection of phosphorylation-induced plasticity changes during ezrin activation. FEBS Lett 581(18):3563–3571
18. Hamada K, Shimizu T, Matsui T, Tsukita S, Hakoshima T (2000) Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J 19(17):4449–4462
19. Fievet BT, Gautreau A, Roy C, Del Maestro L, Mangeat P, Louvard D, Arpin M (2004) Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. J Cell Biol 164(5):653–659
20. Ng T, Parsons M, Hughes WE, Monypenny J, Zicha D, Gautreau A, Arpin M, Gschmeissner S, Verveer PJ, Bastiaens PI, Parker PJ (2001) Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. EMBO J 20(11):2723–2741
21. Pietromonaco SF, Simons PC, Altman A, Elias L (1998) Protein kinase C-theta phosphorylation of moesin in the actin-binding sequence. J Biol Chem 273(13):7594–7603
22. Wald FA, Oriolo AS, Mashukova A, Fregien NL, Langshaw AH, Salas PJ (2008) Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. J Cell Sci 121(Pt 5):644–654
23. Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Tsukita S, Tsukita S (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J Cell Biol 140(3):647–657
24. Cant SH, Pitcher JA (2005) G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. Mol Biol Cell 16(7):3088–3099
25. Nakamura N, Oshiro N, Fukata Y, Amano M, Fukata M, Kuroda S, Matsuura Y, Leung T, Lim L, Kaibuchi K (2000) Phosphorylation of ERM proteins at filopodia induced by Cdc42. Genes Cells 5(7):571–581
26. Baumgartner M, Sillman AL, Blackwood EM, Srivastava J, Madson N, Schilling JW, Wright JH, Barber DL (2006) The Nck-interacting kinase phosphorylates ERM proteins for formation of lamellipodium by growth factors. Proc Natl Acad Sci U S A 103(36):13391–13396
27. Nijhara R, van Hennik PB, Gignac ML, Kruhlak MJ, Hordijk PL, Delon J, Shaw S (2004) Rac1 mediates collapse of microvilli on chemokine-activated T lymphocytes. J Immunol 173(8):4985–4993
28. Liu Y, Belkina NV, Park C, Nambiar R, Loughhead SM, Patino-Lopez G, Ben-Aissa K, Hao JJ, Kruhlak MJ, Qi H, von Andrian UH, Kehrl JH, Tyska MJ, Shaw S (2012) Constitutively active ezrin increases membrane tension, slows migration, and impedes endothelial transmigration of lymphocytes in vivo in mice. Blood 119(2):445–453
29. Chen J, Mandel LJ (1997) Unopposed phosphatase action initiates ezrin dysfunction: a potential mechanism for anoxic injury. Am J Physiol 273(2 Pt 1):C710–C716
30. Kondo T, Takeuchi K, Doi Y, Yonemura S, Nagata S, Tsukita S (1997) ERM (ezrin/radixin/moesin)-based molecular mechanism of microvillar breakdown at an early stage of apoptosis. J Cell Biol 139(3):749–758

31. Ren L, Khanna C (2010) Merlin/NF2 tumor suppressor and ezrin–radixin–moesin (ERM) proteins in cancer development and progression. In: Thomas-Tikhonenko A (ed) Cancer genome and tumor microenvironment. Springer, New York, NY, pp 93–115
32. Tsukita S, Oishi K, Sato N, Sagara J, Kawai A (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J Cell Biol 126(2):391–401
33. Legg JW, Isacke CM (1998) Identification and functional analysis of the ezrin-binding site in the hyaluronan receptor, CD44. Curr Biol 8(12):705–708
34. Legg JW, Lewis CA, Parsons M, Ng T, Isacke CM (2002) A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. Nat Cell Biol 4(6):399–407
35. Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S, Tsukita S (1998) Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxtamembrane cytoplasmic domain of CD44, CD43, and ICAM-2. J Cell Biol 140(4):885–895
36. Serrador JM, Nieto M, Alonso-Lebrero JL, del Pozo MA, Calvo J, Furthmayr H, Schwartz-Albiez R, Lozano F, Gonzalez-Amaro R, Sanchez-Mateos P, Sanchez-Madrid F (1998) CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. Blood 91(12):4632–4644
37. Heiska L, Alfthan K, Gronholm M, Vilja P, Vaheri A, Carpen O (1998) Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate. J Biol Chem 273(34):21893–21900
38. Barreiro O, Yanez-Mo M, Serrador JM, Montoya MC, Vicente-Manzanares M, Tejedor R, Furthmayr H, Sanchez-Madrid F (2002) Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. J Cell Biol 157(7):1233–1245
39. Serrador JM, Vicente-Manzanares M, Calvo J, Barreiro O, Montoya MC, Schwartz-Albiez R, Furthmayr H, Lozano F, Sanchez-Madrid F (2002) A novel serine-rich motif in the intercellular adhesion molecule 3 is critical for its ezrin/radixin/moesin-directed subcellular targeting. J Biol Chem 277(12):10400–10409
40. Lozupone F, Lugini L, Matarrese P, Luciani F, Federici C, Iessi E, Margutti P, Stassi G, Malorni W, Fais S (2004) Identification and relevance of the CD95-binding domain in the N-terminal region of ezrin. J Biol Chem 279(10):9199–9207
41. Brambilla D, Zamboni S, Federici C, Lugini L, Lozupone F, De Milito A, Cecchetti S, Cianfriglia M, Fais S (2012) P-glycoprotein binds to ezrin at amino acid residues 149-242 in the FERM domain and plays a key role in the multidrug resistance of human osteosarcoma. Int J Cancer 130(12):2824–2834
42. Reczek D, Bretscher A (1998) The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. J Biol Chem 273(29):18452–18458
43. Donowitz M, Cha B, Zachos NC, Brett CL, Sharma A, Tse CM, Li X (2005) NHERF family and NHE3 regulation. J Physiol 567(Pt 1):3–11
44. Fievet B, Louvard D, Arpin M (2007) ERM proteins in epithelial cell organization and functions. Biochim Biophys Acta 1773(5):653–660
45. Dransfield DT, Bradford AJ, Smith J, Martin M, Roy C, Mangeat PH, Goldenring JR (1997) Ezrin is a cyclic AMP-dependent protein kinase anchoring protein. EMBO J 16(1):35–43
46. Lamprecht G, Weinman EJ, Yun CH (1998) The role of NHERF and E3KARP in the cAMP-mediated inhibition of NHE3. J Biol Chem 273(45):29972–29978
47. Weinman EJ, Steplock D, Donowitz M, Shenolikar S (2000) NHERF associations with sodium-hydrogen exchanger isoform 3 (NHE3) and ezrin are essential for cAMP-mediated phosphorylation and inhibition of NHE3. Biochemistry 39(20):6123–6129
48. Sun F, Hug MJ, Lewarchik CM, Yun CH, Bradbury NA, Frizzell RA (2000) E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells. J Biol Chem 275(38):29539–29546
49. Luciani F, Matarrese P, Giammarioli AM, Lugini L, Lozupone F, Federici C, Iessi E, Malorni W, Fais S (2004) CD95/phosphorylated ezrin association underlies HIV-1 GP120/IL-2-

induced susceptibility to CD95(APO-1/Fas)-mediated apoptosis of human resting CD4(+)T lymphocytes. Cell Death Differ 11(5):574–582
50. Cha B, Tse M, Yun C, Kovbasnjuk O, Mohan S, Hubbard A, Arpin M, Donowitz M (2006) The NHE3 juxtamembrane cytoplasmic domain directly binds ezrin: dual role in NHE3 trafficking and mobility in the brush border. Mol Biol Cell 17(6):2661–2673
51. Stanasila L, Abuin L, Diviani D, Cotecchia S (2006) Ezrin directly interacts with the alpha1b-adrenergic receptor and plays a role in receptor recycling. J Biol Chem 281(7):4354–4363
52. Zhou R, Cao X, Watson C, Miao Y, Guo Z, Forte JG, Yao X (2003) Characterization of protein kinase A-mediated phosphorylation of ezrin in gastric parietal cell activation. J Biol Chem 278(37):35651–35659
53. Matsui T, Yonemura S, Tsukita S (1999) Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases. Curr Biol 9(21): 1259–1262
54. Fukata Y, Kimura K, Oshiro N, Saya H, Matsuura Y, Kaibuchi K (1998) Association of the myosin-binding subunit of myosin phosphatase and moesin: dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. J Cell Biol 141(2):409–418
55. Shaw RJ, Henry M, Solomon F, Jacks T (1998) RhoA-dependent phosphorylation and relocalization of ERM proteins into apical membrane/actin protrusions in fibroblasts. Mol Biol Cell 9(2):403–419
56. Kotani H, Takaishi K, Sasaki T, Takai Y (1997) Rho regulates association of both the ERM family and vinculin with the plasma membrane in MDCK cells. Oncogene 14(14): 1705–1713
57. Takahashi K, Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Takai Y (1997) Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. J Biol Chem 272(37):23371–23375
58. Jensen PV, Larsson LI (2004) Actin microdomains on endothelial cells: association with CD44, ERM proteins, and signaling molecules during quiescence and wound healing. Histochem Cell Biol 121(5):361–369
59. Zhang L, Xiao R, Xiong J, Leng J, Ehtisham A, Hu Y, Ding Q, Xu H, Liu S, Wang J, Tang DG, Zhang Q (2013) Activated ERM protein plays a critical role in drug resistance of MOLT4 cells induced by CCL25. PLoS One 8(1):e52384
60. Kobori T, Harada S, Nakamoto K, Tokuyama S (2013) Activation of ERM-family proteins via RhoA-ROCK signaling increases intestinal P-gp expression and leads to attenuation of oral morphine analgesia. J Pharm Sci 102(3):1095–1105
61. Batchelor CL, Woodward AM, Crouch DH (2004) Nuclear ERM (ezrin, radixin, moesin) proteins: regulation by cell density and nuclear import. Exp Cell Res 296(2):208–222
62. Sarrio D, Rodriguez-Pinilla SM, Dotor A, Calero F, Hardisson D, Palacios J (2006) Abnormal ezrin localization is associated with clinicopathological features in invasive breast carcinomas. Breast Cancer Res Treat 98(1):71–79
63. Halon A, Donizy P, Surowiak P, Matkowski R (2013) ERM/Rho protein expression in ductal breast cancer: a 15 year follow-up. Cell Oncol (Dordr) 36(3):181–190
64. Di Cristofano C, Leopizzi M, Miraglia A, Sardella B, Moretti V, Ferrara A, Petrozza V, Della Rocca C (2010) Phosphorylated ezrin is located in the nucleus of the osteosarcoma cell. Mod Pathol 23(7):1012–1020
65. Ma L, Jiang T (2013) Clinical implications of Ezrin and CD44 coexpression in breast cancer. Oncol Rep 30(4):1899–1905
66. Gschwantler-Kaulich D, Natter C, Steurer S, Walter I, Thomas A, Salama M, Singer CF (2013) Increase in ezrin expression from benign to malignant breast tumours. Cell Oncol 36(6):485–491
67. Kobel M, Gradhand E, Zeng K, Schmitt WD, Kriese K, Lantzsch T, Wolters M, Dittmer J, Strauss HG, Thomssen C, Hauptmann S (2006) Ezrin promotes ovarian carcinoma cell invasion and its retained expression predicts poor prognosis in ovarian carcinoma. Int J Gynecol Pathol 25(2):121–130

68. Moilanen J, Lassus H, Leminen A, Vaheri A, Butzow R, Carpen O (2003) Ezrin immunoreactivity in relation to survival in serous ovarian carcinoma patients. Gynecol Oncol 90(2):273–281
69. Federici C, Brambilla D, Lozupone F, Matarrese P, de Milito A, Lugini L, Iessi E, Cecchetti S, Marino M, Perdicchio M, Logozzi M, Spada M, Malorni W, Fais S (2009) Pleiotropic function of ezrin in human metastatic melanomas. Int J Cancer 124(12):2804–2812
70. Ilmonen S, Vaheri A, Asko-Seljavaara S, Carpen O (2005) Ezrin in primary cutaneous melanoma. Mod Pathol 18(4):503–510
71. Madan R, Brandwein-Gensler M, Schlecht NF, Elias K, Gorbovitsky E, Belbin TJ, Mahmood R, Breining D, Qian H, Childs G, Locker J, Smith R, Haigentz M Jr, Gunn-Moore F, Prystowsky MB (2006) Differential tissue and subcellular expressionof ERM proteins in normal and malignant tissues: cytoplasmic ezrin expression has prognostic signficance for head and neck squamous cell carcinoma. Head Neck 28(11):1018–1027
72. Schlecht NF, Brandwein-Gensler M, Smith RV, Kawachi N, Broughel D, Lin J, Keller CE, Reynolds PA, Gunn-Moore FJ, Harris T, Childs G, Belbin TJ, Prystowsky MB (2012) Cytoplasmic ezrin and moesin correlate with poor survival in head and neck squamous cell carcinoma. Head Neck Pathol 6(2):232–243
73. Mhawech-Fauceglia P, Dulguerov P, Beck A, Bonet M, Allal AS (2007) Value of ezrin, maspin and nm23-H1 protein expressions in predicting outcome of patients with head and neck squamous-cell carcinoma treated with radical radiotherapy. J Clin Pathol 60(2):185–189
74. Chen QY, Yan J, Hu HZ, Chen FY, Song J, Jiang ZY, Jiao DM, Wu YQ (2012) Expression of ezrin in human non-small cell lung cancer and its relationship with metastasis and prognosis. Zhonghua Zhong Liu Za Zhi 34(6):436–440
75. Lee HW, Kim EH, Oh MH (2012) Clinicopathologic implication of ezrin expression in non-small cell lung cancer. Kr J Pathol 46(5):470–477
76. Zhang XQ, Chen GP, Wu T, Yan JP, Zhou JY (2012) Expression and clinical significance of ezrin in non-small-cell lung cancer. Clin Lung Cancer 13(3):196–204
77. Morales FC, Molina JR, Hayashi Y, Georgescu MM (2010) Overexpression of ezrin inactivates NF2 tumor suppressor in glioblastoma. Neuro Oncol 12(6):528–539
78. Mao J, Yuan XR, Xu SS, Jiang XC, Zhao XT (2013) Expression and Functional Significance of Ezrin in Human Brain Astrocytoma. Cell Biochem Biophys. 67(3):1507–1511
79. Jorgren F, Nilbert M, Rambech E, Bendahl PO, Lindmark G (2012) Ezrin expression in rectal cancer predicts time to development of local recurrence. Int J Colorectal Dis 27(7):893–899
80. Korkeila E, Sundstrom J, Pyrhonen S, Syrjanen K (2012) Main effects and interactions of carbonic anhydrase IX, hypoxia-inducible factor-1alpha, ezrin and glucose transporter-1 in multivariate analysis for disease outcome in rectal cancer. Anticancer Res 32(8):3299–3303
81. Korkeila EA, Syrjanen K, Bendardaf R, Laulajainen M, Carpen O, Pyrhonen S, Sundstrom J (2011) Preoperative radiotherapy modulates ezrin expression and its value as a predictive marker in patients with rectal cancer. Hum Pathol 42(3):384–392
82. Elzagheid A, Korkeila E, Bendardaf R, Buhmeida A, Heikkila S, Vaheri A, Syrjanen K, Pyrhonen S, Carpen O (2008) Intense cytoplasmic ezrin immunoreactivity predicts poor survival in colorectal cancer. Hum Pathol 39(12):1737–1743
83. Patara M, Santos EM, Coudry Rde A, Soares FA, Ferreira FO, Rossi BM (2011) Ezrin expression as a prognostic marker in colorectal adenocarcinoma. Pathol Oncol Res 17(4):827–833
84. Lam EK, Wang X, Shin VY, Zhang S, Morrison H, Sun J, Ng EK, Yu J, Jin H (2011) A microRNA contribution to aberrant Ras activation in gastric cancer. Am J Transl Res 3(2):209–218
85. Li L, Wang YY, Zhao ZS, Ma J (2011) Ezrin is associated with gastric cancer progression and prognosis. Pathol Oncol Res 17(4):909–915
86. Weng WH, Ahlen J, Astrom K, Lui WO, Larsson C (2005) Prognostic impact of immunohistochemical expression of ezrin in highly malignant soft tissue sarcomas. Clin Cancer Res 11(17):6198–6204

87. Carneiro A, Bendahl PO, Akerman M, Domanski HA, Rydholm A, Engellau J, Nilbert M (2011) Ezrin expression predicts local recurrence and development of metastases in soft tissue sarcomas. J Clin Pathol 64(8):689–694
88. Salas S, Bartoli C, Deville JL, Gaudart J, Fina F, Calisti A, Bollini G, Curvale G, Gentet JC, Duffaud F, Figarella-Branger D, Bouvier C (2007) Ezrin and alpha-smooth muscle actin are immunohistochemical prognostic markers in conventional osteosarcomas. Virchows Arch 451(6):999–1007
89. Kim MS, Song WS, Cho WH, Lee SY, Jeon DG (2007) Ezrin expression predicts survival in stage IIB osteosarcomas. Clin Orthop Relat Res 459:229–236
90. Li H, Min D, Zhao H, Wang Z, Qi W, Zheng S, Tang L, He A, Sun Y, Yao Y, Shen Z (2013) The prognostic role of ezrin immunoexpression in osteosarcoma: a meta-analysis of published data. PLoS One 8(6):e64513
91. Boldrini E, Peres SV, Morini S, de Camargo B (2010) Immunoexpression of Ezrin and CD44 in patients with osteosarcoma. J Pediatr Hematol Oncol 32(6):e213–e217
92. Makitie T, Carpen O, Vaheri A, Kivela T (2001) Ezrin as a prognostic indicator and its relationship to tumor characteristics in uveal malignant melanoma. Invest Ophthalmol Vis Sci 42(11):2442–2449
93. Killock DJ, Parsons M, Zarrouk M, Ameer-Beg SM, Ridley AJ, Haskard DO, Zvelebil M, Ivetic A (2009) In vitro and in vivo characterization of molecular interactions between calmodulin, ezrin/radixin/moesin, and L-selectin. J Biol Chem 284(13):8833–8845
94. Lugini L, Lozupone F, Matarrese P, Funaro C, Luciani F, Malorni W, Rivoltini L, Castelli C, Tinari A, Piris A, Parmiani G, Fais S (2003) Potent phagocytic activity discriminates metastatic and primary human malignant melanomas: a key role of ezrin. Lab Invest 83(11): 1555–1567
95. Lugini L, Matarrese P, Tinari A, Lozupone F, Federici C, Iessi E, Gentile M, Luciani F, Parmiani G, Rivoltini L, Malorni W, Fais S (2006) Cannibalism of live lymphocytes by human metastatic but not primary melanoma cells. Cancer Res 66(7):3629–3638
96. Belbin TJ, Singh B, Smith RV, Socci ND, Wreesmann VB, Sanchez-Carbayo M, Masterson J, Patel S, Cordon-Cardo C, Prystowsky MB, Childs G (2005) Molecular profiling of tumor progression in head and neck cancer. Arch Otolaryngol Head Neck Surg 131(1):10–18
97. Bruce B, Khanna G, Ren L, Landberg G, Jirstrom K, Powell C, Borczuk A, Keller ET, Wojno KJ, Meltzer P, Baird K, McClatchey A, Bretscher A, Hewitt SM, Khanna C (2007) Expression of the cytoskeleton linker protein ezrin in human cancers. Clin Exp Metastasis 24(2):69–78
98. Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt SM, Helman LJ (2004) The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 10(2):182–186
99. Yu Y, Khan J, Khanna C, Helman L, Meltzer PS, Merlino G (2004) Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. Nat Med 10(2):175–181
100. Nestl A, Von Stein OD, Zatloukal K, Thies WG, Herrlich P, Hofmann M, Sleeman JP (2001) Gene expression patterns associated with the metastatic phenotype in rodent and human tumors. Cancer Res 61(4):1569–1577
101. Khanna C, Khan J, Nguyen P, Prehn J, Caylor J, Yeung C, Trepel J, Meltzer P, Helman L (2001) Metastasis-associated differences in gene expression in a murine model of osteosarcoma. Cancer Res 61(9):3750–3759
102. Yu Y, Davicioni E, Triche TJ, Merlino G (2006) The homeoprotein six1 transcriptionally activates multiple protumorigenic genes but requires ezrin to promote metastasis. Cancer Res 66(4):1982–1989
103. Krishnan K, Bruce B, Hewitt S, Thomas D, Khanna C, Helman LJ (2006) Ezrin mediates growth and survival in Ewing's sarcoma through the AKT/mTOR, but not the MAPK, signaling pathway. Clin Exp Metastasis 23(3–4):227–236
104. Lun DX, Hu YC, Xu ZW, Xu LN, Wang BW (2013) The prognostic value of elevated ezrin in patients with osteosarcoma. Tumour Biol. 35(2):1263–1266

105. Elliott BE, Qiao H, Louvard D, Arpin M (2004) Co-operative effect of c-Src and ezrin in deregulation of cell-cell contacts and scattering of mammary carcinoma cells. J Cell Biochem 92(1):16–28
106. Endo K, Kondo S, Shackleford J, Horikawa T, Kitagawa N, Yoshizaki T, Furukawa M, Zen Y, Pagano JS (2009) Phosphorylated ezrin is associated with EBV latent membrane protein 1 in nasopharyngeal carcinoma and induces cell migration. Oncogene 28(14):1725–1735
107. Jaroensong T, Endo Y, Lee SJ, Kamida A, Mochizuki M, Nishimura R, Sasaki N, Nakagawa T (2012) Effects of transplantation sites on tumour growth, pulmonary metastasis and ezrin expression of canine osteosarcoma cell lines in nude mice. Vet Comp Oncol 10(4):274–282
108. Ren L, Hong SH, Cassavaugh J, Osborne T, Chou AJ, Kim SY, Gorlick R, Hewitt SM, Khanna C (2009) The actin-cytoskeleton linker protein ezrin is regulated during osteosarcoma metastasis by PKC. Oncogene 28(6):792–802
109. Hong SH, Osborne T, Ren L, Briggs J, Mazcko C, Burkett SS, Khanna C (2011) Protein kinase C regulates ezrin-radixin-moesin phosphorylation in canine osteosarcoma cells. Vet Comp Oncol 9(3):207–218
110. Brown L, Waseem A, Cruz IN, Szary J, Gunic E, Mannan T, Unadkat M, Yang M, Valderrama F, O'Toole EA, Wan H (2013) Desmoglein 3 promotes cancer cell migration and invasion by regulating activator protein 1 and protein kinase C-dependent-Ezrin activation. Oncogene advance online publication, 10 June 2013
111. Hong SH, Ren L, Mendoza A, Eleswarapu A, Khanna C (2012) Apoptosis resistance and PKC signaling: distinguishing features of high and low metastatic cells. Neoplasia 14(3):249–258
112. Rofstad EK, Sundfor K, Lyng H, Trope CG (2000) Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis. Br J Cancer 83(3):354–359
113. Xie K, Huang S (2003) Contribution of nitric oxide-mediated apoptosis to cancer metastasis inefficiency. Free Radic Biol Med 34(8):969–986
114. Brideau G, Makinen MJ, Elamaa H, Tu H, Nilsson G, Alitalo K, Pihlajaniemi T, Heljasvaara R (2007) Endostatin overexpression inhibits lymphangiogenesis and lymph node metastasis in mice. Cancer Res 67(24):11528–11535
115. Medina RA, Owen GI (2002) Glucose transporters: expression, regulation and cancer. Biol Res 35(1):9–26
116. Mendoza M, Khanna C (2009) Revisiting the seed and soil in cancer metastasis. Int J Biochem Cell Biol 41(7):1452–1462
117. Ren L, Hong SH, Chen QR, Briggs J, Cassavaugh J, Srinivasan S, Lizardo MM, Mendoza A, Xia AY, Avadhani N, Khan J, Khanna C (2012) Dysregulation of ezrin phosphorylation prevents metastasis and alters cellular metabolism in osteosarcoma. Cancer Res 72(4):1001–1012
118. Briggs JW, Ren L, Nguyen R, Chakrabarti K, Cassavaugh J, Rahim S, Bulut G, Zhou M, Veenstra TD, Chen Q, Wei JS, Khan J, Uren A, Khanna C (2012) The ezrin metastatic phenotype is associated with the initiation of protein translation. Neoplasia 14(4):297–310
119. Bulut G, Hong SH, Chen K, Beauchamp EM, Rahim S, Kosturko GW, Glasgow E, Dakshanamurthy S, Lee HS, Daar I, Toretsky JA, Khanna C, Uren A (2012) Small molecule inhibitors of ezrin inhibit the invasive phenotype of osteosarcoma cells. Oncogene 31(3):269–281
120. Wan X, Mendoza A, Khanna C, Helman LJ (2005) Rapamycin inhibits ezrin-mediated metastatic behavior in a murine model of osteosarcoma. Cancer Res 65(6):2406–2411
121. Ory B, Moriceau G, Redini F, Heymann D (2007) mTOR inhibitors (rapamycin and its derivatives) and nitrogen containing bisphosphonates: bi-functional compounds for the treatment of bone tumours. Curr Med Chem 14(13):1381–1387
122. Gautreau A, Poullet P, Louvard D, Arpin M (1999) Ezrin, a plasma membrane-microfilament linker, signals cell survival through the phosphatidylinositol 3-kinase/Akt pathway. Proc Natl Acad Sci U S A 96(13):7300–7305

Participation of the Fas/FasL Signaling Pathway and the Lung Microenvironment in the Development of Osteosarcoma Lung Metastases

Gangxiong Huang, Kazumasa Nishimoto, Yuanzheng Yang, and Eugenie S. Kleinerman

Abstract The lungs are the most common site for the metastatic spread of osteosarcoma. Success in using chemotherapy to improve overall survival has reached a plateau. Understanding the biologic properties that permit osteosarcoma cells to grow in the lungs may allow the identification of novel therapeutic approaches—the goal being to alter the tumor cells' expression of cell surface proteins so that there is no longer compatibility with the metastatic niche. We have demonstrated that the Fas Ligand positive ($FasL^+$) lung microenvironment eliminates Fas^+ osteosarcoma cells that metastasize to the lungs. Indeed, osteosarcoma lung metastases from patients are Fas^-, similar to what we found in several different mouse models. The Fas^+ cells are cleared from the lungs through apoptosis induced by the Fas signaling pathway following interaction of Fas on the tumor cell surface with the lung FasL. Blocking the Fas signaling pathway interferes with this process, allowing the Fas^+ cells to grow in the lungs. Our investigations show that Fas expression in osteosarcoma cells is regulated epigenetically by the micro-RNA miR-20a, encoded by the miR-17-92 cluster. Our studies support the feasibility of finding agents that can re-induce Fas expression as a novel therapeutic approach to treat osteosarcoma patients with lung metastases. We have identified two such agents,

G. Huang, M.D. • Y. Yang, Ph.D.
Department of Pediatrics-Research, The University of Texas MD Anderson Cancer Center, Children's Cancer Hospital, 1515 Holcombe – MOD1.002, Houston, TX 77030, USA

K. Nishimoto, M.D., Ph.D.
Department of Orthopedic Surgery, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 162-8582, Japan

E.S. Kleinerman, M.D. (✉)
Division of Pediatrics, Department of Cancer Biology, The Mary V. and John A. Reilly Distinguished Chair, University of Texas M.D. Anderson, Cancer Center, Houston, TX, USA
e-mail: ekleiner@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_11,

the histone deacetylase inhibitor entinostat and the chemotherapeutic agent gemcitabine (GCB). Aerosol GCB and oral entinostat induce the upregulation of Fas and the regression of established osteosarcoma lung metastases. Aerosol GCB was not effective in the FasL-deficient gld mouse confirming that the lung microenvironment was central to the success of this therapy. Our studies establish the critical role of the lung microenvironment in the metastatic process of osteosarcoma to the lungs and suggest an alternative focus for therapy, that is, incorporating the lung microenvironment as part of the treatment strategy against established osteosarcoma disease in the lungs.

Keywords Osteosarcoma • Pulmonary metastasis • Fas • FasL • microRNA-17-92 cluster • miR-20a • Histone deacetylase inhibitors • Entinostat • Gemcitabine • c-FLIP

Introduction

The success or failure of a cancer cell to form a metastasis in a distant organ site depends on its ability to survive in the new microenvironment. Interactions between the primary tumor cells and the normal cells in this new host environment can alter the balance between tumor cell proliferation and tumor cell death. Tumor cells with biologic characteristics that support the adaptation to the new microenvironment will be successful in growing and inducing the vasculature needed to support continued growth. Tumor cells that do not have these needed biologic phenotypes or have phenotypic variations that are not compatible with the new microenvironment will die. Understanding how the microenvironment supports or interferes with tumor cell survival can uncover new strategies for the therapy of metastatic disease.

The most common site of metastases in osteosarcoma is the lungs [1–3]. While the overall survival rate for non-metastatic patients treated with combination chemotherapy and surgery is 60–65 %, this decreases to 10–30 % for patients that present with lung metastases or for those who develop metastases during or after treatment [4, 5]. Salvage chemotherapy has had little impact on this poor patient outcome [6]. In the past 20–25 years, no new drugs have been approved in the USA to treat metastatic osteosarcoma in the lungs. The immune modulator liposomal MTP-PE (MEPACT, mifamirtide) increased both disease-free and overall survival when used in conjunction with chemotherapy in non-metastatic patients [7]. MEPACT has recently been approved by the European Medicine Agency. However, it is not available in the USA and its efficacy in treating relapsed osteosarcoma in the lungs is preliminary [8]. Therefore, at the present time standard chemotherapy combinations and multiple surgical excisions are the only approaches for patients with osteosarcoma lung metastases.

In this chapter, we summarize how Fas expression on osteosarcoma cells, the $FasL^+$ lung microenvironment, and an intact Fas/FasL signaling pathway contribute to the prevention of metastatic spread of osteosarcoma to the lungs. We show how

changing tumor cell properties can allow the tumor microenvironment to induce tumor regression. The studies presented demonstrate the critical role of the lung microenvironment in this metastatic process and offer a different therapeutic focus, which is incorporating the lung microenvironment as part of the strategy to eliminate established metastatic disease.

The Death Receptor Fas and the Fas Signaling Pathway

Fas (CD95, APO-1) is a 48 kDa type I transmembrane protein which belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family [9]. Fas is expressed on a variety of immune cells including T and B cells, as well as being constitutively expressed in numerous other tissues. Its ligand, FasL, is however only expressed in the lungs, small intestine, testes, and anterior chamber of the eye [10, 11]. Interaction of Fas with FasL triggers the apoptotic cascade leading to the activation of Caspase 8/Caspase 3 or the mitochondrial pathway, eventually resulting in cell death. Under normal physiological conditions, the Fas^+ T or B cells will undergo apoptosis when entering the $FasL^+$ testes or anterior chamber of the eye. These are considered "immune privileged" sites. Therefore, the Fas/FasL signaling pathway is critical in regulating immune homeostasis and in maintaining these immune privileged sites in the body. The Fas signaling pathway has been shown to participate in the regulation of tumor development and progression in melanoma and colon carcinoma [12–14].

Fas Expression Inversely Correlates with the Metastatic Potential of Osteosarcoma Cells

We have demonstrated that Fas expression in osteosarcoma cells is inversely correlated with their capability in forming lung metastases. Theoretically, Fas^+ osteosarcoma cells would be eliminated by the $FasL^+$ lung epithelial cells and pneumocytes [15, 16]. Indeed, SAOS-2 human osteosarcoma cells express high levels of Fas and do not form lung metastases following *i.v.* injections [17]. The LM6 and LM7 sublines were developed from SAOS-2 cells by harvesting the tumor cells from lung metastases and reinjecting them into the mouse after culture expansion [16, 17]. This process was repeated six and seven times resulting in the isolation of the LM6 and LM7 sublines. In contrast to the parental SAOS-2 cells, LM6 and LM7 cells express low levels of Fas and formed lung metastases. With each passage, the time from injection to metastasis detection decreased. There was also an increase in both the number and size of the metastases. Transfecting LM7 cells with Fas cDNA upregulated Fas expression and significantly decreased the metastatic potential of these cells compared to control-transfected cells [15, 16]. This phenomenon was also observed in the mouse osteosarcoma model. K7 mouse osteosarcoma cells rarely

form lung metastases and express high levels of Fas. By contrast, K7M3 cells (derived from the K7 cells in a manner similar to the LM7 cells) are highly metastatic and express low levels of Fas [18]. These were the first data suggesting that the presence or absence of Fas expression controlled the metastatic potential of osteosarcoma cells to the lungs and significantly contributed to the ability of osteosarcoma cells to form lung metastases [15, 16].

In addition to the cell lines and animal models described above, we also investigated Fas expression in patient tumor samples [19]. Immunohistochemical analysis of pulmonary metastases from 38 patients revealed that 23 of the 38 patients (60 %) were Fas^- and 12 patients (32 %) were weakly positive. Only one patient sample showed strong positivity for Fas expression. The remaining two patients could not be evaluated due to the extensive necrosis seen in the tumor lesions. These data support the in vivo animal studies cited above.

Given that FasL is constitutively expressed on lung epithelium, we hypothesized that Fas^+ cells will not be able to form pulmonary metastases and that cell death is mediated by the Fas/FasL signaling pathway following the interaction of the receptor on the tumor cell with the FasL on the lung epithelium. However, Fas^- cells can evade this host defense mechanism and be preferentially selected to survive and grow in the lung microenvironment.

To prove this hypothesis, Fas^+ and Fas^- K7M3 cells were injected into wild-type BALB/c and FasL-deficient gld mice [18, 20]. The primary tibia tumors that formed in BALB/c mice were a mixture of Fas^+ and Fas^- cells, whereas the lung metastases were Fas^-. This result is consistent with our observation of the patient lung metastases, further indicating that Fas^- cells are *selected* to form lung metastases. By contrast, the lung metastases in the gld mice were heterogeneous in Fas expression with both Fas^+ and Fas^- cells [18]. This was presumably because there was no FasL on the lung epithelium to interact with the Fas receptor on the Fas^+ tumor cells. This finding demonstrates that FasL expressed in the lungs is *essential* for eliminating Fas^+ cells and supports our hypothesis that Fas expression on osteosarcoma cells and the $FasL^+$ microenvironment contribute to the fate of the tumor cells when they reach the lungs.

An Intact Fas/FasL Signaling Pathway is Essential for the Elimination of Fas^+ Osteosarcoma Cells

The induction of apoptosis depends not only on the expression of the receptor and the ligand but also on a functional Fas signaling pathway within the target cells. Fas^+ cells can escape from apoptosis following engagement of the receptor if the signaling pathway that triggers apoptosis has been disrupted [20]. We have shown the Fas^+ K7 osteosarcoma cells can form lung metastases when the cells were transfected with Fas associated death-domain dominant negative (FDN), which blocks the apoptosis signaling pathway by inhibiting Caspase 8 at the death-inducing signaling complex (DISC). The lung metastases formed following injection of Fas^+ K7 FDN cells were Fas^+. In addition, the retention of FDN-transfected cells in the lungs was significantly longer than control transfected Fas^+ K7 cells [21].

As discussed above, the majority of osteosarcoma lung metastases from patients were Fas^-. However, we also observed the presence of Fas^+ cells in a small percentage of patients [19]. The results described above using the FDN-transfected cells indicate that these Fas^+ osteosarcoma cells may have evaded FasL-induced cell death and survived in the $FasL^+$ lung microenvironment because their Fas signaling pathway was blocked [22, 23].

The expression of c-FLIP is another mechanism that blocks the Fas-signaling pathway and the induction of apoptosis. c-FLIP is the structural homologue of pro-Caspase 8, which functions to block the apoptosis signaling cascade by binding to the Fas-associated death domain (FADD) at the DISC. In an analysis of osteosarcoma patient samples, we found that c-FLIP was upregulated in some of the lung metastases [22]. Similarly, we also found that c-FLIP expression was upregulated in KRIB and CCH-OS-D osteosarcoma lung metastases in our mouse models as compared with the primary tumors in the tibia. Therefore, by inhibiting the apoptosis pathway, c-FLIP may allow the Fas^+ cells to survive and form metastases in the lungs even though the cells express Fas.

Figure 1 summarizes how Fas expression and the Fas/FasL signaling pathway control the metastatic potential of osteosarcoma cells.

Fas Regulation in Osteosarcoma

The important role of Fas in the metastatic process of osteosarcoma to the lungs led us to investigate how Fas expression in these cells was altered. Using the parental non-metastatic SAOS-2 cell line and its metastatic subline LM7, we demonstrated that the Fas gene had not been deleted or mutated but rather had been downregulated. This was done by demonstrating the successful re-expression of Fas in LM7 Fas^- lung metastases following exposure to aerosol interleukin-12 (IL-12) gene therapy or aerosol chemotherapy [15, 24–27]. The re-expression of Fas resulted in tumor regression [25–27]. As IL-12 does not induce cell death, tumor regression could not be attributed to the direct effect of IL-12 on the tumor cells. We further demonstrated that IL-12 upregulated Fas expression by stimulating Fas gene promoter activity [24]. We identified that the kB-Sp1 motif, one of the enhancer elements in the Fas promoter, was responsible for IL-12's activation of the Fas promoter. Treatment of mice with established Fas^- lung metastases with IL-12 gene therapy resulted in increased Fas expression. Similarly, the upregulation of Fas on Fas^- osteosarcoma lung metastases was seen following aerosol gemcitabine (GCB) [26, 27]. The therapeutic activity of aerosol GCB was eliminated in FasL-deficient gld mice. This confirms that the tumor regression induced by aerosol GCB was mediated by the upregulation of Fas on the tumor cell rather than a direct cytotoxic effect of GCB [20]. Several chemotherapeutic agents including cisplatin, doxorubicin, and mitomycin-C have been shown to increase Fas in different tumors [28–30].

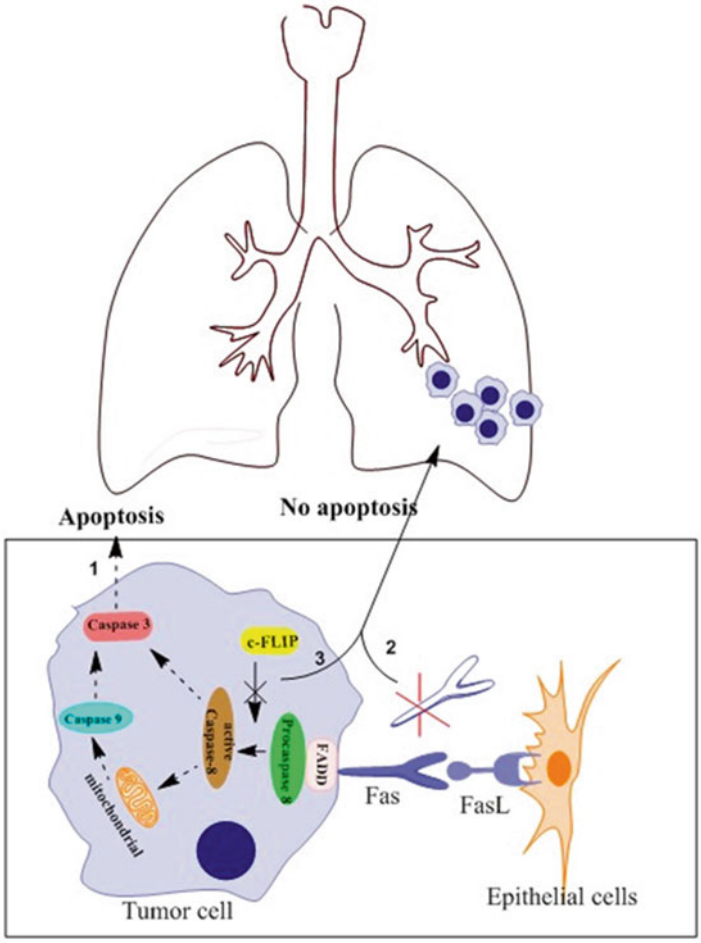


Fig. 1 Fas/FasL signaling pathway and its role in the metastatic potential of osteosarcoma (OS) cells to the lungs. (*1*) The interaction of Fas and FasL initiates the apoptosis cascade resulting in the death of OS tumor cells. (*2*) When OS cells are Fas negative, apoptosis signaling is not triggered. (*3*) When OS cells express c-FLIP, the apoptosis cascade and the signaling pathway are blocked. Therefore, OS tumor cells without Fas expression or those with c-FLIP expression may escape from FasL-induced apoptosis and form lung metastases

The data discussed above show that in osteosarcoma cells, GCB upregulated Fas expression on the cell surface and increased FasL-induced apoptosis in vivo [20].

Osteosarcoma cells have been shown to be heterogeneous in the expression of numerous genes. It is therefore not surprising that the primary bone tumor is comprised of both Fas^+ and Fas^- cells. By contrast, the lung metastases are Fas^- *presumably because the* $FasL^+$ *lung microenvironment eliminated the* Fas^+ *cells*. We demonstrated that these Fas^- cells can be induced to increase Fas expression once they form lung metastases. Therefore, the downregulation of Fas may occur by

an epigenetic mechanism that affects the transcription or posttranscriptional process of the Fas gene. We therefore investigated whether DNA methylation, histone acetylation, or micro-RNAs played a role in the epigenetic silencing of Fas.

Fas Expression in Osteosarcoma Is Not Regulated by Methylation

DNA methylation is one of the important epigenetic mechanisms that regulate gene expression transcriptionally (Fig. 2). Methylation of promoter-associated CpG results in the silencing of genes. Furthermore, the methylation status of a particular gene has been shown to contribute to gene expression in different levels in many types of cancer [31]. Indeed, it has been shown that Fas expression is regulated by gene methylation in antigen-specific cytotoxic T cells, as well as in prostate and colon cancer cells [31–35]. In these cells, the Fas gene is highly methylated resulting in the downregulation of Fas expression which contributes to the cells' anti-apoptotic characteristics. However, we found that methylation mechanism was not responsible for Fas gene silencing in the osteosarcoma cells [36]. Our analysis showed that there were 28 CpGs in the Fas gene promoter region and 46 CpGs in the first intron region. CpG dinucleotides are targets for DNA methylation. Using a PCR-based methylation assay and bisulfite-modified DNA sequencing, we found that more than 99.8 % CPG sites in the Fas promoter and first intron regions were unmethylated. In addition, there was no difference in CpG methylation in the high Fas expressing SAOS-2 cells and the low Fas expressing LM7 cells or cells from the LM7 metastatic lung tumor tissue. Moreover, treatment with the demethylating agent, 5-azadeoxycitidine (AzadC), did not alter Fas expression. Taken together, these results indicated that the downregulation of Fas expression in the metastatic osteosarcoma cells is *not* secondary to DNA methylation.

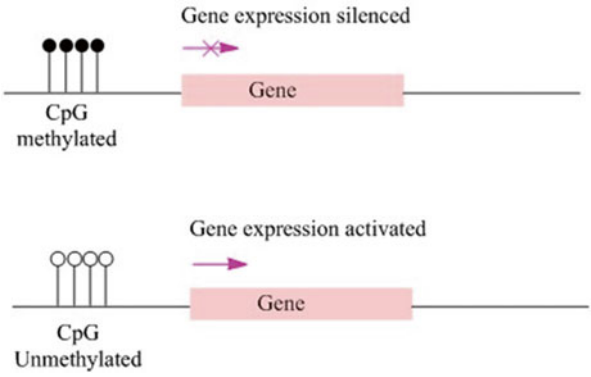


Fig. 2 Methylation regulation of gene expression. DNA methylation occurs in the sites of CpG dinucleotides. DNA methylation of CpG islands in the promoter-associated region results in transcriptional silencing of the gene. Unmethylated CpG islands lead to gene expression

miR-20a Encoded by MicroRNA 17-92 Cluster Regulates Fas Expression

Micro-RNAs (miRNAs) play an important role in gene regulation. More than 30 % of the human coding genes may be regulated by miRNAs [37]. miRNAs are small 21–23 nucleotide noncoding RNAs that function to suppress translation or directly cleave the target mRNA by binding to complementary sequences in the 3′ untranslated regions (3′-UTR) or the coding region of the mRNA (Fig. 3). Thus, miRNAs downregulate the expression of target genes posttranscriptionally. By regulating a variety of genes, miRNAs have been shown to be involved in development, cell differentiation, and regulation [37, 38]. An increasing number of studies have shown that miRNAs are also critical for cancer development and progression. In comparison to normal tissues, the miRNA expression profiles in cancer cells are different. Both overexpression and downregulation have been reported [39]. Many miRNAs have been identified to regulate the genes involving cell differentiation, proliferation, apoptosis, and stress response that are important for the biology of cancer cells. For example, miRNAs can regulate many death receptor signaling proteins including TNF-α, Fas, FADD, ribosome-inactivating protein (RIP), caspase-3, Bcl-2-interacting mediators of cell death (Bim), and p21 [40–45]. Based on their control of particular functions which participate in the initiation and progression of cancers, several miRNAs and their clusters have been classified as oncogene- or tumor suppressor-miRNAs. For example, miR-155 or members of the miR-17-92 cluster have been shown to contribute to tumorigenesis and are considered oncogenic miRNAs, while miR-15a and miR-16-1 function as tumor suppressor-miRNAs [46–49].

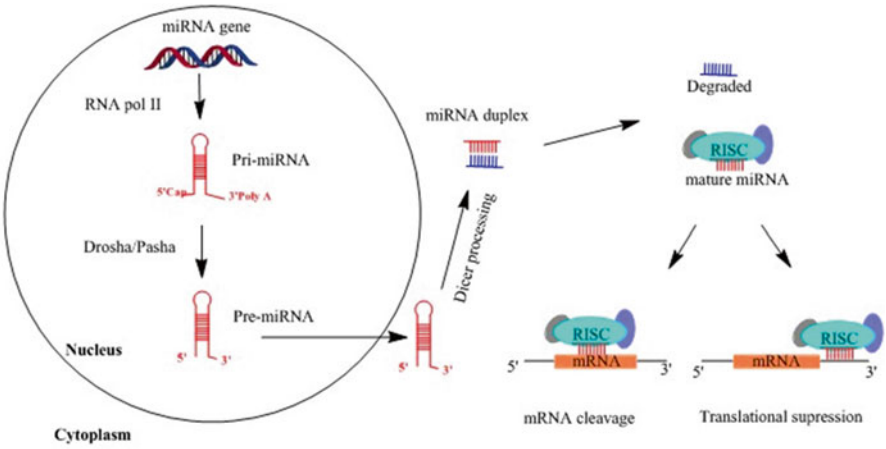


Fig. 3 miRNA processing and gene regulation. Pre-miRNA is transcribed from the miRNA gene by RNA polymerase II, followed by Drosha/Pasha processing to form pre-miRNA. Pre-miRNA is transported from the nucleus to the cytoplasm and processed by Dicer to form the miRNA duplex. Mature miRNA is assembled into the RNA-induced silencing complex (RISC) which is directed to mRNAs of the target gene, resulting in mRNA cleavage or translational suppression

Microarray based miRNA analysis found the dysregulation or overexpression of a dozen of different miRNAs in different osteosarcoma cell lines, suggesting an important role for miRNAs in the pathogenesis and development of osteosarcoma [50, 51]. In order to develop potential therapeutic miRNA targets, the specific miRNAs involved in tumorigenesis, invasion, and metastasis of osteosarcoma must be identified.

The gene for the miR-17-92 cluster is located in the third intron of chromosome 13 (C13orf25) [46]. The human miR-17-92 cluster encodes 6 miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92). The paralogy cluster of miR-17-92 encodes 2 miRNAs, miR-106a, and miR-106b. The amplification of chromosome 13 has been found frequently in B-cell lymphomas. It is therefore consistent that the miR-17-92 cluster was identified in B-cell lymphoma as an oncogenic-miRNA, which functions to promote lymphoma cell growth. Overexpression of miR-17-92 has subsequently been observed in mantle cell lymphoma [46], lung cancer [47], and other solid tumors [48].

We were the first to report that the expression levels of several members of the miR-17-92 and its paralogy clusters (including miR-20a, miR-106a, miR-20b, and miR-19a) are significantly higher in the metastatic LM7 cells than in the parental non-metastatic SAOS-2 cells [52]. Baumhoer et al. later reported that there was a significant upregulation of five members of miR-17-92 and its paralogy clusters (miR-17-5p, miR-18a, miR-93, miR-106a, and miR-106b) in several other osteosarcoma cell lines including HOS-58, U2-OS, MNNG/HOS, and SJSA-1, as compared to normal osteoblasts and mesenchymal stem cells [53]. Namlos et al. reported that all miRNAs in the paralogous clusters miR-17-92, miR-106b-25, and miR-106a were overexpressed in 19 different human osteosarcoma cell lines, as compared to normal bone [51]. These independent results consistently indicate that oncogenic miR-17-92 may play an important role in the development and progression of osteosarcoma.

Our studies also demonstrated that the level of miR-20a inversely correlated with the Fas expression in osteosarcoma patient samples [52]. Further investigations showed that miR-20a induced the downregulation of Fas expression. By contrast, inhibiting miR-20a through the transfection of miR-20a specific inhibitors resulted in the upregulation of Fas. This upregulation of Fas, secondary to the inhibition of miR-20a, led to increased FasL-induced apoptosis and, more importantly, to a significant decrease in the number of lung metastases in our animal model [52]. Taken together, these data confirm the importance of miR-20a in the regulation of Fas expression. Osteosarcoma cells with low miR-20a expressed higher levels of Fas and produced fewer metastases in the lungs presumably due to apoptosis that was induced following the interaction of Fas on the tumor cell with FasL in the lung microenvironment. miR-20a can therefore modulate the metastatic capability of osteosarcoma to the $FasL^+$ lung microenvironment by altering the expression of Fas on the tumor cell. Specific gene expression may be targeted by different miRNAs depending on the type of cells. This is one example of how epigenetic regulation can contribute to the metastatic potential of cancer cells.

The expression of Fas has also been shown to be regulated by other miRNAs such as, miR-21 in glioblastoma, miR-200c in NCI60 cells, and miR-146a in

mesenchymal cells [43, 44, 54]. Therefore, Fas regulation by miRNA may be tumor specific. In osteosarcoma, the ability of miR-20a to promote pulmonary metastasis by downregulating Fas expression is consistent with the oncogenic function of the miR-17-92 cluster. High levels of miR-20a in osteosarcoma cells contribute to metastatic potential by altering tumor cell biology to a phenotype that is more favorable to survive in the lung microenvironment.

We investigated the mechanism of Fas regulation by miR-20a. Typically, miRNAs regulate gene expression through transcriptional repression by interacting with the 3′-UTR region of the specific RNA. This results in the degradation of the mRNA. Bioinformatic analysis revealed two potential binding sites for miR-20a in the 3-UTR' region of Fas mRNA. However, using a luciferase reporter construct assay, we were unable to demonstrate direct targeting of the Fas 3′-UTR by either miR-20a or miR-20a oligonucleotides. We therefore concluded that miR-20a did not regulate Fas expression through translational repression. By contrast, we found that the activity of the Fas promoter was affected by miR-20a. This was determined using reporter assays that evaluated Fas-promoter-driver luciferase expression in the presence of miR-20a or a control in miRNA. We went on to identify a 90 bp region (–240 to –150 bp) that was critical for the miR-20a-mediated downregulation of Fas. Therefore, miR-20a appears to regulate Fas via a transcriptional mechanism. We hypothesize that miR-20a targets transcriptional factor(s) that bind to the Fas promoter leading to the shutdown in Fas expression.

Harnessing the Lung Microenvironment as Part of the Therapy Aimed at Eradicating Osteosarcoma Lung Metastases

We have demonstrated that (a) the presence of the Fas receptor on osteosarcoma cells determines the cell's ability to survive in the $FasL^+$ lung microenvironment; (b) the downregulation of Fas is epigenetic rather than secondary to gene mutation or deletion; (c) the re-expression of Fas on established osteosarcoma lung metastases leads to tumor regression; and (d) this tumor regression is dependent upon a functioning Fas signaling pathway and the expression of FasL in the lungs. Taken together, these findings suggest that a pharmacologic approach aimed at upregulating Fas expression on the tumor cell may allow the lung microenvironment to become part of the therapeutic approach. This requires the identification of agents that can induce the re-expression of Fas, either by targeting miR-20a or having a direct effect on the Fas promoter. To date, we have identified at least two potential agents, the histone deacetylase inhibitor entinostat, and the chemotherapeutic agent gemcitabine (GCB).

In addition to miRNAs, another mechanism for the regulation of gene transcription is chromatin histone acetylation. The function of histone deacetylases (HDACs) is to remove the acetyl groups from the lysine residues in the core histones, which results in the formation of a condensed and transcriptionally inactive chromatin.

HDAC inhibitors can inactivate the function of HDACs, leading to hyperacetylation of histones and transcriptionally active chromatin, resulting in the upregulation of gene expression. Several HDAC inhibitors have been used in clinical trials to treat a variety of hematopoietic malignancies and solid tumors [55]. We demonstrated that the transcription of the Fas gene can be upregulated in human LM7 and CCH-OS-D osteosarcoma cells, and mouse DLM8 osteosarcoma cells by the HDAC 1/3-specific inhibitor, entinostat [56, 57]. This finding was supported by other investigators showing that HDAC-1 or HDAC-3 inhibitors stimulated the upregulation of Fas mRNA in U2OS osteosarcoma cells [58, 59].

Studies have also shown that successful triggering of the Fas/FasL pathway depends on the shifting of the Fas receptor to the lipid rafts in the cell membrane, followed by internalization of the Fas receptor which is mediated by recruiting actin and ezrin [60]. Interestingly, our investigations showed that in addition to the upregulation of Fas expression, entinostat promoted the shifting of the Fas receptor to the lipid rafts in the cell membrane [56]. The increased distribution to the lipid rafts led to the sensitization of osteosarcoma cells to FasL. This process was independent of the upregulation of Fas expression. Therefore, entinostat acts in multiple ways to promote FasL-mediated sensitivity through the regulation of Fas. We found that the upregulation of Fas by entinostat was mediated by decreasing the expression of both miR-17-92 and miR-20a. This was determined by real-time polymerase chain reaction. Finally, we demonstrated that the administration of entinostat to mice with osteosarcoma lung metastases resulted in tumor regression [23]. Entinostat has been used in clinical trials for patients with relapsed acute leukemia, lymphoid malignancies, and other solid tumors including sarcoma [55, 61]. Our data indicate that entinostat may also be a potential agent for the treatment of osteosarcoma lung metastases.

Gemcitabine Upregulates Fas Expression

GCB is a pyrimidine antimetabolite that belongs to the nucleoside analog family, and has been used as a chemotherapeutic agent in small cell lung cancer, pancreatic cancer, and breast cancer. Unfortunately, it has been reported that the efficacy of systemic administration of GCB in the treatment of advanced sarcomas, including osteosarcoma, is poor [62]. However, we have shown that aerosol GCB induced the regression of LM7 or DLM8 osteosarcoma lung metastases in mice [26, 27]. In exploring the underlying mechanism, we found that GCB induced the upregulation of Fas expression on the cell surface of osteosarcoma cells in vitro and on lung metastases in vivo. In vitro, this upregulation of Fas resulted in increased susceptibility to FasL-induced apoptosis. More importantly, the activity of GCB in inducing the regression of osteosarcoma lung metastases was abolished in FasL-deficient gld mice or when the Fas/FasL signaling pathway was blocked [18]. Indeed, the size and number of lung metastases in gld mice following aerosol GCB was increased compared to the control mice. These results indicated that the antitumor effect

induced by aerosol GCB was *not* mediated by a direct effect on the tumor cells but rather secondary to the upregulation of Fas expression followed by the actions of the FasL$^+$ lung microenvironment. These findings further validate that the tumor microenvironment can indeed be part of the therapeutic process.

Summary

Our studies make a strong case that the lung microenvironment, with its constitutive expression of FasL, plays a role in the fate of osteosarcoma cells that migrate to the lungs and whether or not these migrated cells can go on to form lung metastases. The expression of Fas on the cell surface of the osteosarcoma cells is a critical factor. Osteosarcoma cells that have downregulated Fas or a blocked Fas signaling pathway will be able to survive in the FasL$^+$ lung microenvironment. We have also demonstrated that therapeutic interventions that result in the re-expression of Fas can induce the regression of established lung metastases. This is in addition to, or independent of, the agent's ability to directly induce tumor cell apoptosis. It therefore behooves us to consider incorporating potential therapeutic interventions which take advantage of the tumor microenvironment into the therapeutic approach for the treatment of osteosarcoma lung metastases.

References

1. Ritter J, Bielack SS (2010) Osteosarcoma. Ann Oncol 21(Suppl 7):vii320–vii325
2. Jeffree GM, Price CH, Sissons HA (1975) The metastatic patterns of osteosarcoma. Br J Cancer 32(1):87–107
3. Arndt CA, Crist WM (1999) Common musculoskeletal tumors of childhood and adolescence. N Engl J Med 341(5):342–352
4. Harting MT, Blakely ML (2006) Management of osteosarcoma pulmonary metastases. Semin Pediatr Surg 15(1):25–29
5. Longhi A et al (2006) Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 32(6):423–436
6. Kempf-Bielack B et al (2005) Osteosarcoma relapse after combined modality therapy: an analysis of unselected patients in the Cooperative Osteosarcoma Study Group (COSS). J Clin Oncol 23(3):559–568
7. Meyers PA et al (2008) Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival - A report from the Children's Oncology Group. J Clin Oncol 26(4):633–638
8. Chou AJ et al (2009) Addition of muramyl tripeptide to chemotherapy for patients with newly diagnosed metastatic osteosarcoma: a report from the Children's Oncology Group. Cancer 115(22):5339–5348
9. Lee HO, Ferguson TA (2003) Biology of FasL. Cytokine Growth Factor Rev 14(3–4):325–335
10. Algeciras-Schimnich A et al (2002) Molecular ordering of the initial signaling events of CD95. Mol Cell Biol 22(1):207–220
11. Ferguson TA, Green DR (2001) Fas-ligand and immune privilege: the eyes have it. Cell Death Differ 8(7):771–772

12. Moller P et al (1994) Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int J Cancer 57(3):371–377
13. O'Brien DI et al (2005) Targeting the Fas/Fas ligand pathway in cancer. Expert Opin Ther Targets 9(5):1031–1044
14. Owen-Schaub LB et al (1998) Fas and Fas ligand interactions suppress melanoma lung metastasis. J Exp Med 188(9):1717–1723
15. Lafleur EA et al (2004) Increased Fas expression reduces the metastatic potential of human osteosarcoma cells. Clin Cancer Res 10(23):8114–8119
16. Worth LL et al (2002) Fas expression inversely correlates with metastatic potential in osteosarcoma cells. Oncol Rep 9(4):823–827
17. Jia SF, Worth LL, Kleinerman ES (1999) A nude mouse model of human osteosarcoma lung metastases for evaluating new therapeutic strategies. Clin Exp Metastasis 17(6):501–506
18. Koshkina NV et al (2007) Fas-negative osteosarcoma tumor cells are selected during metastasis to the lungs: the role of the Fas pathway in the metastatic process of osteosarcoma. Mol Cancer Res 5(10):991–999
19. Gordon N et al (2005) Fas expression in lung metastasis from osteosarcoma patients. J Pediatr Hematol Oncol 27(11):611–615
20. Gordon N et al (2007) Corruption of the Fas pathway delays the pulmonary clearance of murine osteosarcoma cells, enhances their metastatic potential, and reduces the effect of aerosol gemcitabine. Clin Cancer Res 13(15 Pt 1):4503–4510
21. Gordon N, Kleinerman ES (2009) The role of Fas/FasL in the metastatic potential of osteosarcoma and targeting this pathway for the treatment of osteosarcoma lung metastases. Cancer Treat Res 152:497–508
22. Rao-Bindal K et al (2013) Expression of c-FLIP in pulmonary metastases in osteosarcoma patients and human xenografts. Pediatr Blood Cancer 60(4):575–579
23. Rao-Bindal K et al (2013) The histone deacetylase inhibitor, entinostat (entinostat), downregulates c-FLIP, sensitizes osteosarcoma cells to FasL, and induces the regression of osteosarcoma lung metastases. Curr Cancer Drug Targets 13(4):411–422
24. Zhou Z et al (2005) Interleukin-12 up-regulates Fas expression in human osteosarcoma and Ewing's sarcoma cells by enhancing its promoter activity. Mol Cancer Res 3(12):685–691
25. Duan X et al (2006) Intranasal interleukin-12 gene therapy enhanced the activity of ifosfamide against osteosarcoma lung metastases. Cancer 106(6):1382–1388
26. Gordon N, Kleinerman ES (2010) Aerosol therapy for the treatment of osteosarcoma lung metastases: targeting the Fas/FasL pathway and rationale for the use of gemcitabine. J Aerosol Med Pulm Drug Deliv 23(4):189–196
27. Koshkina NV, Kleinerman ES (2005) Aerosol gemcitabine inhibits the growth of primary osteosarcoma and osteosarcoma lung metastases. Int J Cancer 116(3):458–463
28. Micheau O et al (1997) Sensitization of cancer cells treated with cytotoxic drugs to fas-mediated cytotoxicity. J Natl Cancer Inst 89(11):783–789
29. Poulaki V, Mitsiades CS, Mitsiades N (2001) The role of Fas and FasL as mediators of anticancer chemotherapy. Drug Resist Updat 4(4):233–242
30. Friesen C et al (1996) Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 2(5):574–577
31. Petak I et al (2003) Hypermethylation of the gene promoter and enhancer region can regulate Fas expression and sensitivity in colon carcinoma. Cell Death Differ 10(2):211–217
32. Walker PR et al (2000) Loss of Fas (CD95/APO-1) expression by antigen-specific cytotoxic T cells is reversed by inhibiting DNA methylation. Cell Immunol 206(1):51–58
33. Santourlidis S et al (2001) Hypermethylation of the tumor necrosis factor receptor superfamily 6 (APT1, Fas, CD95/Apo-1) gene promoter at rel/nuclear factor kappa B sites in prostatic carcinoma. Mol Carcinog 32(1):36–43
34. Teodoridis JM et al (2005) CpG island methylation of DNA damage response genes in advanced ovarian cancer. Cancer Res 65(19):8961–8967
35. Butler LM et al (2000) Promoter region methylation does not account for the frequent loss of expression of the Fas gene in colorectal carcinoma. Br J Cancer 82(1):131–135

36. Huang GX, Koshkina NV, Kleinerman ES (2009) Fas expression in metastatic osteosarcoma cells is not regulated by CpG island methylation. Oncol Res 18(1):31–39
37. He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5(7):522–531
38. Shivdasani RA (2006) MicroRNAs: regulators of gene expression and cell differentiation. Blood 108(12):3646–3653
39. Garzon R, Calin GA, Croce CM (2009) MicroRNAs in cancer. Annu Rev Med 60:167–179
40. Inomata M et al (2009) MicroRNA-17-92 down-regulates expression of distinct targets in different B-cell lymphoma subtypes. Blood 113(2):396–402
41. Ovcharenko D et al (2007) Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway. Cancer Res 67(22):10782–10788
42. Petrocca F et al (2008) E2F1-regulated microRNAs impair TGF beta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13(3):272–286
43. Schickel R et al (2010) mir-200c regulates induction of apoptosis through CD95 by targeting FAP-1. Mol Cell 38(6):908–915
44. Suzuki Y et al (2010) Diazoxide potentiates mesenchymal stem cell survival via NF-kappa B-dependent miR-146a expression by targeting Fas. Am J Physiol Heart Circ Physiol 299(4): H1077–H1082
45. Tili E et al (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/ TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179(8):5082–5089
46. Ota A et al (2004) Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res 64(9):3087–3095
47. Hayashita Y et al (2005) A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65(21):9628–9632
48. He L et al (2005) A microRNA polycistron as a potential human oncogene. Nature 435(7043): 828–833
49. Aqeilan RI, Calin GA, Croce CM (2010) miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell Death Differ 17(2):215–220
50. Novello C et al (2013) miRNA expression profile in human osteosarcoma: role of miR-1 and miR-133b in proliferation and cell cycle control. Int J Oncol 42(2):667–675
51. Namlos HM et al (2012) Modulation of the osteosarcoma expression phenotype by microRNAs. PLoS One 7(10):48086
52. Huang G et al (2012) miR-20a encoded by the miR-17-92 cluster increases the metastatic potential of osteosarcoma cells by regulating Fas expression. Cancer Res 72(4):908–916
53. Baumhoer D et al (2012) MicroRNA profiling with correlation to gene expression revealed the oncogenic miR-17-92 cluster to be up-regulated in osteosarcoma. Cancer Genet 205(5): 212–219
54. Papagiannakopoulos T, Shapiro A, Kosik KS (2008) MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res 68(19):8164–8172
55. Glaser KB (2007) HDAC inhibitors: clinical update and mechanism-based potential. Biochem Pharmacol 74(5):659–671
56. Rao-Bindal K, Zhou Z, Kleinerman ES (2012) Entinostat sensitizes osteosarcoma cells to Fas ligand-induced cell death by increasing the localization of Fas in membrane lipid rafts. Cell Death Dis 3:e369
57. Koshkina NV, Rao-Bindal K, Kleinerman ES (2011) Effect of the histone deacetylase inhibitor SNDX-275 on Fas signaling in osteosarcoma cells and the feasibility of its topical application for the treatment of osteosarcoma lung metastases. Cancer 117(15):3457–3467
58. Escaffit F et al (2007) Cleavage and cytoplasmic relocalization of histone deacetylase 3 are important for apoptosis progression. Mol Cell Biol 27(2):554–567
59. Senese S et al (2007) Role for histone deacetylase 1 in human tumor cell proliferation. Mol Cell Biol 27(13):4784–4795

60. Grassme H et al (2001) CD95 signaling via ceramide-rich membrane rafts. J Biol Chem 276(23):20589–20596
61. Ryan QC et al (2005) Phase I and pharmacokinetic study of entinostat, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumors or lymphoma. J Clin Oncol 23(17):3912–3922
62. Okuno S et al (2002) Phase II trial of gemcitabine in advanced sarcomas. Cancer 94(12): 3225–3229

Part III
Unique Osteosarcoma Models

Zebrafish as a Model for Human Osteosarcoma

A.B. Mohseny and P.C.W. Hogendoorn

Abstract For various reasons involving biological comparativeness, expansive technological possibilities, accelerated experimental speed, and competitive costs, zebrafish has become a comprehensive model for cancer research. Hence, zebrafish embryos and full-grown fish have been instrumental for studies of leukemia, melanoma, pancreatic cancer, bone tumors, and other malignancies. Although because of its similarities to human osteogenesis zebrafish appears to be an appealing model to investigate osteosarcoma, only a few osteosarcoma specific studies have been accomplished yet. Here, we review interesting related and unrelated reports of which the findings might be extrapolated to osteosarcoma. More importantly, rational but yet unexplored applications of zebrafish are debated to expand the window of opportunities for future establishment of osteosarcoma models. Accordingly technological advances of zebrafish based cancer research, such as robotic high-throughput multicolor injection systems and advanced imaging methods are discussed. Furthermore, various use of zebrafish embryos for screening drug regimens by combinations of chemotherapy, novel drug deliverers, and immune system modulators are suggested. Concerning the etiology, the high degree of genetic similarity between zebrafish and human cancers indicates that affected regions are evolutionarily conserved. Therefore, zebrafish as a swift model system that allows for the investigation of multiple candidate gene-defects is presented.

Keywords Bone tumor • Metastasis • Angiogenesis • Drug screening • Immune system • Fish

A.B. Mohseny • P.C.W. Hogendoorn, M.D., Ph.D. (✉)
Department of Pathology, Leiden University Medical Center,
P.O. box 9600, H1-Q, Leiden, The Netherlands
e-mail: p.c.w.hogendoorn@lumc.nl

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_12,

General Introduction

The high quantity of worldwide research with dozens of weekly reports registered to PubMed emphasizes the international interest for osteosarcoma investigation. Partly this is explained by the clinical questions which yet need to be answered to improve patient care. On the other hand, it is the complex genesis and pathophysiology of osteosarcoma that attracts researchers from diverse fields of expertise to study this highly malignant bone neoplasm. However, the magnitude of the ongoing research is disproportional to the limited number of satisfying results achieved within the past decades [1, 2]. Here, the complexity of the tumor together with its rareness is the main limiting factor [3, 4]. This chapter provides a brief review of the zebrafish as a model for cancer and more specifically for osteosarcoma and provides some ideas for future zebrafish based studies of osteosarcoma.

Introduction to Zebrafish as a Cancer Model

During recent years zebrafish (*Danio rerio*) models have been increasingly generated to study malignancies, qualifying these fish as illustrative animal systems for the study of human cancer. Some experiment-specific characteristics of the zebrafish make it superior to other model systems; the main advantages include the following. Zebrafish embryos—which in the first few days after fertilization are not larger than just a couple of millimeters—undergo a full external development. Therefore, together with their transparency before pigmentation appears, the embryos provide miniature and optically advantageous model systems. Moreover, the high fecundity and short generation time of these fish make them ideal organisms for in vivo studies [5]. From a genetic point of view, advantageous of zebrafish is that the genome has been fully sequenced, showing many conserved genes as compared to the human genome, and the animals are relatively easily accessible for genetic manipulation [6]. More specifically for osteosarcoma studies it is relevant that zebrafish are vertebrate animals with developmental processes comparable to human osteogenesis. For these reasons, many cancer zebrafish models have been developed [7–11]. Although these models include hematologic (both myeloid and lymphatic lineages) and solid tumors (rhabdomyosarcoma, Ewing's sarcoma, hepatocellular carcinoma and other malignancies), they represent malignancies which are mainly referred to as tumors with relatively simple karyotypic changes [12–14]. This explains the lack of such models and especially transgenic systems for osteosarcoma because of its highly complex genomic alterations and stresses the need for xenograft models.

Miniature Laboratory

Background

The hallmarks of cancer [15] include (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, (6) activating invasion and metastasis, (7) the capability to reprogram or modify cellular metabolism, and (8) evading destruction by the immune system. Broadly these hallmarks can be divided into two groups. The first group including the first four and number 7 contains internal characteristics of malignantly transformed cells while the second group including hallmarks 5, 6, and 8 defines common traits of the interaction between malignant cells and their host. Investigating these hallmarks in osteosarcoma biology shows that many characteristics of the tumor are resulting from a tremendous level of genomic instability. How a normal cell can gain and maintain such genomic instability is not the subject of this chapter; however, data point towards a single master mutation which allows the cell to continuously proliferate accumulating mutations while escaping cell cycle checks and apoptosis [16–20].

Especially for the second group of hallmarks a model is required which would allow for objectification of all the processes which the cancer cells induce inside the body of their host. For this zebrafish embryo models are proven to be ideal as processes like tumor growth, local aggressiveness, angiogenesis, metastases, etc. can be followed in a fast and real-time manner (Fig. 1) [7–9, 21–24]. Regarding osteosarcoma, previously models were established for both groups of hallmarks. First, since osteosarcoma cells show an array of genomic alterations by the time they are isolated from the human tumors that makes them very difficult to study, we established a mouse mesenchymal stem cells (MSCs) based model [25, 26]. This model allowed for addressing processes from the first group of hallmarks such as sustaining proliferative signaling, evading growth suppressors, and resisting cell death [26]. Next, the model was implemented into a zebrafish embryo model system to study aspects from the second group of hallmarks like angiogenesis, migration, and the immune system response of the zebrafish [27]. As for the osteosarcoma cells, any adequate normal cells for comparison on genetic level are lacking, selection for the driver-affected genes would be impossible. Therefore, MSCs were used of which we possessed normal parental cells before transformation and transformed ones, which produced osteosarcoma-like tumors after injection into mice. In short this experiment was conducted as described in Box 1 and Fig. 2.

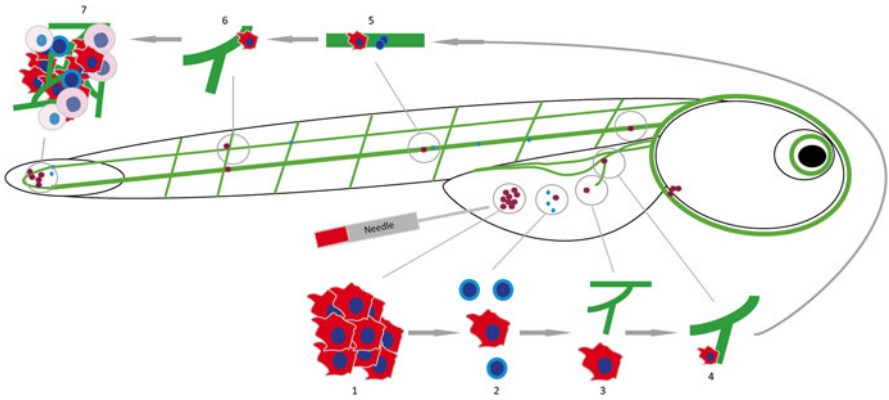


Fig. 1 In vivo tumor progression. A schematic representation of a zebrafish embryo with fluorescent green vasculature is shown. After injection of labeled tumorigenic cells—in this case colored *red*—into the embryos' yolks, important processes of tumor progression can be studied. As indicated by the *arrows*, (*1*) in vivo cell proliferation, (*2*) escaping immune cells (colored *blue*), (*3*) inducing angiogenesis, (*4*) intravasation, (*5*) migration through the circulation, (*6*) extravasation, and (*7*) proliferation at a secondary location can be observed respectively. Depending on how cells are labeled accurate proliferation rates and doubling times can be calculated to compare different cell lines or to examine anti-proliferative effects of selected compounds. Which components of the zebrafish immune system might be encountered by the injected cells is dependent on the embryonic stage. Within a couple of hours after fertilization the innate immune system is functional while the adaptive immunity is detectable within the first weeks. Studying migration of the cells involving angiogenesis, intravasation and extravasation, and homing at a new location is crucial from a clinical point of view as drugs selectively inhibiting any of these aspects might be useful to prevent osteosarcoma cells from metastasizing or to target micro metastases. From a technical point of view, it is important that cells are not directly injected into the blood vessels, as in that case regardless of their intrinsic characteristics cells would travel through the circulation system. Experiments show that "metastases" are most frequently found at the distal end of the tail or the head of the fish. Next to the plausible explanation that cells are entrapped at these locations due to the small vasculature network, it would be interesting to study alternative theories with a role for cytokines and a supportive niche. Please note that if instead of injecting cells, tumor tissue pieces are transplanted into the embryo's, migration of tumor cells and tumor formation elsewhere would be more representative of true metastasis

Cell Lines Versus Primary Tumor and Niche Support

For the model described in Box 1 cultured cells were used to inject into zebrafish embryos. Although within the first hours of injection the cells remain together and proliferate, this clump of cells does not fully represent a true tumor. Inside the yolk the cells lack interaction with stromal cells except for the early immune cells. One way to improve the model would be to inject the cells of interest—either MSCs or osteosarcoma cells—together with stromal cells, for example fibroblasts. Another possibility in case of MSCs would be to inject a mix of normal and transformed MSCs and to hypothesize that the normal MSCs would be stimulated by the transformed ones in a way to provide niche support, maybe by differentiating into other lineages. However, more elegantly, pieces of osteosarcoma directly dissected from patients' tumors could be xenografted into zebrafish embryos. In mouse studies it is shown that such "fresh" pieces of osteosarcoma as well as many osteosarcoma cultured cell lines are able to survive and grow subcutaneously [30, 31]. Unfortunately in those studies the course of tumor growth, angiogenesis, and metastasis could mainly be examined after the

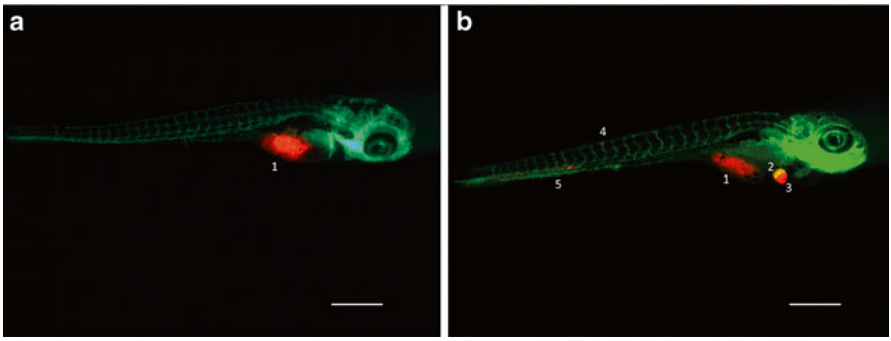


Fig. 2 Normal versus tumorigenic. Two pictures of zebrafish embryos obtained by a camera coupled to a fluorescent microscope are depicted. The transgenic embryos at 2 dpf with green vasculature were injected with cells labeled in *red* and these pictures were taken after 3 days (5 dpf). Magnification bars represent approximately 1 mm. (**a**) A zebrafish embryo injected with normal mouse MSCs is depicted. Despite the strong red signal of the cells (*1*), no signs of angiogenesis were found and the cells remained inside the yolk. (**b**) This embryo was injected with transformed mouse MSCs which were shown to be tumorigenic when transplanted into mice. Already within 24 h sprouting of the subintestinal vein (SIV) was observed indicating angiogenesis (*2*). Furthermore, cells migrated to these vessels (*3*) could be found inside the circulation (*4*) and accumulated at the distal part of the tail (*5*) or the head (not shown here)

animals were sacrificed. By implementing this system of xenotransplantation into zebrafish embryos, it would be possible to image a live tumor piece and to closely follow the subsequent processes in a real-time manner. Currently experiments are ongoing to establish optimal ratios of normal and transformed MSCs when injected together. This will point out if it is possible to create a primary tumor before the processes of angiogenesis and migration take place. Experiments aiming xenotransplantation of human tumors are hampered by technical difficulties due to the small size of the embryos and the aggressiveness of the tumors; however, overcoming these technical problems only seems to be a matter of time.

Immune Response

Another interesting finding from this model leading to new ideas was the fact that the immune response by the fish injected with normal MSCs was clearly different from the immune response of those injected with transformed MSCs as shown by gene expression patterns. The data suggested that the transformed MSCs could regulate the embryos immune system to their advantage. A shortcoming of the model was the lack of a label for the immune cells so this finding could be validated. Therefore, for future studies zebrafish based models are developed with certain labeled immune cells. Fundamental members of the zebrafish immune system, both innate and acquired, are shown to be similar to those in human [32, 33]. This provides the possibility to investigate crucial interactions between tumor cells and macrophages, antigen recognizing lymphocytes and immunoglobulins. Box 2 provides a more detailed overview of the zebrafish immune system in which some players might be important against tumor cells.

Box 1 Zebrafish Progression Model

Towards modeling clinical relevant aspects of osteosarcoma, such as its highly aggressive local growth and its progression in terms of invasion, angiogenesis, and metastasis, zebrafish embryos were used.

The night before the start of the experiment two mature zebrafish of both sexes were put together in an aquarium, but separated by a glass divider so that they could see but not reach each other. One transgenic zebrafish was expressing enhanced green fluorescent protein (EGFP) in all blood vessels [28] and the other was a transparent zebrafish called Casper [29]. Next morning when the divider was removed the female fish almost directly produced about 200–300 eggs which were fertilized by the male resulting into transparent embryos with green blood vessels. Subsequently the embryos started accelerated, simultaneous development outside the mother's body. Meanwhile, two types of MSCs, normal and transformed MSCs which produced osteosarcoma like tumors inside mice were cultured and labeled with a red dye. Next, both type of labeled MSCs were injected into the yolks of zebrafish 2 days post fertilization (dpf) so the red cells could be easily followed inside transparent zebrafish embryos with green vessels (Fig. 3). In contrast to the normal MSCs, the transformed MSCs showed within 3 days after injection excessive proliferation, migration towards the body of the fish, and induced angiogenesis. Whole-genome expression analysis of both the cells and the host showed that angiogenesis and migration-related genes were overexpressed in transformed MSCs as compared to normal MSCs.

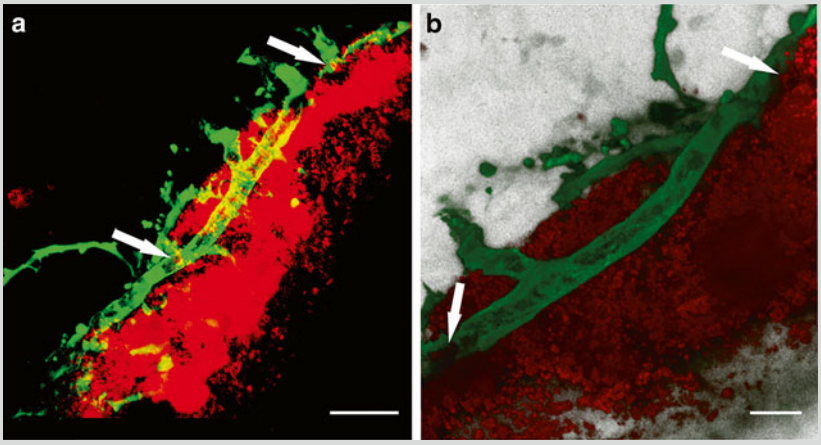


Fig. 3 Intravasation/extravasation of cells. Magnified pictures of the SIV complex—and newly formed vessels—of zebrafish embryos injected by transformed mouse MSCs are shown. Magnification bars represent approximately 100 μm. (**a**) High-resolution image by confocal microscopy depicts intravasation of the cells as indicated by the *white arrows*. (**b**) Three-dimensional reconstruction of (**a**) shows that cells are inside the blood vessels to exclude optical deception when cells and vessels would only overlap

(continued)

Box 1 (continued)

Investigating the host response, embryos injected with transformed MSCs showed decreased expression of immune response-related genes as compared to embryos injected with normal MSCs. The advantages of this model as compared to mouse models were its relatively low costs, its high statistical power by the large group sizes, its high speed—experiments were performed within 5 days—and its advantages in imaging by using transparent fish.

Box 2 Zebrafish Immune System

Zebrafish model systems provide opportunities to identify members of the immune system which play a role in the defense mechanisms against cancer cells. Therefore, it is crucial to know how representative the fish immune system is for the human defense mechanisms.

Although the number of immune organs in zebrafish seems to be limited as compared to mammals and consist only of kidney, thymus, and spleen—so no bone marrow or lymph nodes—zebrafish possess both innate and acquired immunity [34]. The anterior kidney contains developing B lymphoid cells and is mainly involved in antigen processing, IgM formation, and immune memory [35–40]. The spleen plays an essential role in hematopoiesis, antigen trapping and degradation, and antibody production [41, 42]. Erythrocytes destruction by filtrating blood however is accomplished within melanomacrophagic centers in which macrophages are accumulated to capillaries. And finally the T-lymphocytes are produced in the thymus to control many of the previously mentioned effectors of the immune system [43]. The innate—or nonspecific—immune system of the zebrafish includes antibacterial peptides, lectins, and lysozyme expressed in cells of myeloid origin [44]. Furthermore, it is interesting to know that C-reactive protein (CRP) is a highly conserved acute phase protein and present in zebrafish [45]. Also both the classical and alternative complement pathways are comparable to those in human and play an important role in the link between the innate and adaptive immune responses [46–48]. As for the adaptive—or specific—immune response, the overall mechanisms are in zebrafish similar to those in human [49]. Main components of this system in zebrafish include MHC, recombination activating genes (*RAG* 1 and 2 which cause diversity in T cell receptors and antibodies), antigen recognizing lymphocytes and immunoglobulins [49, 50]. Finally important immune modulators such as toll-like receptors and cytokines, including interleukin-1b, TNF-alpha, and IL-6 are found in fish [51–55]. In conclusion, multiple high-quality recent studies have emphasized crucial similarities between the zebrafish and the human immune systems [56, 57]. This opens new windows of opportunity to investigate interactions between osteosarcoma cells—or cancer in general—and components of the immune system with consequences for novel drug screens.

High-Throughput Injection and Imaging

One of the major advantages of using zebrafish embryos as compared to other animal models is related to the small size of the larvae. Keeping 50–100 embryos in one normal sized petri dish or single fish in wells of 96-well plates, provides possibilities to study large groups increasing the power of the experiments. However, at the time the above-mentioned study was performed, which included manual injection of hundreds of embryos, it was clear that for future larger studies alternative methods for injection would be required. Furthermore, handling of the fish after injection and daily imaging to follow the cells were very time-consuming and labor-intensive. For these reasons experiments were performed to assay robotic injections and automated imaging systems. Recently several systems have been reported, by which a combination of robotic injections and several manners of automated imaging guarantee high-throughput screens with less labor intensity [58–60]. One remaining challenge when using robotic arms to inject zebrafish embryos is that the location of injection is less precise. As a consequence embryos might be injected at vital locations and not survive the experiment. Moreover, accidental injections directly inside the blood vessels would bias the results when migration of the cells is a primary outcome. However, robotic injection systems would speed up the experiments to such an extent that it is worthwhile to increase the size of the groups before injection and to select the correctly injected embryos afterwards (Fig. 4).

Drug Screens

Single Drugs

Traditionally zebrafish models were frequently used for screening of chemical compounds to assay carcinogenic effects by looking for tumor formation or mutant fish [61–65]. However, it is only recent that these fish exponentially gained the interest of cancer researchers for screening anticancer drugs. The main reason for this is the establishment of various zebrafish transgenic or xenotransplantation—as exemplified in Box 1—cancer models [61–63, 65–67]. With the availability of such models in which processes related to cancer progression, like angiogenesis and metastasis, can be followed in a live setting, it is only logical to screen drugs which could inhibit these processes. Moreover, when such drug screens are performed in a high-throughput automated fashion, significantly more drugs could be tested. This might decrease the pressure for in vitro selection steps for probable drugs and potentially increase the discovery of novel therapeutics based on in vivo results. Next to the previously mentioned advantages of zebrafish related to imaging, group size and simultaneous development of the embryos, there is another factor which makes the

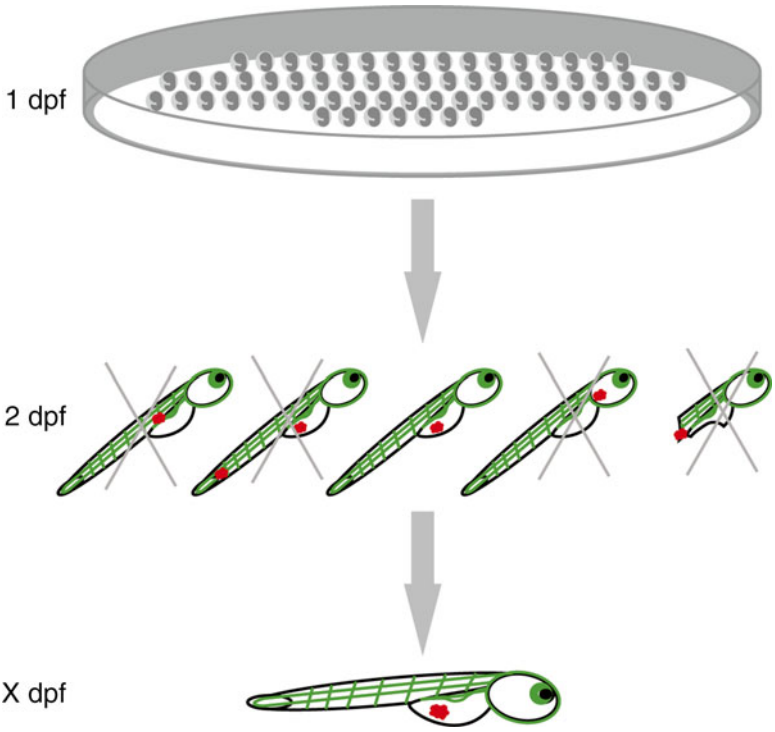


Fig. 4 High-throughput injection and imaging. A schematic work flow is provided for high-throughput injection and screening of zebrafish embryos. At 1 dpf embryos are still inside the chorion and can be mounted in rows in petri dishes coated with agarose. At these stage embryos dot not move, which simplifies robotic injections. Although the embryos will be injected at random locations, its high speed and reduction of labor intensity rationalize robotic injections. The next day embryos which were injected at the right location—in this case only inside the yolk—and without any sign of deformity can be selected. For the following days depending on the duration of the experiment, selected embryos can be imaged on a regular base. Automatic imaging systems could reduce the workload even further and provide a narrower selection for manual screening

fish superior to other animals for drug screening. Namely the drug of interest can be added to the swimming water of the fish which will secure equal uptake by all the members of the experimental group. A drawback here is that other routes of drug delivery, such as micro intravenous injections, are yet hampered by technical difficulties; however, solutions or alternative methods are being developed [59, 68]. Up to today only one report has been published with the specific aim to test anti-osteosarcoma drugs of which the developmental effects were assayed in zebrafish. In this study small molecule inhibitors of Ezrin [69] were shown to inhibit the invasive phenotype of osteosarcoma cells [70].

Combined Therapies

In addition to drug screens for single agents, high-throughput automated zebrafish model systems seem to be amenable for multiple drugs testing which allows for various combinations of different therapeutic regimens. Osteosarcoma patients' outcome considerably improved after the introduction of chemotherapy in the 1970s followed by adjustments in treatment protocols to reduce chemotherapy toxicity as much as possible [71]. However, in terms of survival no significant improvements were accomplished since [2]. Therefore, toxic chemotherapy next to surgery remains the key treatment, which fails in 30–40 % of the patients. Nevertheless, recently alternative or additional therapeutic options were reported to effectively kill osteosarcoma cells and cells of other sarcomas in vitro. Two of the promising protocols include addition of muramyl tripeptide to chemotherapy [72] and the use of anti-EGFR antibody cetuximab to increase the anti-osteosarcomic effect of NK cells [73]. An alternative method to stimulate NK cells cytolytic effect towards tumor cells was shown to be achieved by cytokine stimulation [74, 75]. Next to chemotherapy resistance yet another issue in the lack of specificity in current chemotherapy regimens. Recently the use of gold nanoparticles has been reported by which via the highly expressed folic acid in cancer cells enhanced therapeutic efficacy and reduced side effects were realized [76]. As osteosarcoma cells abundantly express folic acid—in fact methotrexate which is one of the most widely used chemotherapeutics against osteosarcoma is an antifolate—this might allow for better marking of the cancer cells [77]. These novel immune and chemical treatment options are just a few examples, which have proved to be effective for killing cancer cells in vitro. The next logical step is to investigate these alternative therapy regimens wherein combination of the current chemotherapeutics and novel agents can lead to more effective targeted therapy. Zebrafish embryo models provide excellent animal systems to implement such wide drug screens for assaying the effects on human tumor derived cells and even metastases.

Mimicking Human Osteosarcoma

In paradox to the frequent number of attempts, transgenic animal models, which fully represent human conventional osteosarcoma are lacking [78–80]. The main reason for this is the complex karyotype as a result of genomic instability which underlies the disease but is until now indefinable to single mutations which could be applied to animal models [3, 12]. Recently great strides have been made towards a better understanding of the complex pathogenesis of osteosarcoma. Processes likely underlying genomic instability were identified and investigated [17, 19, 26, 81] and possible susceptibility loci were identified [82]. As these type of clues are upcoming, new opportunities for—preferentially conditional—transgenic representative osteosarcoma models are rising. Since the number of possibilities for genomic engineering is

still broad, mouse models would be quite time consuming to start with. Alternatively, in a significantly swifter manner, zebrafish transgenic or knockout lines could be generated, as widely performed for other diseases and cancer [8, 83–95]. Recently detailed protocols have been published [59] for a sequence of experiments for generation of transgenic and knockout zebrafish lines, gene knockdown by using morpholinos, siRNA, or antibodies in a high-throughput manner and more. These protocols shorten the bridge between exciting in vitro data and transgenic zebrafish models.

Conclusion

After some relatively quiet years, great steps have been set towards a better understanding of the complex genomics and pathophysiology of osteosarcoma. Nevertheless, the lack of less toxic and more effective targeted therapy still remains. Therefore, alternative treatment protocols are urgently needed, especially for osteosarcoma patients with chemotherapy insensitive tumors. To accomplish this great challenge for such a multifarious and yet rare disease, the use of representative animal models is unavoidable. Certainly zebrafish embryo models would not always be sufficient as a link between in vitro data and clinical trials. However, current data strongly suggest that these models not only are outstanding alternatives to limit the number of mouse or other animal model studies but also provide new opportunities by allowing for much broader screens. Such screens should start by investigating the progression of osteosarcoma cells live inside the zebrafish bodies. In subsequent steps assaying key players which control tumor cells proliferation, migration through the circulation, metastasis, and the interplay with the immune system could provide novel specific targets for therapy. In addition for recently identified genes which might predispose osteosarcoma, zebrafish provide a speedy method for functional validation. When the targeted genes turn out to indeed be the true driver mutations, simultaneously new representative models are born. Finally all the established xenotransplanted and transgenic models could be utilized for comprehensive drug screens.

Acknowledgements The authors thank Dr. Cleton-Jansen for reading the manuscript and providing valuable comments and Thomas Mohseny for providing fruitful ideas.

References

1. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20(3):776–790
2. Lewis IJ, Nooij MA, Whelan J et al (2007) Improvement in histologic response but not survival in osteosarcoma patients treated with intensified chemotherapy: a randomized phase III trial of the European Osteosarcoma Intergroup. J Natl Cancer Inst 99(2):112–128

3. Sandberg AA, Bridge JA (2003) Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. Cancer Genet Cytogenet 145(1):1–30
4. Cleton-Jansen AM, Buerger H, Hogendoorn PCW (2005) Central high-grade osteosarcoma of bone: diagnostic and genetic considerations. Curr Diagn Pathol 11:390–399
5. Stoletov K, Klemke R (2008) Catch of the day: zebrafish as a human cancer model. Oncogene 27(33):4509–4520
6. Deo RC, MacRae CA (2011) The zebrafish: scalable in vivo modeling for systems biology. Wiley Interdiscip Rev Syst Biol Med 3(3):335–346
7. Merlino G, Khanna C (2007) Fishing for the origins of cancer. Genes Dev 21(11):1275–1279
8. Langenau DM, Keefe MD, Storer NY et al (2007) Effects of RAS on the genesis of embryonal rhabdomyosarcoma. Genes Dev 21(11):1382–1395
9. Feitsma H, Kuiper RV, Korving J et al (2008) Zebrafish with mutations in mismatch repair genes develop neurofibromas and other tumors. Cancer Res 68(13):5059–5066
10. Etchin J, Kanki JP, Look AT (2011) Zebrafish as a model for the study of human cancer. Methods Cell Biol 105:309–337
11. He S, Krens SG, Zhan H et al (2011) A DeltaRaf1-ER-inducible oncogenic zebrafish liver cell model identifies hepatocellular carcinoma signatures. J Pathol 225(1):19–28
12. Bridge JA, Nelson M, McComb E et al (1997) Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. Cancer Genet Cytogenet 95(1):74–87
13. Rosenberg AE, Cleton-Jansen AM, Gd P et al (2013) Conventional osteosarcoma. In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds) WHO classification of tumours of soft tissue and bone. IARC, Lyon, pp 282–289
14. Kuijjer ML, Hogendoorn PC, Cleton-Jansen AM (2013) Genome-wide analyses on high-grade osteosarcoma: making sense of a genomically most unstable tumor. Int J Cancer 133(11):2512–2521
15. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5): 646–674
16. Mohseny AB, Hogendoorn PC (2011) Concise review: mesenchymal tumors: when stem cells go mad. Stem Cells 29(3):397–403
17. Stephens PJ, Greenman CD, Fu B et al (2011) Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 144(1):27–40
18. Forment JV, Kaidi A, Jackson SP (2012) Chromothripsis and cancer: causes and consequences of chromosome shattering. Nat Rev Cancer 12(10):663–670
19. Davoli T, de Lange T (2012) Telomere-driven tetraploidization occurs in human cells undergoing crisis and promotes transformation of mouse cells. Cancer Cell 21(6):765–776
20. Gohring G, Lange K, Hofmann W et al (2012) Telomere shortening, clonal evolution and disease progression in myelodysplastic syndrome patients with 5q deletion treated with lenalidomide. Leukemia 26(2):356–358
21. Stoletov K, Montel V, Lester RD et al (2007) High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. Proc Natl Acad Sci U S A 104(44): 17406–17411
22. Lee LM, Seftor EA, Bonde G et al (2005) The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation. Dev Dyn 233(4):1560–1570
23. Herbomel P, Thisse B, Thisse C (1999) Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126(17):3735–3745
24. Lam SH, Chua HL, Gong Z et al (2004) Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28(1):9–28
25. Tolar J, Nauta AJ, Osborn MJ et al (2007) Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 25(2):371–379
26. Mohseny AB, Szuhai K, Romeo S et al (2009) Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol 219(3): 294–305

27. Mohseny AB, Xiao W, Carvalho R et al (2012) An osteosarcoma zebrafish model implicates Mmp-19 and Ets-1 as well as reduced host immune response in angiogenesis and migration. J Pathol 227(2):245–253
28. Marques IJ, Weiss FU, Vlecken DH et al (2009) Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. BMC Cancer 9:128
29. White RM, Sessa A, Burke C et al (2008) Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2(2):183–189
30. Mayordomo E, Machado I, Giner F et al (2010) A tissue microarray study of osteosarcoma: histopathologic and immunohistochemical validation of xenotransplanted tumors as preclinical models. Appl Immunohistochem Mol Morphol 18(5):453–461
31. Mohseny AB, Machado I, Cai Y et al (2011) Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Lab Invest 91(8): 1195–1205
32. Rauta PR, Nayak B, Das S (2012) Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol Lett 148(1):23–33
33. van der Vaart M, van Soest JJ, Spaink HP et al (2013) Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. Dis Model Mech 6(3):841–854
34. Press CMCL, Evensen O (1999) The morphology of the immune system in teleost fishes. Fish Shellfish Immunol 9:309–318
35. Bromage ES, Kaattari IM, Zwollo P et al (2004) Plasmablast and plasma cell production and distribution in trout immune tissues. J Immunol 173(12):7317–7323
36. Zwollo P, Cole S, Bromage E et al (2005) B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J Immunol 174(11):6608–6616
37. Zwollo P, Haines A, Rosato P et al (2008) Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. Dev Comp Immunol 32(12):1482–1496
38. Zwollo P, Mott K, Barr M (2010) Comparative analyses of B cell populations in trout kidney and mouse bone marrow: establishing "B cell signatures". Dev Comp Immunol 34(12): 1291–1299
39. Brattgjerd S, Evensen O (1996) A sequential light microscopic and ultrastructural study on the uptake and handling of Vibrio salmonicida in phagocytes of the head kidney in experimentally infected Atlantic salmon (Salmo salar L.). Vet Pathol 33(1):55–65
40. Kaattari SL, Irwin MJ (1985) Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. Dev Comp Immunol 9(3):433–444
41. Manning MJ, Nakanishi T (1996) The specific immune system: cellular defense. The fish immune system. Organism, pathogen and environment. Academic, San Diego, CA
42. Press CM, Dannevig BH, Landsverk T (1994) Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon. Fish Shellfish Immunol 4(2):79–93
43. Bowden TJ, Cook P, Rombout JHMW (2005) Development and function of the thymus in teleosts. Fish Shellfish Immunol 19:413–427
44. Hall C, Flores MV, Storm T et al (2007) The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. BMC Dev Biol 7:42
45. Bayne CJ, Gerwick L (2001) The acute phase response and innate immunity of fish. Dev Comp Immunol 25(8–9):725–743
46. Carroll MC (2004) The complement system in regulation of adaptive immunity. Nat Immunol 5(10):981–986
47. Carroll MC (2004) The complement system in B cell regulation. Mol Immunol 41(2–3): 141–146
48. Holland JW, Pottinger TG, Secombes CJ (2002) Recombinant interleukin-1 beta activates the hypothalamic-pituitary-interrenal axis in rainbow trout, Oncorhynchus mykiss. J Endocrinol 175(1):261–267

49. Du Pasquier L (2001) The immune system of invertebrates and vertebrates. Comp Biochem Physiol B Biochem Mol Biol 129(1):1–15
50. Fischer U, Utke K, Somamoto T et al (2006) Cytotoxic activities of fish leucocytes. Fish Shellfish Immunol 20(2):209–226
51. Zou J, Grabowski PS, Cunningham C et al (1999) Molecular cloning of interleukin 1beta from rainbow trout Oncorhynchus mykiss reveals no evidence of an ice cut site. Cytokine 11(8):552–560
52. Bird S, Wang T, Zou J et al (2002) The first cytokine sequence within cartilaginous fish: IL-1 beta in the small spotted catshark (Scyliorhinus canicula). J Immunol 168(7):3329–3340
53. Roca FJ, Mulero I, Lopez-Munoz A et al (2008) Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol 181(7):5071–5081
54. Wang T, Secombes CJ (2009) Identification and expression analysis of two fish-specific IL-6 cytokine family members, the ciliary neurotrophic factor (CNTF)-like and M17 genes, in rainbow trout Oncorhynchus mykiss. Mol Immunol 46(11–12):2290–2298
55. Lee MS, Kim YJ (2007) Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu Rev Biochem 76:447–480
56. Renshaw SA, Trede NS (2012) A model 450 million years in the making: zebrafish and vertebrate immunity. Dis Model Mech 5(1):38–47
57. Mione M, Meijer AH, Snaar-Jagalska BE et al (2009) Disease modeling in zebrafish: cancer and immune responses – a report on a workshop held in Spoleto, Italy, July 20–22, 2009. Zebrafish 6(4):445–451
58. Chang TY, Pardo-Martin C, Allalou A et al (2012) Fully automated cellular-resolution vertebrate screening platform with parallel animal processing. Lab Chip 12(4):711–716
59. Spaink HP, Cui C, Wiweger MI et al (2013) Robotic injection of zebrafish embryos for high-throughput screening in disease models. Methods 62(3):246–254
60. Truong HH, de Sonneville J, Ghotra VP et al (2012) Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens. Biomaterials 33(1):181–188
61. Amatruda JF, Shepard JL, Stern HM et al (2002) Zebrafish as a cancer model system. Cancer Cell 1(3):229–231
62. Smolowitz R, Hanley J, Richmond H (2002) A three-year retrospective study of abdominal tumors in zebrafish maintained in an aquatic laboratory animal facility. Biol Bull 203(2):265–266
63. Stern HM, Zon LI (2003) Cancer genetics and drug discovery in the zebrafish. Nat Rev Cancer 3(7):533–539
64. Berghmans S, Jette C, Langenau D et al (2005) Making waves in cancer research: new models in the zebrafish. Biotechniques 39(2):227–237
65. Goessling W, North TE, Zon LI (2007) New waves of discovery: modeling cancer in zebrafish. J Clin Oncol 25(17):2473–2479
66. Berghmans S, Murphey RD, Wienholds E et al (2005) tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. Proc Natl Acad Sci U S A 102(2):407–412
67. Kari G, Rodeck U, Dicker AP (2007) Zebrafish: an emerging model system for human disease and drug discovery. Clin Pharmacol Ther 82(1):70–80
68. Peng K, Cui C, Tomatsu I et al (2010) Dextran based photodegradable hydrogels formed via a Michael addition. Soft Matter 7:4881–4887
69. Khanna C, Wan X, Bose S et al (2004) The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 10(2):182–186
70. Bulut G, Hong SH, Chen K et al (2012) Small molecule inhibitors of ezrin inhibit the invasive phenotype of osteosarcoma cells. Oncogene 31(3):269–281
71. Anninga JK, Gelderblom H, Fiocco M et al (2011) Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? Eur J Cancer 47(16):2431–2445

72. Chou AJ, Kleinerman ES, Krailo MD et al (2009) Addition of muramyl tripeptide to chemotherapy for patients with newly diagnosed metastatic osteosarcoma: a report from the Children's Oncology Group. Cancer 115(22):5339–5348
73. Buddingh EP, Schilham MW, Ruslan SE et al (2011) Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. Cancer Immunol Immunother 60(4):575–586
74. Lehner M, Gotz G, Proff J et al (2012) Redirecting T cells to Ewing's sarcoma family of tumors by a chimeric NKG2D receptor expressed by lentiviral transduction or mRNA transfection. PLoS One 7(2):e31210
75. Ahn YO, Weigel B, Verneris MR (2010) Killing the killer: natural killer cells to treat Ewing's sarcoma. Clin Cancer Res 16(15):3819–3821
76. Ganeshkumar M, Sathishkumar M, Ponrasu T et al (2013) Spontaneous ultra fast synthesis of gold nanoparticles using Punica granatum for cancer targeted drug delivery. Colloids Surf B Biointerfaces 106:208–216
77. Hagner N, Joerger M (2010) Cancer chemotherapy: targeting folic acid synthesis. Cancer Manag Res 2:293–301
78. Walkley CR, Qudsi R, Sankaran VG et al (2008) Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev 22(12): 1662–1676
79. Parant JM, George SA, Holden JA et al (2010) Genetic modeling of Li-Fraumeni syndrome in zebrafish. Dis Model Mech 3(1–2):45–56
80. Mohseny AB (2008) Bone: conventional osteosarcoma. Atlas Genet Cytogenet Oncol Haematol. http://AtlasGeneticsOncology.org/Tumors/ConvOsteoID5344.html
81. Mohseny AB, Tieken C, van der Velden PA et al (2010) Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. Genes Chromosomes Cancer 49(12):1095–1103
82. Savage SA, Mirabello L, Wang Z et al (2013) Genome-wide association study identifies two susceptibility loci for osteosarcoma. Nat Genet 45(7):799–803
83. Bedell VM, Wang Y, Campbell JM et al (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491(7422):114–118
84. Bill BR, Petzold AM, Clark KJ et al (2009) A primer for morpholino use in zebrafish. Zebrafish 6(1):69–77
85. Cade L, Reyon D, Hwang WY et al (2012) Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. Nucleic Acids Res 40(16): 8001–8010
86. Chen S, Oikonomou G, Chiu CN et al (2013) A large-scale in vivo analysis reveals that TALENs are significantly more mutagenic than ZFNs generated using context-dependent assembly. Nucleic Acids Res 41(4):2769–2778
87. Clark KJ, Voytas DF, Ekker SC (2011) A TALE of two nucleases: gene targeting for the masses? Zebrafish 8(3):147–149
88. Dahlem TJ, Hoshijima K, Jurynec MJ et al (2012) Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genet 8(8):e1002861
89. Huang P, Zhu Z, Lin S et al (2012) Reverse genetic approaches in zebrafish. J Genet Genomics 39(9):421–433
90. Moore FE, Reyon D, Sander JD et al (2012) Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). PLoS One 7(5):e37877
91. Leacock SW, Basse AN, Chandler GL et al (2012) A zebrafish transgenic model of Ewing's sarcoma reveals conserved mediators of EWS-FLI1 tumorigenesis. Dis Model Mech 5(1):95–106
92. Onnebo SM, Condron MM, McPhee DO et al (2005) Hematopoietic perturbation in zebrafish expressing a tel-jak2a fusion. Exp Hematol 33(2):182–188

93. de Andrea CE, Prins FA, Wiweger MI et al (2011) Growth plate regulation and osteochondroma formation: insights from tracing proteoglycans in zebrafish models and human cartilage. J Pathol 224(2):160–168
94. Wiweger MI, Avramut CM, de Andrea CE et al (2011) Cartilage ultrastructure in proteoglycan-deficient zebrafish mutants brings to light new candidate genes for human skeletal disorders. J Pathol 223(4):531–542
95. Wiweger MI, Zhao Z, van Merkesteyn RJ et al (2012) HSPG-deficient zebrafish uncovers dental aspect of multiple osteochondromas. PLoS One 7(1):e29734

Using Canine Osteosarcoma as a Model to Assess Efficacy of Novel Therapies: Can Old Dogs Teach Us New Tricks?

Carlos O. Rodriguez, Jr.

Abstract Since its domestication more than 10,000 years ago, the dog has been the animal that most intimately shares our work and homelife. Interestingly, the dog also shares many of our diseases including cancer such as osteosarcoma. Like the human, osteosarcoma is the most common bone malignancy of the dog and death from pulmonary metastasis is the most common outcome. The incidence of this spontaneous bone neoplasm occurs ten times more frequently that it does so in children with about 8,000–10,000 cases estimated to occur in dogs in the USA. Because there is no "standard of care" in veterinary medicine, the dog can also serve us by being a model for this disease in children. Although the most common therapy for the dog with osteosarcoma is amputation followed by chemotherapy, not all owners choose this route. Consequently, novel therapeutic interventions can be attempted in the dog with or without chemotherapy that could not be done in humans with osteosarcoma due to ethical concerns. This chapter will focus on the novel therapies in the dog that have been reported or are in veterinary clinical trials at the author's institution. It is hoped that collaboration between veterinary oncologists and pediatric oncologists will lead to the development of novel therapies for (micro- or macro-) metastatic osteosarcoma that improve survival and might ultimately lead to a cure in both species.

Keywords Osteosarcoma • Dog cancer model • Large animal model • Novel therapy • Aerosol interleukin-2 • Muramyl tripeptide-phosphatidylethanolamine • Aerosol chemotherapy • Aerosol gemcitabine • Thiomolybdate

C.O. Rodriguez, Jr., D.V.M., Ph.D., D.A.C.V.I.M. (Oncology) (✉)
Department of Veterinary Surgical and Radiological Sciences,
William R. Pritchard Veterinary Medical Teaching Hospital,
School of Veterinary Medicine, University of California, Davis, CA, USA
e-mail: dvmrodriguez@ucdavis.edu

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_13,

In the past 50 years, the dog's status in the USA has risen from that of serving us on the back 40 to a place near the back door, to a warm space in the kitchen, to a dog's bed on the floor of the bedroom, to a space on the actual bed. Indeed, it can be argued there is no other animal that shares both our work and home environment as intimately as does the dog. Interestingly, the dog also shares many of our neoplastic diseases such as mammary cancer (the most common tumor of the dog), diffuse large B cell lymphoma, malignant melanoma, and the subject of this book, osteosarcoma; thus, the dog can again unfailingly serve us, but this time as a relevant spontaneous out-bred large-animal immune-competent tumor model in which to test novel therapies against this formidable foe common to both man and man's best friend: osteosarcoma.

As is the case in children [39], osteosarcoma (OS) is also the most common bone tumor of the dog [45]. It is estimated that 8,000–10,000 cases of canine OS (COS) are diagnosed each year in the USA [45]. While there is no "standard of care," surgery (amputation or limb sparing) plus single agent anthracycline [9, 68] or platinum [10, 92, 98, 105, 113] or anthracycline–platinum combination [4, 5, 16, 17, 47, 73] chemotherapy are considered the mainstays of therapy in COS. The median survival of dogs treated with amputation alone is 4–6 months [73, 109, 111] and the addition of chemotherapy provides median survivals of 11 months or more [4, 5, 7–10, 16, 17, 47, 60, 61, 68, 73, 80, 92, 101, 106, 110, 111, 113, 118]. In the majority of cases, death is attributable to the evolution of the metastatic disease, which is present in 90 % of cases at the time of diagnosis, from occult microscopic disease to clinically detectable diffuse, gross pulmonary metastasis. Euthanasia usually ensues within 60–90 days of the diagnosis of metastasis due to rapid loss of quality of life of the dog [96]. Similarly, despite advances in therapy in pediatric oncology, the 5 year survival rate in children has remained at 67 % for the past 20 years [108] and death from pulmonary metastasis is unfortunately the most common sequelae [12]. It can thus be said with absolute certainty that metastasis are *the* impediment to the cure of OS in both the dog and in humans. Effective therapies targeting metastatic disease are clearly necessary if survivals of either humans or dogs with OS are to be realized. Because of the compressed time-line of the disease from diagnosis to death in the dog and because of the similarities between canine and human OS [88], novel strategies can be quickly assessed for efficacy against COS, and if findings from the canine studies are promising, these clinically useful therapies can be rapidly translated to human trials.

Conventional intravenous anthracycline-, platinum-, or alkylator-based chemotherapy is largely ineffective in the metastatic setting in the dog [6, 13, 41]. Due to the diffuse multifocal nature of the metastatic presentation in the dog, surgery and radiation therapy are rarely useful in the management of COS except in very select cases [84]. The remainder of the chapter will review what has been tried and what we are trying in the veterinary oncology clinic as therapeutic intervention in both the microscopic and macroscopic metastatic setting in the OS-bearing dog with a hope to push the most effective tested therapies into clinical trials in children with OS. The therapies that will be discussed are liposome-encapsulated muramyl tripeptide, tetrathiomolybdate, and aerosol therapy utilizing gemcitabine or interleukin 2 alone or in combination with chemotherapy.

Therapies

Liposome-Encapsulated Muramyl Tripeptide-Phosphatidylethanolamine (L-MTP-PE)

When activated by L-MTP-PE, macrophages and monocytes are primed to eradicate chemotherapy-resistant neoplastic cancer cells in vitro [24, 25, 40] and in vivo in humans [52, 54, 55, 69] and dogs [63, 102, 107]. In murine melanoma models, the in situ pulmonary macrophages are activated and this activation mediates spontaneous regression of pulmonary metastases [26, 27]. In spontaneous COS, two major studies [62, 67] have been completed in the micrometastatic setting based on these data. In the first double-blind placebo-controlled study [67], dogs with previously untreated, histologically confirmed primary osteosarcoma of the extremity that were radiographically free of distant pulmonary metastases were enrolled, underwent complete amputation of the affected limb and were then randomized to receive either L-MTP-PE or empty liposomes immediately postoperatively.

The lyophilized liposomes with or without MTP-PE (CGP19835A lipid) were provided by Ciba-Geigy Limited (Basel, Switzerland). The liposome preparation (L-MTP-PE was 2 mg/m^2) was given twice weekly for 8 weeks by a slow, 5- to 8-min intravenous infusion. A complete physical examination and a complete blood count were performed prior to each liposome treatment and rectal body temperature was monitored hourly for 6 h after each liposome injection. Thoracic radiographs were obtained at 2-month intervals following amputation. Follow-up continued for as long as necessary to determine metastasis-free interval (defined as the time from surgery to evidence of metastasis) and overall survival time (defined as the time from surgery to death or euthanasia due to advanced disease) for each dog.

Twenty-seven dogs with osteosarcoma of the extremities were enrolled in this study. Of these, 14 received L-MTP-PE and 13 received empty/placebo liposomes. The administration was well tolerated; the only observed side effect was a transient elevation in body temperature of 1–2 °C that occurred 1–3 h after the first three to four liposome treatments, and which returned to normal by 6 h post injection in each case in which the fever was observed. While temperature elevations were more frequent in dogs receiving L-MTP-PE than in those receiving empty placebo liposomes the difference was not statistically significant.

Importantly, the median metastasis-free interval for dogs receiving L-MTP-PE or placebo was 168 days and 58 days, respectively ($p = 0.002$). The median overall survival time for the dogs receiving L-MTP-PE or placebo was 222 days and 77 days, respectively ($p < 0.002$). In contrast to the placebo group where all 13 dogs died of metastatic disease, in the L-MTP-PE group, 4 dogs were alive and metastasis free 17, 18.5, 25.5 and 32 months after amputation. This study demonstrated the efficacy, safety and tolerability of L-MTP-PE ALONE in the micrometastatic setting and provided the impetus for the second study in COS [62].

Whereas the first study evaluated L-MTP-PE ALONE in the microscopic metastatic setting, the second double-blind placebo-controlled study [62] combined

this immunotherapy with cisplatin chemotherapy. One hundred eleven dogs with previously untreated, histologically confirmed primary osteosarcoma of the extremity that were radiographically free of distant pulmonary metastases were enrolled in two different trial arms after pre-enrollment health screening and informed owner consent.

In the first arm, within 24 h after surgery, dogs were started on cisplatin chemotherapy (70 mg/m^2 iv) and were scheduled to receive a total of four doses of cisplatin administered once every 28 days. One month after the completion of the cisplatin treatment (4 months after the amputation), the dogs were reevaluated for the presence or absence of metastasis and those that were free of metastatic disease were then block randomized in groups of four to receive either L-MTP-PE or empty placebo liposomes at the same dose, frequency, and administration scheme as described in the first study.

The second arm was a multi-institutional double-blind placebo-controlled study. In this arm, dogs were scheduled to undergo amputation and then receive cisplatin (70 mg/m^2 iv) every 21 days for four treatments as well as either L-MTP-PE or placebo beginning 24 h after the first cisplatin administration on an either once or twice weekly schedule at the same dose and administration scheme as described in the first study. In both arms, the dogs were followed with routine physical exams monthly and thoracic radiographs every 8 weeks following the amputation. Subject evaluations continued as long as necessary to determine both the metastasis free interval and overall survival (both determined as described in the first study) for each dog. Again, as had been noted in the first trial, the only consistently observed side effect was an elevation in the body temperature.

In the first arm, 40 dogs were initially entered into this clinical trial. During the chemotherapy administration period, 15 dogs were excluded from randomization because 13 dogs developed metastasis and 2 died of other causes. Twenty-five dogs were randomized after the completion of 16 weeks of chemotherapy. Fourteen dogs were randomized to receive placebo and eleven dogs were randomized to receive L-MTP-PE. Of the 14 dogs in the placebo group, 13 died of metastasis and 1 died of an unrelated cause (neurological disease). The median metastasis-free interval and overall survival for the dogs in the placebo group was 7.6 months and 9.8 months, respectively. Four dogs survived for more than 1 year. In the L-MTP-PE group, 8 of 11 dogs developed metastasis, two dogs died of unrelated causes (both euthanized for severe arthritis), one was still alive and free of metastasis at the time of the publication of the manuscript, one was alive with metastasis, and one dog with metastasis was lost to follow-up. The median metastasis-free interval and overall survival for the L-MTP-PE group was 11.2 months and 14.4 months, respectively. Seven dogs survived for more than 1 year. Those dogs receiving L-MTP-PE had significantly longer metastasis-free interval ($p < 0.035$) and overall survival time ($p < 0.01$) when compared to dogs given placebo liposomes.

In the second arm, 71 dogs were enrolled but 7 were withdrawn for various reasons. Of the remaining 64 dogs, 21 dogs were randomized to receive L-MTP-PE twice weekly, 21 received L-MTP-PE once weekly, and 22 received placebo

liposomes once weekly. There were no significant differences among the three groups with regard to the metastasis-free intervals or to the overall survival times. A total of 58 dogs completed all four doses of chemotherapy; 6 dogs did not do so due to the evolution of pulmonary metastasis before completion of the 4 planned chemotherapy administrations. Median metastasis free interval for the three groups was 7.5 months, 6.3 months, and 5.8 months, respectively. Median overall survival time for the three groups was 10.3, 10.5, and 7.6 months. In the twice weekly L-MTP-PE group, 19 of 21 dogs developed metastatic disease; 7 dogs survived for 1 year or longer. In the once weekly L-MTP-PE group, 18 of 21 dogs developed metastasis; in this group, 3 dogs with metastasis died of unrelated causes (renal failure, gastric ulcer, and gastric torsion), 1 dog with metastasis was lost to follow-up, and 3 were alive and free of disease; 7 dogs survived for 1 year or longer. In the placebo group, 17 of 22 dogs developed metastasis, 2 dogs died of renal disease with no evidence of metastasis, and 3 were alive and free of disease; 9 dogs survived for more than 1 year. There were no differences in survival among the three treatment groups in the second arm. The median metastasis free survival and overall survival time for all 64 dogs was 6.6 months and 10.3 months, respectively.

Survival times of the 11 dogs receiving twice weekly L-MTP-PE in arm 1 were also compared to those of the 18 dogs in arm 2 receiving twice weekly L-MTP-PE concurrently with four doses of cisplatin. Median overall survival times were 14.4 months and 10.6 months for the dogs in arms 1 and 2, respectively that were free of metastasis at 4 months after surgery, respectively ($p < 0.04$). Survival times of the 14 dogs receiving twice weekly placebo liposomes in arm 1 were compared to those of 16 dogs in arm 2 that received once weekly placebo liposomes concurrently with four doses of cisplatin. There was no difference between the two placebo groups with respect to median survival times (9.8 months and 10.8 months for arm 1 and 2, respectively).

The results from this two arm trial chemo-immunotherapy trial compared favorably with the first trial evaluating postoperative immunotherapy alone where dogs receiving L-MTP-PE had median overall survivals of 7.3 months as compared to 2.5 months of median overall survival for dogs receiving placebo. In the first arm of this trial, cisplatin plus amputation provided a median survival of 9.8 months, which is similar to reports in other canine papers [8, 61, 80, 101, 110, 111]. Contrary to expectations, however, improved survivals were not noted when cisplatin was administered concurrently with L-MTP-PE. A similar inhibitory effect on the L-MTP-PE-mediated macrophage activation against pulmonary metastasis was noted in a B16-F10 mouse melanoma model [11] and may partially explain the negative findings in this COS study.

In comparison, the results of the Phase II trial evaluating L-MTP-PE alone demonstrated a significant increase in both the disease-free and long-term survival of patients with relapsed osteosarcoma [53] and the results of the Phase III trial evaluating L-MTP-PE combined with chemotherapy in newly diagnosed osteosarcoma demonstrated improved 6-year survival [79]. Unfortunately, additional studies with this L-MTP-PE molecule in the dog have been waiting on a ready source of the product.

Tetrathiomolybdate

Targeting neovascularization is another approach to slowing micrometastatic cancer. Because copper has been shown to be an angiogenic stimulant [90, 93], it has been postulated that copper depletion might be a viable approach to the management of cancer [15]. Tetrathiomolybdate (TM) is powerful copper chelator and thus may have utility in the management of micrometastasis of OS. It was originally developed as a well-tolerated oral treatment for the treatment of patients with Wilson's disease, an autosomal recessive disease of copper transport that results in abnormal copper accumulation and toxicity [14]. The ability of TM to reduce total body stores of copper is thought to involve at least two mechanisms. The first involves the formation of a complex together with food proteins in the gastrointestinal tract, and the blocking the absorption of copper from the diet [14]. The second is that the absorbed TM along with copper and albumin in blood form a tripartite complex that renders the copper unavailable for cellular uptake; this sequestration in the complex essentially removes the copper from use in angiogenesis [14]. This newly formed tripartite complex is biologically inert and is slowly cleared in both bile and urine [14]. Tetrathiomolybdate's clinical utility is due to its favorable properties of fast action, copper specificity, and low oral toxicity. This low oral toxicity profile of TM is possible because the level of copper required for angiogenesis is higher than that required for essential copper-dependent cellular processes, such as heme synthesis, the functions of copper/zinc superoxide dismutase (SOD) and cytochrome, and the incorporation into enzymes and other proteins, thus providing a tolerable therapeutic index [14].

Positive studies have been done in rodent models of inflammatory mammary cancer [87], head and neck cancer both in the flank and in the orthotopic setting [18], lung cancer [48], and prostate cancer [114]. We evaluated 13 advanced-stage and metastatic cancer bearing dogs (including COS). Of the nine evaluable dogs, three had stable disease for >4 months and a fourth had a measurable partial response of the metastasis and resolution of the attendant hypertrophic osteopathy while on TM, thus extending the artificial rodent findings to spontaneously occurring cancer in the dog.

These finding provided the impetus to begin a multi-institutional randomized double-blind clinical trial in newly diagnosed COS. The study is evaluating adriamycin (iv q2w×5) with or without TM in the micrometastatic disease setting. This TM-adriamycin couplet has been shown to be effective at restoring doxorubicin sensitivity to cell lines [51]. The postulated and confirmed mechanism of action is through TM-mediated inhibition of Cu-Zn SOD1, an enzyme that protects the cell against reactive oxygen species (ROS). Adriamycin is known to produce ROS, and thus, this combination of a ROS-generating drug with a molecule that can suppress intracellular antioxidant mechanisms may be a useful and powerful therapy. At the time of this writing, 5/40 dogs have been enrolled. Because TM is a drug in common use in humans with Wilson's disease, if positive results are obtained from this trial in COS, then a rationale for the use in children with OSA first as a Phase II and then as a confirmatory Phase III trial is plausible.

Aerosol Therapy

The development of aerosol chemotherapy or aerosol immunotherapy, which targets delivery to the respiratory system where occult micrometastases of OSA are most likely to be found, may provide a noninvasive method of therapy for pulmonary metastasis. To date, there has been some work in the delivery of various chemotherapeutic agents such as gemcitabine and immune modulators such as interleukin-2 to the lungs in an effort to eradicate the micrometastatic or gross metastatic osteosarcoma found in this organ in the mouse model [36, 58], and recently in humans with lung or renal [43, 66] cancer. We have completed one study with aerosol gemcitabine in dogs with gross metastatic disease [96] and are currently utilizing aerosol gemcitabine alone or combined with carboplatin for micrometastatic disease or with ifosfamide for gross metastatic disease. We have also begun therapy with recombinant human interleukin 2 for gross metastatic COS. The remainder of this chapter will focus on these two molecules.

Gemcitabine

Gemcitabine (2′,2′-difluorodeoxcycytidine, Gemzar, dFdC) is a deoxycytidine analogue [42] which has demonstrated clinical utility in the management of diverse human malignancies [1, 32, 35, 70, 76, 77, 85, 86, 91, 94, 95, 99, 115, 121–123], but whose activity has only recently been explored in veterinary oncology [19–21, 23, 28, 29, 44, 46, 56, 65, 71, 72, 74, 75, 103, 112]. In contrast to the large body of preclinical and clinical literature devoted to gemcitabine in the management of carcinomas, relatively fewer studies have been performed in OS in humans [2, 3, 76–78, 83, 85, 86, 117, 120], and to date, one (negative) has been reported in COS [74]. Gemcitabine is routinely administered intravenously as either a 30-min or longer infusion. A major limitation in the control of pulmonary metastasis with the use of the systemic administration of drugs, however, is the reduced drug concentration that is delivered to the lungs due to dilution in the blood volume; this may explain the observed lack of efficacy intravenous therapy for COS. Because aerosol delivery can bypass this limitation and is a strategy that may improve the control of pulmonary metastases, we chose to evaluate aerosol chemotherapy in COS.

In mouse models, aerosol delivery of gemcitabine has been demonstrated to significantly inhibit the growth of primary OS tumors and of established lung metastases in a Fas-dependent manner [33, 34, 59]. Inhaled gemcitabine also prevented metastatic spread, with no evidence of toxicity to normal tissues in the mice. In a relatively larger animal model more reminiscent of the dog or a human, aerosolization of gemcitabine was shown to deposit in a moderate, but significant, quantity in the peripheral lung compartment of the baboon with no evidence of pulmonary or systemic toxicity [31]. Because the clinical feasibility of aerosol delivery in the dog had been demonstrated with other chemotherapeutic and immunomodulatory agents

[38, 49, 50, 100], we completed a study to assess the pulmonary versus systemic toxicity of aerosol gemcitabine delivery, to examine the histopathological effects against gross OS pulmonary metastasis, and to assess the role of the Fas/FasL pathway the dog with gross pulmonary metastasis of OS ultimately with an eye towards pursuing this method of delivery in children with metastatic OS.

Dogs were evaluated at the William R. Prichard Veterinary Medical Teaching Hospital at the University of California by the oncology service. They were custom-fitted with a polyethylene hood and sent home with the owner for desensitization to the wearing of the hood and to the noise of the nebulizer through the use of increased wearing of the hood and food rewards. Baseline hematological and serum biochemical tests were obtained before therapy and then routine clinicopathological (CBC, SBA, ABG) monitoring occurred at 2 weeks, 4 weeks, and monthly thereafter; serial radiographs were obtained monthly.

The caregiver was educated about personal safety (respirator, mask, gloves, and gowns) and trained to administer the therapy to their dog using a Minimate compressor with nebulizer (Precision Medical, Inc., Northhampton, PA). Utilizing this system, aerosol particles containing gemcitabine were delivered that had a mass median aerodynamic diameter of 0.8 mm with GSD 2.1 as measured with the Andersen Cascade Impactor [59]. To prevent occupational exposure to ambient air levels of gemcitabine that may have been propagated during therapy, nebulization was performed outdoors.

The gemcitabine was reconstituted per manufacturer's instructions and administered on a M/W or T/Th schedule. The initial intent was to escalate dogs by 5 mg per week until 25 mg/dog was achieved or when clinicopathological or radiological evidence of toxicity was identified. When no toxicity was found in the first five dogs, subsequent dogs initiated the therapy at 25 mg/dog on the first treatment. Five additional dogs were started at 50 mg/dog total dose twice weekly.

Pet dogs (patients) were exclusively used in this study. The characteristics of aerosol gemcitabine-treated dogs ($n=20$). Osteosarcoma of the skeleton ($n=18$) and osteosarcoma of soft parts ($n=2$) were treated with amputation or radical excision, respectively. Either carboplatin alone ($n=5$) or doxorubicin and carboplatin ($n=11$) was administered in the adjuvant setting to the dogs prior to the detection of metastatic disease. In five dogs, surgery alone was the sole means of control of the primary tumor without additional chemotherapy. Two patients received intravenous chemotherapy (4 and 6 weeks prior to enrollment) for the treatment of their pulmonary metastasis prior to receiving aerosol gemcitabine. All but one patient underwent complete necropsy.

Six hundred seventy-two 1-h doses of aerosol gemcitabine were delivered (mean: 28; range: 2–80) twice weekly. Median duration of treatment was 60 min (range: 45–75). Eleven dogs received 25 mg and seven dogs received 50 mg on schedule. There were no delays in treatments in either group.

CBCs were normal in all dogs at every visit except for a single grade I neutropenia (1,500) episode. Neither thrombocytopenia nor anemia occurred. SBA parameters were within reference range in every patient at every visit. Arterial blood gas and alveolar–arterial gradients did not vary from base line in any dog at any time point.

No gastrointestinal toxicity was reported by any owner. Body weights did not vary from baseline. These findings underscored the systemic safety of this loco-regional approach to gemcitabine chemotherapy in the dog.

To assess the local tissue tolerance, the lungs were examined at necropsy. Mild histological changes were present within the sections of the examined airways that could be directly attributed to aerosol gemcitabine. Four animals exhibited minimal to mild expansion of the submucosa of one or more of their larger conducting airways. The submucosa was thickened by loosely arranged highly vascular connective tissue, which formed short blunt papillary projections that protruded into the airway lumina. No similar lesions were noted among the nontreated animals. Alveoli remained normal in both aerosol-treated and aerosol naïve dogs (Fig. 1a, b). Seven animals, all of which had either pleural effusion or pleural metastases, had marked regionally extensive to generalized chronic proliferative villous pleuritis with prominent, congested vasculature, which was attributable to the pathological process not to the therapy. Six animals had pleural effusion and six animals had pleural metastases; however, only five (of 12) animals possessed both lesions concurrently. Interestingly, in one dog with pleural metastasis, the lungs remained free of pulmonary metastasis. Three dogs with pulmonary and or pleural metastasis developed hypertrophic osteopathy, which was identified antemortem and confirmed postmortem. Alveolar lumina immediately adjacent to metastatic foci were expanded by increased numbers of foamy alveolar macrophages, which frequently possessed abundant intracytoplasmic hemosiderin pigment, indicative of prior hemorrhage. Metastasis was identified in anatomical locations outside of the lungs as well and included liver ($n=3$), spleen ($n=3$), kidney ($n=3$), adrenal ($n=3$), other bones ($n=3$), eye ($n=1$), and skin ($n=1$), which suggests that aerosol therapy should be combined with systemic therapy for maximum long-term survival.

To evaluate the specific effects of aerosol treatment on gross pulmonary metastasis, a series of sections of affected lungs from contemporary but aerosol naïve animals were identified as controls. The degree of necrosis within the metastasis was determined by estimating the overall percentage of necrotic tumor area relative to the total area of the tumor metastases and comparing the results between aerosol-treated and aerosol-naïve lesions (Fig. 1c, d).

When examining the patterns of tumor killing by aerosol gemcitabine, extensive, predominately central, intratumoral necrosis was noted within all (100 %) aerosol-treated dogs (Fig. 1d). There was marked increase in the incidence and severity of necrosis noted among the treated animals compared to the gemcitabine-naive cases. The majority (85 %) of aerosol-treated metastasis exhibited >25 % necrosis. In contrast, only 4 of the 13 (31 %) nontreated animals did so and many foci from aerosol-naïve patients had minimal necrosis (Fig. 1c). Forty-six percent of the aerosol-treated animals exhibited >50 % necrosis, whereas none of the metastasis from naïve control animals exhibited >50 % necrosis. These findings demonstrated the efficacy of metastasis killing by the aerosol delivery of gemcitabine.

Because the status of Fas on tumor metastasis in the lungs may play a role in the evolution of pulmonary metastatic disease in osteosarcoma [33, 34], Fas expression in both the primary tumor (Fig. 2a) and the metastatic foci (Fig. 2b, c) of these

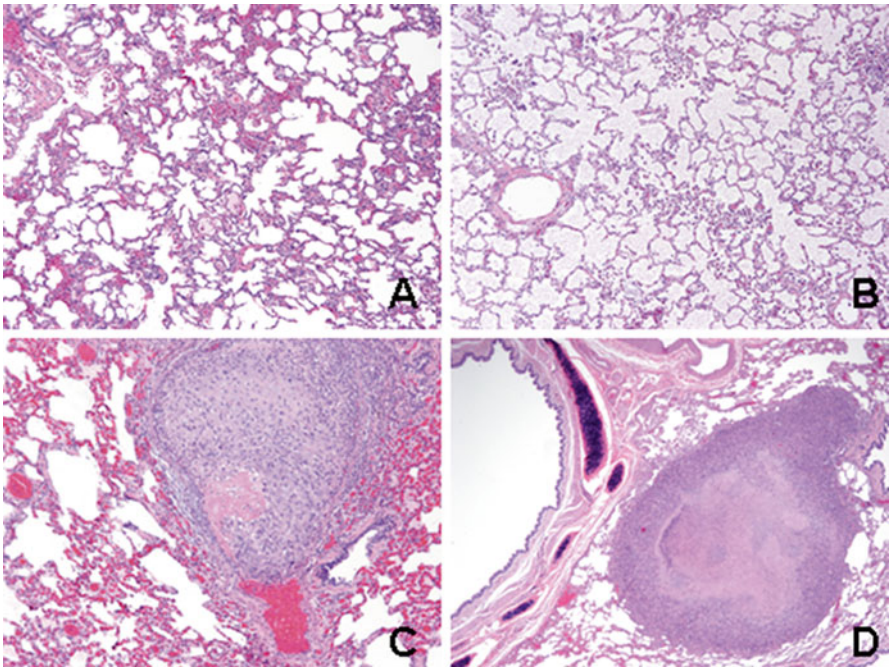


Fig. 1 Representative images of histopathological sections used in evaluating the effect of aerosol gemcitabine on canine pulmonary tissues. Dogs with osteosarcoma that harbored gross pulmonary metastasis were treated with aerosol gemcitabine twice weekly for a median of 7 weeks. Tissues were harvested at necropsy and processed for routine H&E staining. Minimal changes were identified in aerosol gemcitabine-naïve (**a**) or gemcitabine-treated lung (**b**). In contrast to the aerosol gemcitabine-treated metastatic foci (**d**), which demonstrated increased central necrosis, chemotherapy-naïve (**c**) historical controls harbored minimal to no central necrosis. From: J Aerosol Med Pulm Drug Deliv. 2010 Aug; 23(4):197–206

spontaneously arising COS was also examined to determine if this was a possible mechanism of action for the observed tumor cell killing in the metastasis. Canine osteosarcoma ranged in the expression of Fas as detected and measured by immunohistochemistry staining intensity (Fig. 2a). Fas staining intensity did not differ between the primary tumors obtained from aerosol gemcitabine-treated and -naïve dogs ($p > 0.05$; Fig. 2a, compare upper and lower rows). Interestingly, Fas staining intensity was decreased in pulmonary metastases (b) compared to the primary tumor (a), and this difference was statistically significant ($p = 0.008$; photomicrographs: compare a to b; scatter plot: compare Bone and Untreated). Importantly, the Fas staining intensity in pulmonary metastasis from aerosol gemcitabine-treated animals (c) was greater than that found in both the primary tumor ($p = 0.025$; photomicrographs: compare a and c; scatter plot: compare Bone and Aerosol Gemcitabine) and in the gemcitabine-naïve metastases ($p = 0.0075$; Fig. 2, photomicrographs: compare b and c; scatter plot, compare column untreated metastasis and gemcitabine). These data suggested that the Fas/FasL pathway was a potential mechanism of action of cell killing in metastatic osteosarcoma.

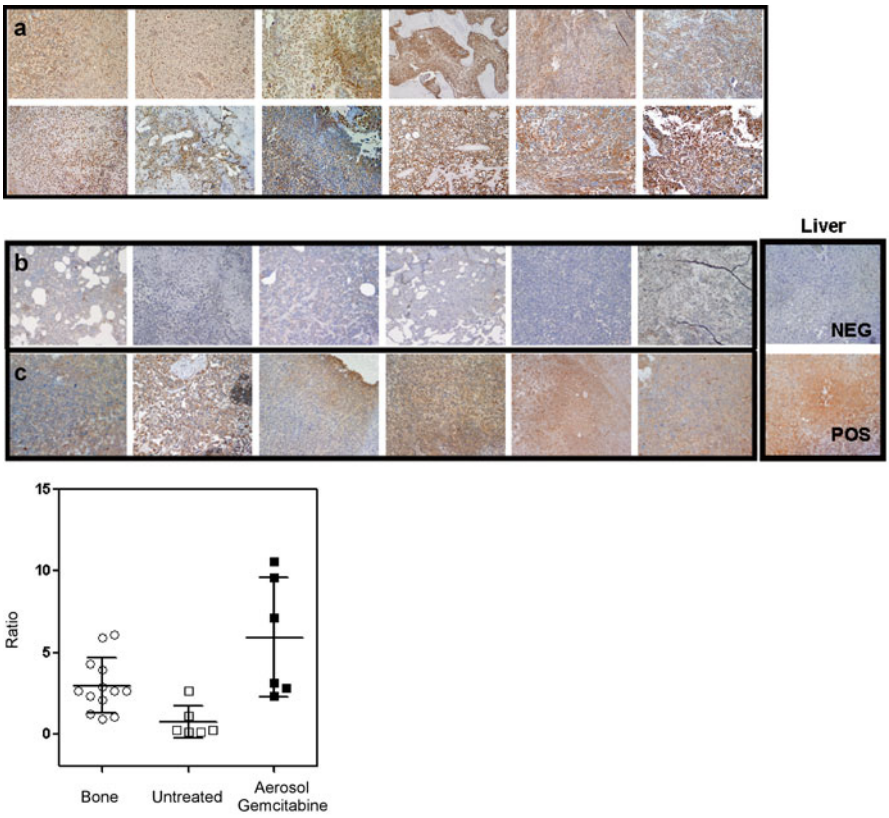


Fig. 2 Fas expression in canine osteosarcoma. Representative sections of primary (**a**) and metastatic (**b**, **c**) osteosarcoma lesions removed from aerosol gemcitabine-naïve (**a**, *top row*, **b**) or aerosol gemcitabine-treated (**a**, *bottom row*, **c**) dogs were harvested at necropsy, formalin fixed and paraffin embedded, and then stained for Fas by immunohistochemistry. The staining in 200 nuclei was quantified and plotted as the ratio of MEAN positive nuclei to total 200 nuclei counted (scatter plot). Canine liver served as the negative (Fas antibody omitted; NEG) and positive (POS) controls. The quantitation of Fas staining intensity (scatter plot) was lower in pulmonary metastases from untreated dogs (*open square*) than in the originating primary osteosarcoma tumor (*open circle*). This difference was significant ($p=0.008$). In contrast, pulmonary metastases obtained from aerosol gemcitabine-treated (*filled square*) dogs demonstrated stronger Fas staining intensity than the in the primary tumor ($p=0.025$). The difference between untreated and treated metastases was also statistically significant ($p=0.0075$). Whisker bars represent SEM. From: J Aerosol Med Pulm Drug Deliv. 2010 Aug; 23(4):197–206

As the Fas/FasL pathway kills metastatic osteosarcoma cells by inducing not necrosis but apoptosis [33, 34, 57, 64, 119], the TUNEL assay was used to determine if the observed increase in Fas expression resulted in an increase in death of tumor cells by apoptosis (Fig. 3) despite the marked background of necrosis. Minimal TUNEL staining was identified in untreated pulmonary metastasis (a). In contrast, TUNEL staining was increased in osteosarcoma cells in the pulmonary metastasis obtained from aerosol gemcitabine-treated dogs (b), and this difference was significant ($p=0.028$).

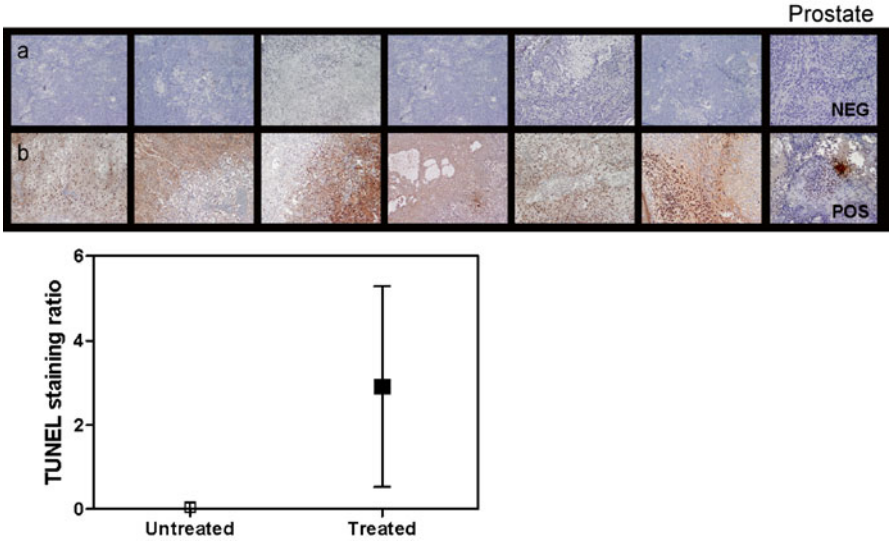


Fig. 3 Apoptosis induced by aerosol gemcitabine in metastatic canine osteosarcoma. Representative sections of metastatic canine osteosarcoma lesions from untreated (**a**) or aerosol gemcitabine-treated (**b**) dogs were harvested at necropsy, formalin-fixed and paraffin embedded, and stained for apoptosis using the TUNEL assay. The staining was quantified as the ratio of MEAN positive nuclei to total 200 nuclei counted (graph). Human prostate served as negative (primary antibody omitted; NEG) and positive (POS) controls. The level of apoptosis was low in untreated pulmonary metastases (*open square*). After exposure to aerosol gemcitabine (*filled square*), there was a marked increase in apoptotic activity in the pulmonary metastases ($p = 0.028$). Whisker bars represent SEM. Where not visible, the bars are contained within the symbol. From: J Aerosol Med Pulm Drug Deliv. 2010 Aug; 23(4):197–206

The findings from this first in dog study confirmed that aerosol gemcitabine is systemically well tolerated, and suggested that local toxicity (manifest as changes in pulmonary function or radiographic appearance) would also be minimal. The clinicopathological and pathological findings confirmed and extended the murine studies [34, 58] in the tumor-bearing dog. The results from this study may provide a rationale for use in humans with OS and can assuage some patient tolerability concerns.

The necropsy findings of metastasis in the abdominal viscera and the brain also underscore the need to treat the patient systemically when using aerosol gemcitabine. As mentioned previously, there is no “standard of care” in veterinary medicine. Thus, we are able to offer single agent aerosol therapy for dogs whose owners refuse conventional therapy (intravenous chemotherapy), something that no ethics committee would allow for in a human trial. This cohort of dogs will allow evaluation of aerosol gemcitabine in the micrometastatic setting, which more closely resembles the murine work that has been performed [34, 58]. To date, we have treated nine dogs with COS in the micrometastatic setting. As was the case in the first trial, the therapy was well tolerated. The median survival of these nine dogs is

8.5 months, or a doubling of survival when amputation is utilized as the only form of therapy for COS (Rodriguez, unpublished data). Additional dogs will be necessary to achieve statistical significance.

Aerosol gemcitabine combined with intravenous chemotherapy is another avenue of exploration in the clinic. We routinely combine aerosol therapy with intravenous carboplatin. Whereas the median survival of dogs treated with intravenous chemotherapy is 11 months [4, 5, 7–10, 16, 17, 47, 60, 61, 68, 73, 80, 92, 101, 106, 110, 111, 113, 118], the combination of aerosol gemcitabine plus carboplatin has resulted in median survivals of 24 months in nine dogs thus treated (Rodriguez, unpublished data). Additional dogs will be necessary to achieve statistical significance.

Finally, for macrometastatic disease, while aerosol gemcitabine appeared to kill cells in the metastatic foci through a Fas/FasL mediated mechanism of action [96], no dog was cured and the overall survival was 75 days. Ifosfamide is a chemotherapeutic agent that can upregulate FasL in pulmonary metastasis of OS in mice [22]. In COS, single agent ifosfamide has shown minimal survival benefit; the dogs lived 95 days [6]. However, because aerosol gemcitabine upregulates Fas and because ifosfamide upregulates FasL, we postulated that the combination of these two therapies would induce the expression of an autocrine death loop on the surface of metastatic COS. To date we have treated seven dogs with macrometastatic COS with a combination of intravenous ifosfamide every 3 weeks along with aerosol gemcitabine delivered twice weekly. The median survival of these dogs is 9.5 months. Additional dogs will be needed to confirm the statistical significance of these findings (Rodriguez, unpublished data). While the pulmonary tissues have been harvested they have not been evaluated for Fas/FasL; it is tempting to speculate that the autocrine death loop will be present.

Interleukin 2

Interleukin 2 (IL-2) was initially identified as a T-cell growth factor [81]. It is produced when T cell recognize antigen-MHC complexes in the face of costimulatory molecules on the surface of antigen-presenting cells. In particular, IL-2 has a pronounced stimulatory effect on natural killer cells [82].

Canine lymphokine activated killer cells were induced in vitro in after exposure to recombinant human IL-2 (rhIL-2) suggesting that the canine IL-2 receptor can be stimulated by the human molecule [30]. An in vivo study confirmed that canine lymphocytes proliferate in response to rhIL-2 [37]. The dogs in this study were infused on four consecutive days with high dose rhIL-2. The side effects included mild gastrointestinal toxicity in all of the dogs. There was however, no evidence of more untoward effects (capillary leak) such as those seen in people treated with high dose IL-2.

Early murine experiments utilizing intraperitoneal administration of recombinant human rhIL-2 demonstrated reductions in growth of pulmonary metastasis of several cancers [89] and when high doses were used, more than 80 % of the

pulmonary lesions were eradicated [97]. Unfortunately, translating the murine high dose systemic therapy comes at a cost to the human patient, which includes pulmonary edema and ascites due to capillary leakage syndrome, hepatocellular necrosis, renal dysfunction, dermatitis, anemia, and thrombocytopenia [104, 116]. Clearly, alternative methods of delivery should be explored.

When rhIL-2 was delivered by aerosol to humans with renal cancer minimal untoward effects were observed [43]. Similarly, when dogs with primary lung cancer or with pulmonary metastasis from COS were treated with aerosol liposomal rhIL-2 no untoward effects were seen [49, 50]. Two of four dogs with metastatic osteosarcoma had complete resolution of the metastasis and the other two had stabilization of the lesions for more than 12 months. To assess the mechanism of action, bronchoalveolar lavage samples were obtained. and the lytic activity of isolated lymphocytes was increased after 15 days of therapy in all nine dogs. There were minimal untoward toxic effects in these dogs. We have treated five dogs with hypertrophic osteopathy due to COS or dogs that have failed other treatments for COS with the commercially available rhIL-2, proleukin (aldesleukin). None of the dogs have achieved a complete response but all dogs with hypertrophic osteopathy have had resolution of their clinical signs and all for dogs have had stable metastatic COS disease for more than 11 months (Rodriguez, unpublished data). The drawback to the use of the rhIL-2 is the development of canine anti rhIL-2 antibodies after 30 days of use (Khanna C, personal communication). We are currently investigating recombinant canine IL-2 in vitro and hope to move this molecule to the clinical setting as it will allow for longer periods of dosing. Importantly, the preclinical murine and canine data have prompted the commencement of an aerosol rhIL-2 trial for metastatic osteosarcoma (Kleinerman, E, personal communication).

In summary, while we share our work and homelife intimately with our dogs, the dog also shares many of our neoplastic diseases including but not limited to osteosarcoma. Because of the similarities between COS and OS in children and because of the compressed timeline of the evolution of COS in the dog, the dog is an excellent model in which to attempt novel therapies aimed at eradicating micrometastatic or macrometastatic osteosarcoma. The dog has served us faithfully since its domestication; perhaps these old dogs can teach us some new tricks.

References

1. Anderson H, Lund B, Bach F et al (1994) Single-agent activity of weekly gemcitabine in advanced non-small-cell lung-cancer - A phase-II study. J Clin Oncol 12:1821–1826
2. Anderson PM, Wiseman GA, Erlandson L et al (2005) Gemcitabine radiosensitization after high-dose samarium for osteoblastic osteosarcoma. Clin Cancer Res 11:6895–6900
3. Ando T, Ichikawa J, Okamoto A et al (2005) Gemcitabine inhibits viability, growth, and metastasis of osteosarcoma cell lines. J Orthop Res 23:964–969
4. Bacon NJ, Ehrhart NR, Dernell WS et al (2008) Use of alternating administration of carboplatin and doxorubicin in dogs with microscopic metastases after amputation for appendicular osteosarcoma: 50 cases (1999-2006). JAVMA 232:1504–1510

5. Bailey D, Erb H, Williams L et al (2003) Carboplatin and doxorubicin combination chemotherapy for the treatment of appendicular osteosarcoma in the dog. J Vet Intern Med 17:199–205
6. Batschinski K, Dervisis NG, Kitchell BE (2012) Evaluation of ifosfamide salvage therapy for metastatic canine osteosarcoma. Vet Comp Oncol. 2012 Sep 18. DOI: 10.1111/j.1476-5829.2012.00355
7. Berg J, Gebhardt MC, Rand WM (1997) Effect of timing of postoperative chemotherapy on survival of dogs with osteosarcoma. Cancer 79:1343–1350
8. Berg J, Weinstein MJ, Schelling SH et al (1992) Treatment of dogs with osteosarcoma by administration of cisplatin after amputation or limb-sparing surgery: 22 cases (1987-1990). J Am Vet Med Assoc 200:2005–2008
9. Berg J, Weinstein MJ, Springfield DS et al (1995) Results of surgery and doxorubicin chemotherapy in dogs with osteosarcoma. J Am Vet Med Assoc 206:1555–1560
10. Bergman PJ, Macewen EG, Kurzman ID et al (1996) Amputation and carboplatin for treatment of dogs with osteosarcoma: 48 cases (1991 to 1993). J Vet Intern Med 10:76–81
11. Bezault J, Walsh C, Tarcsay L et al (1993) Analysis of the antimetastatic effects of synthetic muramyl tripeptide (CGP 19835A) encapsulated in liposomes in combination with other immunomodulatory agents and chemotherapeutic drugs. In Vivo 7:487–491
12. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20:776–790
13. Boston SE, Ehrhart NP, Dernell WS et al (2006) Evaluation of survival time in dogs with stage III osteosarcoma that undergo treatment: 90 cases (1985-2004). J Am Vet Med Assoc 228:1905–1908
14. Brewer GJ, Dick RD, Yuzbasiyan-Gurkin V et al (1991) Initial therapy of patients with Wilson's disease with tetrathiomolybdate. Arch Neurol 48:42–47
15. Brewer GJ, Merajver SD (2002) Cancer therapy with tetrathiomolybdate: antiangiogenesis by lowering body copper – a review. Integr Cancer Ther 1:327–337
16. Chun R, Garrett LD, Henry C et al (2005) Toxicity and efficacy of cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma. J Am Anim Hosp Assoc 41:382–387
17. Chun R, Kurzman ID, Couto CG et al (2000) Cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma: a pilot study. J Vet Intern Med 14:495–498
18. Cox C, Teknos TN, Barrios M et al (2001) The role of copper suppression as an antiangiogenic strategy in head and neck squamous cell carcinoma. Laryngoscope 111:696–701
19. Cozzi PJ, Bajorin DF, Tong W et al (1999) Toxicology and pharmacokinetics of intravesical gemcitabine: a preclinical study in dogs. Clin Cancer Res 5:2629–2637
20. De Brito Galvao JF, Kisseberth WC, Murahari S et al (2012) Effects of gemcitabine and gemcitabine in combination with carboplatin on five canine transitional cell carcinoma cell lines. Am J Vet Res 73:1262–1272
21. Dominguez PA, Dervisis NG, Cadile CD et al (2009) Combined gemcitabine and carboplatin therapy for carcinomas in dogs. J Vet Intern Med 23:130–137
22. Duan X, Jia SF, Koshkina N et al (2006) Intranasal interleukin-12 gene therapy enhanced the activity of ifosfamide against osteosarcoma lung metastases. Cancer 106:1382–1388
23. Elpiner AK, Brodsky EM, Hazzah TN et al (2011) Single-agent gemcitabine chemotherapy in dogs with hepatocellular carcinomas. Vet Comp Oncol 9:260–268
24. Fidler IJ, Kleinerman ES (1984) Lymphokine-activated human-blood monocytes destroy tumor-cells but not normal-cells under cocultivation conditions. J Clin Oncol 2:937–943
25. Fidler IJ, Schroit AJ (1988) Recognition and destruction of neoplastic cells by activated macrophages: discrimination of altered self. Biochim Biophys Acta 948:151–173
26. Fidler IJ, Sone S, Fogler WE et al (1981) Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous-injection of liposomes containing muramyl dipeptide. Proc Natl Acad Sci U S A 78:1680–1684
27. Fidler IJ, Sone S, Fogler WE et al (1982) Efficacy of liposomes containing a lipophilic muramyl di peptide derivative for activating the tumoricidal properties of alveolar macrophages in-vivo. J Biol Response Mod 1:43–56

28. Freise KJ, Martin-Jimenez T (2006) Pharmacokinetics of gemcitabine and its primary metabolite in dogs after intravenous bolus dosing and its in vitro pharmacodynamics. J Vet Pharmacol Ther 29:137–145
29. Freise KJ, Martin-Jimenez T (2006) Pharmacokinetics of gemcitabine and its primary metabolite in dogs after intravenous infusion. J Vet Pharmacol Ther 29:147–152
30. Funk J, Schmitz G, Failing K et al (2005) Natural killer (NK) and lymphokine-activated killer (LAK) cell functions from healthy dogs and 29 dogs with a variety of spontaneous neoplasms. Cancer Immunol Immunother 54:87–92
31. Gagnadoux F, Leblond V, Vecellio L et al (2006) Gemcitabine aerosol: in vitro antitumor activity and deposition imaging for preclinical safety assessment in baboons. Cancer chemotherapy and pharmacology 58:237–244
32. Gandhi V, Plunkett W, Du M et al (2002) Prolonged infusion of gemcitabine: clinical and pharmacodynamic studies during a phase I trial in relapsed acute myelogenous leukemia. J Clin Oncol 20:665–673
33. Gordon N, Arndt CS, Hawkins DS et al (2005) Fas expression in lung metastasis from osteosarcoma patients. J Pediat Hematol Oncol 27:611–615
34. Gordon N, Koshkina NV, Jia S-F et al (2007) Corruption of the Fas pathway delays the pulmonary clearance of murine osteosarcoma cells, enhances their metastatic potential, and reduces the effect of aerosol gemcitabine. Clin Cancer Res 13:4503–4510
35. Grunewald R, Kantarjian H, Du M et al (1992) Gemcitabine in leukemia - a phase-I clinical, plasma, and cellular pharmacology study. J Clin Oncol 10:406–413
36. Guma SR, Lee DA, Yu L et al (2013) Natural killer cell therapy and aerosol interleukin-2 for the treatment of osteosarcoma lung metastasis. Pediat Blood Cancer 61(4):618–626
37. Helfand SC, Soergel SA, Macwilliams PS et al (1994) Clinical and immunological effects of human recombinant interleukin-2 given by repetitive weekly infusion to normal dogs. Cancer Immunol Immunother 39:84–92
38. Hershey AE, Kurzman ID, Forrest LJ et al (1999) Inhalation chemotherapy for macroscopic primary or metastatic lung tumors: proof of principle using dogs with spontaneously occurring tumors as a model. Clin Cancer Res 5:2653–2659
39. Herzog CE (2005) Overview of sarcomas in the adolescent and young adult population. J Pediat Hematol Oncol 27:215–218
40. Hibbs JB (1974) Discrimination between neoplastic and non-neoplastic cells invitro by activated macrophages. J Natl Cancer Inst 53:1487–1492
41. Hillers KR, Dernell WS, Lafferty MH et al (2005) Incidence and prognostic importance of lymph node metastases in dogs with appendicular osteosarcoma: 228 cases (1986-2003). J Am Vet Med Assoc 226:1364–1367
42. Huang P, Chubb S, Hertel LW et al (1991) Action of 2′,2′-difluorodeoxycytidine on dna-synthesis. Cancer Res 51:6110–6117
43. Huland E, Heinzer H, Jorres RA et al (2004) Therapeutic approaches in metastatic renal cell carcinoma: local immunotherapy. Der Urologe Ausg A 43(Suppl 3):S140–S144
44. Jones PD, De Lorimier LP, Kitchell BE et al (2003) Gemcitabine as a radiosensitizer for nonresectable feline oral squamous cell carcinoma. J Am Anim Hosp Assoc 39:463–467
45. Jongeward SJ (1985) Primary bone-tumors. Vet Clin Small Anim Pract 15:609–641
46. Kawai M, Esumi Y, Ishizaki M et al (1995) Metabolism of gemcitabine in rat and dog. Xenobiotica 25:405–416
47. Kent MS, Strom A, London CA et al (2004) Alternating carboplatin and doxorubicin as adjunctive chemotherapy to amputation or limb-sparing surgery in the treatment of appendicular osteosarcoma in dogs. J Vet Intern Med 18:540–544
48. Khan MK, Miller MW, Taylor J et al (2002) Radiotherapy and antiangiogenic TM in lung cancer. Neoplasia 4:164–170
49. Khanna C, Hasz DE, Klausner JS et al (1996) Aerosol delivery of interleukin 2 liposomes is nontoxic and biologically effective: canine studies. Clin Cancer Res 2:721–734
50. Khanna C, Waldrep JC, Anderson PM et al (1997) Nebulized interleukin 2 liposomes: aerosol characteristics and biodistribution. J Pharm Pharmacol 49:960–971

51. Kim KK, Kawar NM, Singh RK et al (2011) Tetrathiomolybdate induces doxorubicin sensitivity in resistant tumor cell lines. Gynecol Oncol 122:183–189
52. Kleinerman ES, Erickson KL, Schroit AJ et al (1983) Activation of tumoricidal properties in human-blood monocytes by liposomes containing lipophilic muramyl tripeptide. Cancer Res 43:2010–2014
53. Kleinerman ES, Gano JB, Johnston DA et al (1995) Efficacy of liposomal muramyl tripeptide (Cgp 19835a) in the treatment of relapsed osteosarcoma. Am J Clin Oncol 18:93–99
54. Kleinerman ES, Jia SF, Griffin J et al (1992) Phase-II study of liposomal muramyl tripeptide in osteosarcoma - the cytokine cascade and monocyte activation following administration. J Clin Oncol 10:1310–1316
55. Kleinerman ES, Murray JL, Snyder JS et al (1989) Activation of tumoricidal properties in monocytes from cancer-patients following intravenous administration of liposomes containing muramyl tripeptide phosphatidylethanolamine. Cancer Res 49:4665–4670
56. Kosarek CE, Kisseberth WC, Gallant SL et al (2005) Clinical evaluation of gemcitabine in dogs with spontaneously occurring malignancies. J Vet Intern Med 19:81–86
57. Koshkina NV, Khanna C, Mendoza A et al (2007) Fas-negative osteosarcoma tumor cells are selected during metastasis to the lungs: the role of the Fas pathway in the metastatic process of osteosarcoma. Mol Cancer Res 5:991–999
58. Koshkina NV, Kleinerman ES (2005) Aerosol gemcitabine inhibits the growth of primary osteosarcoma and osteosarcoma lung metastases. Int J Cancer 116:458–463
59. Koshkina NV, Waldrep JC, Roberts LE et al (2001) Paclitaxel liposome aerosol treatment induces inhibition of pulmonary metastases in murine renal carcinoma model. Clin Cancer Res 7:3258–3262
60. Kozicki AR, Robat C, Chun R et al (2013) Adjuvant therapy with carboplatin and pamidronate for canine appendicular osteosarcoma. Vet Comp Oncol. 10 MAY 2013, DOI: 10.1111/vco.12040
61. Kraegel SA, Madewell BR, Simonson E et al (1991) Osteogenic sarcoma and cisplatin chemotherapy in dogs: 16 cases (1986-1989). J Am Vet Med Assoc 199:1057–1059
62. Kurzman ID, Macewen EG, Rosenthal RC et al (1995) Adjuvant therapy for osteosarcoma in dogs: results of randomized clinical trials using combined liposome-encapsulated muramyl tripeptide and cisplatin. Clin Cancer Res 1:1595–1601
63. Kurzman ID, Shi F, Macewen EG (1993) In-vitro and in-vivo canine mononuclear cell production of tumor-necrosis-factor induced by muramyl peptides and lipopolysaccharide. Vet Immunol Immunopathol 38:45–56
64. Lafleur EA, Koshkina NV, Stewart J et al (2004) Increased Fas expression reduces the metastatic potential of human osteosarcoma cells. Clin Cancer Res 10:8114–8119
65. Leblanc AK, Ladue TA, Turrel JM et al (2004) Unexpected toxicity following use of gemcitabine as a radiosensitizer in head and neck carcinomas: a veterinary radiation therapy oncology group pilot study. Vet Radiol Ultrasound 45:466–470
66. Lemarie E, Vecellio L, Hureaux J et al (2011) Aerosolized gemcitabine in patients with carcinoma of the lung: feasibility and safety study. J Aerosol Med Pulm Drug Deliv 24:261–270
67. Macewen EG, Kurzman ID, Rosenthal RC et al (1989) Therapy for osteo-sarcoma in dogs with intravenous-injection of liposome-encapsulated muramyl tripeptide. J Natl Cancer Inst 81:935–938
68. Madewell BR, Leighton RL, Theilen GH (1978) Amputation and doxorubicin for treatment of canine and feline osteogenic-sarcoma. Eur J Cancer 14:287–293
69. Maeda M, Knowles RD, Kleinerman ES (1991) Muramyl tripeptide phosphatidylethanolamine encapsulated in liposomes stimulates monocyte production of tumor-necrosis-factor and interleukin-1 invitro. Cancer Commun 3:313–321
70. Marchi E, Alinari L, Tani M et al (2005) Gemcitabine as frontline treatment for cutaneous T-cell lymphoma: phase II study of 32 patients. Cancer 104:2437–2441

71. Marconato L, Lorenzo RM, Abramo F et al (2008) Adjuvant gemcitabine after surgical removal of aggressive malignant mammary tumours in dogs. Vet Comp Oncol 6:90–101
72. Marconato L, Zini E, Lindner D et al (2011) Toxic effects and antitumor response of gemcitabine in combination with piroxicam treatment in dogs with transitional cell carcinoma of the urinary bladder. J Am Vet Med Assoc 238:1004–1010
73. Mauldin GN, Matus RE, Withrow SJ et al (1988) Canine osteosarcoma. Treatment by amputation versus amputation and adjuvant chemotherapy using doxorubicin and cisplatin. J Vet Intern Med 2:177–180
74. Mcmahon M, Mathie T, Stingle N et al (2011) Adjuvant carboplatin and gemcitabine combination chemotherapy postamputation in canine appendicular osteosarcoma. J Vet Intern Med 25:511–517
75. Mcmahon MB, Bear MD, Kulp SK et al (2010) Biological activity of gemcitabine against canine osteosarcoma cell lines in vitro. Am J Vet Res 71:799–808
76. Merimsky O, Meller I, Flusser G et al (2000) Gemcitabine in soft tissue or bone sarcoma resistant to standard chemotherapy: a phase II study. Cancer Chemother Pharmacol 45:177–181
77. Merimsky O, Meller I, Kollender Y et al (1998) Palliative effect of gemcitabine in osteosarcoma resistant to standard chemotherapy. Eur J Cancer 34:1296–1297
78. Merimsky O, Meller I, Kollender Y et al (2000) Gemcitabine in bone sarcoma resistant to Doxorubicin-based chemotherapy. Sarcoma 4:7–10
79. Meyers PA, Schwartz CL, Krailo MD et al (2008) Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival – a report from the Children's Oncology Group. J Clin Oncol 26:633–638
80. Morello E, Buracco P, Martano M et al (2001) Bone allografts and adjuvant cisplatin for the treatment of canine appendicular osteosarcoma in 18 dogs. J Small Anim Pract 42:61–66
81. Morgan DA, Ruscetti FW, Gallo R (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193:1007–1008
82. Mule JJ, Yang JC, Afreniere RL et al (1987) Identification of cellular mechanisms operational in vivo during the regression of established pulmonary metastases by the systemic administration of high-dose recombinant interleukin 2. J Immunol 139:285–294
83. Navid F, Willert JR, Mccarville MB et al (2008) Combination of gemcitabine and docetaxel in the treatment of children and young adults with refractory bone sarcoma. Cancer 113:419–425
84. O'brien MG, Straw RC, Withrow SJ et al (1993) Resection of pulmonary metastases in canine osteosarcoma: 36 cases (1983-1992). Vet Surg 22:105–109
85. Okuno S, Edmonson J, Mahoney M et al (2002) Phase II trial of gemcitabine in advanced sarcomas. Cancer 94:3225–3229
86. Okuno S, Ryan LM, Edmonson JH et al (2003) Phase II trial of gemcitabine in patients with advanced sarcomas (E1797): a trial of the Eastern Cooperative Oncology Group. Cancer 97:1969–1973
87. Pan Q, Kleer CG, Van Golen KL et al (2002) Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. Cancer Res 62:4854–4859
88. Paoloni M, Davis S, Lana S et al (2009) Canine tumor cross-species genomics uncovers targets linked to osteosarcoma progression. BMC Genomics 10:625
89. Papa MZ, Mule JJ, Rosenberg SA (1986) Antitumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 in vivo: successful immunotherapy of established pulmonary metastases from weakly immunogenic and nonimmunogenic murine tumors of three district histological types. Cancer Res 46:4973–4978
90. Parke A, Bhattacherjee P, Palmer RM et al (1988) Characterization and quantification of copper sulfate-induced vascularization of the rabbit cornea. Am J Pathol 130:173–178
91. Perez-Manga G, Lluch A, Alba E et al (2000) Gemcitabine in combination with doxorubicin in advanced breast cancer: final results of a phase II pharmacokinetic trial. J Clin Oncol 18:2545–2552
92. Phillips B, Powers BE, Dernell WS et al (2009) Use of single-agent carboplatin as adjuvant or neoadjuvant therapy in conjunction with amputation for appendicular osteosarcoma in dogs. J Am Anim Hosp Assoc 45:33–38

93. Raju KS, Alessandri G, Ziche M et al (1982) Ceruloplasmin, copper ions, and angiogenesis. J Natl Cancer Inst 69:1183–1188
94. Rizzieri DA, Bass AJ, Rosner GL et al (2002) Phase I evaluation of prolonged-infusion gemcitabine with mitoxantrone for relapsed or refractory acute leukemia. J Clin Oncol 20:674–679
95. Rizzieri DA, Ibom VK, Moore JO et al (2003) Phase I evaluation of prolonged-infusion gemcitabine with fludarabine for relapsed or refractory acute myelogenous leukemia. Clin Cancer Res 9:663–668
96. Rodriguez CO, Crabbs TA, Wilson DW et al (2010) Aerosol gemcitabine: preclinical safety and in vivo antitumor activity in osteosarcoma-bearing dogs. J Aerosol Med Pulm Drug Deliv 23:197–206
97. Rosenberg SA, Mule JJ, Spiess PJ et al (1985) Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. J Exp Med 161:1169–1188
98. Saam DE, Liptak JM, Stalker MJ et al (2011) Predictors of outcome in dogs treated with adjuvant carboplatin for appendicular osteosarcoma: 65 cases (1996-2006). J Am Vet Med Assoc 238:195–206
99. Santoro A, Bredenfeld H, Devizzi L et al (2000) Gemcitabine in the treatment of refractory Hodgkin's disease: results of a multicenter phase II study. J Clin Oncol 18:2615–2619
100. Selting K, Essman S, Reinero C et al (2011) Targeted combined aerosol chemotherapy in dogs and radiologic toxicity grading. J Aerosol Med Pulm Drug Deliv 24:43–48
101. Shapiro W, Fossum TW, Kitchell BE et al (1988) Use of cisplatin for treatment of appendicular osteosarcoma in dogs. J Am Vet Med Assoc 192:507–511
102. Shi FS, Macewen EG, Kurzman ID (1993) In-vitro and in-vivo effect of doxorubicin combined with liposome-encapsulated muramyl tripeptide on canine monocyte activation. Cancer Res 53:3986–3991
103. Shipley LA, Brown TJ, Cornpropst JD et al (1992) Metabolism and disposition of gemcitabine, and oncolytic deoxycytidine analog, in mice, rats, and dogs. Drug Metab Dispos 20:849–855
104. Siegel JP, Puri RK (1991) Interleukin-2 toxicity. J Clin Oncol 9:694–704
105. Simcock JO, Withers SS, Prpich CY et al (2012) Evaluation of a single subcutaneous infusion of carboplatin as adjuvant chemotherapy for dogs with osteosarcoma: 17 cases (2006-2010). J Am Vet Med Assoc 241:608–614
106. Skorupski KA, Uhl JM, Szivek A et al (2013) Carboplatin versus alternating carboplatin and doxorubicin for the adjuvant treatment of canine appendicular osteosarcoma: a randomized, phase III trial. Vet Comp Oncol. 2013 Oct 4. DOI: 10.1111/vco.12069
107. Smith BW, Kurzman ID, Schultz KT et al (1993) Muramyl peptides augment the in vitro and in vivo cytostatic activity of canine plastic-adherent mononuclear cells against canine osteosarcoma cells. Cancer Biother 8:137–144
108. Smith MA, Seibel NL, Altekruse SF et al (2010) Outcomes for children and adolescents with cancer: challenges for the twenty-first century. J Clin Oncol 28:2625–2634
109. Spodnick GJ, Berg J, Rand WM et al (1992) Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978-1988). J Am Vet Med Assoc 200:995–999
110. Straw RC, Withrow SJ, Richter SL et al (1991) Amputation and cisplatin for treatment of canine osteosarcoma. J Vet Intern Med 5:205–210
111. Thompson JP, Fugent MJ (1992) Evaluation of survival times after limb amputation, with and without subsequent administration of cisplatin, for treatment of appendicular osteosarcoma in dogs: 30 cases (1979-1990). J Am Vet Med Assoc 200:531–533
112. Turner AI, Hahn KA, Rusk A et al (2006) Single agent gemcitabine chemotherapy in dogs with spontaneously occurring lymphoma. J Vet Intern Med 20:1384–1388
113. Vail DM, Kurzman ID, Glawe PC et al (2002) STEALTH liposome-encapsulated cisplatin (SPI-77) versus carboplatin as adjuvant therapy for spontaneously arising osteosarcoma (OSA) in the dog: a randomized multicenter clinical trial. Cancer Chemother Pharmacol 50:131–136

114. Van Golen KL, Bao L, Brewer GJ et al (2002) Suppression of tumor recurrence and metastasis by a combination of the PHSCN sequence and the antiangiogenic compound tetrathiomolybdate in prostate carcinoma. Neoplasia 4:373–379
115. Van Moorsel CJ, Peters GJ, Pinedo HM (1997) Gemcitabine: future prospects of single-agent and combination studies. Oncologist 2:127–134
116. Vial T, Descotes J (1992) Clinical toxicity of interleukin-2. Drug Saf 7:417–433
117. Wagner-Bohn A, Paulussen M, Vieira Pinheiro JP et al (2006) Phase II study of gemcitabine in children with solid tumors of mesenchymal and embryonic origin. Anticancer Drugs 17:859–864
118. Withrow SJ, Thrall DE, Straw RC et al (1993) Intraarterial cisplatin with or without radiation in limb-sparing for canine osteosarcoma. Cancer 71:2484–2490
119. Worth LL, Lafleur EA, Jia SF et al (2002) Fas expression inversely correlates with metastatic potential in osteosarcoma cells. Oncol Rep 9:823–827
120. Zak D, Styler MJ, Rosenbluth JZ et al (2005) Combination of gemcitabine and irinotecan for recurrent metastatic osteogenic sarcoma. Clin Adv Hematol Oncol 3:297–299, discussion 300-292
121. Zinzani PL, Baliva G, Magagnoli M et al (2000) Gemcitabine treatment in pretreated cutaneous T-cell lymphoma: experience in 44 patients. J Clin Oncol 18:2603–2606
122. Zinzani PL, Bendandi M, Stefoni V et al (2000) Value of gemcitabine treatment in heavily pretreated Hodgkin's disease patients. Haematologica 85:926–929
123. Zinzani PL, Magagnoli M, Bendandi M et al (1998) Therapy with gemcitabine in pretreated peripheral T-cell lymphoma patients. Ann Oncol 9:1351–1353

Part IV
Novel Therapeutic Approaches Based on Biology: Tumor Targeted Therapy

Oncolytic Viruses for Potential Osteosarcoma Therapy

Pooja Hingorani, Valerie Sampson, Christina Lettieri, and E. Anders Kolb

Abstract Since the first anecdotal reports of dramatic tumor responses following a viral infection in early 1900s, the field of oncolytic virotherapy has evolved at a rapid pace finally making its way into clinical trials. A large number of both wild-type and genetically altered viruses with a preferential replication-competency for tumor cells have been studied in tissue cultures, animal models and in humans, with an ever increasing repertoire of new viruses being added to this pool. Although oncolytic viruses have caused dramatic antitumor responses in cell cultures and mouse models, their clinical effects in humans have been modest. Therefore, the current research is focused on understanding the mechanisms by which viruses kill tumor cells, the barriers to successful viral delivery and penetration into tumor cells, the role of the immune system in viral oncolysis and generating stronger target specific and replication competent viruses. Osteosarcoma is a challenging malignancy to identify novel targets for therapy due to its complex genetic make-up. Oncolytic virotherapy may be a promising approach as a novel therapeutic, not dependent on consistent expression of a single target. In this review we summarize the supportive evidence and rationale for use of viral oncolysis in osteosarcoma along with the specific challenges it may face.

P. Hingorani, M.D. • C. Lettieri, M.D.
Center for Cancer and Blood Disorders, Phoenix Children's Hospital, 1919 E Thomas Road, Phoenix, AZ 85016, USA
e-mail: phingorani@phoenixchildrens.com

V. Sampson, Ph.D.
Alfred I. duPont Hospital for Children, 1701, Rockland Rd, Wilmington, DE 19803, USA

E.A. Kolb, M.D. (✉)
Alfred I. duPont Hospital for Children, 1600, Rockland Rd, Wilmington, DE 19803, USA

Nemours Center for Childhood Cancer and Blood Disorders, Alfred. I. duPont Hospital for Children, 1600 Rockland Road, Wilmington, DE 19803, USA
e-mail: eakolb@nemours.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_14,

Keywords Osteosarcoma • Oncolytic viruses • Virotherapy • RNA virus • DNA virus • Replication-competent • Cancer stem cells

Introduction

Historical reports of dramatic remissions of cancer following a viral illness date back to early 1900s [22, 25]. These observations led to the idea of using viruses to treat cancer in the 1950s [51, 81]. With the advent of cell culture technologies and animal models, testing viruses in cancer models became possible. Several publications reported decreased growth or complete tumor regressions of implantable tumors in mice after virus therapy but with significant toxicity [79, 80, 82]. Around the 1960s–1970s, unmodified and impure viruses with low pathogenicity were used to treat hundreds of patients with cancer via various routes [99]. In the majority of patients, viruses were cleared by the immune system without any effect on the cancer growth. However, cancer regressions were noted in a few patients with severely immunocompromised host systems but were associated with significant morbidity and mortality secondary to overwhelming viral infection in these patients. These animal and human experiments dampened the enthusiasm of oncolytic virus therapy until in early 1990s; the first report of a genetically engineered attenuated HSV was reported in a murine glioblastoma model [72]. Over the past two decades, the advances in genetic engineering technologies as well as viral delivery systems have rapidly increased the pace of clinical development of viral therapies in cancer patients with hundreds of patients treated on clinical trials with a variety of DNA and RNA viruses via intratumoral or intravenous routes. More than 25 clinical trials using oncolytic viruses are currently open for cancer patients across the country (www.clinicaltrials.gov). The safety profile of oncolytic viruses has been excellent across all trials and all routes of administration. The clinical efficacy of most oncolytic viruses given alone has been below what was expected from the preclinical response data due to unique challenges of this therapy in humans. However, a few recent trials of intratumoral injection of a genetically modified HSV and vaccinia virus (JX594) that express GM-CSF, in patients with melanoma and hepatocellular carcinoma respectively, have demonstrated compelling evidence of single agent virus therapy leading to objective responses in patients [88, 96]. These data support the belief that although anticancer viral therapy may not have yet yielded the expected outcomes, its full potential is yet to be harnessed. Some of the challenges and novel approaches to overcome these are discussed later in this chapter.

Metastatic osteosarcoma is an extremely challenging disease to cure with 5-year event-free survival being less than 20 %. Conventional chemotherapy and surgery have remained the mainstay of therapy for the past 4–5 decades. Novel therapeutic approaches which may include a combination of new cytotoxics, agents targeting specific pathway abnormalities, immunotherapies, genomic approaches, or virotherapy are all potential options to improve the dismal outcome in these patients. In this chapter, we specifically review the role of viral therapies in OS as a potential therapeutic option.

Oncolytic DNA Viruses in Osteosarcoma

The main strategy for virotherapy in osteosarcoma is to optimize the oncolytic potential of naturally or genetically rendered oncolytic viruses that target aberrantly expressed molecules and pathways (e.g., Rb/E2F/p16, p53, IFNs, ILs, PKR, EGFR, Ras) in tumor cells. The following sections describe the DNA viruses that have been evaluated preclinically and clinically in osteosarcoma. These are adenovirus (AdV), herpes simplex virus (HSV), and vaccinia virus (Table 1 lists the preclinical and clinical trials of DNA viruses in OS).

Adenovirus

AdV is a non-enveloped, dsDNA virus with a linear genome of 36 kb that causes mild respiratory and alimentary tract infections. Wild type (WT) AdV has many appealing characteristics of a therapeutic viral vector, including the ability to infect both dividing and non-dividing cells by inducing an S-Phase-like state [106], rapid increase in viral titers [89], transduction of numerous cell types, and non-integration of the viral genome into the host genome, to reduce risk of mutagenic effects [45]. Accordingly, AdVs are the first commercially developed oncolytic virus and remain among the most promising. Adenoviral-2 and -5 serotypes of subgroup C are the most common strains used in research.

Following infection, the fiber protein of the AdV particle binds the fiber receptor of the host cell, the coxsackievirus and AdV receptor (CAR), and the viral penton base attaches to the integrin receptor of the cell membrane [102]. This permits internalization by receptor-mediated endocytosis, and the release of dsDNA into the nucleus where viral early-region 1A (E1A) or early-region 1B (E1B) genes are transcribed by RNA polymerase II [16, 67]. These viral proteins inactivate cellular retinoblastoma protein (Rb) and p53 tumor suppressor protein and induce cell cycle progression and promote replication [67]. E1A binds and inhibits the function of Rb. In normal cells, Rb-E2F complex formation suppresses the cell from entering the S Phase of the cell cycle. The binding of E1A to pRb and the subsequent release of E2F1 are required for stimulating cell cycle progression for effective viral replication to occur. E1B is a 55 kDa protein that binds and inactivates p53. The E1B-p53 complex binds DNA and regulates transcriptional activity leading to cell cycle progression and replication of WT AdVs [12].

Table 1 Oncolytic DNA viruses evaluated in osteosarcoma

DNA virus	Trials with osteosarcoma	Citation
Adenovirus	Preclinical	[63, 111]
	Phase I	[84, 85]
Herpes simplex virus	Preclinical	[11]
	Phase I	[66, 87]
Vaccinia virus	Preclinical	[41]

The emphasis of AdV in virotherapy has been on conditionally replicating AdV vectors (CRAds). Tumor targeted conditional replication can be achieved with partial deletions of essential early genes. Deletions in E1A or E1B genes result in attenuated mutants that cannot bind normal cellular proteins that drive gene expression initiating and maintaining cellular proliferation to restrict productive virus infection in normal cells. CRAds are replication-competent in cancer cells which lack functional Rb and p53, allowing in situ amplification and intratumoral spread of infection. ONYX-015 (dl1520) is a chimeric human group AdV, engineered to suppress expression of E1B and is the first CRAd to enter clinical trials [36]. This agent has been evaluated with systemic chemotherapy in patients with advanced sarcomas [85]. Replication following intratumoral administration was generally low and when applied with standard sarcoma chemotherapy [85] one partial response (PR) was observed in a patient with malignant peripheral nerve sheath tumors. While these reports were promising, concerns for tumor targeting, antitumor activity, and host immune response were highlighted in these early studies.

Inactivating p53 and Rb mutations are common in osteosarcoma [76, 112]. In Rb-deficient patients, osteosarcoma is the second most common neoplasm, after retinoblastoma. The CRAd, AdΔ24 (Addl922–947) contains a 24-bp deletion in the E1A region to abolish binding to pRb [31] for permissive replication in cells with a defective Rb pathway. When tested in human osteosarcoma cell lines, primary cell cultures, as well as subcutaneous osteosarcoma xenografts, AdΔ24 demonstrated limited antitumor responses at low-doses of the virus [111]. One limitation in the use of AdVs is that they bind directly to CAR and many malignant cells, including osteosarcoma, express low levels of CAR [20] which limits entry of viral particles into tumor cells [26].

Another approach to designing a conditionally replicative CRAd has been to place genes essential for replication under the control of a tumor marker-specific promoter which results in the expression of critical genes (Fig. 1). While there is no tumor marker for osteosarcoma, this method has been applied using osteocalcin, a bone protein highly expressed in osteotropic and numerous solid tumors, including osteosarcoma and prostate cancer [56, 57]. A Phase I/II clinical trial for treatment of metastasized osteosarcoma using the osteocalcin promoter expressing the E1A gene (Ad-OC-E1A) was planned but the study is unpublished [9]. Ad-OC-E1A showed activity in preclinical models of osteosarcoma and its pulmonary metastasis [64] suggesting this therapeutic strategy could be explored in bone sarcomas and cancers that metastasize to the bone. An AdV using the osteocalcin promoter to drive expression of the suicide gene herpes simplex virus-thymidine kinase (HSV-1 TK) also showed antitumor activity in bone tumor xenografts with low IC50 values [15]. This approach is particularly attractive as doses may be of low magnitude to prevent secondary effects in patients. Human telomerase and the catalytic subunit telomerase reverse transcriptase (hTERT), a polymerase that stabilizes telomere lengths, are highly expressed and activated in a large number of cancers, but not in normal cells [55, 97]. Telomelysin (OB-301) is a replication-defective CRAd that utilizes the hTERT promoter to restrict viral replication to telomerase expressing tumor cells [55]. Telomelysin was tested in a Phase I clinical trial for solid tumors and demonstrated cytolytic properties in osteosarcoma both in vitro and in vivo [63].

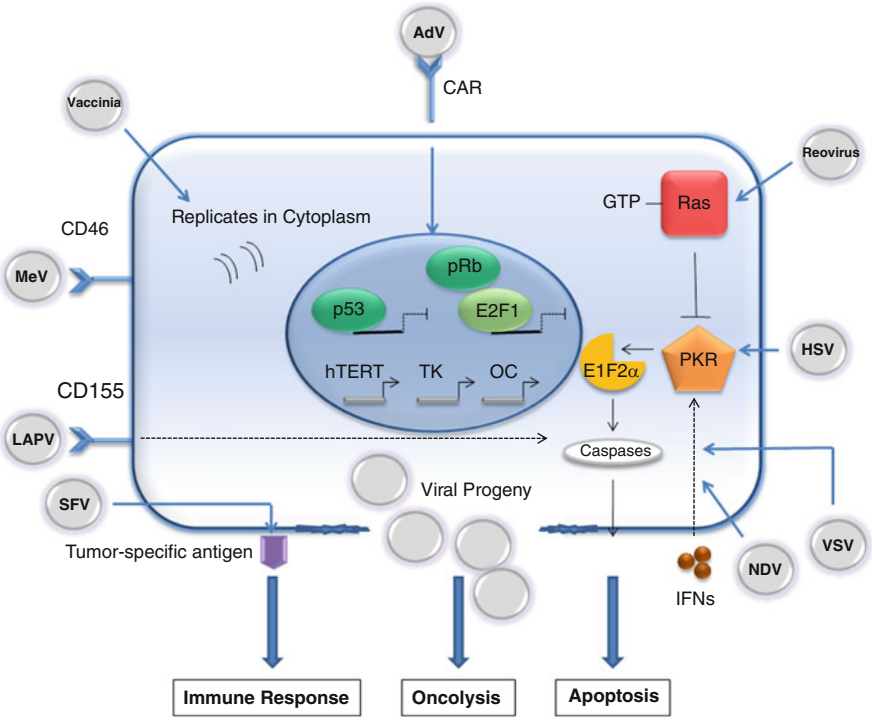


Fig. 1 Targets for oncolytic virotherapy in osteosarcoma. Schematic representation of various DNA and RNA viruses studied to date in osteosarcoma. The figure depicts (**a**) Various modes of entry of viruses into the tumor cell (receptor dependent, e.g., AdV, MeV, LAPV; receptor independent, e.g., reovirus, VSV, SFV, vaccinia) and (**b**) Various modes of tumor kill (viral replication mediated cell oncolysis, e.g., vaccinia, reovirus, AdV; caspase induced apoptosis, e.g., LAPV, HSV; immune mediated tumor kill, e.g., SFV). IFN mediated antiviral response is impaired in several tumor cells aiding in entry and replication of several viruses causing oncolysis (e.g., NDV, VSV). *AdV* adenovirus, *HSV* herpes simplex virus, *VSV* vesicular stomatitis virus, *NDV* Newcastle disease virus, *MeV* measles virus, *SFV* Semliki forest virus, *LAPV* live attenuated polio virus, *IFN* interferon. *Solid blue arrows* represent viral penetration; *Solid black arrows* represent activated signaling pathway or transcription; *Solid blunt lines* represent inhibition of signaling pathway or transcription; *Dotted black lines* represent indirect signaling pathways

Experimental studies showed virulence in multiple bone and soft tissue sarcoma cell lines [95] as well as safety in a Phase I trial of patients with advanced solid tumors including sarcomas [84].

Herpes Simplex Virus

HSV is a large, enveloped, dsDNA virus with a 152 kb genome that encodes more than 80 genes, approximately half of which are involved in replication. HSV type 1

(HSV-1) and type 2 (HSV-2) belong to the herpesviridae family. Oral ulcerations (HSV-1) and genital ulcerations (HSV-1 or HSV-2) are the most common manifestations of the disease. Viral attachment is mediated via interactions of glycoproteins with heparan sulfate proteoglycans (HSPGs) present on host cells [43]. Fusion of the virion envelope with the plasma membrane and release of tegument proteins permit penetration and viral DNA is released for replication. Immediate early α proteins enter the nucleus and activate gene transcription, early β proteins direct DNA replication, and late γ proteins form viral structural proteins. Following primary infection, viruses are transported along the sensory nerves to sensory nerve cell bodies, where they become latent. Neutralizing antibodies are produced by the immune system but the virus is not cleared from the body. Although the majority of the population has preexisting anti-HSV immunity, oncolytic virotherapy with HSV is effective in many different types of tumors including colorectal cancer and melanoma [52, 75].

HSV was the first genetically engineered, replication-selective oncolytic virus tested in the laboratory [72]. In addition to ease of cell entry and robust viral replication in cells, the use of HSV as an oncolytic agent has many other distinct advantages. The large viral DNA genome provides opportunities for deletion of replication and nonessential genes (~ 30 kb) and insertion of transgenes. HSV-1 virus is unique, in that the spread of infection is not dependent on a hematogenous or lymphatic route but rather by cell-to-cell contact, which may promote intratumoral spread and confinement [24]. The natural infection of HSV can cause lethal encephalitis, and thus, it was necessary to attenuate the virus with deletions of the viral neuropathogenicity gene, $\gamma_1$34.5, an inhibitor of cellular apoptosis, to minimize morbidity. Upon HSV infection, the innate activation of dsRNA-dependent protein kinase (PKR) phosphorylates the translation initiation factor eIF-2a and terminates host protein synthesis to prevent viral replication. ICP34.5, the viral protein product of the $\gamma_1$34.5 gene overcomes the PKR pathway host defense mechanism by recruiting protein phosphatase-1a to dephosphorylate eIF2a, permitting viral protein synthesis and translation. eIF2a is expressed in normal cells but attenuated in tumor cells with an aberrant PKR pathway [17, 107]. The Ras/Mitogen activated protein kinase kinase (MEK) pathway is often activated in cancer cells including osteosarcoma, which suppresses PKR (Fig. 1). This enables $\gamma_1$34.5-deficient HSV replication in these cancers [98]. In the event of adverse effects, uncontrolled replication can be terminated with anti-HSV drugs such as ganciclovir and acyclovir.

The UL39 gene encodes ICP6 protein, the large subunit of ribonucleotide reductase (RR), an enzyme required for viral DNA replication which is highly expressed in rapidly dividing tumor cells [66, 77]. Inactivation of ICP6 results in selective HSV-1 replication in tumors but not in quiescent cells. As a consequence, UL39 and $\gamma_1$34.5 deletions to HSV strains confer oncolytic activity in tumor tissue while sparing normal cells. HSV-TK is encoded by viral gene UL23; which phosphorylates thymidine (and other nucleosides). HSV-TK is able to phosphorylate the drug ganciclovir, resulting in a toxic derivative that is incorporated into DNA to enhance cell killing [74]. Treatment of experimental osteosarcoma tumors in rats by HSV-TK gene transfer and ganciclovir appears promising for rapidly proliferating tumors such as osteosarcoma [15].

Although HSV is a neurotropic virus, it is efficacious in numerous cancer types, including sarcomas, melanomas, colon, breast, lung, prostate, and hepatic tumors, and demonstrates safety and antitumor effects in preclinical and clinical studies of these malignancies [66]. Experimental studies to evaluate the efficacy of oncolytic HSV in osteosarcoma were performed with NV1020 and G207 viral strains genetically engineered from HSV-1 [11]. NV1020 is a multimodal HSV-1 virus, containing an HSV-2 glycoprotein insertion (to attenuate potency in normal cells), gene deletion of one copy of ICP34.5 (to enhance potency in tumors) and an HSV-1 TK gene driven by the viral α4 promoter (to promote deoxyribonucleotide synthesis during cell division to facilitate viral DNA replication). G207 is another conditionally replicating HSV-1 vector with viral gene deletions of UL39 and both copies of ICP34.5. High preclinical efficacy of NV1020 and G207 was demonstrated in pediatric rhabdomyosarcoma (RMS) and malignant fibrous histiocytoma cells and modest sensitivity in osteosarcoma cells [11].

HSV1716 (SEPREHVIR (R)), which has a single viral gene deletion (ICP34.5) has been applied in several clinical studies. Preclinical data suggest intratumoral HSV injection induces an immune response against tumor antigens, effectively acting as an in situ cancer vaccine [87]. An ongoing Phase I clinical study evaluating direct intratumoral injection of HSV in children and young adults with non-central nervous system tumors including patients with rhabdomyosarcoma, osteosarcoma, and Ewing's sarcoma has recently been expanded to evaluate intravenous administration of the virus (NCT00931931). Systemic delivery will be required for treatment of metastatic cancer. The trial involves a single treatment at one of two dose levels and requires additional consent for multiple injections if there is a partial or complete response. Like AdVs, numerous oncolytic HSV-1 strains are being constructed containing several genetic modifications. HSV-1 containing the human calponin promoter restricts viral replication to specific tissues [114]. Calponin mRNA and protein are aberrantly expressed in a variety of human soft tissue and bone tumors and could support the replication of this transcriptional targeting virus, resulting in tumor specific oncolysis.

Vaccinia Virus

Vaccinia virus is a member of the poxvirus family. This is a large, enveloped virus containing a linear, dsDNA genome of about 190 kb, which encodes approximately 250 genes. The vaccinia virus was first used as a vaccination for smallpox, making this the first successfully eradicated human disease. WT vaccinia virus has exhibited a natural affinity for preferentially infecting malignant tumors. At least sixteen poxvirus proteins are required for viral entry into the host, 4 for attachment and 12 for penetration [60]. Unlike many viruses, which rely on cellular machinery to replicate, vaccinia replicates virtually as an independent unit. The virus remains exclusively in the cytoplasm and uses virally encoded polymerases to replicate which eliminates the possibility of chromosomal integration. Vaccinia induces tumor killing by replicative necrosis. The vaccinia growth factor (VGF) is a secreted virulence factor that binds to and activates the epidermal growth factor receptor (EGFR), which is critical for viral spread in untransformed tissues [21].

Vaccinia virus has a wide host range and natural tumor tropism and is able to infect most mammalian cell lines. Vaccinia has shown antitumor activity in clinical trials for chronic lymphocytic leukemia [40], metastatic malignant melanoma [115], and prostate cancer (NCT00108732). This virus has been preclinically tested against fibrosarcoma, osteosarcoma, fibrohistiocytoma, and rhabdomyosarcoma cell lines and shows high cytotoxicity against osteosarcoma [41]. An attenuated strain was created with a TK gene deletion to prevent deoxyribonucleotide synthesis during replication. This limits viral replication to cells with high levels of TK, which is typical in cancer cells with a mutated RAS or p53 gene. This viral strain was first shown to selectively replicate in sarcomas [92]. A double-deleted vaccinia virus (vvDD) containing deletions TK and of VGF [21] created a more effective systemic agent, JX-963. Additional selectivity is achieved with JX-963 by expressing combined granulocyte–macrophage colony-stimulating factor (GM-CSF) to initiate an antitumor immune response [100].

JX-594 is an oncolytic vaccinia poxvirus expressing human GM-CSF and with TK gene deletion. A planned Phase 1 dose escalation study of JX-594, administered by intratumoral injection in pediatric patients with unresectable refractory solid tumors is being evaluated (NCT01169584). Tumors include neuroblastoma, lymphoma, Wilms' tumor, rhabdomyosarcoma, Ewing's sarcoma, osteosarcoma, non-rhabdomyosarcoma, soft tissue sarcomas, and malignant peripheral nerve sheath tumors. Although vaccinia induces a strong immune response and can cause encephalitis in immunocompromised patients, the virus is generally safe. Neutralizing antibodies do not hinder antitumoral activity of the virus over multiple injection cycles. A Phase 2b randomized trial evaluating JX-594 plus best supportive care versus best supportive care in patients with advanced hepatocellular carcinoma who have failed sorafenib treatment is also in progress (NCT01387555).

Oncolytic RNA Viruses in Osteosarcoma

Several RNA viruses are being analyzed as oncolytic agents for the treatment of osteosarcoma including reovirus [44], Semliki forest virus (SFV) [53], vesicular stomatitis virus (VSV) [86], measles virus [10], and Newcastle disease virus (NDV) [93] (Table 2 lists the RNA viruses tested in preclinical and clinical trials in OS).

Reovirus

Reovirus is a double stranded RNA virus ubiquitous in the environment that is a member of the reoviridae virus family along with orbivirus and rotavirus. Reovirus usually causes no clinical sequelae but can be isolated from the respiratory

Table 2 Oncolytic RNA viruses evaluated in osteosarcoma

RNA virus	Trials with osteosarcoma	Citation
Reovirus	Preclinical	[44, 78, 108]
	Phase I	
Semliki forest virus	Preclinical	[53]
Vesicular stomatitis virus	Preclinical	[59, 86]
Measles virus	Preclinical	[10]
Poliovirus	Preclinical	[6]
Newcastle disease virus	Preclinical	[69, 93]
	Phase I	
	Phase II	

and gastrointestinal tract of humans [50]. Reovirus selectivity is imparted by its reliance on cells with an activated Ras-signaling pathway, which is present in many human cancers including osteosarcoma [83]. Reolysin is a formulation of reovirus type 3, Dearing strain that has been developed by Oncolytics Biotech and is the only therapeutic reovirus currently being utilized in therapeutic trials. Reolysin showed significant effect in sarcoma cell lines in preclinical studies in vivo and in vitro. All sarcoma cell lines treated with 1–10 virus particles per cell at the time of plating had a decrease in cell viability with 120 h of continuous exposure to the virus, and human osteosarcoma xenografts implanted subcutaneously into the flanks of athymic nu/nu mice responded to treatment with Reolysin (5×10^9 pfu) given intravenously every other day for 3 days starting on Day 1 and Day 22. Reovirus infection was confirmed with visualization of viral particles by electron microscopy after 48 h in the osteosarcoma implanted tumors. Reolysin showed significant antitumor activity in the sarcoma models when compared to the controls, and there was statistically significant improvement in survival for the osteosarcoma models. In addition, stable disease was achieved in mice treated with Reolysin alone and when combined with cisplatin treatment a partial response (greater than 50 % regression in tumor volume but tumor mass remaining >0.1 cm^3) was obtained [44]. More than 16 adult clinical trials using Reolysin have been completed to date. In the Phase I trials in which the virus was given intravenously, Reolysin was found to be very well tolerated with no maximum tolerated dose up to 3×10^{10} $TCID_{50}$ [108]. The most common symptoms were mild flu-like complaints and did not require treatment. In 2007, a Phase II trial began which utilized Reolysin as monotherapy for patients with bone and soft tissue sarcomas metastatic to the lungs. Accrual is ongoing; however, at last publication, seven patients with osteosarcoma have been enrolled. Primary efficacy goals have been met, and thus far Reolysin has shown to be well tolerated in this population and shows promise for the treatment of metastatic sarcoma [78]. A Phase I trial of Reolysin alone and in combination with cyclophosphamide in pediatric patients with relapsed or refractory solid tumors is ongoing (NCT01240538). Accrual to this study is also ongoing.

Semliki Forest Virus

SFV is an enveloped alphavirus of the family Togaviridae with neurotropic properties that normally infects rodents causing fatal encephalitis but in humans is considered nonpathogenic [8, 105]. An avirulent strain of the virus, SFVA7(74), was modified into an attenuated vector that expressed green fluorescent protein, VA7-EGFP, and tested side by side with another widely studied oncolytic virus, conditionally replicating AdV Ad5Δ24, against osteosarcoma xenografts. In vitro, VA7-EGFP grew in four out of five osteosarcoma cell lines efficiently with less time, lower multiplicities of infection (MOI), and more extensive cell death than the AdV. When human osteosarcoma xenografts were implanted into nude mice, both oncolytic viruses were highly effective in shrinking subcutaneous tumors when given intratumorally, although VA7-EGFP showed slightly more potent antitumor effect when compared with Ad5Δ24 and significantly improved the survival of the animals [53].

Vesicular Stomatitis Virus

VSV is an RNA virus that infects cattle, horses, and swine, but causes rare insignificant sequelae with human infection. VSV replicates rapidly in sarcomas with progeny virus beginning to emerge within a few hours of infection. It has innate selectivity due to its sensitivity to the effects of interferon. It also has the ability to replicate in hypoxic areas of tumors. An oncolytically enhanced mutant of VSV, VSV-G/GFP, has a green fluorescent protein gene embedded to allow better visualization of infected cells. VSV has been shown to be ten times more infective in sarcoma cell lines than in normal mesoderm and caused rapid killing of most types of sarcoma, including three osteosarcoma cell lines treated with 5 pfu/cell. One osteosarcoma cell line was among those classified as highly susceptible to infection [86]. When reovirus was compared to VSV, a single dose of VSV completely infected 12 of 13 sarcoma cell lines within 36 h and showed 100 % killing by 48 h compared to reovirus which inhibited sarcoma growth by 50 % after 5 days after multiple doses at comparable MOI [86]. Using an isolated limb perfusion approach to minimize systemic exposure of the virus, the technique of isolated limb perfusion was utilized for hind leg osteosarcoma, and VSV was given directly into a canalized femoral artery of the isolated hind limb. Four weeks after tumor inoculation, the tumor volumes were significantly smaller in the treated group than the control group. In addition, viral gene expression was limited to osteosarcoma cells and not found in nearby perfused leg muscle, non-perfused leg muscle, brain, lung, or liver. In this study, all rats survived without loss of body weight, limb viability, or infections after the treatment [59].

Measles Virus

An oncolytic measles virus (MeV) has been evaluated in multiple sarcoma cell lines including osteosarcoma. When tested, the osteosarcoma cell line was found to be resistant to oncolysis; resistance was due to inhibition of viral replication and not viral entry. Resistance to the virus was found to be at least in part due to elevated cytoplasmic pathogen receptors and interferon stimulating genes because resistant cell lines such as osteosarcoma displayed higher basal mRNA expression of these proteins. Indeed, levels of interferon-β were increased in the osteosarcoma cell culture supernatant after infection with MeV, but when cells were treated with a neutralizing antibody to IFN-β, they observed no improvement in the susceptibility to the MeV, indicating that the differences accounting for resistance to MeV are located upstream of interferon production [10].

Poliovirus

Poliovirus is a non-enveloped RNA virus which infects the nervous system of humans and can lead to the neurologic disorder of poliomyelitis. Due to its dependence on CD155 for host cell binding and infection [73], poliovirus has been tested as oncovirotherapy in several neuroectodermal malignancies. However, CD155 expression was discovered in many bone and soft tissue sarcoma cell lines, and in fact infection with live attenuated poliovirus has been shown to induce apoptosis in several osteosarcoma cell lines in vitro through activation of the caspase cascade. When subcutaneous fibrosarcoma xenografts implanted in mice were treated with intratumoral injections of the virus (1×10^6 $TCID_{50}$) daily for 3 days, 40 % tumor growth inhibition was seen [6].

Newcastle Disease Virus

Only two RNA viruses, reovirus [108] and NDV [69], have been tested in patients with osteosarcoma in Phase I studies and have been proven to be safely administered intravenously. NDV is an avian paramyxovirus not known to cause disease in humans [14]. PV701 is an attenuated strain of NDV that selectively lyses tumor cells based on tumor-specific defects in the interferon signaling. The virus has been shown to infect osteosarcoma cell lines in plaque assays while sparing normal fibroblast cells in culture [93]. NDV was tested in over 113 patients with solid tumors and was very well tolerated, especially after the first dose desensitization. In addition, six major and four minor responses were seen among 95 heavily pretreated patients [69].

A Phase I and II NCI trial is studying the effect of NDV on patients with advanced glioblastoma multiforme, soft and bone sarcomas and neuroblastoma (NCT01174537). At the time of this writing, there are no patients currently enrolled.

General Challenges and Novel Approaches of Virotherapy in Humans

Preclinical and clinical studies have provided scientific and medical evidence to support the use of oncolytic virotherapy as a viable, new treatment modality for cancer. These targeting strategies hold great promise to significantly impact both outcomes and quality of life for patients. In light of these findings, studies addressing the effects of viral infection and improving the efficacy, safety, and applicability of virotherapy are ongoing. This section highlights some of the general challenges encountered in the use of viruses as targeted therapies (Fig. 2).

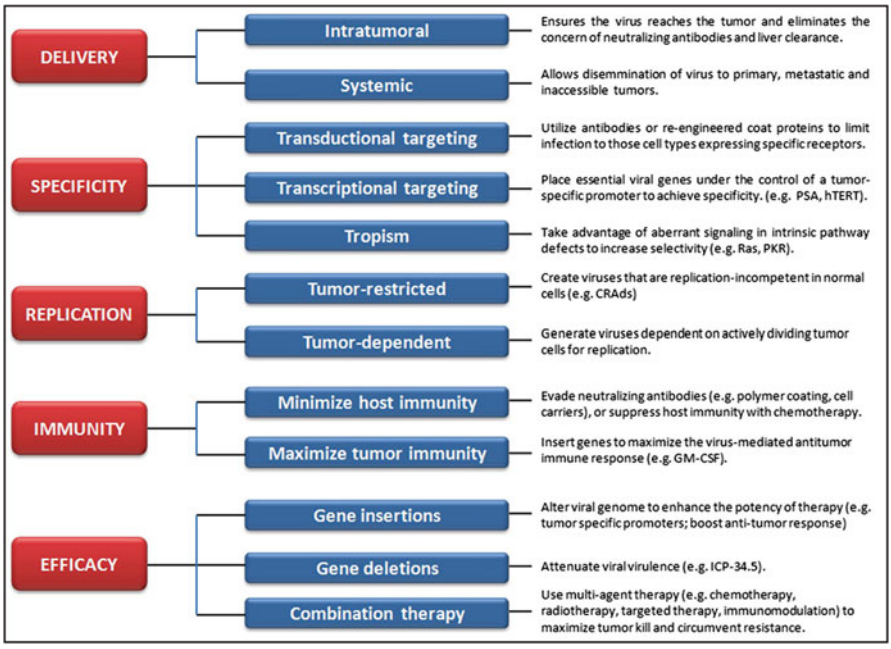


Fig. 2 Strategies to resolve challenges in oncolytic virotherapy. Schematic summary of the general challenges of oncolytic virotherapy as a cancer therapeutic and strategies being employed to address those challenges

Tumor Specificity

When targeting an oncolytic virus to selectively attack a cancer cell, specificity is paramount in order to maintain clinical safety. Tumor specificity is either intrinsic to viruses or engineered by genetic alterations to the viral genome to achieve a better therapeutic effect. RNA viruses have small genomes with relative intrinsic tumor selectivity, such as the reovirus which targets Ras-transformed cells [44]. Others like the MeV virus possess specific tropism based on the expression of cell surface receptors (SLAM, CD46) that are unique to cancer cells [117]. Meanwhile, lack of the CAR receptor in ovarian cancer, melanoma, colon cancer, and also osteosarcoma has been attributed to limited efficacy of adenoviral treatments in these diseases.

There are multiple strategies to enhance tumor selectivity and infection of malignant cells while sparing normal tissues. Transductional retargeting strategies such as modifying the AdV fiber knob with an integrin-binding arginine–glycine–aspartate (RGD) peptide motif into the HI-loop of the fiber knob region [113] and masking the capsid with polymers (e.g., PEG or PHMA) enhance virus attachment and entry into the cell [19]. Transcriptional targeting strategies use tumor marker specific promoters to express critical genes in cancer cells. AdV strains containing the human prostate-specific antigen (PSA) promoter [94] to drive E1A gene expression, successfully attenuated growth of PSA-expressing prostate cancer cells in vitro. Double targeting with both transductional and transcriptional strategies is expected to be additive over any one method. A more recently developed approach has been to deliver oncolytic viruses to the tumors by carrier cells [110]. Cellular carriers including mesenchymal stem cells (MSCs), dendritic cells, T-cells, and intrinsic blood carriers such as peripheral blood mononuclear cells (PBMCs) [1] have all been shown in different models to enhance viral delivery to tumors by decreasing the viral neutralization in the blood [47, 48, 58, 70]. In addition, specific tumor targeting in this manner may also limit off-target side effects of oncolytic viruses. MSCs are specifically of interest in solid tumors due to their inherent tendency to home towards the tumor stroma. This has been demonstrated in preclinical models of renal cancer, ovarian cancer, melanoma and gliomas [3, 13, 44, 70]. A small case series of use of autologous MSCs infected with an oncolytic AdV in patients with relapsed neuroblastoma showed good safety profile and evidence of antitumor activity [38].

Neutralizing Antibodies

Most of the general population is seropositive against many common viruses including AdV, HSV, MeV, vaccinia virus, and reovirus. Naturally acquired immunity is not restrictive to virotherapy, as most oncolytic viruses are generally replication-competent in the presence of preexisting host antibodies. Viral replication is either

inherent or altered through genetic modifications and is the prerequisite for tumor cell lysis and increase in viral progeny by lateral spread throughout the tumor. However, intracellular viral proteins can be processed by the host and presented on major histocompatibility type I (MHC-I) proteins as viral antigens. Viral spread may be restricted by this activation of the immune response during the initial stages of infection with rapid increases in interferons and natural killer cells for prompt viral clearance. Clinical studies have demonstrated that antibody titers increase dramatically within weeks after both intratumoral delivery of reovirus in gliomas [30] and intravenous administration in patients with metastatic melanoma [33]. Other studies have shown that the presence of antibodies in immunocompetent hosts impair effective systemic delivery of oncolytic AdV [103] and VSV [90], limiting the efficacy of virotherapy. It is also apparent, that a dramatic immune response positively impacts therapy by inducing potent antitumoral immune effectors that destroy cancer cells which are not directly lysed by virus. At present, the positive and negative effects of the immune response to viruses and tumors in virotherapy are still ambiguous. Ideally, it would be beneficial to minimize immune responses against the virus and maximize this response against the tumor.

The use of attenuated viral strains (e.g., deletion of the matrix protein of VSV, the NS1 protein of influenza virus, the C and V proteins of paramyxovirus family members, the HSV γ34.5 protein and the proteins encoded in the E1 and E3 regions of the AdV genome) can suppress the magnitude of the antibody response in patients to allow viral replication and spread within the tumor, and multiple treatments. In a syngeneic rat glioma model, pretreatment with cyclophosphamide enhances HSV replication and oncolysis and reduces an HSV-mediated increase in CD68+ and CD163+ cells and intratumoral IFN-γ [32]. Carrier cell-based delivery has also been demonstrated to circumvent antiviral immunity [90] and the strategy of viral cloaking using genetic modifications of the capsid has been suggested to limit AdV clearance and promote tumor targeting [5, 27].

To enhance the immunotherapeutic potential of oncolytic viruses immune regulatory genes are inserted into viral genomes. Granulocyte-monocyte colony stimulating factor (GM-CSF) promotes the differentiation of progenitor cells into dendritic cells, and has been successfully used in strategies to generate tumor-reactive cytotoxic lymphocytes [27]. Strains of vaccinia virus, measles, HSV, and AdVs have all been engineered to incorporate GM-CSF [39, 54, 62, 65]. In a model of bilateral flank lymphoma treated with HSV expressing murine GM-CSF, tumor regression was observed when compared with treatments by control HSV lacking GM-CSF and this response was associated with enhanced splenocyte production of IFN-γ on tumor stimulation [65]. Measles virus expressing murine GM-CSF also showed greater efficacy compared with the vector lacking GM-CSF after intratumoral injection [39]. The cytotoxic T lymphocyte antigen CTLA-4 downregulates T cells and anti-CTLA-4 antibody prolongs T cell activation. Upon intracerebral administration, oncolytic HSV-1 G207 preferentially replicates within glioma cells, which may elicit tumor-specific systemic immune and cytotoxic T lymphocyte (CTL) responses in addition to direct cytopathic effects. Further, combination therapy of anti-CTLA-4 antibody with VSV, enhanced antitumor effects in a mammary

tumor model, in a CD4 and CD8 T cell dependent manner [37]. Although Treg cells suppress the generation of adaptive responses, Treg cell depletion after VSV therapy was found to have a negative therapeutic effect, stimulating an antiviral immune response and leading to rapid viral clearance [23]. This highlights the importance of investigating antiviral as well as antitumor immune responses.

Combination Therapy

Oncolytic virotherapy in preclinical and clinical cancer models have shown few examples of single-agent tumor eradication. Moreover, because tumors are typically heterogeneous genetically and therapy selects for resistant phenotypes, single agents are not usually completely effective. Combination therapies with other anti-cancer modalities (standard chemotherapy, radiotherapy, targeted therapy, immuno-modulators) are the next strategies that are being developed. The benefits of this multi-agent approach with several chemotherapeutic treatments have already been shown to suppress the neutralizing effect of antibodies [32] and enhance cell killing and antitumor effects [42]. In multiple Phase I clinical trials, WT reovirus (Reolysin) was administered in combination with docetaxel [18], gemcitabine [68] and carboplatin and paclitaxel [49] and disease control was exhibited for the majority of patients at a well-tolerated dose. Combinations of vascular endothelial growth factor (VEGF) and paclitaxel or cisplatin increased the vascular permeability of the tumor endothelium and improved the delivery of Sindbis vector to tumors [104]. The VEGF pathway is involved in angiogenesis and is crucial for tumor growth and progression. The sequence of administration of agents in combination therapy is also important. While pretreatment with cyclophosphamide enhances HSV replication and oncolysis [32], maximal efficacy was observed when cisplatin was administered concomitantly with or subsequently to ONYX-015 [42]. It is further anticipated that by acting on different cell populations within the tumor, these multimodal strategies will achieve a greater therapeutic benefit at lower drug toxicities.

Risks of Virotherapy

Viral infection proceeds through three major stages: (1) Initial (acute) infection is associated with rapid viral replication and dissemination, which is often accompanied by a transient period of disease; (2) This is followed by a latent period, during which the virus is brought under immune control and no disease occurs; (3) High levels of viral replication could resume at some later time, leading to disease. Viruses that do not integrate into the host cell DNA or replicate only in the cytoplasm do not carry this latter risk, precluding the risk of mutation (e.g., vaccinia, HSV). Developing new viruses and modifying their genes presents a greater risk of creating undesirable mutations or dangerous new diseases altogether.

To date, the oncolytic viruses tested in clinical trials are well tolerated and have good safety profiles. Newly engineered viral strains are preclinically evaluated in robust and reliable preclinical models of disease providing insights into the virulence of therapies and to inform early-phase clinical testing of agents in patients in an efficient manner. AdVs, HSV, poxviruses, and reoviruses have been all tested in preclinical mouse models. While xenograft tumors allow the investigation of viral efficacy directly in cancer cells of human origin, immunodeficient animals do not allow the study of therapies in the context of an intact immune system. Nonetheless, these studies are necessary to generate safety, toxicity, and efficacy profiles.

Specific Challenges of Virotherapy in Osteosarcoma

Most of the above-discussed challenges with virotherapy are applicable in patients with osteosarcoma. Although elegant work using different approaches to enhance viral oncolytic effects have been described in preclinical cell culture or mouse model systems of cancers including osteosarcoma, these preclinical model systems have major limitations, and therefore, the results do not translate equivalently into human trials. The current preclinical model systems use immunodeficient mice lacking a host immune system that does not address the issues of viral clearance in an immunocompetent host. Specifically, ideal preclinical models would be those that are immunocompetent, are comparable to human pathogenesis and are susceptible to the oncolytic virus being studied. In osteosarcoma, establishment of lung metastases is the major cause of mortality. Hence, it is imperative that the preclinical model is able to replicate the disease process. Canine model mirrors the human host most closely both in terms of immune competence as well as osteosarcoma growth and metastasis. Canine specific conditionally replicative AdV has shown efficacy in canine osteosarcoma cells in vitro [61]. More recently, successful delivery of the canine specific AdV in dogs with neutralizing antibodies by using osteosarcoma tumor cells as carriers has been demonstrated [4]. No clinical trials of oncolytic viruses in dogs are currently ongoing but if conducted, will provide valuable insights into promoting virotherapy in osteosarcoma.

The other major challenge in solid tumors is delivery and penetration of enough virus into the tumor tissue. This can be specifically challenging for osteosarcoma that usually contains a dense osteoid matrix and high interstitial tumor pressure. To reduce viral neutralization after systemic administration by serum neutralizing antibodies and enhance viral delivery into the tissues, different viral carriers have been tested in preclinical models. A recent study showed effective delivery of porphyrin loaded nanoparticles using MSCs into osteosarcoma cells causing photoinduced cell death in vitro [28]. Thus, MSCs may serve as a good delivery system in osteosarcoma for other agents including oncolytic viruses and should be further evaluated.

Most solid tumors are believed to have a peripheral zone of newly developing microvasculature and proliferating tumor cells and a core center of necrosis with

hypoxic conditions and minimal vasculature. Hence, delivery of drugs or viruses is limited to the peripheral zone with inadequate penetration into the central core of the tumor. Attempts at increasing tumor penetration of oncolytic viruses include targeting of the virus to receptors on endothelial cells by attaching specific receptor antibodies on the virus [7], enhancing the permeability of the tumor blood vessels by either giving chemotherapy or radiation or targeted agents such as vascular endothelial growth factor [104] and decreasing the tumor interstitial pressure by chemotherapy or radiation or agents that target the tumor stroma such as collagenase or hyaluronidase [34, 116]. All of these strategies are likely very relevant in osteosarcoma and a combination approach would potentially be most beneficial. Combination of chemotherapy and potentially radiation therapy prior to oncolytic virus administration may enhance the effects of viral oncolysis as demonstrated in preclinical models of pediatric sarcomas [44]. Hyaluronidase has been shown to induce a transcapillary pressure gradient and improve the distribution and uptake of liposomal doxorubicin in human osteosarcoma xenografts [29]. However, some of these methodologies such as intratumoral administration of collagenase or hyaluronidase are challenging to achieve in humans especially in metastatic settings. In addition, none of these approaches address the issue of being able to ensure delivery of oncolytic viruses to every tumor cell, specifically the ones in the hypoxic central areas. In recent years, hypoxia-activated prodrugs (HAPs) such as TH-302 and PR-104 have been developed that specifically target the hypoxic areas of the tumor and are in clinical trials involving sarcomas [35]. Combination of HAPs with other therapies including oncolytic viruses may be one potential rational strategy.

Another challenge that several solid tumors including sarcomas face is the existence of cancer stem cells (CSCs) which are believed to be cells that possess the ability to self-renew and give rise to other tumor cells. CSCs are a subgroup of cells that exist within the tumor in a distinct microenvironment and possess the characteristic of resistance to chemotherapy and radiation therapy thus being responsible for tumor dormancy and relapse or progression of tumors after standard treatment modalities. CSCs have been identified in osteosarcoma and are characterized by the presence of cell surface markers such as CD133, CD117, and Stro-1 and enzymes such as ALDH [2, 101, 109]. This subpopulation of cells has increased tumorigenic abilities, self-renewal capacity and metastatic potential in preclinical models. While traditional therapeutic strategies do not work for this subpopulation of cells, oncolytic viruses may have the ability to attack these cells either in their unmodified form or when modified to specifically target these cells. Although no specific studies exist of targeting osteosarcoma CSCs by oncolytic viruses, studies in other tumor types including neuroblastoma and rhabdomyosarcoma have shown equal sensitivity of CD133+ CSCs and CD133− tumor cells to modified oncolytic HSV-1 virus [71, 91].

All of the above studies highlight the elegant work that has accomplished remarkable results in preclinical studies with oncolytic viruses using different strategies to ranging from delivery of the virus to final effective tumor killing. Perhaps, the biggest challenge in osteosarcoma is the rarity of the disease and limited number of patients

that would be eligible for clinical trials with oncolytic viruses. To be able to choose the most effective oncolytic virus and strategy from scores of different possibilities that will work in humans will be a daunting task especially in light of the current clinical trial designs that require a significant number of patients to determine safety and efficacy of a new agent. Newer, more flexible trial designs using innovative statistical strategies will be needed to be able to test multiple different oncolytic viruses in this patient population.

Future Directions

Despite tremendous progress made in the field of oncolytic virotherapy in both adult and pediatric cancers, limited data exists in osteosarcoma. The need for more robust research using novel therapies such as viruses in osteosarcoma, where overall survival has not changed for several decades cannot be overstated. The future of oncolytic viruses in osteosarcoma will depend on continuing preclinical research studies involving a model host such as the canine that mirrors human disease closely, testing novel viral delivery, tumor penetration, and replication strategies, harnessing the host immune system to aid in viral killing and specifically targeting the resistant CSC population that is likely responsible for relapse and progression in most patients. Data from such studies will guide the choice and technique of viruses to be used in clinical trials. Finally, novel clinical trial designs that address the uniqueness of viral therapy and not just view them as another new drug would be needed to impact patient outcome.

References

1. Adair RA, Roulstone V, Scott KJ, Morgan R, Nuovo GJ, Fuller M, Beirne D, West EJ, Jennings VA, Rose A, Kyula J, Fraser S, Dave R, Anthoney DA, Merrick A, Prestwich R, Aldouri A, Donnelly O, Pandha H, Coffey M, Selby P, Vile R, Toogood G, Harrington K, Melcher AA (2012) Cell carriage, delivery, and selective replication of an oncolytic virus in tumor in patients. Sci Transl Med 4:138ra77
2. Adhikari AS, Agarwal N, Wood BM, Porretta C, Ruiz B, Pochampally RR, Iwakuma T (2010) CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. Cancer Res 70:4602–4612
3. Ahmed AU, Tyler MA, Thaci B, Alexiades NG, Han Y, Ulasov IV, Lesniak MS (2010) A comparative study of neural and mesenchymal stem cell-based carriers for oncolytic adenovirus in a model of malignant glioma. Mol Pharm 8:1559–1572
4. Alcayaga-Miranda F, Cascallo M, Rojas JJ, Pastor J, Alemany R (2010) Osteosarcoma cells as carriers to allow antitumor activity of canine oncolytic adenovirus in the presence of neutralizing antibodies. Cancer Gene Ther 17:792–802
5. Alemany R, Curiel DT (2001) CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther 8:1347–1353
6. Atsumi S, Matsumine A, Toyoda H, Niimi R, Iino T, Nakamura T, Matsubara T, Asanuma K, Komada Y, Uchida A, Sudo A (2012) Oncolytic virotherapy for human bone and soft tissue sarcomas using live attenuated poliovirus. Int J Oncol 41:893–902

7. Bachtarzi H, Stevenson M, Subr V, Ulbrich K, Seymour LW, Fisher KD (2011) Targeting adenovirus gene delivery to activated tumour-associated vasculature via endothelial selectins. J Control Release 150:196–203
8. Barrett PN, Sheahan BJ, Atkins GJ (1980) Isolation and preliminary characterization of Semliki Forest virus mutants with altered virulence. J Gen Virol 49:141–147
9. Benjamin R, Helman L, Meyers P, Reaman G, (2001) A phase I/II dose escalation and activity study of intravenous injections of OCaP1 for subjects with refractory osteosarcoma metastatic to lung. Hum Gene Ther Aug 10; 12(12):1591–1593
10. Berchtold S, Lampe J, Weiland T, Smirnow I, Schleicher S, Handgretinger R, Kopp HG, Reiser J, Stubenrauch F, Mayer N, Malek NP, Bitzer M, Lauer UM (2013) Innate immune defense defines susceptibility of sarcoma cells to measles vaccine virus-based oncolysis. J Virol 87:3484–3501
11. Bharatan NS, Currier MA, Cripe TP (2002) Differential susceptibility of pediatric sarcoma cells to oncolysis by conditionally replication-competent herpes simplex viruses. J Pediatr Hematol Oncol 24:447–453
12. Bischoff A (2003) Viruses, bacteria and parasites. Attack on the fetus. MMW Fortschr Med 145:4–8
13. Bolontrade MF, Sganga L, Piaggio E, Viale DL, Sorrentino MA, Robinson A, Sevlever G, Garcia MG, Mazzolini G, Podhajcer OL (2012) A specific subpopulation of mesenchymal stromal cell carriers overrides melanoma resistance to an oncolytic adenovirus. Stem Cells Dev 21:2689–2702
14. Cassel WA, Garrett RE (1965) Newcastle disease virus as an antineoplastic agent. Cancer 18:863–868
15. Charissoux JL, Grossin L, Leboutet MJ, Rigaud M (1999) Treatment of experimental osteosarcoma tumors in rat by herpes simplex thymidine kinase gene transfer and ganciclovir. Anticancer Res 19:77–80
16. Chu RL, Post DE, Khuri FR, Van Meir EG (2004) Use of replicating oncolytic adenoviruses in combination therapy for cancer. Clin Cancer Res 10:5299–5312
17. Coffey MC, Strong JE, Forsyth PA, Lee PW (1998) Reovirus therapy of tumors with activated Ras pathway. Science 282:1332–1334
18. Comins C, Spicer J, Protheroe A, Roulstone V, Twigger K, White CM, Vile R, Melcher A, Coffey MC, Mettinger KL, Nuovo G, Cohn DE, Phelps M, Harrington KJ, Pandha HS (2010) REO-10: a phase I study of intravenous reovirus and docetaxel in patients with advanced cancer. Clin Cancer Res 16:5564–5572
19. Coughlan L, Alba R, Parker AL, Bradshaw AC, McNeish IA, Nicklin SA, Baker AH (2010) Tropism-modification strategies for targeted gene delivery using adenoviral vectors. Viruses 2:2290–2355
20. Cripe TP, Dunphy EJ, Holub AD, Saini A, Vasi NH, Mahller YY, Collins MH, Snyder JD, Krasnykh V, Curiel DT, Wickham TJ, DeGregori J, Bergelson JM, Currier MA (2001) Fiber knob modifications overcome low, heterogeneous expression of the coxsackievirus-adenovirus receptor that limits adenovirus gene transfer and oncolysis for human rhabdomyosarcoma cells. Cancer Res 61:2953–2960
21. de Magalhaes JC, Andrade AA, Silva PN, Sousa LP, Ropert C, Ferreira PC, Kroon EG, Gazzinelli RT, Bonjardim CA (2001) A mitogenic signal triggered at an early stage of vaccinia virus infection: implication of MEK/ERK and protein kinase A in virus multiplication. J Biol Chem 276:38353–38360
22. DePace N (1912) Sulla Scomparsa di un enorme cancro begetante del callo dell'utero senza cura chirurgica. Ginecoligia 9:82
23. Diaz RM, Galivo F, Kottke T, Wongthida P, Qiao J, Thompson J, Valdes M, Barber G, Vile RG (2007) Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. Cancer Res 67:2840–2848
24. Dingwell KS, Johnson DC (1998) The herpes simplex virus gE-gI complex facilitates cell-to-cell spread and binds to components of cell junctions. J Virol 72:8933–8942
25. Dock G (1904) The influence of complicating diseases upon leukaemia. Am J Med Sci 127:561–592

26. Douglas JT, Kim M, Sumerel LA, Carey DE, Curiel DT (2001) Efficient oncolysis by a replicating adenovirus (ad) in vivo is critically dependent on tumor expression of primary ad receptors. Cancer Res 61:813–817
27. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci U S A 90:3539–3543
28. Duchi S, Sotgiu G, Lucarelli E, Ballestri M, Dozza B, Santi S, Guerrini A, Dambruoso P, Giannini S, Donati D, Ferroni C, Varchi G (2013) Mesenchymal stem cells as delivery vehicle of porphyrin loaded nanoparticles: effective photoinduced in vitro killing of osteosarcoma. J Control Release 168:225–237
29. Eikenes L, Tari M, Tufto I, Bruland OS, de Lange Davies C (2005) Hyaluronidase induces a transcapillary pressure gradient and improves the distribution and uptake of liposomal doxorubicin (Caelyx) in human osteosarcoma xenografts. Br J Cancer 93:81–88
30. Forsyth P, Roldan G, George D, Wallace C, Palmer CA, Morris D, Cairncross G, Matthews MV, Markert J, Gillespie Y, Coffey M, Thompson B, Hamilton M (2008) A phase I trial of intratumoral administration of reovirus in patients with histologically confirmed recurrent malignant gliomas. Mol Ther 16:627–632
31. Fueyo J, Gomez-Manzano C, Alemany R, Lee PS, McDonnell TJ, Mitlianga P, Shi YX, Levin VA, Yung WK, Kyritsis AP (2000) A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene 19:2–12
32. Fulci G, Breymann L, Gianni D, Kurozomi K, Rhee SS, Yu J, Kaur B, Louis DN, Weissleder R, Caligiuri MA, Chiocca EA (2006) Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. Proc Natl Acad Sci U S A 103:12873–12878
33. Galanis E, Markovic SN, Suman VJ, Nuovo GJ, Vile RG, Kottke TJ, Nevala WK, Thompson MA, Lewis JE, Rumilla KM, Roulstone V, Harrington K, Linette GP, Maples WJ, Coffey M, Zwiebel J, Kendra K (2012) Phase II trial of intravenous administration of Reolysin((R)) (Reovirus Serotype-3-dearing Strain) in patients with metastatic melanoma. Mol Ther 20:1998–2003
34. Ganesh S, Gonzalez-Edick M, Gibbons D, Van Roey M, Jooss K (2008) Intratumoral coadministration of hyaluronidase enzyme and oncolytic adenoviruses enhances virus potency in metastatic tumor models. Clin Cancer Res 14:3933–3941
35. Ganjoo KN, Cranmer LD, Butrynski JE, Rushing D, Adkins D, Okuno SH, Lorente G, Kroll S, Langmuir VK, Chawla SP (2011) A phase I study of the safety and pharmacokinetics of the hypoxia-activated prodrug TH-302 in combination with doxorubicin in patients with advanced soft tissue sarcoma. Oncology 80:50–56
36. Ganly I, Kirn D, Eckhardt G, Rodriguez GI, Soutar DS, Otto R, Robertson AG, Park O, Gulley ML, Heise C, Von Hoff DD, Kaye SB (2000) A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. Clin Cancer Res 6:798–806
37. Gao Y, Whitaker-Dowling P, Griffin JA, Barmada MA, Bergman I (2009) Recombinant vesicular stomatitis virus targeted to Her2/neu combined with anti-CTLA4 antibody eliminates implanted mammary tumors. Cancer Gene Ther 16:44–52
38. Garcia-Castro J, Alemany R, Cascallo M, Martinez-Quintanilla J, Arriero Mdel M, Lassaletta A, Madero L, Ramirez M (2010) Treatment of metastatic neuroblastoma with systemic oncolytic virotherapy delivered by autologous mesenchymal stem cells: an exploratory study. Cancer Gene Ther 17:476–483
39. Grote D, Cattaneo R, Fielding AK (2003) Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. Cancer Res 63:6463–6468
40. Hansen RM, Libnoch JA (1978) Remission of chronic lymphocytic leukemia after smallpox vaccination. Arch Intern Med 138:1137–1138
41. He S, Li P, Chen CH, Bakst RL, Chernichenko N, Yu YA, Chen N, Szalay AA, Yu Z, Fong Y, Wong RJ (2012) Effective oncolytic vaccinia therapy for human sarcomas. J Surg Res 175:e53–e60

42. Heise C, Lemmon M, Kirn D (2000) Efficacy with a replication-selective adenovirus plus cisplatin-based chemotherapy: dependence on sequencing but not p53 functional status or route of administration. Clin Cancer Res 6:4908–4914
43. Herold BC, Visalli RJ, Susmarski N, Brandt CR, Spear PG (1994) Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J Gen Virol 75(Pt 6):1211–1222
44. Hingorani P, Zhang W, Lin J, Liu L, Guha C, Kolb EA (2011) Systemic administration of reovirus (Reolysin) inhibits growth of human sarcoma xenografts. Cancer 117:1764–1774
45. Hitt MM, Addison CL, Graham FL (1997) Human adenovirus vectors for gene transfer into mammalian cells. Adv Pharmacol 40:137–206
46. Hsiao WC, Sung SY, Liao CH, Wu HC, Hsieh CL (2012) Vitamin D3-inducible mesenchymal stem cell-based delivery of conditionally replicating adenoviruses effectively targets renal cell carcinoma and inhibits tumor growth. Mol Pharm 9:1396–1408
47. Ilett EJ, Barcena M, Errington-Mais F, Griffin S, Harrington KJ, Pandha HS, Coffey M, Selby PJ, Limpens RW, Mommaas M, Hoeben RC, Vile RG, Melcher AA (2011) Internalization of oncolytic reovirus by human dendritic cell carriers protects the virus from neutralization. Clin Cancer Res 17:2767–2776
48. Ilett EJ, Prestwich RJ, Kottke T, Errington F, Thompson JM, Harrington KJ, Pandha HS, Coffey M, Selby PJ, Vile RG, Melcher AA (2009) Dendritic cells and T cells deliver oncolytic reovirus for tumour killing despite pre-existing anti-viral immunity. Gene Ther 16:689–699
49. Karapanagiotou EM, Roulstone V, Twigger K, Ball M, Tanay M, Nutting C, Newbold K, Gore ME, Larkin J, Syrigos KN, Coffey M, Thompson B, Mettinger K, Vile RG, Pandha HS, Hall GD, Melcher AA, Chester J, Harrington KJ (2012) Phase I/II trial of carboplatin and paclitaxel chemotherapy in combination with intravenous oncolytic reovirus in patients with advanced malignancies. Clin Cancer Res 18:2080–2089
50. Kasel JA, Rosen L, Evans HE (1963) Infection of human volunteers with a reovirus of bovine origin. Proc Soc Exp Biol Med 112:979–981
51. Kelly E, Russell SJ (2007) History of oncolytic viruses: genesis to genetic engineering. Mol Ther 15:651–659
52. Kemeny N, Brown K, Covey A, Kim T, Bhargava A, Brody L, Guilfoyle B, Haag NP, Karrasch M, Glasschroeder B, Knoll A, Getrajdman G, Kowal KJ, Jarnagin WR, Fong Y (2006) Phase I, open-label, dose-escalating study of a genetically engineered herpes simplex virus, NV1020, in subjects with metastatic colorectal carcinoma to the liver. Hum Gene Ther 17:1214–1224
53. Ketola A, Hinkkanen A, Yongabi F, Furu P, Maatta AM, Liimatainen T, Pirinen R, Bjorn M, Hakkarainen T, Makinen K, Wahlfors J, Pellinen R (2008) Oncolytic Semliki forest virus vector as a novel candidate against unresectable osteosarcoma. Cancer Res 68:8342–8350
54. Kim MO, Kim SH, Lee SR, Kim KS, Min KS, Lee HT, Kim SJ, Ryoo ZY (2006) Transgene expression of biological active recombinant human granulocyte-colony stimulating factor (hG-CSF) into mouse urine. Life Sci 78:1003–1009
55. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015
56. Ko SC, Cheon J, Kao C, Gotoh A, Shirakawa T, Sikes RA, Karsenty G, Chung LW (1996) Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. Cancer Res 56:4614–4619
57. Koeneman KS, Kao C, Ko SC, Yang L, Wada Y, Kallmes DF, Gillenwater JY, Zhau HE, Chung LW, Gardner TA (2000) Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. World J Urol 18:102–110
58. Komarova S, Kawakami Y, Stoff-Khalili MA, Curiel DT, Pereboeva L (2006) Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. Mol Cancer Ther 5:755–766
59. Kubo T, Shimose S, Matsuo T, Fujimori J, Sakaguchi T, Yamaki M, Shinozaki K, Woo SL, Ochi M (2011) Oncolytic vesicular stomatitis virus administered by isolated limb perfusion suppresses osteosarcoma growth. J Orthop Res 29:795–800

60. Laliberte JP, Weisberg AS, Moss B (2011) The membrane fusion step of vaccinia virus entry is cooperatively mediated by multiple viral proteins and host cell components. PLoS Pathog 7:e1002446
61. Le LP, Rivera AA, Glasgow JN, Ternovoi VV, Wu H, Wang M, Smith BF, Siegal GP, Curiel DT (2006) Infectivity enhancement for adenoviral transduction of canine osteosarcoma cells. Gene Ther 13:389–399
62. Lei N, Shen FB, Chang JH, Wang L, Li H, Yang C, Li J, Yu DC (2009) An oncolytic adenovirus expressing granulocyte macrophage colony-stimulating factor shows improved specificity and efficacy for treating human solid tumors. Cancer Gene Ther 16:33–43
63. Li G, Kawashima H, Ogose A, Ariizumi T, Xu Y, Hotta T, Urata Y, Fujiwara T, Endo N (2011) Efficient virotherapy for osteosarcoma by telomerase-specific oncolytic adenovirus. J Cancer Res Clin Oncol 137:1037–1051
64. Li X, Jung C, Liu YH, Bae KH, Zhang YP, Zhang HJ, Vanderputten D, Jeng MH, Gardner TA, Kao C (2006) Anti-tumor efficacy of a transcriptional replication-competent adenovirus, Ad-OC-E1a, for osteosarcoma pulmonary metastasis. J Gene Med 8:679–689
65. Liu BL, Robinson M, Han ZQ, Branston RH, English C, Reay P, McGrath Y, Thomas SK, Thornton M, Bullock P, Love CA, Coffin RS (2003) ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther 10:292–303
66. Liu S, Dai M, You L, Zhao Y (2013) Advance in herpes simplex viruses for cancer therapy. Sci China Life Sci 56:298–305
67. Liu TC, Kirn D (2005) Viruses with deletions in antiapoptotic genes as potential oncolytic agents. Oncogene 24:6069–6079
68. Lolkema MP, Arkenau HT, Harrington K, Roxburgh P, Morrison R, Roulstone V, Twigger K, Coffey M, Mettinger K, Gill G, Evans TR, de Bono JS (2011) A phase I study of the combination of intravenous reovirus type 3 Dearing and gemcitabine in patients with advanced cancer. Clin Cancer Res 17:581–588
69. Lorence RM, Pecora AL, Major PP, Hotte SJ, Laurie SA, Roberts MS, Groene WS, Bamat MK (2003) Overview of phase I studies of intravenous administration of PV701, an oncolytic virus. Curr Opin Mol Ther 5:618–624
70. Mader EK, Maeyama Y, Lin Y, Butler GW, Russell HM, Galanis E, Russell SJ, Dietz AB, Peng KW (2009) Mesenchymal stem cell carriers protect oncolytic measles viruses from antibody neutralization in an orthotopic ovarian cancer therapy model. Clin Cancer Res 15:7246–7255
71. Mahller YY, Williams JP, Baird WH, Mitton B, Grossheim J, Saeki Y, Cancelas JA, Ratner N, Cripe TP (2009) Neuroblastoma cell lines contain pluripotent tumor initiating cells that are susceptible to a targeted oncolytic virus. PLoS One 4:e4235
72. Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM (1991) Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science 252:854–856
73. Mendelsohn CL, Wimmer E, Racaniello VR (1989) Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell 56:855–865
74. Mesnil M, Yamasaki H (2000) Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. Cancer Res 60:3989–3999
75. Miller CG, Fraser NW (2000) Role of the immune response during neuro-attenuated herpes simplex virus-mediated tumor destruction in a murine intracranial melanoma model. Cancer Res 60:5714–5722
76. Miller CW, Aslo A, Tsay C, Slamon D, Ishizaki K, Toguchida J, Yamamuro T, Lampkin B, Koeffler HP (1990) Frequency and structure of p53 rearrangements in human osteosarcoma. Cancer Res 50:7950–7954
77. Mineta T, Rabkin SD, Martuza RL (1994) Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. Cancer Res 54:3963–3966

78. Mita AC, Sankhala K, Sarantopoulos J et al (2009) A phase II study of intravenous (IV) wild-type reovirus (Reolysin) in the treatment of patients with bone and soft tissue sarcomas metastatic to the lung. ASCO meeting abstracts. J Clin Oncol 27:10524
79. Moore AE (1949) The destructive effect of the virus of Russian Far East encephalitis on the transplantable mouse sarcoma 180. Cancer 2:525–534
80. Moore AE (1951) Inhibition of growth of five transplantable mouse tumors by the virus of Russian Far East encephalitis. Cancer 4:375–382
81. Moore AE (1952) Viruses with oncolytic properties and their adaptation to tumors. Ann N Y Acad Sci 54:945–952
82. Moore AE, O'Connor S (1950) Further studies on the destructive effect of the virus of Russian Far East encephalitis on the transplantable mouse sarcoma 180. Cancer 3:886–890
83. Na KY, Kim YW, Park YK (2012) Mitogen-activated protein kinase pathway in osteosarcoma. Pathology 44:540–546
84. Nemunaitis J, Tong AW, Nemunaitis M, Senzer N, Phadke AP, Bedell C, Adams N, Zhang YA, Maples PB, Chen S, Pappen B, Burke J, Ichimaru D, Urata Y, Fujiwara T (2010) A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. Mol Ther 18:429–434
85. Opyrchal M, Aderca I, Galanis E (2009) Phase I clinical trial of locoregional administration of the oncolytic adenovirus ONYX-015 in combination with mitomycin-C, doxorubicin, and cisplatin chemotherapy in patients with advanced sarcomas. Methods Mol Biol 542:705–717
86. Paglino JC, van den Pol AN (2011) Vesicular stomatitis virus has extensive oncolytic activity against human sarcomas: rare resistance is overcome by blocking interferon pathways. J Virol 85:9346–9358
87. Papanastassiou V, Rampling R, Fraser M, Petty R, Hadley D, Nicoll J, Harland J, Mabbs R, Brown M (2002) The potential for efficacy of the modified (ICP 34.5(-)) herpes simplex virus HSV1716 following intratumoural injection into human malignant glioma: a proof of principle study. Gene Ther 9:398–406
88. Park BH, Hwang T, Liu TC, Sze DY, Kim JS, Kwon HC, Oh SY, Han SY, Yoon JH, Hong SH, Moon A, Speth K, Park C, Ahn YJ, Daneshmand M, Rhee BG, Pinedo HM, Bell JC, Kirn DH (2008) Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. Lancet Oncol 9:533–542
89. Pesonen S, Kangasniemi L, Hemminki A (2010) Oncolytic adenoviruses for the treatment of human cancer: focus on translational and clinical data. Mol Pharm 8:12–28
90. Power AT, Wang J, Falls TJ, Paterson JM, Parato KA, Lichty BD, Stojdl DF, Forsyth PA, Atkins H, Bell JC (2007) Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. Mol Ther 15:123–130
91. Pressey JG, Haas MC, Pressey CS, Kelly VM, Parker JN, Gillespie GY, Friedman GK (2013) CD133 marks a myogenically primitive subpopulation in rhabdomyosarcoma cell lines that are relatively chemoresistant but sensitive to mutant HSV. Pediatr Blood Cancer 60:45–52
92. Puhlmann M, Brown CK, Gnant M, Huang J, Libutti SK, Alexander HR, Bartlett DL (2000) Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. Cancer Gene Ther 7:66–73
93. Reichard KW, Lorence RM, Cascino CJ, Peeples ME, Walter RJ, Fernando MB, Reyes HM, Greager JA (1992) Newcastle disease virus selectively kills human tumor cells. J Surg Res 52:448–453
94. Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR (1997) Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res 57:2559–2563
95. Sasaki T, Tazawa H, Hasei J, Kunisada T, Yoshida A, Hashimoto Y, Yano S, Yoshida R, Uno F, Kagawa S, Morimoto Y, Urata Y, Ozaki T, Fujiwara T (2011) Preclinical evaluation of telomerase-specific oncolytic virotherapy for human bone and soft tissue sarcomas. Clin Cancer Res 17:1828–1838
96. Senzer NN, Kaufman HL, Amatruda T, Nemunaitis M, Reid T, Daniels G, Gonzalez R, Glaspy J, Whitman E, Harrington K, Goldsweig H, Marshall T, Love C, Coffin R, Nemunaitis JJ (2009) Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-

encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. J Clin Oncol 27:5763–5771
97. Shay JW, Bacchetti S (1997) A survey of telomerase activity in human cancer. Eur J Cancer 33:787–791
98. Smith KD, Mezhir JJ, Bickenbach K, Veerapong J, Charron J, Posner MC, Roizman B, Weichselbaum RR (2006) Activated MEK suppresses activation of PKR and enables efficient replication and in vivo oncolysis by Deltagamma(1)34.5 mutants of herpes simplex virus 1. J Virol 80:1110–1120
99. Southam CM (1960) Present status of oncolytic virus studies. Trans N Y Acad Sci 22:657–673
100. Thorne SH, Hwang TH, O'Gorman WE, Bartlett DL, Sei S, Kanji F, Brown C, Werier J, Cho JH, Lee DE, Wang Y, Bell J, Kirn DH (2007) Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. J Clin Invest 117:3350–3358
101. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, Fazioli F, Pirozzi G, Papaccio G (2011) Human primary bone sarcomas contain CD133+ cancer stem cells displaying high tumorigenicity in vivo. FASEB J 25:2022–2030
102. Tomko RP, Xu R, Philipson L (1997) HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci U S A 94:3352–3356
103. Tsai V, Johnson DE, Rahman A, Wen SF, LaFace D, Philopena J, Nery J, Zepeda M, Maneval DC, Demers GW, Ralston R (2004) Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model. Clin Cancer Res 10:7199–7206
104. Tseng JC, Granot T, DiGiacomo V, Levin B, Meruelo D (2010) Enhanced specific delivery and targeting of oncolytic Sindbis viral vectors by modulating vascular leakiness in tumor. Cancer Gene Ther 17:244–255
105. Vaha-Koskela MJ, Tuittila MT, Nygardas PT, Nyman JK, Ehrengruber MU, Renggli M, Hinkkanen AE (2003) A novel neurotropic expression vector based on the avirulent A7(74) strain of Semliki Forest virus. J Neurovirol 9:1–15
106. Van Dyke TA (1994) Analysis of viral-host protein interactions and tumorigenesis in transgenic mice. Semin Cancer Biol 5:47–60
107. Varghese S, Newsome JT, Rabkin SD, McGeagh K, Mahoney D, Nielsen P, Todo T, Martuza RL (2001) Preclinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. Hum Gene Ther 12:999–1010
108. Vidal L, Pandha HS, Yap TA, White CL, Twigger K, Vile RG, Melcher A, Coffey M, Harrington KJ, DeBono JS (2008) A phase I study of intravenous oncolytic reovirus type 3 Dearing in patients with advanced cancer. Clin Cancer Res 14:7127–7137
109. Wang L, Park P, Zhang H, La Marca F, Lin CY (2011) Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity. Int J Cancer 128:294–303
110. Willmon C, Harrington K, Kottke T, Prestwich R, Melcher A, Vile R (2009) Cell carriers for oncolytic viruses: Fed Ex for cancer therapy. Mol Ther 17:1667–1676
111. Witlox AM, Van Beusechem VW, Molenaar B, Bras H, Schaap GR, Alemany R, Curiel DT, Pinedo HM, Wuisman PI, Gerritsen WR (2004) Conditionally replicative adenovirus with tropism expanded towards integrins inhibits osteosarcoma tumor growth in vitro and in vivo. Clin Cancer Res 10:61–67
112. Wong FL, Boice JD Jr, Abramson DH, Tarone RE, Kleinerman RA, Stovall M, Goldman MB, Seddon JM, Tarbell N, Fraumeni JF Jr, Li FP (1997) Cancer incidence after retinoblastoma. Radiation dose and sarcoma risk. JAMA 278:1262–1267
113. Yamamoto M, Curiel DT (2010) Current issues and future directions of oncolytic adenoviruses. Mol Ther 18:243–250
114. Yamamura H, Hashio M, Noguchi M, Sugenoya Y, Osakada M, Hirano N, Sasaki Y, Yoden T, Awata N, Araki N, Tatsuta M, Miyatake SI, Takahashi K (2001) Identification of the transcriptional regulatory sequences of human calponin promoter and their use in targeting a

conditionally replicating herpes vector to malignant human soft tissue and bone tumors. Cancer Res 61:3969–3977
115. Zajac P, Oertli D, Marti W, Adamina M, Bolli M, Guller U, Noppen C, Padovan E, Schultz-Thater E, Heberer M, Spagnoli G (2003) Phase I/II clinical trial of a nonreplicative vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes and costimulatory molecules in metastatic melanoma patients. Hum Gene Ther 14:1497–1510
116. Zheng X, Goins BA, Cameron IL, Santoyo C, Bao A, Frohlich VC, Fullerton GD (2011) Ultrasound-guided intratumoral administration of collagenase-2 improved liposome drug accumulation in solid tumor xenografts. Cancer Chemother Pharmacol 67:173–182
117. Zhou Q, Schneider IC, Gallet M, Kneissl S, Buchholz CJ (2011) Resting lymphocyte transduction with measles virus glycoprotein pseudotyped lentiviral vectors relies on CD46 and SLAM. Virology 413:149–152

IL-11Rα: A Novel Target for the Treatment of Osteosarcoma

Valerae O. Lewis

Abstract Recent advances have shown that cell surface receptors are expressed differentially in normal and pathological conditions. Novel organ specific and disease specific proteins expressed on tumor vasculature have been identified by in vivo phage display technology and the diversity of the tumor-associated vasculature has provided the basis for the development of targeted therapeutics. Investigators recently screened a phage display library in a human cancer patient. An IL-11 mimic phage displaying the cyclic peptide CGRRAGGSC (single letter amino acid code) specifically bound to immobilized IL-11Rα. It has been demonstrated that the expression of the IL-11Rα is increased in several other types of tumors including osteosarcoma. The ability to selectively target the IL-11Rα may provide an alternative treatment of for a disease where new treatment options are truly needed.

Introduction

Osteosarcoma is the most common primary tumor of bone. Although modern chemotherapy introduced in the 1980s improved survival from 20 to 70 %, the survival rate has plateaued. Death from pulmonary failure, secondary to progression of pulmonary metastatic disease, remains a significant problem. The initial hope that poor responders, those patients who did not respond to neoadjuvant chemotherapy, could be salvaged by adding new chemotherapeutic agents or new chemotherapy regimens has not been realized. New treatment options are needed. As the understanding of osteosarcoma biology at the cellular and subcellular level increases, investigators are pursing possible osteosarcoma tumor targets for selective therapy.

V.O. Lewis, M.D. (✉)
Department of Orthopaedic Oncology, MD Anderson Cancer Center,
P.O. Box 301402, Unit 1448, Houston, TX 77230-1402, USA
e-mail: volewis@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_15,

There is a clear need for improved biomarkers of tumor cell growth and potential targets in osteosarcoma. In vivo phage display has been used to isolate probes that home selectively to different vascular beds and target tissues. Both tissue-specific and angiogenesis-related vascular ligand-receptor pairs have been identified with this technology. Targeted delivery of cytotoxic drugs, proapoptotic peptides, fluorophores, or cytokines to the vasculature generally improved selectivity and/or therapeutic windows in animal models [1–3]. Researchers recently screened a phage display library in a human cancer patient. The published large-scale survey of motifs that localized to different organs showed that the tissue distribution of circulating peptides was not random [4]. To gain insight into candidate native ligands, a panel of proteins that contained the selected peptides was identified by homology blast against established protein databases. Among the selected peptide sequences, the motif Arg-Arg-Ala-Gly-Gly-Ser had homology to interleukin 11 (IL-11). An IL-11 mimic phage displaying the cyclic peptide CGRRAGGSC (single letter amino acid code) specifically bound to immobilized IL-11Rα.

Studies with archival human tissues revealed that the expression of the IL-11Rα is increased in several other types of tumors such as colon cancer, gastric cancer, breast cancer, prostate cancer, and osteosarcoma. Morphologic and functional analyses revealed IL-11Rα to be a potential target for intervention in human prostate cancer [5–11].

IL-11 is a multifunctional cytokine first isolated in 1990 from bone marrow stromal cells. A member of the gp130 family of cytokines, that includes IL-6, leukemia-inhibitory factor, and oncostatin M, it has multiple effects on the hematopoietic and nonhematopoietic systems. The binding of IL-11 to IL-11Rα mediates the assembly of a multisubunit transmembrane receptor and this receptor complex initiates intracellular signaling by association with the transmembrane signal transducer glycoprotein gp-130 [12, 13]. IL-11/IL-11Rα binding and downstream signaling via signal transduction and activator of transcription 3 activation has been proposed as a leading molecular pathway in several biological activities such as adipogenesis, osteoclastogenesis, neurogenesis, and hematopoiesis and metastasis [13, 14].

IL-11Rα in Osteosarcoma

Researchers demonstrated that IL-11Rα is expressed in several osteosarcoma cell lines [11, 12]. Immunofluorescence analysis revealed that a IL11 mimic phage, a phage displaying CGRRAGGSC, specifically bound to the IL-11Rα and could be internalized by the receptor into osteosarcoma cells. These data supported IL-11Rα as a viable candidate target for ligand-directed delivery to osteosarcoma tumor cells. In vivo orthotopic mice models of osteosarcoma were then used to examine the in vivo expression of IL-11Rα. Immunohistochemical staining of formalin-fixed, paraffin-embedded osteosarcoma tumors at 2, 3, 5, and 7 weeks postinjection revealed that IL-11Rα staining was strongly localized to the intratibial lesions, then at later time points, limited to the periphery of the lesions. Staining of the surrounding

medullary bone was not present. IL-11Rα staining was most prominent in viable tumor areas and as the central areas of the tumors became necrotic, staining was no longer present in this area. It has been suggested that the biologic characteristics of the metastatic nodules may differ from those of the primary tumor, so it was important to assess whether IL-11Rα expression was present in the lung metastases. Since these in vivo models of osteosarcoma spontaneously developed lung metastases, the pulmonary metastases were examined for IL-11Rα expression. Strong expression of IL-11Rα was noted in the pulmonary metastases but not in the control or normal lung parenchyma.

Having demonstrated the presence of a functionally active IL-11Rα on the primary and lung metastases, researchers were then able to target the receptor. Systemic administration of an IL-11Rα-targeting phage displaying the cyclic nonapeptide CGRRAGGSC resulted in strong and selective accumulation of IL-11Rα-homing phage particles both in the primary and the lung metastasis in the osteosarcoma but not in several control organs including brain, lung, heart, and kidney [11]. Administration of a chimeric peptide, composed of the IL-11Rα homing peptide and a proapoptotic domain, CGRRAGGSC-D(KLAKLAK)2, to a mouse orthotopic model of osteosarcoma, caused regression of the primary osteosarcoma and its pulmonary metastasis (unpublished data).

To evaluate whether expression of IL-11Rα in the murine tumor models translated to human osteosarcoma, a large panel of human primary and metastatic (lung) tumors was evaluated. Expression of IL-11Rα was noted in all primary osteosarcoma samples. In addition, IL-11Rα was noted to be expressed on the endothelial cells within the tumors. Interestingly, only the small caliber blood vessels within the tumor positively expressed the IL-11Rα, whereas large tumor blood vessels did not express the receptor at detectable levels. All pulmonary metastases were positive for IL-11Rα expression, but control and normal lung parenchyma was negative for IL-11Rα expression. This data taken together suggests that therapeutic targeting of IL-11Rα could potentially act as an antitumor, antiangiogenesis, and antimetastatic agent for the management of human osteosarcoma.

Therapeutic Activity

Microscopic osteosarcoma lung metastases have been shown to be sensitive to immune-based therapy [15, 16]. This data taken together with the success of liposome-encapsulated MTP-PE (L-MTP-PE) supported the investigation of the use of engineered T cells to combat the pulmonary metastases that develop in osteosarcoma. Researchers, armed with the knowledge that IL-11Rα is expressed in pulmonary metastases, developed IL-11Rα-CAR-specific T cells aimed at eradicating osteosarcoma lung metastases.

IL-11Rα-CAR-specific T cells were created by transfecting human peripheral blood T cells with an IL-11R-CAR transposon and then propagating the transfected T cells ex vivo with aAPCs. IL-11Rα-CAR expression was confirmed by western

blot analysis and flow cytometry. These T cells were cytotoxic to several osteosarcoma cells lines in vitro, including SAOS-7, LM0-7 and KRIB cells. Following injection of the IL-11Rα-CAR-specific T cells into orthotopic models of osteosarcoma, the IL-11Rα-CAR-specific T cells accumulated in the osteosarcoma lung metastases but not in the normal surrounding tissues. The IL-11Rα-CAR-specific T cells selectively infiltrated lung metastases and induced apoptosis thereby causing regression of osteosarcoma pulmonary metastases. The use of IL-11Rα-CAR-specific T cells in osteosarcoma patients may have additional relevance since not only do the T-cell infusions increase the total number of T cells, which has been shown to be beneficial to patients, but it can recruit other cytotoxic effector cells which has been hypothesized to have some benefit as well [15, 17, 18].

Conclusion

Histologic and functional data has established that the IL-11/IL-11Rα system acts as a bona fide ligand receptor pair in osteosarcoma. Data also demonstrates that the receptor can be selectively targeted and effectively cause regression in primary and metastatic osteosarcoma. The fact that this has been done with genetically altered T-cells and chimeric peptides aimed at the IL-11Rα reinforces the therapeutic potential of the IL-11/IL-11Rα system. The toxicities and side effects of systemic chemotherapy are significant and debilitating, and a new approach to the treatment of osteosarcoma which is not chemotherapy based will provide a positive impact on the osteosarcoma patient.

References

1. Pasqualini R, Ruoslahti E (1996) Organ targeting in vivo using phage display peptide libraries. Nature 380:364–366
2. Pasqualini R, Arap W (2004) Hybridoma-free generation of monoclonal antibodies. Proc Natl Acad Sci U S A 101:257–259
3. Marchio S, Lahdenranta J, Schlingemann RO, Valdembri D, Wesseling P, Arap MA et al (2004) Aminopeptidase A is a functional target in angiogenic blood vessels. Cancer Cell 5:151–162
4. Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ et al (2002) Steps toward mapping the human vasculature by phage display. Nat Med 8:121–127
5. Kiessling S, Muller-Newen G, Leeb SN, Hausmann M, Rath HC, Strater J et al (2004) Functional expression of the interleukin-11 receptor alpha-chain and evidence of antiapoptotic effects in human colonic epithelial cells. J Biol Chem 279:10304–10315
6. Schwertschlag US, Trepicchio WL, Dykstra KH, Keith JC, Turner KJ, Dorner AJ (1999) Hematopoietic, immunomodulatory and epithelial effects of interleukin-11. Leukemia 13:1307–1315
7. Teramura M, Kobayashi S, Yoshinaga K, Iwabe K, Mizoguchi H (1996) Effect of interleukin 11 on normal and pathological thrombopoiesis. Cancer Chemother Pharmacol 38(Suppl):S99–S102

8. Zurita AJ, Troncoso P, Cardo-Vila M, Logothetis CJ, Pasqualini R, Arap W (2004) Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. Cancer Res 64:435–439
9. Hanavadi S, Martin TA, Watkins G, Mansel RE, Jiang WG (2006) Expression of interleukin 11 and its receptor and their prognostic value in human breast cancer. Ann Surg Oncol 13:802–808
10. Nakayama T, Yoshizaki A, Izumida S, Suehiro T, Miura S, Uemura T et al (2007) Expression of interleukin-11 (IL-11) and IL-11 receptor alpha in human gastric carcinoma and IL-11 upregulates the invasive activity of human gastric carcinoma cells. Int J Oncol 30:825–833
11. Lewis VO, Ozawa MG, Deavers MT, Wang G, Shintani T, Arap W et al (2009) The interleukin-11 receptor alpha as a candidate ligand-directed target in osteosarcoma: consistent data from cell lines, orthotopic models, and human tumor samples. Cancer Res 69:1995–1999
12. Huang G, Yu L, Cooper LJ, Hollomon M, Huls H, Kleinerman ES (2012) Genetically modified T cells targeting interleukin-11 receptor alpha-chain kill human osteosarcoma cells and induce the regression of established osteosarcoma lung metastases. Cancer Res 72:271–281
13. Putoczki T, Ernst M (2010) More than a sidekick: the IL-6 family cytokine IL-11 links inflammation to cancer. J Leukoc Biol 88:1109–1117
14. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C et al (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549
15. Kleinerman ES, Gano JB, Johnston DA, Benjamin RS, Jaffe N (1995) Efficacy of liposomal muramyl tripeptide (CGP 19835A) in the treatment of relapsed osteosarcoma. Am J Clin Oncol 18:93–99
16. Meyers PA, Schwartz CL, Krailo MD, Healey JH, Bernstein ML, Betcher D et al (2008) Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival – a report from the Children's Oncology Group. J Clin Oncol 26:633–638
17. Moore C, Eslin D, Levy A, Roberson J, Giusti V, Sutphin R (2010) Prognostic significance of early lymphocyte recovery in pediatric osteosarcoma. Pediatr Blood Cancer 55:1096–1102
18. Levine BL, Bernstein WB, Aronson NE, Schlienger K, Cotte J, Perfetto S et al (2002) Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. Nat Med 8:47–53

Bone-Seeking Radiopharmaceuticals as Targeted Agents of Osteosarcoma: Samarium-153-EDTMP and Radium-223

Peter M. Anderson, Vivek Subbiah, and Eric Rohren

Abstract Osteosarcoma is a cancer characterized by formation of bone by malignant cells. Routine bone scan imaging with Tc-99m-MDP is done at diagnosis to evaluate primary tumor uptake and check for bone metastases. At time of relapse the Tc-99m-MDP bone scan also provides a specific means to assess formation of bone by malignant osteosarcoma cells and the potential for bone-seeking radiopharmaceuticals to deliver radioactivity directly into osteoblastic osteosarcoma lesions. This chapter will review and compare a bone-seeking radiopharmaceutical that emits beta-particles, samarium-153-EDTMP, with an alpha-particle emitter, radium-223. The charged alpha particles from radium-223 have far more mass and energy than beta particles (electrons) from Sm-153-EDTMP. Because radium-223 has less marrow toxicity and more radiobiological effectiveness, especially if inside the bone forming cancer cell than samarium-153-EDTMP, radium-223 may have greater potential to become widely used against osteosarcoma as a targeted therapy. Radium-223 also has more potential to be used with chemotherapy against osteosarcoma and bone metastases. Because osteosarcoma makes bone and radium-223 acts like calcium, this radiopharmaceutical could possibly become a new targeted means to achieve safe and effective reduction of tumor burden as well as facilitate better surgery and/or radiotherapy for difficult to resect large, or metastatic tumors.

Keywords Osteosarcoma • Internal radiotherapy • Radium-223 • Samarium-153 • Alpha particle • Beta particle • Bone scan for screening • Double strand DNA breaks • Resistance is futile • Radiobiological effectiveness (RBE)

P.M. Anderson, M.D., Ph.D. (✉)
Levine Children's Hospital, Charlotte, NC USA

Carolinas Healthcare System, Levine Children's Hospital and Levine Cancer Institute, 1001 Blythe Blvd, Medical Center Plaza Suite 601, Charlotte, NC 28203, USA
e-mail: peter.anderson@carolinashealthcare.org

V. Subbiah, M.D. • E. Rohren, M.D., Ph.D.
MD Anderson Cancer Center, Houston, TX, USA

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_16,

Osteosarcoma Biology Favors Use of Bone-Seeking Radiopharmaceuticals

The pathologic diagnosis of osteosarcoma is characterized by formation of bone [1]. For detection of new bone formation by osteosarcoma, the "routine" ^{99m}Tc-MDP bone scan is the best screening test. Because osteoblastic osteosarcoma tumors make new bone, the ^{99m}Tc-MDP bone scan is a specific and sensitive test. This should be routinely done at diagnosis and after relapse in patients with osteosarcoma. Avid uptake of the bone-seeking ^{99m}Tc-MDP radioactive tracer into osteosarcoma lesions identifies the possibility of using a bone-seeking radiopharmaceutical for targeted therapy. Although currently bone-seeking radiopharmaceuticals are used in the setting of palliative care for patients with bone metastases this chapter will review principles for not only current use, but also future use of internal radiation to control osteosarcoma. Preclinical work and human studies have provided information to understand the advantages and limitations of beta emitters such as samarium-153-EDTMP compared to a new bone-seeking alpha emitter, radium-223 [2].

Radiation for Osteosarcoma Cancer Control

The use of radiation for local control of osteosarcoma has been a controversial topic. Early studies with radiation alone resulted in a high rate of osteosarcoma local relapse and lack of durable local control [3]. Radiotherapy of osteosarcoma can also result in skin toxicity, wound complications, and increased risk of infection [4]. Proton irradiation, carbon ion radiotherapy, and photons using intensity-modulated radiation therapy (IMRT) have been shown to provide some benefit for axial osteosarcoma and metastatic osteosarcoma tumors which are difficult or impossible to resect [5–13]. Stereotactic radiotherapy (i.e., 1–5 large fractions of radiation) has been useful for metastases of brain, spine [14] and in lungs [15, 16]. Because patients with osteosarcoma metastases and/or axial sites have very high rates of relapse and poor prognosis, new and better means of definitive local control are needed [17, 18]. Radiotherapy of osteosarcoma lesions is likely most effective when combined with chemotherapy [13, 19–23].

The Problem of Multiple Bone and/or Metastatic Sites of Osteosarcoma

Osteosarcoma bone metastases at diagnosis are associated with a very poor prognosis [24, 25]. Although the use of ifosfamide was helpful in this group [25], patients with high alkaline phosphatase or metastatic disease in two organs had less than a 5 %

survival in the French series [24]. Combined lung and bone metastases and/or relapse at the site of primary tumor sometimes contribute to treatment failure and death from osteosarcoma because of difficulty in local control of multiple sites. Bone-seeking radiopharmaceuticals can offer a potential means to simultaneously treat multiple osseous and osteoblastic non-osseous sites of osteosarcoma (Table 1). This is because lung or other visceral metastases of osteosarcoma can be osteoblastic and thus incorporate bone-seeking radiopharmaceutical. As shared earlier, the bone scan with avid uptake of ^{99m}Tc-MDP is the best screening test to identify potential candidates for this approach.

Properties of Samarium-153-EDTMP, a Beta-Emitting Radiopharmaceutical

Samarium-153 manufacture occurs by placing a capsule of samarium-152 oxide into a nuclear reactor. Neutron capture produces the unstable samarium-153 isotope. Decay of samarium-153 to stable europium-153 produces a beta particle (electron) and a photon (gamma ray) which is also useful for gamma camera imaging (Tables 1 and 2) [26, 27]. Samarium-153-EDTMP has been studied since early work by William Goeckeler in 1987 showing that the ethylene diamine tetramethylene phosphonate (EDTMP) chelate was not only one of the most effective chelates to deliver the beta-emitting samarium-153 isotope to the bones, but also was also associated with very little release from bone once it was deposited in the bone mineral hydroxyapatite [28].

Table 1 Alpha has high energy and short range

			Effective	
Bone-seeking radiopahrmaceutical	Particles emitted	Energy (MeV)	Range (um)	Half-life (days)
Radium-223	Alpha	27.8	~50	11.4
Samarium-153	Beta	0.2	~500	1.9

Table 2 Gamma imaging using bone-seeking radiopharmaceuticals

Radiopharmaceutical imaging agent	Energy (keV)	Decay (%abundance)	Gamma camera imaging
Tc-99m-MDP	141	(89 %)	Yes
Samarium-153-EDTMP	103	(29 %)	Yes
Radium-223	81	(15 %)	Yes
	84	(26 %)	Yes
	269	(14 %)	Yes

Preclinical Studies of Samarium-153-EDTMP in Relation to Osteosarcoma

The potential usefulness of samarium-153-EDTMP for treating osteosarcoma was first described by Lattimer et al. in dogs with spontaneously occurring osteosarcoma primary tumors [29]. Dogs with smaller osteoblastic tumors had more durable responses than dogs with larger tumors; this may be due to more intense and uniform deposition of the samarium-153-EDTMP radiopharmaceutical. Aas et al. showed that a dose of 36–57 MBq/kg (1–1.5 mCi/kg) samarium-153-EDTMP provided approximately 20 Gy to primary osteosarcoma tumors in dogs with reduction in pain as well as delaying the onset of metastatic disease [30]. It is not known whether treatment efficacy was due to rapid reduction of tumor burden or treatment of micrometastases already in the lungs at the time of presentation.

The biodistribution of samarium-153-EDTMP is almost exclusively skeletal with rapid blood clearance and bone lesion to normal bone ratio of 17:1; unbound radiopharmaceutical is eliminated in the urine [28]. Because growth plates are sites of active deposition of hydroxyapatite, juvenile 8-week-old rabbits were used to investigate potential effects of samarium-153-EDTMP on epiphyses [31]. Clinically significant damage was seen at a dose of 1 mCi/kg when the rabbits were evaluated 8 weeks later (age = 16 weeks). Although no long-term studies of the effects of samarium-153-EDTMP on prepubertal bone growth and repair have been reported, samarium-153-EDTMP can facilitate bone healing of bones involved in older cancer patients indicating potential for healing after damage by internal radiation.

Samarium-153-EDTMP Experience Against Cancer in Humans

This radiopharmaceutical has been available for palliative treatment of bone metastases including osteosarcoma [32] since the mid 1990s. The most extensive use of samarium-153-EDTMP has been in prostate cancer [33–35]. Although the dose limiting toxicity is thrombocytopenia, repeated doses of samarium-153-EDTMP have been safely given to men with prostate cancer [36, 37]. The samarium-153-EDTMP radiopharmaceutical also has been used with docetaxel successfully [38, 39].

Samarium-153-EDTMP has also been used in standard doses (1–1.5 mCi/kg) in osteosarcoma [26, 27, 32, 40, 41]. Because of the heterogeneity of deposition and/or difficulty of standard doses to produce durable responses, samarium-153-EDTMP has also been combined with radiotherapy [42]. A method for dose calculations for combined external beam and internal samarium-153-EDTMP radiotherapy in osteosarcoma tumors has recently been published [43]. Once samarium-153-EDTMP is administered and unbound drug is eliminated in the urine (this occurs within 6 h), then a “bone-specific” radiosensitization chemotherapy drug can be given [22]. The principle is that once the radiopharmaceutical is bound to

the target (bone/bone-forming tumor) and unbound ^{153}Sm-EDTMP is eliminated into the urine, then the radiosensitization effects of chemotherapy are localized to regions of bound radiopharmaceutical because visceral organs (e.g., lungs, heart, liver, intestines, brain) have very low amounts of bound samarium-153-EDTMP radiopharmaceutical.

High-Dose Samarium-153-EDTMP

Avid and specific skeletal and bone-forming tumor localization of samarium-153-EDTMP allowed for a 30-fold dose escalation in osteosarcoma [44]. High-dose samarium-153-EDTMP, with or without chemotherapy, requires stem cell support because of the potential for prolonged thrombocytopenia, as shown by Turner et al. [45, 46]. High-dose samarium-153-EDTMP has been used by different investigators to treat osteosarcoma [41, 44, 47–50]. Although increased radiographic responses were seen using gemcitabine radiosensitization 1 day after samarium-153-EDTMP infusion, the durability of response against osteosarcoma metastases was not improved [47]. To summarize, it would appear that samarium-153-EDTMP is useful in the relatively limited osteosarcoma situations: (a) palliation of bone metastases, (b) palliation of metastases of tumors that form bone (i.e., positive on bone scan), and (c) in conjunction with external beam radiotherapy for control of unresectable osteosarcoma.

Advantages of Radium-223, an Alpha Particle Emitting Bone-Seeking Radiopharmaceutical Compared to the Beta Emitter, Samarium-153-EDTMP

Once a radionuclide is deposited in bone and/or in or near a cancer cell or tumor vessel in bone, the rate of rate of radioactive emissions (half-life), range, and energy of particle emissions (MeV) are quite different within the target zone for alpha versus beta emitters [51–54]. Energy, tissue penetration range, gamma camera imaging, and physical characteristics of these bone-seeking radiopharmaceuticals are a summarized in Tables 1, 2, and 3, respectively. Figure 1 depicts mass and energy characteristics of ionizing radiation (gamma rays, electrons or beta particles, protons, and alpha particles) as well as different type of DNA damage from the ionizing radiation particles. Figure 2 illustrates the radioactive decay cascade of radium-223.

All radium isotopes are unstable and decay to produce radiation. Prior experience with radium for treatment of cancer in the early twentieth century used radium-226 which has long half-life and significant safety problems associated with decay to long-lived radon daughters (i.e., radioactive radon gas) and off-target radiation side effects from radioactive radon (Fig. 3). Hence, the radium-226 isotope is now

Table 3 ^{153}Sm-EDTMP and Radium-223: physical characteristics

Radiopharmaceutical	Samarium-153-EDTMP	Radium-223
Half-life ($t_{1/2}$)	46 h	11.4 days
Radiation emitted	Beta (electron)	Alpha (×4)
Relative mass	1	7,000
MeV particle emission	0.66 max	27.8
Energy	0.22 average	
Linear energy transfer (LET)	0.015–0.4 keV/um	60–230 keV/um
Type of DNA damage	Single strand breaks	Double strand breaks
Cytotoxic to G_0 cells (dormant metastases)	No	Yes
# Hits to kill cancer cells	100–1,000	1–4
Effective range (um)	>500	50–100
Elimination of unbound	Urine—within 4–6 h	GI tract (1–3 days)
Bone–red marrow ratio	4.4	10.3
Safety	Medium	VERY High

considered unsuitable for safe internal radiotherapy [55]. However, radium-223 has favorable decay characteristics: radon daughter decay is rapid (4 s), providing much less of a chance for "off target" radon diffusion (Fig. 3).

Preclinical studies of radium-223. Production and characterization of clinical grade radium-223 has been previously described in detail [55, 56]. Because radium-223 is an alkaline earth metal, it acts like calcium. The radium-223 isotope has been shown to specifically deposit alpha particles at sites inside the intended skeletal metastases and/or bone-forming osteosarcoma target lesions [56–60]. Preclinical studies in rodents with radium-223 showed avid skeletal deposition, relative sparing of the bone marrow, and nearly no soft tissue uptake [57, 61]. Extremely high doses of radium-223 in Balb c mice [1,250, 2,500, and 3,750 kBq/kg (25–75× the recommended monthly dose of 50 kBq/kg)] caused some effects on marrow, but the 4-week LD50 was not reached [62]. In this study, the greatest effect was on osteoblasts and osteocytes; it also confirmed marrow sparing and inability of the short-range alpha particles from radium-223 to completely ablate radiation-sensitive hematopoietic stem cells.

Experience with radium-223 in a phase I [59] and a randomized phase II trial in men with metastatic prostate cancer confirmed excellent activity against bone metastases and a low toxicity profile (i.e., a high therapeutic index) [58–60, 63]. Using doses of 5, 25, 50, or 100 kBq/kg, a dose response relationship was seen in pain index at week 2 [60] and the highest dose group also had significantly decreased levels of alkaline phosphatase. Two-year follow-up of the phase II trial shows overall survival benefit of 65 weeks vs 46 weeks comparing radium-223 versus placebo (HR 0.476; cox regression $p=0.017$). There were no long-term hematologic toxicities or secondary malignancies reported in this small phase II cohort ($N=33$) [63]. Results of a randomized phase III, double-blind, placebo controlled trial of [2, 64] radium-223 in prostate cancer at a dose of 50 kBq/kg monthly × 6 and 2:1 randomization between active and placebo ($N=921$) were presented at ASCO 2012 [64] and recently published in the New England Journal of Medicine. This study resulted

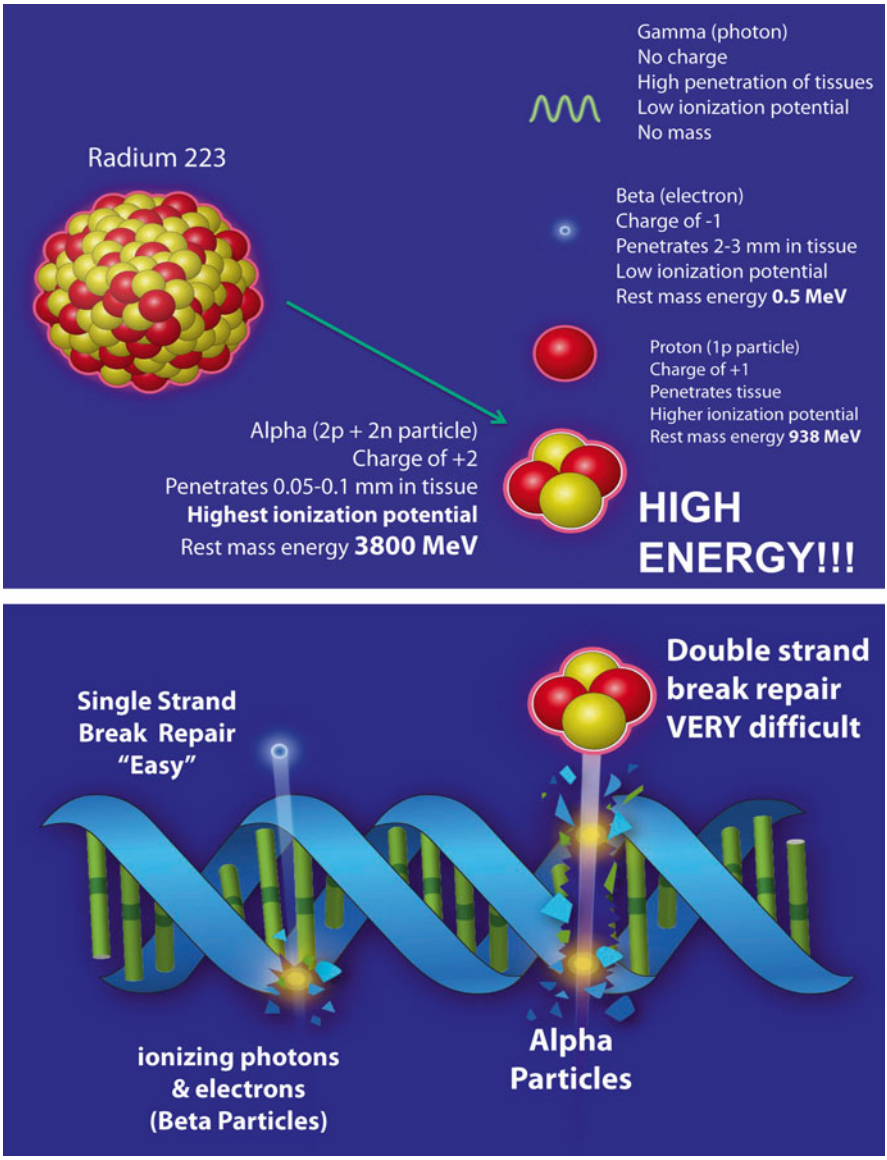


Fig. 1 Radioactive particle mass, energy, and DNA damage. *Top*: photons have no mass; protons have ¼ the mass energy of alpha particles. Thus, alpha particles have much greater mass and energy than electrons (beta particles). *Bottom*: Graphic representation of the high energy of alpha particles causing double strand breaks which are more difficult for cancer cells to repair than single stand breaks

in the FDA approval of radium-223 in May 2013. Compared to placebo radium-223 was associated with significantly improved overall survival (median, 14.9 months vs. 11.3 months; hazard ratio, 0.70; 95 % CI, 0.58–0.83; $P < 0.001$) and was also associated with prolonged time to first skeletal-related event (median 15.6 months

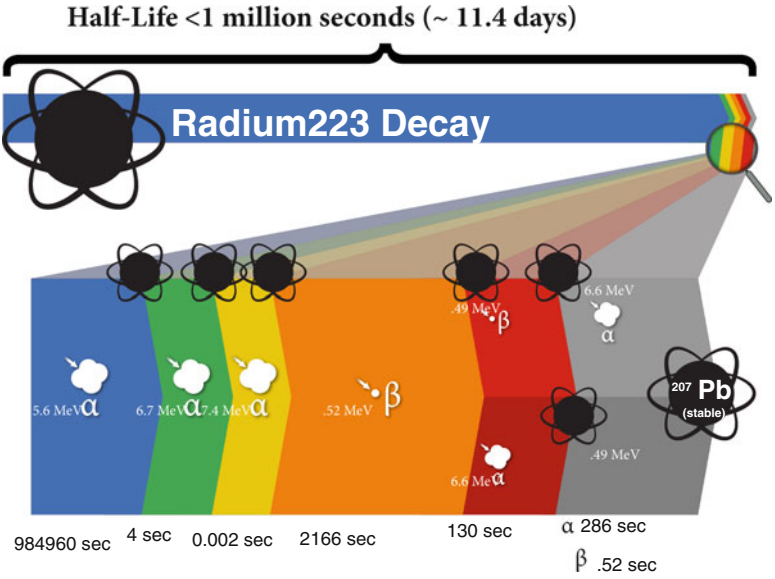


Fig. 2 Radium-223 decay cascade. On average, the initial ejection of the high LEt alpha particle takes a relatively long time ($t_{1/2}$ 11.4 days is almost a million seconds). Subsequent quick decay of unstable isotopes of radon (4 s), polonium (2 ms), lead (2,166 s) bismuth (130 s), and polonium or thallium isotopes (287 s) yields an additional three alpha particles + two beta particles in the same before the stable Pb-207 isotope is finally formed. Alpha particle emissions account for about 94 % of the emitted energy of radium-223. In 1 month (<3 half-lives) ~10 % of radioactivity remains; in 7 weeks (6 half-lives) only about 1/64 (<2 %) of initial radium-223 radioactivity remains

vs 9.8 months, respectively; HR = 0.658; 95 % CI, 0.522–0.830; $p = 0.00037$). Hematologic adverse events were uncommon (any grade 3 or 4 neutropenia in 2.2 % and 0.7 % and any grade 3 or 4 thrombocytopenia in 6.3 % and 2 % of the radium-223 and placebo groups, respectively). Although targeting of osteoblastic osteosarcoma tumors would expected to be much more specific than prostate cancer, currently this is an unlabeled use of the radiopharmaceutical.

At MD Anderson Cancer Center a single osteosarcoma patient with head, neck, and skull base osteosarcoma with skeletal metastases was provided 2 doses of radium-223 in December 2009 and January 2010 [65]. Decrease in alkaline phosphatase and improvement in pain for approximately 2 months was seen. Bone scan showing the clinical response of this patient is illustrated in Fig. 4. At MD Anderson Cancer Center, a phase I dose trial in osteosarcoma is open to accrual (www.clinicaltrials.gov # NCT01833520). The purpose is to determine safety of escalating doses of radium-223 in osteosarcoma patients with osteoblastic tumors as well as to determine best quantitative imaging to evaluate responses using Tc-99m-MDP Spect-CT, NaF-18 PET, and F-18 deoxyglucose.

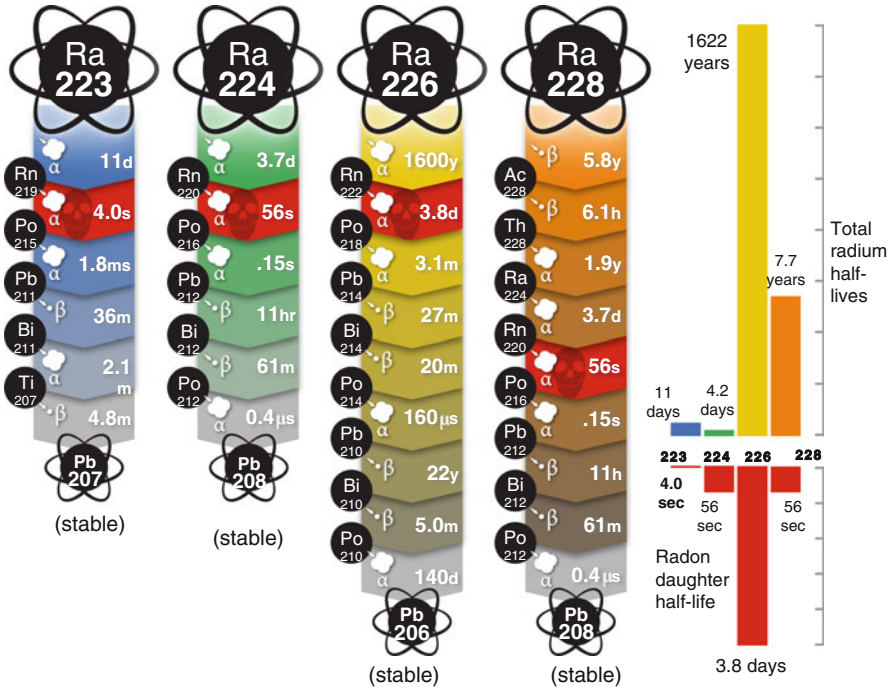


Fig. 3 Safety of Radium-223 compared to other radium isotopes is graphically depicted. Radon (Rn) daughter decay is in *red*. The very short half-life of Rn daughter for radium-223 (4 s) limits amount of diffusion away from the targeted bone tumor deposition of radium-223. In contrast in the early twentieth century radium-226 was used clinically. This isotope was less safe and is no longer in clinical use because of the radon daughter $t_{1/2}$ of 3.8 days resulted in off-target radiation side effects

Possible Roles of Bone-Seeking Radiopharmaceuticals in Osteosarcoma Therapy

Palliation of painful bone metastases can be accomplished in a number of ways: medical treatment (opiates), or using local control measures including surgery, radiofrequency ablation, and/or radiotherapy. Thus, the use of external beam radiotherapy for treatment of painful osseous metastases is a widely accepted medical practice. Techniques are improving and stereotactic radiotherapy for spine metastases has become a frontline strategy [14, 66]. Larger single fractions seem to be more effective; this has been reviewed in meta-analyses of more than 25 clinical trials [67–71]. Because of internal lesion deposition and low marrow toxicity the usefulness of radium-223 and external beam radiotherapy for control of osteoblastic osteosarcoma remains to be determined, but is a strategy that may yield more durable control, particularly if combined with chemotherapy after localization of the bone-seeking isotope to the target lesion(s).

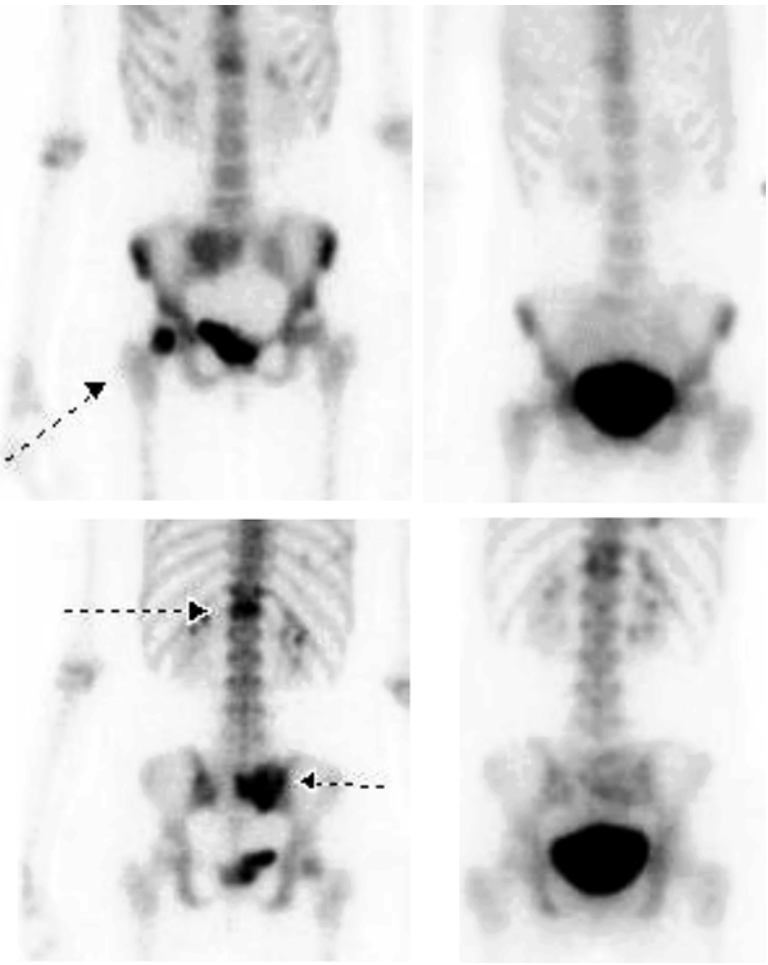


Fig. 4 Improvement in 3 distant osteosarcoma skeletal metastases after 50 kBq/kg radium-223 × 2 doses 1 month apart. The bone scan shows supine (*top*) and prone (*bottom*) views: note the less avid Tc-99m-MDP uptake of T12 spine, right acetabular, and sacral osteosarcoma bone metastases comparing before (*right* with *arrows*) to after treatment (*left* without *arrows*). This patient also had improved pain at these sites and serum alkaline phosphatase decrease from 964 to 276 in 7 weeks after radium-223 administration

Experience with combined use of radiopharmaceuticals with chemotherapy: Combining ^{153}Sm-EDTMP with docetaxel has been reported to have synergy in prostate cancer [39], and with bortezomib in myeloma [72]. Unfortunately, because of delayed thrombocytopenia (usually ~3–6 weeks after a dose), the combination of ^{153}Sm-EDTMP in routine osteosarcoma is probably not feasible in many patients.

Would Low Marrow Toxicity of Radium-223 Allow Concurrent Use with Osteosarcoma Chemotherapy?

Radium-223 should be suitable for use in combination with chemotherapy, but additional work needs to be done. If the experience with humans is the same as the experience of dogs with osteosarcoma treated with samarium-153-EDTMP who had a delayed development of lung metastases [30], it is possible that early treatment with radium-223 could affect control in lung metastases. Thus far, the evidence suggests that radium-223 should have a higher therapeutic index (low marrow toxicity, more effect on malignant bone-forming cells that take up the radiopharmaceutical) than samarium-153-EDTMP. Because of current poor survival, patients likely to benefit are those with bone metastases [24] or axial tumors [17, 18]. Benefit in these very high-risk groups could then provide the rationale for randomized clinical trials and wider application of this targeted radiopharmaceutical against osteosarcoma.

Conclusion

Samarium-153-EDTMP has modest efficacy in the setting of palliative treatment of osteosarcoma metastases, but it is sometimes difficult to use repeated doses or with chemotherapy. The path length (range) of radium-223 is shorter, and thus, there is less hematologic toxicity because fewer marrow stem cells are "innocent bystanders." It is the author's view that radium-223 has the potential to significantly improve effectiveness of osteosarcoma chemotherapy as well as external beam radiation of unresectable tumors. Radium-223 may also possibly provide rapid control of initial pain and could possibly contribute to increased necrosis of osteoblastic tumors. Furthermore, because radium-223 has the potential to reduce viability of lung osteosarcoma micro-metastases, it also has potential to impact survival and reduce the incidence of relapses in the lungs as well as in the bones.

Acknowledgements Peter M. Anderson acknowledges Greg Wiseman and Oyvind Bruland for their advice and sharing ideas during in the development of bone-seeking radiopharmaceuticals for osteosarcoma and Norman Jaffe for his mentorship when working with metastatic osteosarcoma patients. Research has been supported by the Shannon Wilkes Osteosarcoma fund, and Lauren Edwards Behr sarcoma research fund, Sarah's Garden of Hope. The University of Texas MD Anderson Cancer Center is supported by Cancer Center Support Grant No. CA 016672. Dr. Anderson was supported by the Curtis Distinguished Professorship and is currently partially supported by Levine Cancer Institute.

References

1. Raymond AK, Jaffe N (2009) Osteosarcoma multidisciplinary approach to the management from the pathologist's perspective. Cancer Treat Res 152:63–84
2. Parker C et al (2013) Alpha emitter radium-223 and survival in metastatic prostate cancer. N Engl J Med 369(3):213–223

3. Beck JC et al (1976) The role of radiation therapy in the treatment of osteosarcoma. Radiology 120(1):163–165
4. Trapeznikov, N.N., et al., *[Treatment of limb osteosarcoma at the turn of the century (half century of experience in research)].* Vestn Ross Akad Med Nauk, 2001(9): p. 46-9.
5. Ciernik IF et al (2011) Proton-based radiotherapy for unresectable or incompletely resected osteosarcoma. Cancer 117(19):4522–4530
6. DeLaney TF et al (2009) Phase II study of high-dose photon/proton radiotherapy in the management of spine sarcomas. Int J Radiat Oncol Biol Phys 74(3):732–739
7. DeLaney TF et al (2005) Radiotherapy for local control of osteosarcoma. Int J Radiat Oncol Biol Phys 61(2):492–498
8. Mahajan A et al (2008) Multimodality treatment of osteosarcoma: radiation in a high-risk cohort. Pediatr Blood Cancer 50(5):976–982
9. Matsunobu A et al (2012) Impact of carbon ion radiotherapy for unresectable osteosarcoma of the trunk. Cancer 118(18):4555–4563
10. Imai R et al (2006) Cervical spine osteosarcoma treated with carbon-ion radiotherapy. Lancet Oncol 7(12):1034–1035
11. Schwarz R et al (2009) The role of radiotherapy in oseosarcoma. Cancer Treat Res 152:147–164
12. Wagner TD et al (2009) Combination short-course preoperative irradiation, surgical resection, and reduced-field high-dose postoperative irradiation in the treatment of tumors involving the bone. Int J Radiat Oncol Biol Phys 73(1):259–266
13. Hernberg MM et al (2011) Chemoradiotherapy in the treatment of inoperable high-grade osteosarcoma. Med Oncol 28(4):1475–1480
14. Wang XS et al (2012) Stereotactic body radiation therapy for management of spinal metastases in patients without spinal cord compression: a phase 1-2 trial. Lancet Oncol 13(4):395–402
15. Ricardi U et al (2012) Stereotactic body radiation therapy for lung metastases. Lung Cancer 75(1):77–81
16. Dhakal S et al (2012) Stereotactic body radiotherapy for pulmonary metastases from soft-tissue sarcomas: excellent local lesion control and improved patient survival. Int J Radiat Oncol Biol Phys 82(2):940–945
17. Ozaki T et al (2003) Osteosarcoma of the pelvis: experience of the Cooperative Osteosarcoma Study Group. J Clin Oncol 21(2):334–341
18. Ozaki T et al (2002) Osteosarcoma of the spine: experience of the Cooperative Osteosarcoma Study Group. Cancer 94(4):1069–1077
19. Machak GN et al (2003) Neoadjuvant chemotherapy and local radiotherapy for high-grade osteosarcoma of the extremities. Mayo Clin Proc 78(2):147–155
20. Anderson P, Salazar-Abshire M (2006) Improving outcomes in difficult bone cancers using multimodality therapy, including radiation: physician and nursing perspectives. Curr Oncol Rep 8(6):415–422
21. Anderson PM (2003) Effectiveness of radiotherapy for osteosarcoma that responds to chemotherapy. Mayo Clin Proc 78(2):145–146
22. Anderson P et al (2008) Outpatient chemotherapy plus radiotherapy in sarcomas: improving cancer control with radiosensitizing agents. Cancer Control 15(1):38–46
23. Dincbas FO et al (2005) The role of preoperative radiotherapy in nonmetastatic high-grade osteosarcoma of the extremities for limb-sparing surgery. Int J Radiat Oncol Biol Phys 62(3):820–828
24. Mialou V et al (2005) Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome – the French pediatric experience. Cancer 104(5):1100–1109
25. Goorin AM et al (2002) Phase II/III trial of etoposide and high-dose ifosfamide in newly diagnosed metastatic osteosarcoma: a pediatric oncology group trial. J Clin Oncol 20(2):426–433
26. Anderson P, Nunez R (2007) Samarium lexidronam (153Sm-EDTMP): skeletal radiation for osteoblastic bone metastases and osteosarcoma. Expert Rev Anticancer Ther 7(11):1517–1527
27. Anderson P (2006) Samarium for osteoblastic bone metastases and osteosarcoma. Expert Opin Pharmacother 7(11):1475–1486

28. Goeckeler WF et al (1987) Skeletal localization of samarium-153 chelates: potential therapeutic bone agents. J Nucl Med 28(4):495–504
29. Lattimer JC et al (1990) Clinical and clinicopathologic response of canine bone tumor patients to treatment with samarium-153-EDTMP. J Nucl Med 31(8):1316–1325
30. Aas, M., et al., *Internal radionuclide therapy of primary osteosarcoma in dogs, using 153Sm-ethylene-diamino-tetramethylene-phosphonate (EDTMP).* Clin Cancer Res, 1999. **5**(10 Suppl): p. 3148 s-3152s.
31. Essman SC et al (2003) Effects of 153Sm-ethylenediaminetetramethylene phosphonate on physeal and articular cartilage in juvenile rabbits. J Nucl Med 44(9):1510–1515
32. Bruland OS et al (1996) Targeted radiotherapy of osteosarcoma using 153 Sm-EDTMP. A new promising approach. Acta Oncol 35(3):381–384
33. Sandeman TF, Budd RS, Martin JJ (1992) Samarium-153-labelled EDTMP for bone metastases from cancer of the prostate. Clin Oncol (R Coll Radiol) 4(3):160–164
34. Sartor O (2004) Overview of samarium sm 153 lexidronam in the treatment of painful metastatic bone disease. Rev Urol 6(Suppl 10):S3–S12
35. Sartor O et al (2004) Samarium-153-Lexidronam complex for treatment of painful bone metastases in hormone-refractory prostate cancer. Urology 63(5):940–945
36. Higano CS et al (2008) Safety analysis of repeated high doses of samarium-153 lexidronam in men with hormone-naive prostate cancer metastatic to bone. Clin Genitourin Cancer 6(1):40–45
37. Menda Y et al (2000) Efficacy and safety of repeated samarium-153 lexidronam treatment in a patient with prostate cancer and metastatic bone pain. Clin Nucl Med 25(9):698–700
38. Morris MJ et al (2009) Phase I study of samarium-153 lexidronam with docetaxel in castration-resistant metastatic prostate cancer. J Clin Oncol 27(15):2436–2442
39. Tu SM et al (2009) Phase I study of concurrent weekly docetaxel and repeated samarium-153 lexidronam in patients with castration-resistant metastatic prostate cancer. J Clin Oncol 27(20):3319–3324
40. Loeb DM et al (2009) Dose-finding study of 153Sm-EDTMP in patients with poor-prognosis osteosarcoma. Cancer 115(11):2514–2522
41. Loeb DM et al (2010) Tandem dosing of samarium-153 ethylenediamine tetramethylene phosphoric acid with stem cell support for patients with high-risk osteosarcoma. Cancer 116(23):5470–5478
42. Hobbs RF et al (2011) A treatment planning method for sequentially combining radiopharmaceutical therapy and external radiation therapy. Int J Radiat Oncol Biol Phys 80(4):1256–1262
43. Senthamizhchelvan S et al (2012) Tumor dosimetry and response for 153Sm-ethylenediamine tetramethylene phosphonic acid therapy of high-risk osteosarcoma. J Nucl Med 53(2):215–224
44. Anderson PM et al (2002) High-dose samarium-153 ethylene diamine tetramethylene phosphonate: low toxicity of skeletal irradiation in patients with osteosarcoma and bone metastases. J Clin Oncol 20(1):189–196
45. Turner JH et al (1992) 153Sm-EDTMP and melphalan chemoradiotherapy regimen for bone marrow ablation prior to marrow transplantation: an experimental model in the rat. Nucl Med Commun 13(5):321–329
46. Turner JH et al (1993) Radiopharmaceutical therapy of 5 T33 murine myeloma by sequential treatment with samarium-153 ethylenediaminetetramethylene phosphonate, melphalan, and bone marrow transplantation. J Natl Cancer Inst 85(18):1508–1513
47. Anderson PM et al (2005) Gemcitabine radiosensitization after high-dose samarium for osteoblastic osteosarcoma. Clin Cancer Res 11(19 Pt 1):6895–6900
48. Franzius C et al (2001) High-activity samarium-153-EDTMP therapy followed by autologous peripheral blood stem cell support in unresectable osteosarcoma. Nuklearmedizin 40(6):215–220
49. Franzius C et al (1999) High-activity samarium-153-EDTMP therapy in unresectable osteosarcoma. Nuklearmedizin 38(8):337–340
50. Franzius C, Schuck A, Bielack SS (2002) High-dose samarium-153 ethylene diamine tetramethylene phosphonate: low toxicity of skeletal irradiation in patients with osteosarcoma and bone metastases. J Clin Oncol 20(7):1953–1954

51. Kassis AI (2008) Therapeutic radionuclides: biophysical and radiobiologic principles. Semin Nucl Med 38(5):358–366
52. Zhu X et al (2010) Solid-tumor radionuclide therapy dosimetry: new paradigms in view of tumor microenvironment and angiogenesis. Med Phys 37(6):2974–2984
53. Huang CY et al (2012) Microdosimetry for targeted alpha therapy of cancer. Comput Math Methods Med 2012:153212
54. Baidoo KE, Yong K, Brechbiel MW (2013) Molecular pathways: targeted alpha-particle radiation therapy. Clin Cancer Res 19(3):530–537
55. Bruland OS et al (2008) Radium-223: from Radiochemical Development to Clinical Applications in Targeted Cancer Therapy. Curr Radoipharmaceut 1(1):203–208
56. Bruland, O.S., et al., *High-linear energy transfer irradiation targeted to skeletal metastases by the alpha-emitter 223Ra: adjuvant or alternative to conventional modalities?* Clin Cancer Res, 2006. **12**(20 Pt 2): p. 6250 s-6257s.
57. Henriksen G et al (2003) Targeting of osseous sites with alpha-emitting 223Ra: comparison with the beta-emitter 89Sr in mice. J Nucl Med 44(2):252–259
58. Nilsson S et al (2007) Bone-targeted radium-223 in symptomatic, hormone-refractory prostate cancer: a randomised, multicentre, placebo-controlled phase II study. Lancet Oncol 8(7): 587–594
59. Nilsson S et al (2005) First clinical experience with alpha-emitting radium-223 in the treatment of skeletal metastases. Clin Cancer Res 11(12):4451–4459
60. Nilsson S et al (2012) A randomized, dose-response, multicenter phase II study of radium-223 chloride for the palliation of painful bone metastases in patients with castration-resistant prostate cancer. Eur J Cancer 48(5):678–686
61. Henriksen G et al (2002) Significant antitumor effect from bone-seeking, alpha-particle-emitting (223)Ra demonstrated in an experimental skeletal metastases model. Cancer Res 62(11):3120–3125
62. Larsen RH et al (2006) Radiotoxicity of the alpha-emitting bone-seeker 223Ra injected intravenously into mice: histology, clinical chemistry and hematology. In Vivo 20(3):325–331
63. Nilsson S et al (2013) Two-year survival follow-up of the randomized, double-blind, placebo-controlled phase II study of radium-223 chloride in patients with castration-resistant prostate cancer and bone metastases. Clin Genitourin Cancer 11(1):20–26
64. Parker, C., et al., *Updated analysis of the phase III, double-blind, randomized, multinational study of radium-223 chloride in castration-resistant prostate cancer (CRPC) patients with bone metastases (ALSYMPCA).* J Clin Oncol, 2012. **30**(suppl; abstr LBA4512).
65. Anderson P (2011) Osteosarcoma: an opportunity for targeted radiotherapy. In: Speer TW (ed) Targeted radionuclide therapy. Lippincott Williams & Wilkins, Wolters Kluwer Health, Philadelphia, PA, pp 473–477
66. Garg AK et al (2012) Phase 1/2 trial of single-session stereotactic body radiotherapy for previously unirradiated spinal metastases. Cancer 118(20):5069–5077
67. Sze WM et al (2004) Palliation of metastatic bone pain: single fraction versus multifraction radiotherapy - a systematic review of the randomised trials. Cochrane Database Syst Rev (2): CD004721
68. Sze WM et al (2003) Palliation of metastatic bone pain: single fraction versus multifraction radiotherapy – a systematic review of randomised trials. Clin Oncol (R Coll Radiol) 15(6):345–352
69. Wu JS et al (2004) Radiotherapy fractionation for the palliation of uncomplicated painful bone metastases - an evidence-based practice guideline. BMC Cancer 4:71
70. Wu JS et al (2003) Meta-analysis of dose-fractionation radiotherapy trials for the palliation of painful bone metastases. Int J Radiat Oncol Biol Phys 55(3):594–605
71. Chow E et al (2007) Palliative radiotherapy trials for bone metastases: a systematic review. J Clin Oncol 25(11):1423–1436
72. Goel A et al (2006) Synergistic activity of the proteasome inhibitor PS-341 with non-myeloablative 153-Sm-EDTMP skeletally targeted radiotherapy in an orthotopic model of multiple myeloma. Blood 107(10):4063–4070

Part V
Novel Therapeutic Approaches Based on Biology: Immunotherapy

Muramyl Tripeptide-Phosphatidyl Ethanolamine Encapsulated in Liposomes (L-MTP-PE) in the Treatment of Osteosarcoma

Paul A. Meyers and Alexander J. Chou

Abstract Bacille Calmette-Guerin (BCG) has been used for decades as an immune stimulant to treat cancer. Early work by Fidler and Kleinerman identified muramyl dipeptide (MDP) as a critical component of the BCG cell wall which retained most of the immunostimulatory properties of the native BCG. Addition of a peptide to MDP resulted in muramyl tripeptide (MTP) which allowed incorporation into liposomal membranes. The resulting pharmaceutical, liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE or mifamurtide) showed activity in preclinical models of human cancers. Phase I studies documented the safety of the compound for human administration. These trials did not reach a maximally tolerated dose (MTD), and the dose chosen for phase II trials was a biologically optimized dose, not an MTD. Phase II studies showed decreased risk of further recurrence in patients who received mifamurtide after surgical ablation of metastatic osteosarcoma. A phase III prospective randomized trial demonstrated a statistically significant reduction in the risk of death from osteosarcoma when MTP was added to systemic chemotherapy for the treatment of localized osteosarcoma. The same trial allowed treatment of patients who presented with initially metastatic disease. While the overall and event-free survival was improved in patients with metastatic osteosarcoma who received L-MTP-PE, the sample size was small and the improvement did not achieve conventional statistical significance. From 2008 to 2012, patients with metastatic and recurrent osteosarcoma were given L-MTP-PE in an expanded access trial, and the results suggest a decreased risk of subsequent recurrence and death with the inclusion of L-MTP-PE in the treatment strategy for these high-risk patients.

P.A. Meyers, M.D. (✉) • A.J. Chou, M.D.
Department of Pediatrics, Weill Cornell Medical College, New York, NY USA

Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10065, USA
e-mail: meyersp@mskcc.org; choua@mskcc.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_17,

Introduction

The concept of using immunotherapy to treat chemotherapy-resistant tumors has been around for several decades. The use of T-cells, lymphokine-activated killer cells, interferon, and NK cells have been explored for the treatment of solid tumors including melanoma, brain tumors, hepatoblastoma, and lymphoma. While there have been reported successes particularly in the use of α-interferon (α-IFN) to treat metastatic melanoma, improvement in survival for large numbers of patients with other solid tumors has been modest. Furthermore, there is no standard of care treatment that combines cytokines, T-cells, or NK cells with chemotherapy for newly diagnosed patients.

The one immune cell that has largely been ignored in terms of its potential in cancer treatment is the macrophage. In this chapter, the history and development of the macrophage-activating agent, L-MTP-PE, will be traced from the first concept through preclinical studies, phase I, phase II, and phase III trials. The phase III trial demonstrated for the first time that an agent that targets and activates macrophages can be successfully combined with chemotherapy to achieve an improvement in long-term outcome as measured by a significant decrease in the mortality rate at 6–8 years. The use of L-MTP-PE together with chemotherapy in newly diagnosed nonmetastatic osteosarcoma patients resulted in a decrease in the death rate as well as an improvement in both the progression-free and long-term survival of patients with this disease. Targeting the macrophage and activating its tumoricidal function is therefore an approach that warrants more focus and additional clinical investigations.

Background

Bacille Calmette-Guerin (BCG) was isolated after hundreds of passages to create a vaccine against tuberculosis. As early as the 1930s, BCG was used to stimulate the immune system in patients with cancer in hopes that their enhanced immunity would lead to regression of cancer. BCG remains in use to the present as an adjuvant for superficial bladder cancer. Injection of BCG into superficial bladder cancers leads to tumor regression [1].

Zwilling and Campolito demonstrated that BCG could activate alveolar macrophages to become tumoricidal to autologous tumor cells [2]. Japanese investigators localized this macrophage-activating activity to the cell-wall skeleton [3]. A synthetic peptidoglycan, *N*-acetyl muramyl-L-alanine-D-isoglutamine, or muramyl dipeptide (MDP) was formulated to correspond to a component found in a water-soluble extract of cell wall of mycobacteria [4]. Benacerraf and colleagues demonstrated that MDP could serve as an adjuvant to enhance immune responses [5]. Fidler and colleagues demonstrated that encapsulating lymphokines in liposomes resulted in more efficient activation of macrophages [6]. The same group showed that liposome-encapsulated MDP could result in activation of tumoricidal properties in rat alveolar

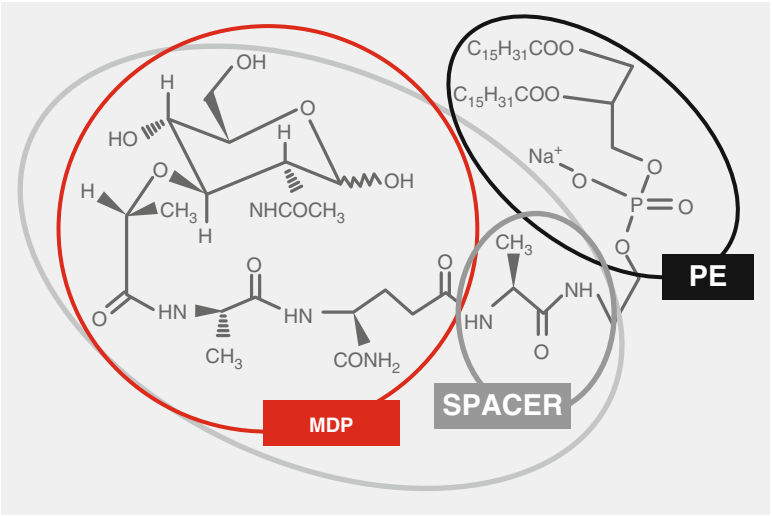


Fig. 1 Muramyl dipeptide (MDP) is the component of the BCG cell wall which retains the immune-stimulating properties of BCG. MTP-PE was created by adding a peptide spacer and binding the resulting tripeptide to phosphatidyl ethanolamine to improve lipid solubility. Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

macrophages [7]. It was shown that unmodified MDP was eliminated from the systemic circulation very rapidly [8]. Fidler and colleagues demonstrated that intravenous injection of liposomes containing MDP could eradicate spontaneous metastases and activate alveolar macrophages in a murine model [9].

It was shown that low-molecular-weight compounds such as MDP could leak from liposomes. Fidler's group demonstrated that modification of MDP by the addition of a third peptide to create muramyl tripeptide (MTP) followed by incorporation into multi-lamellar liposomes enhanced macrophage activation [10] (Figs. 1 and 2). Kleinerman and Fidler initiated work using liposome-encapsulated muramyl tripeptide (L-MTP-PE) in human cells [11]. They demonstrated that human blood monocytes could be rendered tumoricidal after activation with L-MTP-PE.

Early Clinical Investigation

The first studies of L-MTP-PE in humans were performed at the MD Anderson Cancer Center (MDACC). The results of the first phase 1 trials were reported in 1989 [12]. Toxicity was moderate, with the most common side effects reported including chills, fever, malaise, and nausea. The maximum tolerated dose (MTD) was reported to be 6 mg/m^2. Imaging studies of radiolabeled L-MTP-PE showed rapid uptake in the spleen, liver, lungs, nasopharynx, and, in two patients, tumor.

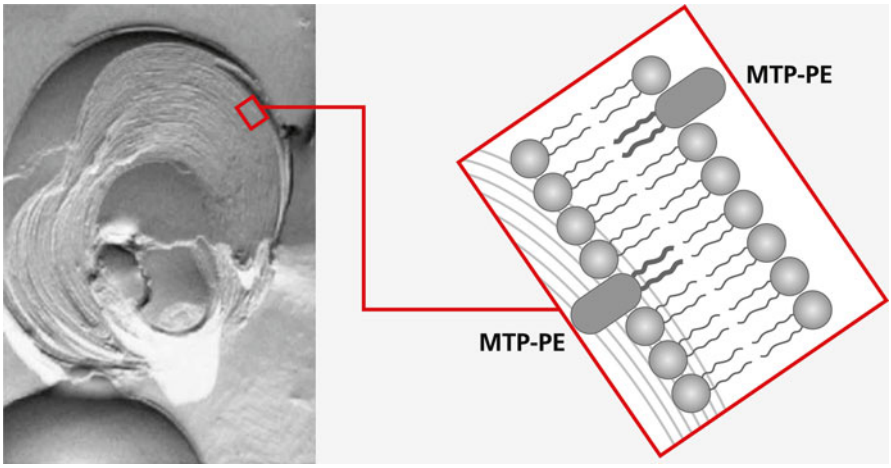


Fig. 2 When muramyl tripeptide-phosphatidyl ethanolamine is incorporated into liposomes, the compound intercalates into the membrane bilayers of the liposome to create the pharmaceutical L-MTP-PE. Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

Kleinerman reported the tumoricidal properties of peripheral blood monocytes from the patients who were the subjects of that phase I study [13]. She reported that activation of monocyte-mediated tumorilytic activity was found in 24 of 28 patients at some point during therapy. While the MTD for the clinical trial was reported to be 4–6 mg/m^2, the optimal biological dose for macrophage activation was 0.5–2.0 mg/m^2. This concept that optimal biological dose may be lower than MTD has been confirmed in many studies of biological agents for the treatment of cancer [14].

L-MTP-PE had been shown to be capable of inducing lung-resident alveolar macrophages to become tumoricidal. It had been shown to prevent tumor cells from developing into pulmonary metastases in murine models. These observations suggested that L-MTP-PE might be useful in preventing the progression of microscopic metastases in the lung to clinically detectable size, making L-MTP-PE particularly interesting in osteosarcoma. At initial presentation, most patients with osteosarcoma do not have clinically detectable metastatic disease. In the absence of systemic therapy, 90 % of these patients will go on to develop clinical metastases, and the great majority of these metastases will appear first in the lung [15]. This makes osteosarcoma a good candidate disease in which to study an agent which activates pulmonary macrophages to become tumoricidal.

Many studies of new anticancer drugs are performed in models in which human tumor cell lines are grown in immunodeficient mice. These heterotopic xenografts are an imperfect model because human tumor cell lines are often significantly mutated from the tumor of origin, tumors grow in compartments which are different from the compartments in which they spontaneously arise, and the lack of an immune system makes it impossible to test therapies that incorporate host immune responses. Dogs develop osteosarcoma spontaneously. Osteosarcoma in dogs arises

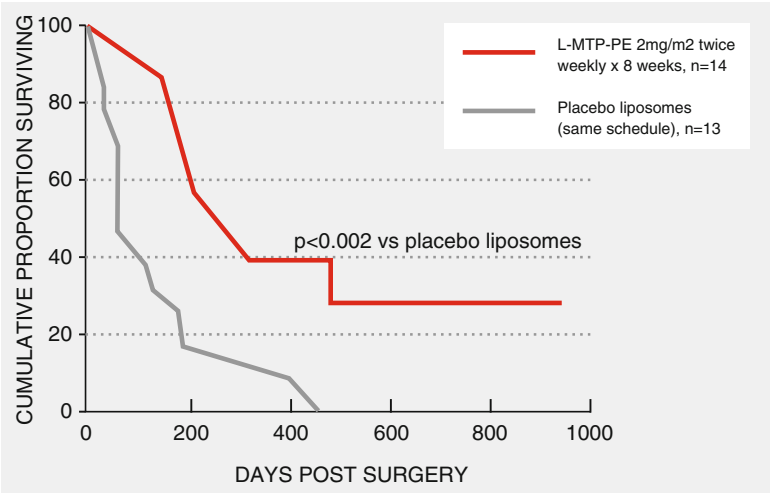


Fig. 3 MacEwen et al. conducted a prospective randomized double-blind study of L-MTP-PE as adjuvant therapy in dogs with osteosarcoma following amputation of the extremity with the primary tumor. Adjuvant L-MTP-PE resulted in a statistically significant improvement in the overall survival and the apparent cure of some animals. Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

in the long bones and metastasizes to the lungs, recapitulating human disease. Osteosarcoma in dogs is an excellent natural model in which to study new agents for the treatment of human osteosarcoma.

MacEwen and colleagues conducted a double-blinded placebo-controlled trial of L-MTP-PE in dogs with osteosarcoma [16]. All the dogs had osteosarcoma without clinically detectable metastatic disease. All dogs underwent amputation of the tumor-bearing limb. Dogs were randomly assigned to receive L-MTP-PE or a placebo consisting of empty liposomes. Historic data suggested that all dogs with osteosarcoma treated with amputation alone would rapidly develop metastatic disease and die. The prospective randomized study confirmed the historical experience. All of the dogs assigned to receive placebo developed metastasis, and the median survival time was 77 days. Median survival for the dogs treated with L-MTP-PE was 222 days, a statistically significant improvement, and 4 of 14 treated dogs remained free of recurrent osteosarcoma 1 year following amputation (Fig. 3). These encouraging results supported the conduct of subsequent phase II trials in human patients and ultimately the randomized phase III trial.

Investigators at the MDACC conducted a phase II study of L-MTP-PE in patients with osteosarcoma which had recurred with pulmonary metastases after initial therapy with surgery and combination cytotoxic chemotherapy [17]. All patients were rendered disease free by surgical resection of pulmonary metastases. L-MTP-PE was administered twice weekly for 12 weeks (group 1). An additional cohort of patients received L-MTP-PE twice weekly for 12 weeks and then once weekly for an additional 12 weeks for a total of 24 weeks of treatment (group 2).

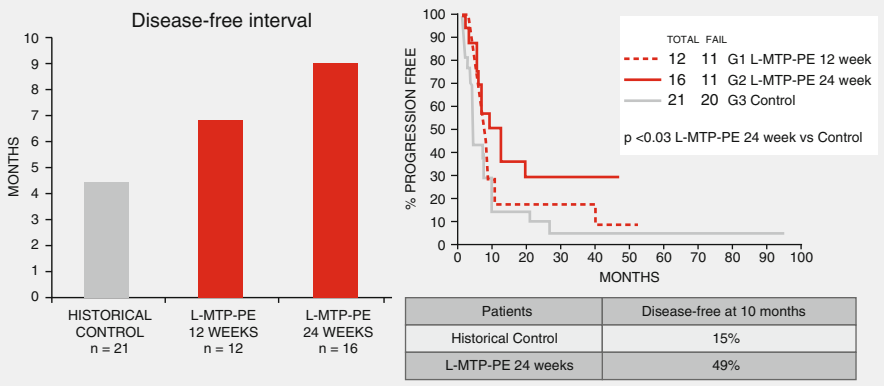


Fig. 4 Investigators at the MD Anderson Cancer Center performed a phase 2 trial of adjuvant L-MTP-PE in patients with metastatic recurrent osteosarcoma whose pulmonary metastases were resected. The addition of L-MTP-PE resulted in progression-free survival, and that survival was better when L-MTP-PE was administered for a longer period. Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

Progression-free survival was compared to a similar historical control group treated at MDACC with surgery and chemotherapy (Fig. 4). The median time to relapse for group 2 patients was 9 months compared to 4.5 months for the historical control group. Additionally, group 2 patients had a better outcome than group 1, supporting the concept that longer duration therapy with L-MTP-PE was superior. Since all patients who entered the trial had undergone resection of pulmonary metastases prior to study entry, it was possible to compare the histology of pulmonary metastases resected from study participants after treatment with L-MTP-PE to their own pulmonary metastases prior to the administration of L-MTP-PE [18]. Nodules resected following treatment showed peripheral fibrosis surrounding the tumor and inflammatory cell infiltration. This was evidence that L-MTP-PE had a biological effect on the tumor metastases.

Chemotherapy has been shown to be an essential component of the treatment of osteosarcoma [15]. If we wished to administer L-MTP-PE and chemotherapy concurrently to patients, we needed to show that chemotherapy did not interfere with the macrophage activation caused by L-MTP-PE and the L-MTP-PE did not interfere with chemotherapy. In in vitro studies adding monocytes activated by L-MTP-PE to cultures of tumor cells with serial concentrations of doxorubicin, there was no modification of the tumor response [19]. L-MTP-PE and chemotherapy were administered concurrently in three murine syngeneic models, and no additive toxicity was observed. Similar antitumor effects of chemotherapy were observed with and without L-MTP-PE [20].

Most importantly, Kleinerman and colleagues showed that doxorubicin did not interfere with cytokine release or activation of monocyte tumoricidal function by L-MTP-PE [19, 21]. They studied monocytes obtained from patients before, during, and after chemotherapy administration and showed no differences in the response to L-MTP-PE [22].

Investigators at MDACC and the Memorial Sloan-Kettering Cancer Center (MSKCC) performed a phase II study of concurrent administration of ifosfamide and L-MTP-PE in patients with metastatic pulmonary osteosarcoma that had recurred after initial therapy with surgery and multi-agent chemotherapy that did not include ifosfamide [23]. There was no increase in the anticipated toxicity of ifosfamide and no delays in administration of ifosfamide. Increases in cytokines following L-MTP-PE were similar to those seen when L-MTP-PE was administered as a single agent. Tumors removed from the lungs of patients following chemotherapy and L-MTP-PE showed both necrosis typically associated with chemotherapy and fibrosis and inflammatory changes previously reported following the administration of L-MTP-PE.

Prospective Randomized Phase III Trial

L-MTP-PE had demonstrated safety in a phase I trial. It had shown improved outcome compared to historical controls in a phase II trial. It was safe to administer concurrently with chemotherapy. It had shown significant improvement in survival in a prospective, randomized, double-blinded study in dogs with osteosarcoma. This evidence supported the development and design of a randomized phase III trial in osteosarcoma.

At the time that the phase III study was being designed there was another question of importance to the pediatric oncology community. Ifosfamide had been shown to be an active agent in patients with osteosarcoma which recurred following initial therapy, and objective responses were reported in 30–50 % of patients [24, 25]. Many investigators were using three chemotherapy agents to treat osteosarcoma: cisplatin, doxorubicin, and high-dose methotrexate. We designed a trial to answer two questions:

1. Would the addition of ifosfamide to cisplatin, doxorubicin, and high-dose methotrexate for the treatment of osteosarcoma improve the outcome?
2. Would the addition of L-MTP-PE to chemotherapy improve the outcome?

Osteosarcoma is a rare disease. In order to answer both questions in a reasonable period of time it was necessary to use a factorial design. In a factorial design, patients are randomly assigned for each intervention, but each intervention is analyzed for its effect on the entire population. Therefore, all patients who received ifosfamide (four-drug chemotherapy) would be compared to all patients who did not receive ifosfamide (three-drug chemotherapy), without considering whether or not they had been assigned to receive L-MTP-PE. All patients assigned to receive L-MTP-PE would be compared to all patients assigned not to receive L-MTP-PE, without considering whether they had been assigned to receive three- or four-drug chemotherapy. These marginal analyses rely on the assumption that no interaction exists between the two study interventions. No preclinical or clinical evidence suggested that there would be an interaction between the two study interventions and there was no plausible biological basis to suggest an interaction [22]. The final analysis at the completion of the randomized prospective phase III trial detected no interaction.

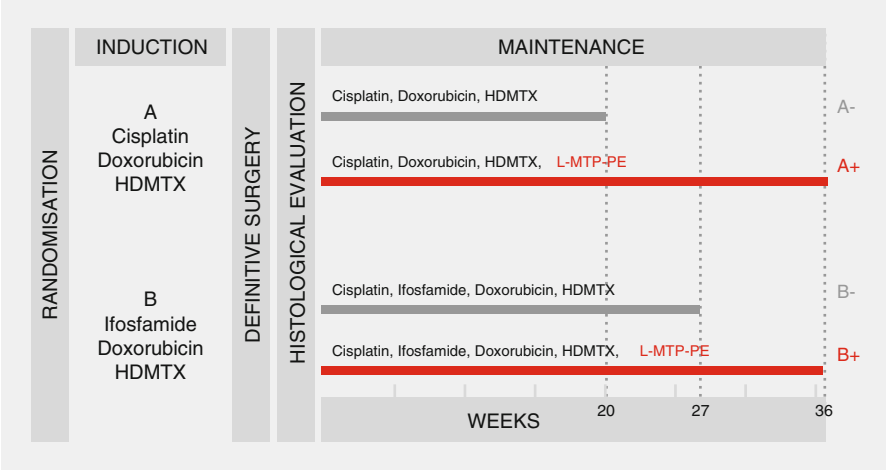


Fig. 5 The North American pediatric cooperative groups performed a prospective randomized trial in patients with osteosarcoma. Patients were assigned to receive chemotherapy with cisplatin, doxorubicin, and HD methotrexate (regimen A) or the same three agents with the addition of ifosfamide (regimen B). Total doses of cisplatin, doxorubicin, and HD methotrexate were identical in both regimens. Patients were randomly assigned at study entry to receive or not to receive L-MTP-PE, and L-MTP-PE was begun following surgical resection of the primary tumor and any sites of macroscopic metastatic disease. Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

The study design for the chemotherapy question was an addition study (Fig. 5). Patients assigned to treatment arm A received cisplatin, doxorubicin, and high-dose methotrexate. Patients assigned to treatment arm B received the same agents with the addition of ifosfamide. Patients received an initial period of chemotherapy followed by definitive surgical resection of the primary tumor followed by additional adjuvant chemotherapy. Assessment of necrosis in the primary tumor after the initial period of systemic chemotherapy was performed as there is a strong correlation between the degree of necrosis in the primary tumor following initial therapy and outcome [26]. The duration of the chemotherapy prior to definitive surgery can influence the degree of necrosis observed at the time of definitive surgery [27]. Therefore the duration of the initial period of chemotherapy was the same in both regimen A and regimen B.

When to initiate L-MTP-PE therapy was a critical issue. All the preclinical and early clinical studies suggested that the efficacy of L-MTP-PE is linked to tumor burden and that maximum activity is seen in the setting of minimal residual disease [9, 23]. Roughly 80 % of patients with osteosarcoma present without clinically detectable metastatic disease as determined by conventional imaging. However, clinical studies clearly show that close to 90 % of these "non-metastatic patients" have microscopic metastases. This is the rationale for continuing chemotherapy following removal of the primary tumor. Unfortunately, despite the use of adjuvant chemotherapy following surgery 30–35 % of patients will relapse in the lung within 2 years.

Since L-MTP-PE has its maximum effect against minimal residual disease, L-MTP-PE therapy was initiated following surgical resection of the primary tumor. There were thus four treatment arms A, A+, B, and B+. Patients assigned to regimen A received chemotherapy with cisplatin, doxorubicin, and high-dose methotrexate. Patients assigned to regimen B received chemotherapy with the same three drugs with the addition of ifosfamide. Patients assigned to receive L-MTP-PE were designated with the addition of a plus sign to the chemotherapy regimen; 677 patients were randomly assigned to one of the four treatment regimens at the time of study enrollment. In retrospect, this was an error in study design, because it allowed for an imbalance in the assignment of poor-prognosis patients (as determined by % necrosis) to one arm, an error that ultimately masked the treatment success of L-MTP-PE in the three-drug plus L-MTP-PE group (A+) as discussed below.

There was no difference in the frequency of greater and lesser necrosis following initial chemotherapy between the patients assigned to chemotherapy regimens A and B. Toxicities were very similar among the arms of the study and were as anticipated from the chemotherapy regimen. We saw no increased toxicity from the addition of L-MTP-PE.

Analysis of the results of the study approximately 9 years after the last patient was enrolled (13 years after enrollment of the first patient) revealed the following:

1. Treatment with three chemotherapy drugs (regimen A) and four chemotherapy drugs (regimen B) achieved the same probability for both event-free and overall survival.
2. All patients assigned to receive L-MTP (with three- or four-drug chemotherapy) showed an improvement in event-free survival compared to those that received three- or four-drug chemotherapy alone. The probability for event-free survival 6 years from study entry was 67 % with L-MTP-PE and 61 % without. The *p*-value for this difference was 0.08.
3. The same comparison showed a statistically significant improvement in the overall survival. The probability for overall survival 6 years from study entry was 78 % with L-MTP-PE and 70 % without. The *p*-value for this difference was 0.03 (Fig. 6).
4. The hazard ratio for death from osteosarcoma comparing treatment with L-MTP-PE to treatment without was 0.7.

Necrosis following initial chemotherapy in the randomized prospective trial was analyzed according to the method described by Huvos [26]. Less necrosis (Huvos grade 1 and 2 necrosis) was associated with a higher probability for recurrence and death than more necrosis (Huvos grade 3 and 4). When we analyzed the frequency of greater and lesser necrosis among the patients assigned to receive each of the four possible randomized therapies, we observed an excess of patients with less necrosis assigned to receive three-drug chemotherapy in combination with L-MTP-PE (regimen A+) (Table 1). Since the observation of less necrosis strongly correlates with a higher probability for recurrence, this imbalance could explain the apparent failure to observe an improved outcome for event-free survival among the patients receiving three-drug chemotherapy who were assigned to receive L-MTP-PE.

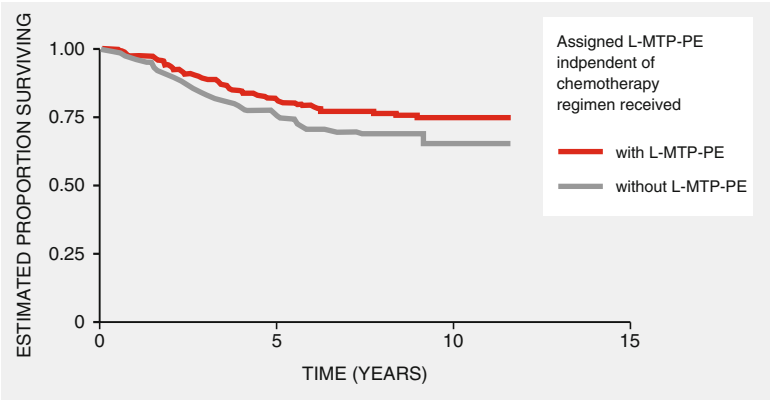


Fig. 6 Administration of L-MTP-PE was associated with improved overall survival. Patients assigned to receive L-MTP-PE following definitive surgery had a 78 % probability of survival of 6 years following study entry, compared with a 70 % probability for patients not assigned to receive L-MTP-PE ($p=0.03$). Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

Table 1 Prospective randomized phase 3 trial: Imbalance in necrosis among patients assigned to treatment arms

Regimen	Huvos grade 1 and 2 necrosis Unfavorable	Huvos grade 3 and 4 necrosis Favorable	Not reported[a]	Total
A	78 (45 %)	71 (41 %)	25 (14 %)	174
A + L-MTP-PE	93 (56 %)	52 (31 %)	22 (13 %)	167
B	78 (47 %)	68 (41 %)	20 (12 %)	166
B + L-MTP-PE	73 (43 %)	72 (42 %)	26 (15 %)	171
Total	322	263	93	678

For the comparison between unfavorable and favorable necrosis among the treatment arms $p=0.08$
[a]Includes patients who progressed prior to definitive surgery or for whom data was not reported

Further analysis of the imbalance in necrosis revealed that by chance most of the imbalance took place in patients older than 16 at study entry. For patients aged less than 16 at study entry, there was better balance among the study arms in the frequency of patients with greater and lesser necrosis following initial chemotherapy (Table 2). This allowed us to examine the effect of the addition of L-MTP-PE to chemotherapy in 496 patients free from the confounding effect of an excess of patients with poor necrosis in one study arm. For this group of 496 children, the addition of L-MTP-PE to chemotherapy resulted in improved event-free survival (Fig. 7). The improvement was seen with both chemotherapy regimens to the same degree. There was no interaction between the two study questions. For this group, the addition of L-MTP-PE to chemotherapy resulted in improved overall survival (Fig. 8). The improvement was exactly the same for both chemotherapy regimens.

Table 2 Prospective randomized phase 3 trial: Balance in necrosis among patients less than 16 assigned to treatment arms

Regimen	Huvos grade 1 and 2 necrosis Unfavorable	Huvos grade 3 and 4 necrosis Favorable	Not reported[a]	Total
A	56 (43 %)	55 (42 %)	20 (15 %)	131
A + L-MTP-PE	63 (51 %)	43 (36 %)	16 (13 %)	121
B	60 (48 %)	52 (42 %)	12 (10 %)	124
B + L-MTP-PE	47 (39 %)	56 (48 %)	15 (13 %)	120
Total	225	208	96	495

[a]Includes patients who progressed prior to definitive surgery or for whom data was not reported

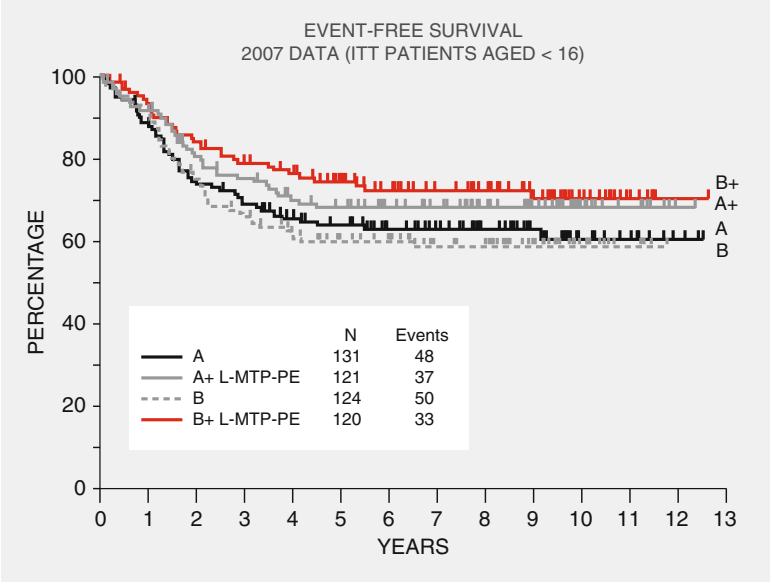


Fig. 7 Among 496 patients aged less than 16 years in whom there was no imbalance in necrosis following initial chemotherapy, administration of L-MTP-PE was associated with improved event-free survival with both chemotherapy regimens, and there was no interaction between the two study interventions. Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

The hazard ration for death associated with the addition of L-MTP-PE was 0.5 ($p = 0.001$). This analysis of 496 children in a prospective randomized trial represents one of the largest experiences ever reported for osteosarcoma and demonstrates a clinically and statistically significant improvement for both event-free and overall survival when L-MTP-PE is added to chemotherapy. The benefit was independent of the chemotherapy regimen to which the patients were assigned.

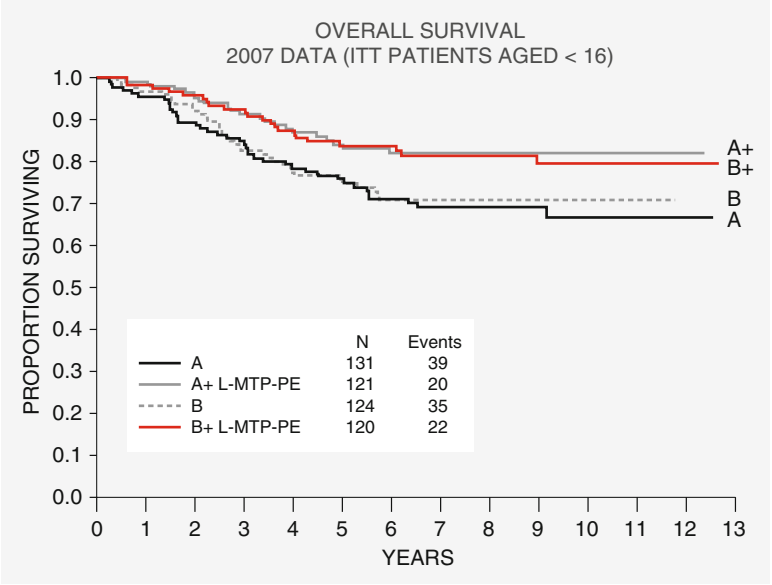


Fig. 8 Among 496 patients aged less than 16 years in whom there was no imbalance in necrosis following initial chemotherapy, administration of L-MTP-PE was associated with improved overall survival with both chemotherapy regimens, and there was no interaction between the two study interventions. The hazard ratio for death among this population in whom there was no confounding influence from an imbalance of patients with poor necrosis in one study arm was 0.5 ($p = 0.001$). Copyright of the image is held by Paul Meyers. Used with permission from Paul Meyers

Phase III Randomized Trial for Patients with Metastatic Disease at Initial Presentation

For patients who do present with clinically detectable metastasis, the great majority have metastatic disease detected only in the lung. These patients can also be considered to have minimal residual disease burden after surgical removal of the primary tumor and all palpable pulmonary nodules. We elected to begin L-MTP-PE therapy for these patients at the time they were rendered free of clinically detectable metastatic disease, following surgical resection of the primary tumor, and pulmonary nodules for those patients. The results of the trial for this stratum were reported in 2009 [28]. The small number of patients enrolled in this stratum decreased the power of this stratum to detect statistically significant differences between treatments. We reported the following observations:

1. Similar to what we found in the study for "non-metastatic" patients, there was no interaction between the two study interventions seen in the patients in the metastatic stratum.
2. There was no difference in outcome between three-drug and four-drug regimens for either event-free or overall survival.

3. Both event-free and overall survival were superior for the patients who received L-MTP-PE than for the patients who did not, although neither analysis achieved a conventional level of statistical significance.
4. The hazard ratio for death from osteosarcoma comparing treatment with L-MTP-PE to treatment without was 0.7, exactly the same as the hazard ratio for patients with localized osteosarcoma.

Compassionate Access Trials

From 2008 to 2012 L-MTP-PE was administered to 205 patients with either osteosarcoma with metastases at initial presentation or patients with metastatic recurrent osteosarcoma after prior treatment with surgery and multi-agent chemotherapy. This was part of a compassionate access clinical protocol [29]. Patients received either L-MTP-PE as a single agent or L-MTP-PE concurrently with chemotherapy. Among 50 patients whose disease was completely resected, more than 50 % remained alive for more than 2 years from study entry. Many of these patients were treated following two or more recurrences after treatment with ≥2 prior lines of therapy.

Regulatory Status of L-MTP-PE

L-MTP-PE, marketed as MEPACT or mifamurtide, was approved by the European Medicines Agency in 2008 for newly diagnosed, non-metastatic osteosarcoma in conjunction with chemotherapy [30]. L-MTP-PE is currently included in the treatment of osteosarcoma in many countries in Europe, Central and South America, Israel, and Turkey. It remains an investigational agent in the United States.

L-MTP-PE is the only immune therapy to date to have received global approval for the treatment of a newly diagnosed sarcoma in conjunction with chemotherapy. The use of L-MTP-PE has had a clinically and statistically significant impact on the long-term survival of hundreds of children with osteosarcoma. Its toxicity profile is minimal when compared to chemotherapy. Its success warrants further investigation in other types of sarcomas and solid tumors that metastasize to the lung. The clinical success of L-MTP-PE also supports more focus on the macrophage as an immune cell target for therapy.

References

1. Houghton BB, Chalasani V, Hayne D, Grimison P, Brown CS, Patel MI et al (2013) Intravesical chemotherapy plus bacille Calmette-Guerin in non-muscle invasive bladder cancer: a systematic review with meta-analysis. BJU Int 111(6):977–983. doi:10.1111/j.1464-410X.2012.11390.x, PubMed PMID: 23253618

2. Zwilling BS, Campolito LB (1977) Destruction of tumor cells by BCG-activated alqolar macrophages. J Immunol 119(3):838–841, PubMed PMID: 330756
3. Namba M, Ogura T, Hirao F, Azuma I, Yamamura Y (1978) Antitumor activity of peritoneal exudate cells induced by cell-wall skeleton of Mycobacterium bovis BCG. Gann 69(6):831–834, PubMed PMID: 374173
4. Ellouz F, Adam A, Ciorbaru R, Lederer E (1974) Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem Biophys Res Commun 59(4):1317–1325, PubMed PMID: 4606813
5. Sugimoto M, Germain RN, Chedid L, Benacerraf B (1978) Enhancement of carrier-specific helper T cell function by the synthetic adjuvant, N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP). J Immunol 120(3):980–982, PubMed PMID: 344799
6. Sone S, Poste G, Fidler IJ (1980) Rat alveolar macrophages are susceptible to activation by free and liposome-encapsulated lymphokines. J Immunol 124(5):2197–2202, PubMed PMID: 7365253
7. Sone S, Fidler IJ (1980) Synergistic activation by lymphokines and muramyl dipeptide of tumoricidal properties in rat alveolar macrophages. J Immunol 125(6):2454–2460, PubMed PMID: 7430635
8. Parant M, Parant F, Chedid L, Yapo A, Petit JF, Lederer E (1979) Fate of the synthetic immunoadjuvant, muramyl dipeptide (14C-labelled) in the mouse. Int J Immunopharmacol 1(1):35–41, PubMed PMID: 551094
9. Fidler IJ, Sone S, Fogler WE, Barnes ZL (1981) Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous injection of liposomes containing muramyl dipeptide. Proc Natl Acad Sci U S A 78(3):1680–1684, PubMed PMID: 6940181; PubMed Central PMCID: PMC319196
10. Schroit AJ, Fidler IJ (1982) Effects of liposome structure and lipid composition on the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. Cancer Res 42(1):161–167, PubMed PMID: 7053846
11. Kleinerman ES, Erickson KL, Schroit AJ, Fogler WE, Fidler IJ (1983) Activation of tumoricidal properties in human blood monocytes by liposomes containing lipophilic muramyl tripeptide. Cancer Res 43(5):2010–2014, PubMed PMID: 6831430
12. Murray JL, Kleinerman ES, Cunningham JE, Tatom JR, Andrejcio K, Lepe-Zuniga J et al (1989) Phase I trial of liposomal muramyl tripeptide phosphatidylethanolamine in cancer patients. J Clin Oncol 7(12):1915–1925, PubMed PMID: 2479721
13. Kleinerman ES, Murray JL, Snyder JS, Cunningham JE, Fidler IJ (1989) Activation of tumoricidal properties in monocytes from cancer patients following intravenous administration of liposomes containing muramyl tripeptide phosphatidylethanolamine. Cancer Res 49(16):4665–4670, PubMed PMID: 2787207
14. Marshall JL (2012) Maximum-tolerated dose, optimum biologic dose, or optimum clinical value: dosing determination of cancer therapies. J Clin Oncol 30(23):2815–2816. doi:10.1200/JCO.2012.43.4233, PubMed PMID: 22753919
15. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB et al (1986) The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med 314(25):1600–1606. doi:10.1056/NEJM198606193142502, PubMed PMID: 3520317
16. MacEwen EG, Kurzman ID, Rosenthal RC, Smith BW, Manley PA, Roush JK et al (1989) Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide. J Natl Cancer Inst 81(12):935–938, PubMed PMID: 2733037
17. Kleinerman ES, Gano JB, Johnston DA, Benjamin RS, Jaffe N (1995) Efficacy of liposomal muramyl tripeptide (CGP 19835A) in the treatment of relapsed osteosarcoma. Am J Clin Oncol 18(2):93–99, PubMed PMID: 7900714
18. Kleinerman ES, Raymond AK, Bucana CD, Jaffe N, Harris MB, Krakoff IH et al (1992) Unique histological changes in lung metastases of osteosarcoma patients following therapy with liposomal muramyl tripeptide (CGP 19835A lipid). Cancer Immunol Immunother 34(4):211–220, PubMed PMID: 1537053

19. Hudson MM, Snyder JS, Jaffe N, Kleinerman ES (1988) In vitro and in vivo effect of adriamycin therapy on monocyte activation by liposome-encapsulated immunomodulators. Cancer Res 48(18):5256–5263, PubMed PMID: 3261632
20. Killion JJ, Kleinerman ES, Wilson MR, Tanaka M, Fidler IJ (1992) Sequential therapy with chemotherapeutic drugs and liposome-encapsulated muramyl tripeptide: determination of potential interactions between these agents. Oncol Res 4(10):413–418, PubMed PMID: 1292756
21. Asano T, Fujimaki W, McWatters A, An T, Matsushima K, Kleinerman ES (1993) Effect of Adriamycin on liposomal muramyl tripeptide's ability to up-regulate monocyte cytokine expression. Cancer Immunol Immunother 37(6):408–411, PubMed PMID: 8242665
22. Kleinerman ES, Snyder JS, Jaffe N (1991) Influence of chemotherapy administration on monocyte activation by liposomal muramyl tripeptide phosphatidylethanolamine in children with osteosarcoma. J Clin Oncol 9(2):259–267, PubMed PMID: 1988574
23. Kleinerman ES, Meyers PA, Raymond AK, Gano JB, Jia SF, Jaffe N (1995) Combination therapy with ifosfamide and liposome-encapsulated muramyl tripeptide: tolerability, toxicity, and immune stimulation. J Immunother Emphasis Tumor Immunol 17(3):181–193, PubMed PMID: 7613644
24. Miser JS, Kinsella TJ, Triche TJ, Tsokos M, Jarosinski P, Forquer R et al (1987) Ifosfamide with mesna uroprotection and etoposide: an effective regimen in the treatment of recurrent sarcomas and other tumors of children and young adults. J Clin Oncol 5(8):1191–1198, PubMed PMID: 3114435
25. Pratt CB, Horowitz ME, Meyer WH, Etcubanas E, Thompson EI, Douglass EC et al (1987) Phase II trial of ifosfamide in children with malignant solid tumors. Cancer Treat Rep 71(2):131–135, PubMed PMID: 3100034
26. Meyers PA, Heller G, Healey J, Huvos A, Lane J, Marcove R et al (1992) Chemotherapy for nonmetastatic osteogenic sarcoma: the Memorial Sloan-Kettering experience. J Clin Oncol 10(1):5–15, PubMed PMID: 1370176
27. Meyers PA, Gorlick R, Heller G, Casper E, Lane J, Huvos AG et al (1998) Intensification of preoperative chemotherapy for osteogenic sarcoma: results of the Memorial Sloan-Kettering (T12) protocol. J Clin Oncol 16(7):2452–2458, PubMed PMID: 9667263
28. Chou AJ, Kleinerman ES, Krailo MD, Chen Z, Betcher DL, Healey JH et al (2009) Addition of muramyl tripeptide to chemotherapy for patients with newly diagnosed metastatic osteosarcoma: a report from the Children's Oncology Group. Cancer 115(22):5339–5348. doi:10.1002/cncr.24566, PubMed PMID: 19637348; PubMed Central PMCID: PMC2783515
29. Anderson PM, Meyers P, Kleinerman E, Vankatakrishnan K, Hughes D, Herzog C, Huh W, Sutphin R, Vyas Y, Chen V, Oliva C, Wang B, Liu Y, Chou A (2013) Mifamurtide in metastatic and recurrent osteosarcoma: a patient access study with pharmacokinetic, pharmacodynamic, and safety assessments. Pediatr Blood Cancer 61(2):238–244
30. NICE. Mifamurtide for the treatment of osteosarcoma 2011. www.nice.org.uk/ta235

Genetically Modified T-Cell Therapy for Osteosarcoma

Christopher DeRenzo and Stephen Gottschalk

Abstract T-cell immunotherapy may offer an approach to improve outcomes for patients with osteosarcoma, who fail current therapies. In addition, it has the potential to reduce treatment-related complications for all patients. Generating tumor-specific T cells with conventional antigen presenting cells *ex vivo* is time consuming and often results in T-cell products with a low frequency of tumor-specific T cells. In addition, the generated T cells remain sensitive to the immunosuppressive tumor microenvironment. Genetic modification of T cells is one strategy to overcome these limitations. For example, T cells can be genetically modified to render them antigen specific, resistant to inhibitory factors, or increase their ability to home to tumor sites. Most genetic modification strategies have only been evaluated in preclinical models, however early phase clinical trials are in progress. In this chapter we review the current status of gene-modified T-cell therapy with special focus on osteosarcoma, highlighting potential antigenic targets, preclinical and clinical studies, and strategies to improve current T-cell therapy approaches.

C. DeRenzo
Center for Cell and Gene Therapy, Houston Methodist, Texas Children's Hospital, Baylor College of Medicine, 1102 Bates Street, Suite 1770, Houston, TX 77030, USA

Texas Children's Cancer Center, Texas Children's Hospital, Baylor College of Medicine, Houston, TX 77030, USA

Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA

S. Gottschalk (✉)
Center for Cell and Gene Therapy, Houston Methodist, Texas Children's Hospital, Baylor College of Medicine, 1102 Bates Street, Suite 1770, Houston, TX 77030, USA

Texas Children's Cancer Center, Texas Children's Hospital, Baylor College of Medicine, Houston, TX 77030, USA

Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA

Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX 77030, USA
e-mail: smgottsc@txch.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_18,

Keywords Pediatric cancer • Osteosarcoma • Cancer immunotherapy • T-cell therapy • Gene therapy • Chimeric antigen receptors • Tumor antigens

Introduction

Adoptive T-cell therapy refers to the isolation of allogeneic or autologous T cells, followed by *ex vivo* manipulation, and subsequent infusion into patients for therapeutic gain [101]. Channeling the cytotoxic killing and specific targeting ability of T cells through adoptive transfer has the potential to improve outcomes for patients with osteosarcoma. An early example of adoptive T-cell therapy for osteosarcoma was reported by Sutherland et al. [113]. A 14-year-old girl, who had the same human leukocyte antigen (HLA) type as her mother received unmanipulated, maternal lymphocytes. Lymphocytes isolated from the patient post infusion killed osteosarcoma cells in vitro, but the patient had only a minimal clinical response prior disease progression and death. Since Sutherland's report, significant advances in immunotherapeutic techniques have taken place.

Cell therapy with conventional T cells has shown promise in several clinical settings [11, 52, 101]. Examples include donor lymphocyte infusions (DLI) after stem cell transplantation to treat CML relapse [61], infusion of Epstein-Barr virus (EBV)-specific T lymphocytes to treat EBV-related lymphomas and nasopharyngeal carcinoma [5, 7, 24, 72, 110], infusion of tumor infiltrating lymphocytes (TILs) to treat melanoma [31, 101], and the infusion of virus-specific T cells to prevent and treat viral-associated disease in immunocompromised patients [42, 64, 65].

Since the *ex vivo* generation of T cells specific for tumor associated antigens (TAA) is often cumbersome, investigators have developed genetic modification strategies to render T cells TAA specific [52, 101, 104]. For example, infusion of T cells genetically modified with chimeric antigen receptors (CAR) specific for GD2 or CD19 has shown promise in early clinical studies for neuroblastoma and CD19-positive hematological malignancies including acute lymphoblastic leukemia and lymphoma [12, 39, 54, 60, 71, 92, 93, 105]. Besides rendering T cells tumor-specific, genetic modifications enable the generation of T cells with enhanced effector functions (Table 1). While these approaches have been mainly evaluated in preclinical models, some are already being actively explored in the clinic. In this chapter we review the current status of gene-modified T-cell therapy for osteosarcoma, highlighting potential antigenic targets, preclinical and clinical studies, and strategies to improve T-cell therapeutic approaches.

T-Cell Therapy Targets for Osteosarcoma

Developing successful antigen-specific T-cell therapy depends on the availability of specific TAA. Once a TAA is identified, TAA-specific T cells can be either generated using conventional antigen presenting cells or by gene transfer to recognize and induce killing of TAA-positive osteosarcoma.

Table 1 Genetic modifications for T-cell therapy for osteosarcoma

Goal	Introduced gene class	Example
Antigen-specificity	Receptors	αβ TCR, CAR
T-cell expansion	Costim molecules	CD80, 41BBL
	Domains of costim molecules	CD27, CD28, 41BB, OX40
	Cytokines	IL12, IL15
Resistance to inhibitory tumor environment	Costim molecules	CD80, 41BBL
	Domains of costim molecules	CD27, CD28, 41BB, OX40
	Cytokines	IL12, IL15
	Dominant negative receptors	DN TGFβ receptor
	Chimeric cytokine receptors	IL4/IL2, L4/IL7
	shRNAs	FAS
	Constitutive activated kinases	AKT
Improve T-cell homing to tumor sites	Chemokine receptors	CCR2b or CXCR2
Safety	Inducible suicide genes	HSV-tk; caspase 9
	Cell surface markers	CD20

DN dominant negative, *HSV-tk* Herpes simplex virus thymidine kinase, *IL* interleukin, *TGFβ* transforming growth factor β

TAA are potential candidates for immunotherapy, including T-cell therapy, if they are (1) expressed at higher than normal levels on tumor cells compared to nonmalignant host cells, (2) are normally only expressed during fetal development or at immunoprivileged sites, such as the testes, (3) contain novel peptide sequences created by gene mutation, (4) are viral antigens, (5) are antigens produced by epigenetic changes, (6) or are antigens on non-transformed cells in the tumor microenvironment [15, 98, 121]. Unaltered tissue-differentiation antigens on tumors can also be targets for T-cell immunotherapy, but only if the associated tissues are not essential for life and/or their products can be replaced [121]. For example, CD19-specific T-cell therapy induces regression of CD19-positive malignancies, but also leads to long-term depletion of normal, CD19-positive B cells, which can be remedied by the infusion of intravenous immunoglobulin (IVIG) [12, 39, 54, 60, 92, 105].

For osteosarcoma, numerous TAA have been described that are summarized in Table 2. These include human epidermal growth factor receptor 2 (HER2) [2, 38], interleukin 11 receptor alpha (IL11Rα) [46] , melanoma associated antigen (MAGE) and g melanoma antigen (GAGE) family members [49], GD2 (a disialoganglioside; not a protein tumor associated antigen) [129], New York esophageal squamous cell carcinoma 1 (NY-ESO-1) [49], clusterin-associated protein 1 (CLUAP1) [48], papillomavirus binding factor [118], fibroblast activation protein (FAP) [130], tumor endothelial marker 1 (TEM1) [103], and B7-H3 [75]. Other TAA for osteosarcoma-targeted T-cell therapy are rapidly being elucidated. Orentas et al. described several potential targets shared amongst multiple pediatric tumors, such as melanoma cell adhesion molecule (MCAM) and glypican-2, which are present on six different pediatric solid tumors, but not expressed in normal tissues [83, 84]. While gene expression data was used to identify these targets, and additional studies are needed to confirm expression on a protein level, the work demonstrates the use of gene expression array data to identify new TAAs.

Table 2 Tumor associated antigens expressed in osteosarcoma

Target antigen	Cell surface expression	Preclinical in vivo studies[a]	Clinical studies[b]
B7-H3	+	–	–
CLUAP1	–	–	–
FAP	+	–	–
GAGE 1,2,8	–	–	–
GD2	+	–	–
Glypican-2	–	–	–
HER2	+	+	+
IL-11Rα	+	+	–
MAGE A1-6,10, 12; C2	–	–	–
MCAM	–	–	–
NY-ESO-1	–	–	–
Papillomavirus binding factor	–	–	–
TEM1	+	–	–

CLUAP1 clusterin-associated protein 1, *FAP* fibroblast activation protein, *GAGE* G melanoma antigen, *GD2* disialoganglioside, *HER2* human epidermal growth factor receptor 2, *IL11Rα* interleukin 11 receptor α, *MAGE* melanoma associated antigen, *MCAM* melanoma cell adhesion molecule, *NY-ESO-1* new York esophageal squamous cell carcinoma 1, *TEM1* tumor endothelial marker 1

[a]Using an osteosarcoma model

[b]Including osteosarcoma patients

Genetic Approaches to Render T Cells Specific for Osteosarcoma

Since the *ex vivo* generation of conventional antigen-specific T cells is often cumbersome and unreliable, investigators have developed genetic approaches to rapidly generate antigen-specific T cells. These include the forced expression of α/β T-cell receptors (TCRs) and CARs.

α/β TCR Modified T-Cells

Conventional TCRs are composed of α and β chains that form heterodimers. TCRs recognize peptides, which are derived from proteins and are presented on major histocompatibility complex (MHC) molecules on the cell surface. Isolating TCRs for adoptive T-cell therapy requires the generation of TAA-specific T-cell clones and subsequent isolation and cloning of the TCR α and β chains [120]. In general, a large number of T-cell clones need to be screened, and isolated TCRs often are of low affinity requiring additional affinity maturation. Following isolation, genes encoding the α and β chains are cloned into retroviral or lentiviral vectors, and then used to transduce T cells [98]. Since T cells express endogenous α/β TCRs, mispairing between endogenous α/β and transgenic α/β TCR chain is a common problem.

Several approaches have been developed to overcome this limitation, including the introduction of disulfide bonds or use of murine sequences to favor dimerization of transgenic α/β TCR chains [23, 37]. Silencing the expression of endogenous α/β TCR by shRNAs or zinc-finger nucleases is another option [82, 116].

α/β TCRs have been isolated for several TAA including CEA, GP100, MAGEA3, MART1, and NY-ESO-1 [51, 76, 77, 87, 99]. So far the safety and efficacy of α/β TCR T-cell therapy has been evaluated mainly in melanoma patients, but studies have also been conducted for patients with sarcoma, colon cancer and multiple myeloma. One of the first studies in humans with transgenic α/β TCR T cells was conducted by Morgan et al. and demonstrated that the infusion of autologous polyclonal T cells expressing MART1-specific α/β TCRs was safe and induced objective tumor responses in 2 out of 15 lymphodepleted melanoma patients [77]. To increase the response rates, the same group infused T cells expressing high affinity MART1- and gp100-specific α/β TCRs. While response rates increased, several patients developed toxicities including skin rash, uveitis, and/or hearing loss that were not associated with antitumor responses [51]. Recognition of normal tissues expressing low levels of CEA has also been reported for the adoptive transfer of CEA-specific α/β TCR T cells [87]. In contrast, infusion of NY-ESO-1-specific α/β TCR T cells was well tolerated with a response in 4/6 patients with synovial cell sarcoma and in 5/11 patients with melanoma. In addition, an ongoing clinical study indicates that NY-ESO-1-specific α/β TCR T cells induce clinical responses in patients with multiple myeloma without off-targets effects [68]. As mentioned above, affinity maturation is frequently used to increase the activity of α/β TCRs. However, this can lead to recognition of related antigens resulting in severe adverse events [74, 76]. For example, infusion of MAGE A3-specific α/β TCR T cells caused fatal neurotoxicity due to recognition of MAGE A12 as well as fatal cardiac toxicities due to recognition of titin.

Thus clinical studies so far have not only demonstrated the potency of adoptively transferred α/β TCR-modified T cells but also their clinical limitations. Nevertheless, active exploration of α/β TCR-modified T-cell therapy is warranted for osteosarcoma.

CAR-Modified T Cells

Antigen-specific T cells can also be generated by the transfer of genes encoding CARs [32, 73, 104]. CARs consist of an ectodomain that confers antigen specificity, a hinge, a transmembrane domain, and an endodomain that contains signaling domains derived from the T-cell receptor CD3-ζ chain and co-stimulatory molecules such as CD27, CD28, 41BB, or OX40. Depending on the number of co-stimulatory domains, CARs are referred to as first generation (no), second generation (one), or third generation (two) CARs. CARs targeting multiple pediatric malignancies have been developed [2, 16, 39, 44, 46, 86, 93, 102, 106]. CAR ectodomains are most commonly generated by joining the heavy and light chain variable regions of a monoclonal antibody (MAb), expressed as a single-chain Fv (scFv) molecule.

CARs recognize unprocessed antigen on tumor cell surfaces, and do not require peptide presentation on MHC molecules.

CAR T-cell therapy has several advantages compared to α/β T-cell therapy. Because CARs do not require antigen presentation on MHC molecules, generation of CAR T cells for patients does not require HLA matching. This property also renders CAR T cells resistant to tumor escape mechanisms, such as downregulation of HLA molecules and defects in the MHC class I processing pathway. A second advantage is that MAbs already exist for numerous surface antigens, obviating the need of cumbersome α/β TCR isolation. Additionally, CAR T cells recognize carbohydrate and glycolipid antigens, in addition to protein antigens [73, 104]. Furthermore, CARs confer T-cell specificity in a single molecule unlike artificial α/β TCRs, which require the expression of two molecules that are prone to heterodimerization with the endogenously expressed α/β TCR chains. A potential drawback of CARs is that in general only cell surface molecules are recognized. However, the isolation of scFvs that recognize HLA-molecule/peptide complexes has allowed the generation of CARs that recognize peptides derived from intracellular proteins [107, 127].

Of the TAA expressed in osteosarcoma, GD2, HER2, IL11Rα, and FAP are expressed on the cell surface, and have been targeted with CAR T cells in preclinical animal models and/or clinical studies. Pule et al. expressed a first generation GD2-specific CAR on EBV-specific T cells and gave them to 11 children with advanced neuroblastoma [71, 93]. Three of them had complete responses (sustained in two) while an additional two with bulky tumors showed substantial tumor necrosis. Follow up studies are in progress [80], and these encouraging results should justify the exploration of GD2-specific CAR T cells for patients with osteosarcoma.

While HER2 is not gene amplified in osteosarcoma, 60–70 % of osteosarcoma are HER2 positive and HER2-positivity is associated with poor outcomes [38, 79]. T cells expressing a second generation CAR with a CD28.ζ-endodomain showed promising antitumor activity in preclinical animal models [2]. In addition, HER2-CAR T cells had potent antitumor activity against osteosarcoma sarcospheres, which are enriched in osteosarcoma-initiating cells [97]. However, safety concerns have been raised in regards to targeting HER2 with CAR T cells in humans. One patient, who received high dose chemotherapy followed by the infusion of 1×10^{10} T cells expressing a third generation HER2-specific CAR and IL2 developed respiratory failure within 12 h of infusion and died [78]. Subsequently, up to $1 \times 10^{8}/m^{2}$ T cells expressing a second generation CAR were given to pediatric and adolescent sarcoma patients. While the infusions were safe, infused T cells did not expand significantly post infusion, and antitumor activity of the infused T cells was limited [1].

To target IL11Rα, Huang and colleagues developed a second generation CAR that contains the natural ligand (IL11) as a CAR ectodomain [46]. IL11Rα-specific CAR T cells recognized and killed IL11Rα-positive osteosarcoma cells, and caused

regression of lung metastases in the KRIB metastatic osteosarcoma model in vivo. Lastly, T cells expressing a second generation FAP-specific CAR, which not only target FAP-positive osteosarcoma cells but also FAP-positive stromal cells [53], have shown promising antitumor activity in preclinical models, which are discussed in the section "Genetic Modification to Overcome Tumor-Mediated Immunosuppression."

In summary CAR T cells have shown promising antitumor activity in preclinical animal models, and the initial clinical experience is encouraging. However, several challenges remain including in vivo T-cell expansion and persistence, the inhibitory tumor microenvironment, T-cell trafficking to tumor sites, and safety. As reviewed in the next section (Table 1), we and others believe that additional genetic modifications of T cells have the potential to overcome these obstacles.

Genetic Approaches to Enhance the Effector Function of Osteosarcoma-Specific T Cells

Enhancing T-Cell Expansion and Persistence In Vivo

Dramatic T-cell expansion and long-term persistence post infusion of adoptively transferred T cells has been observed in lymphodepleted patients post hematopoietic stem cell transplantation or in patients that have been lymphodepleted with chemotherapy and/or radiation prior to T-cell transfer [31, 42]. Since T-cell expansion post antigen recognition requires the provision of costimulation, investigators have included signaling domains in CAR endodomains derived from costimulatory molecules including CD27, CD28, 4-1BB, and OX40. Numerous preclinical studies have documented the benefit of added costimulation [13, 14, 94, 109], however only one study so far has done a "head to head comparison" in humans. Savoldo et al. compared CD19-specific CARs with a ζ– or CD28.ζ-domain [105]. While CD28 costimulation resulted in expansion of adoptively transferred CAR.CD28.ζ T cells in contrast to CAR.ζ T cells, the effect was limited. Limited expansion was also observed in our clinical trial with HER2.CAR.CD28.ζ T cells for patients with sarcoma [1]. Both of these studies were conducted without lymphodepleting chemotherapy and/or radiation. Thus, aggressive lymphodepletion prior to T-cell transfer might enhance CAR.CD28.ζ T-cell expansion in vivo. Other options include vaccination post infusion to boost T-cell expansion. Lastly, most studies have been conducted with unselected T cells. Recent studies indicate that it might be advantageous to express CARs in T cells that are specific for viruses, so that infused cells could be boosted by vaccination (e.g., influenza) [25] or by viruses, which are present latently in humans (e.g., EBV) [93]. In addition, expressing CARs in T-cell subsets like effector memory T cells has the potential to enhance T-cell persistence [3, 114].

Genetic Modifications to Overcome Tumor-Mediated Immunosuppression

Malignant cells including osteosarcoma and their supporting stroma have developed an intricate system to suppress the immune system [4, 34, 36, 96, 119]. They (1) secrete immunosuppressive cytokines such as transforming growth factor (TGFβ) or IL10, (2) attract immunosuppressive cells such regulatory T cells (Tregs) or myeloid derived suppressor cells (MSDCs), (3) inhibit dendritic cell maturation, (4) express molecules on the cell surface that suppress immune cells including FAS ligand (FAS-L) and PD-L1, and (5) create a metabolic environment (e.g., high lactate, low tryptophan) that is immunosuppressive.

Three broad approaches have been developed to overcome tumor immunosuppression [66]: (1) increasing CAR T-cell activation, for example by enhanced co-stimulation (discussed above) or by local production of transgenic cytokines, (2) engineering CAR T cells to be resistant to the immune evasion strategies used by the tumor, or (3) targeting the cellular components of the tumor stroma. Any one may affect more than one mechanism of tumor immunosuppression.

CAR T cells can be engineered to produce immunostimulatory cytokines by transgenic expression of cytokines such as IL-15 [45, 95], which improves CAR T-cell expansion and persistence in vivo. In addition, it renders T cells resistant to the inhibitory effects of Tregs by activation of the phosphoinositide 3-kinase (PI3K) pathway [90]. Alternatively, transgenic expression of IL12 in CAR T cells acts directly to enhance T-cell activity [18, 56, 89, 131]. In addition it reverses the immunosuppressive tumor environment by triggering the apoptosis of inhibitory tumor-infiltrating macrophages, dendritic cells, and MDSCs through a FAS-dependent pathway [56], resulting in enhanced antitumor activity of adoptively transferred T cells in several preclinical animal models. While there are safety concerns in regards to constitutive IL12 expression, there are several mechanisms available to restrict IL12 production to activated T cells at the tumor site by using inducible expression systems [131].

Conversely, instead of themselves being engineered to produce cytokines, CAR T cells can be engineered to be resistant to cytokines such as IL4 and TGFβ that inhibit their cytolytic function. TGFβ is widely used by tumors as an immune evasion strategy [128], since it promotes tumor growth, limits effector T-cell function, and activates Tregs. These detrimental effects of TGFβ can be negated by modifying T cells to express a dominant-negative TGFβ receptor type II (DNR), which lacks most of the cytoplasmic kinase domain [8, 33]. DNR expression interferes with TGFβ-signaling and restores T-cell effector function in the presence of TGFβ, and a clinical study evaluating this strategy is in progress for patients with EBV-positive lymphomas [6].

It is also possible to engineer T cells to actively benefit from the inhibitory signals generated by the tumor environment, by converting inhibitory into stimulatory signals [67, 123, 125]. For example, linking the extracellular domain of the TGFβ RII to the endodomain of toll-like receptor (TLR) 4 results in a chimeric receptor that not only renders T cells resistant to TGFβ, but also induces T-cell activation and

expansion [123]. Chimeric IL4 receptors are another example of these "signal converters." Many tumors secrete IL4 to create a TH2-polarized environment, and two groups of investigators have shown that expression of chimeric IL4 receptors consisting of the ectodomain of the IL4 receptor and the endodomain of the IL-7Rα or the IL-2Rβ chain enable T cells to proliferate in the presence of IL4 and retain their effector function including TH1-polarization [67, 125].

Silencing genes that render T cells susceptible to inhibitory signals in the tumor microenvironment may also improve T-cell function. For example, many tumor cells express FAS ligand, and silencing FAS in T cells prevents FAS-induced apoptosis [30]. Besides silencing genes, expression of a constitutively active form of serine/threonine AKT (caAKT), which is a major component of the PI3K pathway, in T cells has also been shown to improve their function [112]. caAKT-expressing T cells sustained higher levels of NF-κB and had elevated levels of anti-apoptotic genes such as Bcl2, resulting in resistance to Tregs and TGFβ.

Lastly, most solid tumors have a stromal compartment that supports tumor growth directly through paracrine secretion of cytokines, growth factors, and provision of nutrients, and contributes to tumor-induced immunosuppression [22, 40, 91]. For example, we have shown in preclinical studies that T cells expressing CARs specific for FAP expressed on cancer associated fibroblasts (CAFs) have potent antitumor effects [53]. In addition, combining tumor-specific CAR T cells with FAP-specific CAR T cells enhanced antitumor effects. While recently some concerns have been raised in regards to targeting FAP [100, 117], our findings indicate that targeting CAFs has the potential to improve the antitumor effects of adoptively transferred CAR T cells. Targeting the tumor vasculature with CARs to enhance T-cell therapy for solid tumors has been explored by others [19, 81]. Targeting the tumor vasculature with vasculature endothelial growth factor receptor (VEGFR)2-specific CAR T cells in addition to tumor cells synergized in inducing tumor regression in several syngeneic, preclinical tumor models [17]. In addition, transgenic expression of VEGFR2-specific CARs and IL12 in T cells was sufficient to eradicate tumors, indicating that combining countermeasures might potentiate effects [18].

While many of the discussed genetic modification strategies have not been explored in osteosarcoma models, these strategies could be readily integrated in current T-cell therapy approaches for osteosarcoma.

Genetic Modification of T Cells to Improve Homing to Tumor Sites

T-cell homing to solid tumor sites might be limited. For example, Kershaw et al. evaluated the safety and efficacy of first generation folate receptor (FR)-α CAR T cells in patients with ovarian cancer [58]. Infused T cells persisted less than 3 weeks in all but one patient, and did not specifically home to tumor sites as judged by ^{111}Indium scintigraphy. No antitumor activity was observed. Since then, several investigators have shown in preclinical models that the expression of chemokine receptors in CAR T cells that recognize chemokines secreted by solid tumors can

enhance T-cell homing. For example, transgenic expression of chemokine receptors CCR2b or CXCR2 in T cells enhances trafficking to CCL2- or CXCL1-secreting solid tumors including melanoma and neuroblastoma [26, 57].

Improving Safety of T-Cell Therapy

Toxicities can be divided into four categories: (1) toxicities due to genetic modification (which have not been observed with genetically modified T cells in humans so far [9]), (2) "on target organ" toxicities (e.g., depletion of normal B cells post CD19-CAR T cells [54]), (3) "on target, off organ" toxicities (e.g., liver toxicity of carbonic anhydrase IX CAR T cells to target renal cell carcinoma [62]), and (4) systemic inflammatory syndromes [39, 54, 92].

Genetic safety switches have been developed to selectively destroy genetically modified T cells once adverse events occur. The most widely used suicide gene strategy for T-cell therapy is to introduce the herpes simplex virus thymidine kinase (HSV-tk) gene into the T cells. HSV-tk phosphorylates acyclovir, valacyclovir, and ganciclovir to toxic nucleosides [21]. T cells transduced with HSV-tk are robustly killed in the presence of these medications and clinical studies demonstrate effectiveness of the strategy. A drawback to utilizing HSV-tk as a safety switch for T-cell therapy is the immunogenicity of HSV-tk, and that some patients require acyclovir, valacyclovir, or ganciclovir to treat herpetic diseases. Therefore, genetic safety switches using non-immunogenic human components have been developed, such as inducible caspase 9 that can be activated by a dimerizer [28, 111]. Once exposed to the dimerizer, genetically modified T cells rapidly undergo apoptosis. Another approach includes the transgenic expression of CD20, rendering T cells sensitive to the clinically approved CD20 MAb rituximab [47]. While suicide gene switches can selectively kill infused cells, systemic inflammatory syndromes might be difficult to control with this approach since resident immune cells, which are activated by the infused T cells, most likely contribute. Recent studies indicate that IL6 plays a critical role in these syndromes, and the infusion of the IL6 receptor MAb (tocilizumab) alone or in combinations with steroids and TNFα MAbs (infliximab) proved to be effective [39, 54, 92].

While suicide switches are one strategy to prevent "on target, off organ" toxicities, other strategies include the generation of T cells that are only fully activated if they encounter a unique "antigen address" at the tumor site. Examples include the development of T cells expressing two CARs in which one TAA-specific CAR has an endodomain with a ζ-signaling domain, and a second CAR, specific for another TAA, provides costimulation [59, 63, 126].

Combinatorial T-Cell Therapy

As for other cancer therapies, combinatorial therapies hold the promise to improve T-cell therapy for cancer [122]. These can be divided into approaches that (1) kill tumor cells without affecting T cells, (2) enhance the expression of TAA,

(3) improve T-cell expansion and persistence, and (4) reverse the inhibitory tumor microenvironment. For example the BRAF inhibitor vemurafenib has no adverse effects on T-cell function, and combining vemurafenib with adoptive transfer of T cells enhances antitumor effects in preclinical animal models of melanoma [29, 69]. Increasing the expression of TAA in cancer cells can be achieved with epigenetic modifiers such decitabine [20, 27].

Combining T-cell therapy with blocking antibodies specific for negative regulators of T-cell responses such as the cytotoxic T-lymphocyte-associated protein (CTLA-4) and programmed cell death 1 (PD-1) is one strategy to increase their function [55, 88, 115, 124]. The role of CTLA-4 as a negative regulator of T-cell responses has been well demonstrated in CTLA-4-deficient mice and preclinical tumor models. Based on these studies, an antibody to block human CTLA-4, ipilimumab, was developed, and a phase III randomized clinical trial showed that 23 % patients with metastatic melanoma survive more than 4 years following ipilimumab treatment, leading to FDA approval in March 2011 [43].

Similarly, combining T-cell therapy with MAbs that block PD-1 and/or its ligands (PD-L1 and PD-L2) is another promising approach. A recent clinical trial evaluating the safety and efficacy of a PD-L1 antibody reported encouraging objective clinical response rates in patients with advanced melanoma, renal cell carcinoma, and non-small-cell lung cancer [10]. In addition, a recent report demonstrated the benefit of combing PD-1 blockade with the adoptive transfer of HER2-CAR T cells in a preclinical melanoma model [50].

As mentioned in section "'Enhancing T-Cell Expansion and Persistence In Vivo," the administration of vaccines is an attractive strategy to boost adoptively transferred T cells in vivo. Several groups have shown that vaccines augment the effectiveness of adoptive T-cell therapy in preclinical animal models [70, 85, 108]. Besides provision of antigen, providing potent co-stimulation, and/or cytokines was critical for the observed effects. However, limited experience is available in humans except for an ongoing clinical trial in which patients are vaccinated with an autologous DC vaccine post α/β TCR T-cell transfer.

Lastly, reversing the immunosuppressive tumor microenvironment with small molecule inhibitors is another approach to enhance the antitumor activity of adoptively transferred T cells. For example blocking STAT3 in combination with the adoptive transfer of T cells resulted in enhanced antitumor effects [35, 41].

Conclusions

T-cell therapy has shown promising results in early phase clinical studies especially for hematological malignancies. For solid tumors, including osteosarcoma, T-cell therapy has shown promise in preclinical studies but formidable challenges remain in developing safe and effective T-cell therapies for patients with osteosarcoma. These include target antigen selection, limited in vivo T-cell expansion and persistence, T-cell trafficking to tumor sites, and the hostile tumor microenvironment. Genetic modification of T cells and combining T-cell transfer with other therapies holds the promise to overcome some of these obstacles.

Acknowledgement The authors are supported by NIH grants 1R01CA148748-01A1, 1R01CA173750-01, P01CA094237, CPRIT RP101335, Alex's Lemonade Stand Foundation, The V Foundation, and Cookies for Kid's Cancer.

Conflict of interest. The Center for Cell and Gene Therapy has a research collaboration with Celgene and bluebird bio. CD and SG have patent applications in the field of T-cell and gene-modified T-cell therapy for cancer.

References

1. Ahmed N, Brawley V, Diouf O et al (2012) T cells redirected against HER2 for the adoptive immunotherapy for HER2-positive osteosarcoma. Cancer Res 72(8 Suppl 1):Abstract
2. Ahmed N, Salsman VS, Yvon E et al (2009) Immunotherapy for osteosarcoma: genetic modification of T cells overcomes low levels of tumor antigen expression. Mol Ther 17(10):1779–1787
3. Berger C, Jensen MC, Lansdorp PM et al (2008) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest 118(1):294–305
4. Biller BJ, Guth A, Burton JH et al (2010) Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma. J Vet Intern Med 24(5):1118–1123
5. Bollard CM, Aguilar L, Straathof KC et al (2004) Cytotoxic T lymphocyte therapy for epstein-barr virus+ Hodgkin's disease. J Exp Med 200(12):1623–1633
6. Bollard CM, Dotti G, Gottschalk S et al (2012) Administration of TGF-beta resistant tumor-specific CTL to patients with EBV-associated HL and NHL. Mol Ther 20(Suppl 1):S22
7. Bollard CM, Gottschalk S, Leen AM et al (2007) Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. Blood 110(8):2838–2845
8. Bollard CM, Rossig C, Calonge MJ et al (2002) Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. Blood 99(9):3179–3187
9. Bonini C, Brenner MK, Heslop HE et al (2011) Genetic modification of T cells. Biol Blood Marrow Transplant 17(1 Suppl):S15–S20
10. Brahmer JR, Tykodi SS, Chow LQ et al (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366(26):2455–2465
11. Brenner MK, Heslop HE (2010) Adoptive T cell therapy of cancer. Curr Opin Immunol 22(2):251–257
12. Brentjens RJ, Davila ML, Riviere I et al (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med 5(177):177ra38
13. Brentjens RJ, Latouche JB, Santos E et al (2003) Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. Nat Med 9(3):279–286
14. Carpenito C, Milone MC, Hassan R et al (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A 106(9):3360–3365
15. Cheever MA, Allison JP, Ferris AS et al (2009) The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res 15(17):5323–5337
16. Cheung NK, Guo HF, Modak S et al (2003) Anti-idiotypic antibody facilitates scFv chimeric immune receptor gene transduction and clonal expansion of human lymphocytes for tumor therapy. Hybrid Hybridomics 22(4):209–218

17. Chinnasamy D, Tran E, Yu Z et al (2013) Simultaneous targeting of tumor antigens and the tumor vasculature using T lymphocyte transfer synergize to induce regression of established tumors in mice. Cancer Res 73(11):3371–3380
18. Chinnasamy D, Yu Z, Kerkar SP et al (2012) Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. Clin Cancer Res 18(6):1672–1683
19. Chinnasamy D, Yu Z, Theoret MR et al (2010) Gene therapy using genetically modified lymphocytes targeting VEGFR-2 inhibits the growth of vascularized syngenic tumors in mice. J Clin Invest 120(11):3953–3968
20. Chou J, Voong LN, Mortales CL et al (2012) Epigenetic modulation to enable antigen-specific T-cell therapy of colorectal cancer. J Immunother 35(2):131–141
21. Ciceri F, Bonini C, Stanghellini MT et al (2009) Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. Lancet Oncol 10(5):489–500
22. Cirri P, Chiarugi P (2011) Cancer associated fibroblasts: the dark side of the coin. Am J Cancer Res 1(4):482–497
23. Cohen CJ, Li YF, El-Gamil M et al (2007) Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. Cancer Res 67(8):3898–3903
24. Comoli P, Pedrazzoli P, Maccario R et al (2005) Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes. J Clin Oncol 23(35):8942–8949
25. Cooper LJ, Al Kadhimi Z, Serrano LM et al (2005) Enhanced antilymphoma efficacy of CD19-redirected influenza MP1-specific CTLs by cotransfer of T cells modified to present influenza MP1. Blood 105(4):1622–1631
26. Craddock JA, Lu A, Bear A et al (2010) Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. J Immunother 33(8): 780–788
27. Cruz CR, Gerdemann U, Leen AM et al (2011) Improving T-cell therapy for relapsed EBV-negative Hodgkin lymphoma by targeting upregulated MAGE-A4. Clin Cancer Res 17(22):7058–7066
28. Di Stasi A, Tey SK, Dotti G et al (2011) Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med 365(18):1673–1683
29. Donia M, Fagone P, Nicoletti F et al (2012) BRAF inhibition improves tumor recognition by the immune system: potential implications for combinatorial therapies against melanoma involving adoptive T-cell transfer. Oncoimmunology 1(9):1476–1483
30. Dotti G, Savoldo B, Pule M et al (2005) Human cytotoxic T lymphocytes with reduced sensitivity to Fas-induced apoptosis. Blood 105(12):4677–4684
31. Dudley ME, Yang JC, Sherry R et al (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 26(32):5233–5239
32. Eshhar Z, Waks T, Gross G et al (1993) Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc Natl Acad Sci U S A 90(2):720–724
33. Foster AE, Dotti G, Lu A et al (2008) Antitumor activity of EBV-specific T lymphocytes transduced with a dominant negative TGF-beta receptor. J Immunother 31(5):500–505
34. Franchi A, Arganini L, Baroni G et al (1998) Expression of transforming growth factor beta isoforms in osteosarcoma variants: association of TGF beta 1 with high-grade osteosarcomas. J Pathol 185(3):284–289
35. Fujita M, Zhu X, Sasaki K et al (2008) Inhibition of STAT3 promotes the efficacy of adoptive transfer therapy using type-1 CTLs by modulation of the immunological microenvironment in a murine intracranial glioma. J Immunol 180(4):2089–2098
36. Gajewski TF, Meng Y, Blank C et al (2006) Immune resistance orchestrated by the tumor microenvironment. Immunol Rev 213:131–145

37. Goff SL, Johnson LA, Black MA et al (2010) Enhanced receptor expression and in vitro effector function of a murine-human hybrid MART-1-reactive T cell receptor following a rapid expansion. Cancer Immunol Immunother 59(10):1551–1560
38. Gorlick R, Huvos AG, Heller G et al (1999) Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 17(9):2781–2788
39. Grupp SA, Kalos M, Barrett D et al (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368(16):1509–1518
40. Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21(3):309–322
41. Herrmann A, Kortylewski M, Kujawski M et al (2010) Targeting Stat3 in the myeloid compartment drastically improves the in vivo antitumor functions of adoptively transferred T cells. Cancer Res 70(19):7455–7464
42. Heslop HE, Slobod KS, Pule MA et al (2010) Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 115(5):925–935
43. Hodi FS, O'Day SJ, McDermott DF et al (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8):711–723
44. Hombach A, Heuser C, Sircar R et al (1998) An anti-CD30 chimeric receptor that mediates CD3-zeta-independent T-cell activation against Hodgkin's lymphoma cells in the presence of soluble CD30. Cancer Res 58(6):1116–1119
45. Hoyos V, Savoldo B, Quintarelli C et al (2010) Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia 24(6):1160–1170
46. Huang G, Yu L, Cooper LJ et al (2012) Genetically modified T cells targeting interleukin-11 receptor alpha-chain kill human osteosarcoma cells and induce the regression of established osteosarcoma lung metastases. Cancer Res 72(1):271–281
47. Introna M, Barbui AM, Bambacioni F et al (2000) Genetic modification of human T cells with CD20: a strategy to purify and lyse transduced cells with anti-CD20 antibodies. Hum Gene Ther 11(4):611–620
48. Ishikura H, Ikeda H, Abe H et al (2007) Identification of CLUAP1 as a human osteosarcoma tumor-associated antigen recognized by the humoral immune system. Int J Oncol 30(2): 461–467
49. Jacobs JF, Brasseur F, Hulsbergen-van de Kaa CA et al (2007) Cancer-germline gene expression in pediatric solid tumors using quantitative real-time PCR. Int J Cancer 120(1):67–74
50. John LB, Devaud C, Duong CM et al (2013) Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. Clin Cancer Res 19(20): 5636–5646
51. Johnson LA, Morgan RA, Dudley ME et al (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114(3):535–546
52. June C, Rosenberg SA, Sadelain M et al (2012) T-cell therapy at the threshold. Nat Biotechnol 30(7):611–614
53. Kakarla S, Chow KK, Mata M et al (2013) Antitumor effects of chimeric receptor engineered human T cells directed to tumor stroma. Mol Ther 21:1611–1620
54. Kalos M, Levine BL, Porter DL et al (2011) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 3(95):95ra73
55. Keir ME, Butte MJ, Freeman GJ et al (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677–704
56. Kerkar SP, Leonardi AJ, van Panhuys N (2013) Collapse of the tumor stroma is triggered by IL-12 induction of Fas. Mol Ther 21(7):1369–1377
57. Kershaw MH, Wang G, Westwood JA et al (2002) Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum Gene Ther 13(16): 1971–1980

58. Kershaw MH, Westwood JA, Parker LL et al (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res 12(20 Pt 1):6106–6115
59. Kloss CC, Condomines M, Cartellieri M et al (2013) Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat Biotechnol 31(1):71–75
60. Kochenderfer JN, Dudley ME, Feldman SA et al (2012) B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood 119(12):2709–2720
61. Kolb HJ (2008) Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood 112(12):4371–4383
62. Lamers CH, Sleijfer S, Vulto AG et al (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol 24(13):e20–e22
63. Lanitis E, Poussin M, Klattenhof A et al (2013) Chimeric antigen receptor T cells with dissociated signaling domains exhibit focused antitumor activity with reduced potential for toxicity *in vivo*. Cancer Immunol Res (OnLine First April 7, 2013)
64. Leen AM, Bollard CM, Mendizabal AM et al (2013) Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. Blood 121(26):5113–5123
65. Leen AM, Myers GD, Sili U et al (2006) Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat Med 12(10):1160–1166
66. Leen AM, Rooney CM, Foster AE (2007) Improving T cell therapy for cancer. Annu Rev Immunol 25:243–265
67. Leen A, Katari U, Keiman J et al (2011) Improved expansion and anti-tumor activity of tumor-specific CTLs using a transgenic chimeric cytokine receptor. Mol Ther 19(S1):S194
68. Levine BL, Rapoport AP, Stadtmauer EA et al (2013) Safety and correlates of clinical response in an early phase clinical trial in multiple myeloma patients post auto-SCT and adoptive immunotherapy with engineered T cells expressing an HLA-A2 restricted affinity-enhanced TCR for LAGE-1 and NY-ESO-1. Mol Ther 21(S1):S114
69. Liu C, Peng W, Xu C et al (2013) BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. Clin Cancer Res 19(2):393–403
70. Lou Y, Wang G, Lizee G et al (2004) Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. Cancer Res 64(18):6783–6790
71. Louis CU, Savoldo B, Dotti G et al (2011) Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 118(23): 6050–6056
72. Louis CU, Straathof K, Bollard CM et al (2010) Adoptive transfer of EBV-specific T cells results in sustained clinical responses in patients with locoregional nasopharyngeal carcinoma. J Immunother 33(9):983–990
73. Maher J (2012) Immunotherapy of malignant disease using chimeric antigen receptor engrafted T cells. ISRN Oncol 2012:278093
74. Maus MV, Linette GP, Stadtmauer EA et al (2013) Cardiovascular toxicity and Titin cross-reactivity of affinity-enhanced TCR-engineered T cells against HLA-A1 restricted MAGE-A3 antigen. Mol Ther 21(suppl):S24
75. Modak S, Kramer K, Gultekin SH et al (2001) Monoclonal antibody 8H9 targets a novel cell surface antigen expressed by a wide spectrum of human solid tumors. Cancer Res 61(10):4048–4054
76. Morgan RA, Chinnasamy N, Abate-Daga D et al (2013) Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother 36(2):133–151
77. Morgan RA, Dudley ME, Wunderlich JR et al (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314(5796):126–129

78. Morgan RA, Yang JC, Kitano M et al (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 18(4):843–851
79. Morris CD, Gorlick R, Huvos G et al (2001) Human epidermal growth factor receptor 2 as a prognostic indicator in osteogenic sarcoma. Clin Orthop Relat Res 382:59–65
80. Myers D, Jun J, Rooney C et al (2013) Administration of GD2 chimeric antigen receptor modified, tri-virus specific cytotoxic T lymphocytes after HLA mismatched allogeneic stem cell transplantation for relapsed, refractory neuroblastoma. Mol Ther 22(S1):S5–S6
81. Niederman TM, Ghogawala Z, Carter BS et al (2002) Antitumor activity of cytotoxic T lymphocytes engineered to target vascular endothelial growth factor receptors. Proc Natl Acad Sci U S A 99(10):7009–7014
82. Okamoto S, Mineno J, Ikeda H et al (2009) Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR. Cancer Res 69(23):9003–9011
83. Orentas RJ (2013) Reading the tea leaves of tumor-mediated immunosuppression. Clin Cancer Res 19(5):955–957
84. Orentas RJ, Lee DW, Mackall C (2012) Immunotherapy targets in pediatric cancer. Front Oncol 2:3
85. Overwijk WW, Theoret MR, Finkelstein SE et al (2003) Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. J Exp Med 198(4):569–580
86. Park JR, Digiusto DL, Slovak M et al (2007) Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. Mol Ther 15(4):825–833
87. Parkhurst MR, Yang JC, Langan RC et al (2011) T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther 19(3):620–626
88. Peggs KS, Quezada SA, Allison JP (2009) Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. Clin Exp Immunol 157(1):9–19
89. Pegram HJ, Lee JC, Hayman EG et al (2012) Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. Blood 119(18):4133–4141
90. Perna SK, De AB, Pagliara D et al (2013) Interleukin 15 provides relief to CTLs from regulatory T cell-mediated inhibition: implications for adoptive T cell-based therapies for lymphoma. Clin Cancer Res 19(1):106–117
91. Polyak K, Haviv I, Campbell IG (2009) Co-evolution of tumor cells and their microenvironment. Trends Genet 25(1):30–38
92. Porter DL, Levine BL, Kalos M et al (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365(8):725–733
93. Pule MA, Savoldo B, Myers GD et al (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 14(11):1264–1270
94. Pule MA, Straathof KC, Dotti G et al (2005) A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther 12(5): 933–941
95. Quintarelli C, Vera JF, Savoldo B et al (2007) Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. Blood 110(8): 2793–2802
96. Rabinovich GA, Gabrilovich D, Sotomayor EM (2007) Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 25:267–296
97. Rainusso N, Brawley VS, Ghazi A et al (2012) Immunotherapy targeting HER2 with genetically modified T cells eliminates tumor-initiating cells in osteosarcoma. Cancer Gene Ther 19(3):212–217
98. Restifo NP, Dudley ME, Rosenberg SA (2012) Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol 12(4):269–281

99. Robbins PF, Morgan RA, Feldman SA et al (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29(7):917–924
100. Roberts EW, Deonarine A, Jones JO et al (2013) Depletion of stromal cells expressing fibroblast activation protein-alpha from skeletal muscle and bone marrow results in cachexia and anemia. J Exp Med 210(6):1137–1151
101. Rosenberg SA, Restifo NP, Yang JC et al (2008) Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 8(4):299–308
102. Rossig C, Bollard CM, Nuchtern JG et al (2001) Targeting of G(D2)-positive tumor cells by human T lymphocytes engineered to express chimeric T-cell receptor genes. Int J Cancer 94(2):228–236
103. Rouleau C, Curiel M, Weber W et al (2008) Endosialin protein expression and therapeutic target potential in human solid tumors: sarcoma versus carcinoma. Clin Cancer Res 14(22):7223–7236
104. Sadelain M, Brentjens R, Riviere I (2013) The basic principles of chimeric antigen receptor design. Cancer Discov 3(4):388–398
105. Savoldo B, Ramos CA, Liu E et al (2011) CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. J Clin Invest 121(5):1822–1826
106. Savoldo B, Rooney CM, Di Stasi A et al (2007) Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30zeta artificial chimeric T-cell receptor for immunotherapy of Hodgkin disease. Blood 110(7):2620–2630
107. Schuberth PC, Jakka G, Jensen SM et al (2013) Effector memory and central memory NY-ESO-1-specific re-directed T cells for treatment of multiple myeloma. Gene Ther 20(4):386–395
108. Song XT, Turnis M, Zhou X et al (2010) A Th1-inducing adenoviral vaccine for boosting adoptively transferred T cells. Mol Ther 19(1):211–217
109. Stephan MT, Ponomarev V, Brentjens RJ et al (2007) T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection. Nat Med 13(12):1440–1449
110. Straathof KC, Bollard CM, Popat U et al (2005) Treatment of nasopharyngeal carcinoma with Epstein-Barr Virus-specific T lymphocytes. Blood 105:1898–1904
111. Straathof KC, Pule MA, Yotnda P et al (2005) An inducible caspase 9 safety switch for T-cell therapy. Blood 105(11):4247–4254
112. Sun J, Dotti G, Huye LE et al (2010) T cells expressing constitutively active Akt resist multiple tumor-associated inhibitory mechanisms. Mol Ther 18(11):2006–2017
113. Sutherland CM, Krementz ET, Hornung MO et al (1976) Transfer of in vitro cytotoxicity against osteogenic sarcoma cells. Surgery 79(6):682–685
114. Terakura S, Yamamoto TN, Gardner RA et al (2012) Generation of CD19-chimeric antigen receptor modified CD8+ T cells derived from virus-specific central memory T cells. Blood 119(1):72–82
115. Tivol EA, Borriello F, Schweitzer AN et al (1995) Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 3(5):541–547
116. Torikai H, Reik A, Liu PQ et al (2012) A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. Blood 119(24):5697–5705
117. Tran E, Chinnasamy D, Yu Z et al (2013) Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia. J Exp Med 210(6):1125–1135
118. Tsukahara T, Kawaguchi S, Torigoe T et al (2008) Prognostic impact and immunogenicity of a novel osteosarcoma antigen, papillomavirus binding factor, in patients with osteosarcoma. Cancer Sci 99(2):368–375
119. Urakawa H, Nishida Y, Nakashima H et al (2009) Prognostic value of indoleamine 2,3-dioxygenase expression in high grade osteosarcoma. Clin Exp Metastasis 26(8):1005–1012

120. Uttenthal BJ, Chua I, Morris EC et al (2012) Challenges in T cell receptor gene therapy. J Gene Med 14(6):386–399
121. van Der Bruggen P, Zhang Y, Chaux P et al (2002) Tumor-specific shared antigenic peptides recognized by human T cells. Immunol Rev 188:51–64
122. Vanneman M, Dranoff G (2012) Combining immunotherapy and targeted therapies in cancer treatment. Nat Rev Cancer 12(4):237–251
123. Watanabe N, Anurathapan U, Brenner M et al (2013) Transgenic expression of a novel immunosuppressive signal converter on T cells. Mol Ther 22(S1):S153
124. Waterhouse P, Penninger JM, Timms E et al (1995) Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 270(5238):985–988
125. Wilkie S, Burbridge SE, Chiapero-Stanke L et al (2010) Selective expansion of chimeric antigen receptor-targeted T-cells with potent effector function using interleukin-4. J Biol Chem 285(33):25538–25544
126. Wilkie S, van Schalkwyk MC, Hobbs S et al (2012) Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. J Clin Immunol 32(5):1059–1070
127. Willemsen RA, Debets R, Hart E et al (2001) A phage display selected fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes. Gene Ther 8(21):1601–1608
128. Yang L, Pang Y, Moses HL (2010) TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. Trends Immunol 31(6):220–227
129. Yu AL, Uttenreuther-Fischer MM, Huang CS et al (1998) Phase I trial of a human-mouse chimeric anti-disialoganglioside monoclonal antibody ch14.18 in patients with refractory neuroblastoma and osteosarcoma. J Clin Oncol 16(6):2169–2180
130. Yuan D, Liu B, Liu K et al (2013) Overexpression of fibroblast activation protein and its clinical implications in patients with osteosarcoma. J Surg Oncol 108(3):157–162
131. Zhang L, Kerkar SP, Yu Z et al (2011) Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment. Mol Ther 19(4):751–759

Natural Killer Cells for Osteosarcoma

Nidale Tarek and Dean A. Lee

Abstract Natural killer (NK) cells are lymphocytes of the innate immune system that have the ability to recognize malignant cells through detection of a variety of cell-surface indicators of stress and danger. Once activated through such recognition, NK cells release cytokines and induce target cell lysis through a variety of mechanisms. NK cells are increasingly recognized as important mediators of other immunotherapeutic modalities, including cytokines, antibodies, immunomodulators, and stem cell transplantation. Adoptive immunotherapies with NK cells are being tested in early-stage clinical trials, and recent advances in manipulating their number and function have caused a renewed emphasis on this cancer-fighting cell. In this chapter we address the evidence for NK cell recognition of osteosarcoma in vitro and in vivo, discuss new therapies that are directly or indirectly dependent on NK cell function, and describe potential approaches for manipulating NK cell number and function to enhance therapy against osteosarcoma.

Keywords Natural killer cells • Adoptive immunotherapy • Immunomodulation • Monoclonal antibodies • NKG2D

Brief Overview of NK Cell Biology

NK cells are lymphocytes of the innate immune system with cytotoxic and regulatory functions, which are critical mediators of immune responses against infections and malignancies [1]. Unlike adaptive T and B lymphocytes, NK cells are characterized by their ability to recognize such targets without prior sensitization. Human NK cells comprise approximately 10–15 % of all peripheral blood lymphocytes and are

N. Tarek, M.D. • D.A. Lee, M.D., Ph.D. (✉)
Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX, USA
e-mail: NTarek@mdanderson.org; dalee@mdanderson.org

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7_19,

identified by the lack of CD3 and the presence of CD56 and/or CD16. NK cells express several classes of activating receptors that recognize proteins which are upregulated by cell stress or are of nonself origin, and are negatively regulated by inhibitory receptors that primarily bind human leukocyte antigens (HLA) as a mechanism of self-recognition. NK-cell effector function, including target cytotoxicity, is triggered when the balance of activating and inhibiting signals is tipped towards activation.

Activating and Inhibitory Receptors

Several families of activating receptors have been characterized, including CD16 (FcRγIIIa), natural cytotoxicity receptors (NCR), and NK Group 2 (NKG2)-family lectin-like receptors. CD16 is the low-affinity Fc receptor which binds the Fc portion of human IgG1 and IgG3, mediating antibody-dependent cell cytotoxicity (ADCC) of antibody-labeled cells [2]. The NCRs (NKp30, NKp44, and NKp46) are activating receptors that bind virus- and stress-related proteins, with expression mostly restricted to NK cells [3]. The receptors of the NKG2 family are expressed as heterodimers with CD94, except for NKG2D which is expressed as homodimer [4]. NKG2D, the major activating receptor in this family, recognizes MHC-class-I-related Chain A or B (MICA/B) and members of the UL-16 binding protein (ULBP)-family, which are increased in response to cellular stress.

Killer Immunoglobulin-like Receptors (KIR) have cytoplasmic domains comprised of a short immunoreceptor tyrosine-based activating motif (ITAM) or a long immunoreceptor tyrosine-based inhibition motif (ITIM) [4]. The primary inhibitory receptors in NK cells are the long-tailed KIRs and NKG2A, which serve to recognize self by binding to HLA class I molecules, preventing NK-mediated lysis of cells with normal HLA expression.

Fifteen closely related KIR genes are located on chromosome 19q13.4 and are present or absent in many haplotype combinations such that most individuals lack one or more KIR genes. In addition to their haplotype variability, KIR genes are highly polymorphic, are variably expressed between NK cells, and functional reactivity is educated by interaction with the host HLA haplotypes. Thus, the NK-cell repertoire varies greatly between individuals. The allelic variations in KIR have been grouped into A and B haplotypes [5], with B haplotypes having greater numbers of activating KIR genes. Allogeneic transplants from donors with the "B" haplotype are predicted to have superior NK cell-mediated antitumor effects [6].

Inhibitory KIRs are specific for HLA isotypes on the basis of conserved amino acid residues at position 80. Approximately half of HLA-C alleles have the amino acid asparagine (N) at residue 80—referred to as Group C1—which confers binding to KIR2DL2 and KIR2DL3. The other half of the C alleles codes for lysine (K) at residue 80 (Group C2), which confers binding to KIR2DL1. Similarly, about 40 % of HLA-B alleles carry the supertypic serologic epitope HLA-Bw4 (defined primarily by threonine (T) at residue 80), which confers binding to KIR3DL1.

The presence of the HLA ligand regulates the activity of these KIRs during NK cell development through a process called licensing. Thus, given both parental alleles it is possible for the HLA type of an individual to restrict NK cell licensing to as few as one (e.g., C2/C2 homozygous and Bw4–) or as many as three (C1/C2 heterozygous and Bw4+) inhibitory KIR.

This HLA-biased education without HLA-restricted antigen recognition (as for T cells), gave rise to the "missing-self hypothesis," which postulates that NK cells recognize and destroy autologous cells with lost or altered self-HLA class I molecules [7]. However, classical HLA class I is not always required to protect from NK-cell mediated cytotoxicity, nor is it always sufficient to prevent NK-cell cytotoxicity [8].

Mechanisms of NK Cell-Mediated Killing

Upon receiving a predominance of activating signals, NK cells release granules containing perforin and granzymes directed towards the target cell. The perforins form a pore in the cell membrane, allowing entry of the granzymes to the cytoplasm to induce apoptosis by direct activation of caspase-3 [9]. NK cell activation also results in increased expression of death receptor ligands on the NK cell, such as Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [10], which induce apoptosis via associated death receptors on target cells [11–13]. In addition to these pathways, NK cells also produce several cytokines such as IFN-γ, which are important in mediating the adaptive immune response against cancer [14].

Evidence for NK Cell Activity in Osteosarcoma

NK Cell Function in Osteosarcoma Patients

NK cells play a critical role in tumor surveillance and low NK cell cytotoxicity in older adults is associated with increased risk of developing cancer [15]. Clinical data show that NK cells may play an important role in osteosarcoma (OS) prevention and treatment response. In patients with OS, a lower number of circulating NK cells was observed in peripheral blood compared to normal controls [16] suggesting that NK cells play a preventive role in OS tumor development. Furthermore, patients undergoing treatment for OS demonstrate better survival outcome with faster absolute lymphocyte recovery compared to patient with slow lymphocyte recovery [17], denoting the antitumor role of the immune system in treatment response. Finally, patients with OS treated with Il-2 in addition to polychemotherapy and surgery showed augmentation in the number and activity of NK cells, the magnitude of which correlated with an improved clinical outcome [18].

Whereas NK cells in patients with several types of cancer have been shown to have poor function, NK cells isolated from patients with OS were shown to be functionally and phenotypically unimpaired, have intact IFN signaling, and demonstrated cytolytic activity against autologous and allogeneic OS cells and other target cells [19, 20].

Expression of NK Ligands

The susceptibility of tumor cells to NK cell lysis is regulated by the proportion of inhibiting and activating signals perceived upon interaction of NK cells with the target cell. It correlates negatively with expression of HLA class I antigens and positively with intercellular adhesion molecules and activating ligands on the surface of tumor cells.

Downregulation of HLA class I antigens on the cell surface can be induced by stress conditions and is correlated with increased susceptibility to NK cell killing through decreased signaling by inhibitory KIRs, a phenomenon described as "missing-self." In vitro experiments with OS cell lines of varying levels of HLA class I antigen expression show that OS cells with surface expression of HLA are less susceptible to killing by NK cells compared to cells lacking cell surface HLA; moreover, downregulation of cell-surface HLA enhances the sensitivity of NK-resistant OS cells to NK killing. Similarly, OS target cell killing correlates with their degree of KIR-HLA incompatibility with the NK cells [21]. In vivo, OS primary and metastatic tumors have been shown to lose or downregulate HLA class I expression, thus becoming more susceptible to NK cell killing [22].

Expression of cell adhesion molecules renders tumor cells more susceptibility to NK-mediated lysis; these molecules fortify cell-to-cell interactions and provide co-stimulatory signals that enhance the cytotoxic activity of NK cells [23, 24]. Expression of the adhesion molecules CD54 and CD58 increases the bond between target and effector cells and correlates positively with the susceptibility of OS cells to NK lysis [25–27]. In vivo, lack of CD54 expression allows the circulation of tumor cells, avoids establishing stable cytolytic conjugates, and provides means of evading NK spontaneous lysis [28].

Several activating receptor–ligand interactions have been implicated in the interaction of NK cells with OS cells. Ligands for NKG2D and DNAM-1 activating receptors (MICA/B, ULBP, PVR, and nectin-2) are widely expressed on OS cell lines and OS tumor samples [19, 29], rendering them more sensitive to NK recognition and killing. Cytolysis of OS cells is dependent on NKG2D and DNAM-1 pathways and blockade of both pathways is required for optimal inhibition of activated NK cells; activation through NKG2D and DNAM-1 pathways also overcomes inhibition of NK cells mediated by KIR–HLA interaction [19]. In vivo, the level of MICA expression on OS cells has been correlated with staging; expression of MICA is higher in patients with early stage disease compared to late stage, suggesting a role for MICA-NKG2D mediated NK control of OS [29], and downregulation of MICA appears to be a common immune escape mechanism [30]. Unlike other

tumor types, MICA expression on OS tumor cells is unaltered by exposure to chemotherapy [19]. NK cell recognition of OS tumor cell has also been described via the NCR receptors, although the ligands on OS cells for these receptors is unknown.

Mechanisms of Killing

NK cells exert direct and indirect antitumor activity and kill target tumor cells via release of perforin and granzyme containing granules, secretion of cytokines and effector molecules, ligation of death receptors, and ADCC through the CD16 receptor.

OS cell lines and freshly isolated OS tumor cells are susceptible to the cytolytic activity of activated NK cells [31]. The release of granules containing granzyme B (GrB) into the target cell cytoplasm is a predominant pathway involved in NK cell killing of OS cells; blocking GrB results in complete abrogation of NK-mediated OS lysis [19]. NK cytotoxicity to OS cells is enhanced by Fc–FcγR interaction; epidermal growth factor receptor (EGFR)-expressing OS cells are more susceptible to NK killing in the presence anti-EGFR monoclonal antibody (MoAb) compared to EGFR-negative OS cells [32]. The Fas-Fas ligand mediated apoptosis, an important process of target killing by NK cells, appears at least in vitro to play only a minor role in the interaction between NK cells and OS cells [19].

Mechanisms of Immune Escape

Tumor cells may acquire diverse mechanisms to evade NK cell recognition [33]. No or low expression of adhesion molecules or ligands for activating receptors and/or increased expression of ligands for inhibitory receptors are described mechanisms adapted by tumor cells to evade NK cell surveillance. In addition, shedding of NKG2D ligands (soluble sMICA) from the membrane of tumor cells can impair NKG2D-mediated cytotoxicity by blocking the NKG2D receptors on NK cells. Furthermore, secretion of immunosuppressive cytokines and transforming growth factor-β has been associated with defective NK cell function, restricting tumor cell recognition and killing.

Both classical and nonclassical HLA class I molecules, which are ligands for inhibitory KIR and CD94/NKG2A receptors, are expressed on some OS naïve tumors and may be increased in OS cells when exposed to chemotherapy [19].

OS cell lines and tumor sample show higher expression of surface MICA compared to normal bone tissue and benign bone tumors making them theoretically more susceptible to NK cells killing. However, soluble MICA was detected in the serum of some patient with OS resulting in diminished NKG2D expression on NK cells and decreased tumor cell killing. Clinical correlation showed that in patients with OS, elevated MICA expression combined with increased soluble MICA was

associated with decreased NKG2D expression on PBMC, and this combination correlated significantly with advanced and metastatic disease [29, 34]. With progression of OS, expression of MICA decreases, soluble MICA increases, and expression of NKG2D on NK cells decreases [29].

Indirect Activation of NK Cell Function

In patients with OS, NK cell abnormalities have been described including lower numbers of circulating cells and decreased expression of activating receptors; these NK cells, however, have normal cytolytic activity following stimulation in vitro. The antitumor activity of NK cells can be modulated and enhanced by monoclonal antibodies, cytokines, or immunomodulators.

Monoclonal Antibodies

ADCC by NK cells requires interaction between the Fc receptor (CD16) on NK cells and the Fc region of an antibody binding to an antigen on the tumor cell surface, resulting in NK cell activation and degranulation toward the target cell.

EGFR is expressed on 90 % of OS tumor samples [35]. Cetuximab, a MoAb targeting EGFR, increases NK-dependent lysis of EGFR-expressing sarcomas. Importantly, the sensitivity to cetuximab-enhanced lysis by resting NK cells is comparable among most EGFR-expressing cell lines, including chemotherapy-resistant OS cells [32]. Although prolonged OS/NK cell cocultures and excess of tumor cells in culture results in diminished NK cell cytotoxicity secondary to downregulation of activating receptors on NK cell surface, ADCC killing of OS by NK cells is unaltered by this suppressive mechanism [36].

Cytokines

Cytokines may act directly on tumor cells as anti-proliferative agents and indirectly via activation of cellular immune agents such as NK cells leading to increased lysis of tumor cells.

Interleukin (IL)-15 potentiates the cytolytic activity of NK cells by increasing NKG2D expression on cell surface and enhancing GrB release upon activation. IL-15 activation reverses impaired expression of NKG2D and DNAM-1 and impaired NK cell cytotoxicity induced by prolonged cocultures of NK cells with OS cells, and NK cells activated with IL-15 prior to coculture with OS cells do not downregulate activating receptors and preserve functional activity despite prolonged exposure to target cells [36]. IL-2 and IL-12 increase cytotoxicity of NK cells to NK-sensitive and NK-resistant OS cell lines by increasing the density of CD18 and

CD2 receptors on the NK cell surface, enhancing the conjugate-forming capacity of NK cells to OS targets [37]. Importantly, targeted application of IL-2 to the lung by aerosolized delivery markedly improves the migration of adoptively transferred NK cells into lung metastasis, resulting in enhanced control of metastatic disease [38].

IL-12 increases expression of ICAM-1 (a ligand for CD18) on OS cell lines cocultured with PBMCs in cell-to-cell contact [39]. In a mouse model of metastatic osteosarcoma, mice bearing pulmonary metastasis treated with IL-12 showed decreased number and size of pulmonary metastasis mediated by NK cells [40]. IFN potentiates NK-mediated lysis of OS cell lines, IFN-conjugated antibodies specifically localize tumor cells in a mouse xenograft tumor model and further increase NK cell activation and tumor cell lysis [41, 42]. IL-17 augments expression of fibronectin on OS cell lines that express the IL-17 receptor, mediating increased adhesion of NK cells to OS cells and thus enhancing NK cytotoxicity. IL-17 has no direct effect on NK cells function [43].

Chemotherapy

As mentioned above, chemotherapy appears to increase expression of inhibitory ligands, but does not increase MICA [19]. Chemotherapy does increase sensitivity to ADCC by NK cells [36], and both gemcitabine [44] and cisplatin [45] may increase sensitivity of OS to direct NK cell lysis by upregulation of Fas or downregulation of anti-apoptotoic proteins.

Immunomodulators

In addition to MoAbs and cytokines, a variety immunomodulatory drugs have been successfully combined with NK cells to potentiate their antitumor activity and treat human malignancies [46–48].

In the setting of OS, the activity of NK cells may be weakened or enhanced by immune-modulating agents. Sodium valproate (an HDAC inhibitor) and hydralazine (a DNA-methylation inhibitor) increase the expression of MICA and MICB on OS cells, but not sMICA in serum, and therefore increase the susceptibility of tumor cells to NK cell lysis [49, 50]. Moreover, hydralazine increases cell surface expression of Fas and augments Fas-induced OS cell death, whereas valproic acid sensitizes OS cells to Fas-mediated cell death and decreases production of soluble Fas [49, 50], thus further potentiating OS sensitivity to NK cell killing. However, both HDAC inhibition [51] and DNA hypomethylation [52] can have an adverse direct effect on NK cell function, necessitating approaches that sequence drug therapy and cell therapy. A narrow-spectrum HDAC inhibitor, SNDX-275, has been shown to increase osteosarcoma killing through upregulation of Fas [53], c-FLIP [54], and MICA [52], and also augments NK cell function through upregulation of NKG2D [30].

Lenalidomide is an immunomodulatory thalidomide derivative with activity against a wide variety of cancers. Lenalidomide may enhance NK cell number and maturation through increased IL-15 levels [55]. Lenalidomide augments the activity of NK cells by enhancing ADCC of MoAbs against solid tumors [56], including trastuzumab and cetuximab activity against bone sarcomas [57]. Mifamurtide (MTP-PE), discussed extensively in chapter "IL-11Rα: A Novel Target for the Treatment of Osteosarcoma," may exert some of its anticancer effect by enhancing NK cell activity [58].

Heat treatment of OS cell lines increases their susceptibility to NK-cell-mediated lysis through upregulation heat-shock-protein 72 (HSP72) expression [59]. Hypoxia decreases the expression of MICA on OS cell lines in a hypoxia-inducible factor 1α (HIF-1α)-dependent manner and consequently decreases the susceptibility of tumor cells to NK cell lysis [60]. However, hypoxia does not interfere with MoAb-mediated target cell killing by ADCC [61].

NK Cell Adoptive Immunotherapy

Clinical NK Cell Sources and Trials

NK cells may be obtained in numbers sufficient for clinical use in adoptive immunotherapy by apheresis and CD3 depletion, or by *ex vivo* expansion. NK cells have been successfully expanded from peripheral blood, cord blood, and pluripotent or embryonic stem cells. Expansion methods have included various combinations of cytokines, cytokine fusion proteins, cytokines and OKT3, cytokines and stromal support, antibody-coated beads, and feeder cells obtained from peripheral blood or derived from EBV-lymphoblastoid cell lines or K562 (reviewed in [62]).

NK cells have been delivered by adoptive transfer to very few patients with osteosarcoma. Expanded NK cells were given as adjuvant immunotherapy after matched allogeneic transplant (C. Mackall, personal communication—ClinicalTrials.gov Identifier NCT01287104). As mentioned above, KIR-ligand incompatibility is associated with increased NK cell activity against osteosarcoma cell lines [21]. Thus, similar to the observed benefit in AML, it is likely that approaches using mismatched allogeneic donors for NK cell therapy of osteosarcoma will have greater antitumor effect than matched or autologous NK cells. NK92 is a cell line derived from a patient with NK cell leukemia and has NK cell-like activity against tumor cell lines. Clinical grade irradiated NK92 cells have been infused in a patient with advanced osteosarcoma, though no response to treatment was observed [63].

Future Approaches

The recent availability of clinically viable approaches for obtaining large number of NK cells now enables the clinical testing of combination therapies to enhance NK cell function and osteosarcoma sensitivity. Genetic modification of NK cells may

further enhance their activity against osteosarcoma. The antigen-binding domains of all of the MoAbs mentioned above have been identified and genetically manipulated to generate chimeric antigen receptors (CAR) that mediate enhanced killing by T cells, as described in chapter "Using Canine Osteosarcoma as a Model to Assess Efficacy of Novel Therapies: Can Old Dogs Teach Us New Tricks?" These CAR also have potential application for clinical development in NK cells, and CAR with NKG2D-like specificity can further improve the NK cell immunotherapy of osteosarcoma in murine models [64]. The ability to deliver large cell doses, combination with sensitizing chemotherapy, radiation, or immunomodulatory drugs, and genetic modifications will be the subjects of cutting edge trials in the decade to come.

References

1. Raulet DH, Guerra N (2009) Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nat Rev Immunol 9(8):568–580. doi:10.1038/nri2604
2. Trinchieri G (1989) Biology of natural killer cells. Adv Immunol 47:187–376
3. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19:197–223. doi:10.1146/annurev.immunol.19.1.197, 19/1/197 [pii]
4. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH (2011) Activating and inhibitory receptors of natural killer cells. Immunol Cell Biol 89(2):216–224. doi:10.1038/icb.2010.78
5. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, Lanier LL, Parham P (1997) Human diversity in killer cell inhibitory receptor genes. Immunity 7(6):753–763
6. McQueen KL, Dorighi KM, Guethlein LA, Wong R, Sanjanwala B, Parham P (2007) Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. Hum Immunol 68(5):309–323. doi:10.1016/j.humimm.2007.01.019
7. Ljunggren HG, Karre K (1990) In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today 11(7):237–244
8. Farag SS, Caligiuri MA (2006) Human natural killer cell development and biology. Blood Rev 20(3):123–137. doi:10.1016/j.blre.2005.10.001, S0268-960X(05)00055-X [pii]
9. Browne KA, Blink E, Sutton VR, Froelich CJ, Jans DA, Trapani JA (1999) Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol 19(12):8604–8615
10. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B (1998) Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. J Exp Med 188(12):2375–2380
11. Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, Okamura H, Nakanishi K, Okumura K, Yagita H (1999) Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. J Immunol 163(4):1906–1913
12. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, Yagita H, Okumura K (2001) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J Exp Med 193(6):661–670
13. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Yamaguchi N, Yagita H, Okumura K (2001) Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated

and IFN-gamma-dependent suppression of subcutaneous tumor growth. Cell Immunol 214(2): 194–200. doi:10.1006/cimm.2001.1896
14. Smyth MJ, Hayakawa Y, Takeda K, Yagita H (2002) New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer 2(11):850–861. doi:10.1038/nrc928
15. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K (2000) Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet 356(9244):1795–1799. doi:10.1016/S0140-6736(00)03231-1, S0140-6736(00)03231-1 [pii]
16. Markiewicz K, Zeman K, Kozar A, Golebiowska-Wawrzyniak M, Wozniak W (2012) Evaluation of selected parameters of cellular immunity in children with osteosarcoma at diagnosis. Med Wieku Rozwoj 16(3):212–221
17. Moore C, Eslin D, Levy A, Roberson J, Giusti V, Sutphin R (2010) Prognostic significance of early lymphocyte recovery in pediatric osteosarcoma. Pediatr Blood Cancer 55(6):1096–1102. doi:10.1002/pbc.22673
18. Luksch R, Perotti D, Cefalo G, Gambacorti Passerini C, Massimino M, Spreafico F, Casanova M, Ferrari A, Terenziani M, Polastri D, Gambirasio F, Podda M, Bozzi F, Ravagnani F, Parmiani G, Fossati Bellani F (2003) Immunomodulation in a treatment program including pre- and post-operative interleukin-2 and chemotherapy for childhood osteosarcoma. Tumori 89(3):263–268
19. Buddingh EP, Schilham MW, Ruslan SE, Berghuis D, Szuhai K, Suurmond J, Taminiau AH, Gelderblom H, Egeler RM, Serra M, Hogendoorn PC, Lankester AC (2011) Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. Cancer Immunol Immunother 60(4):575–586. doi:10.1007/s00262-010-0965-3
20. Buddingh EP, Ruslan SE, Berghuis D, Gelderblom H, Anninga JK, Hogendoorn PC, Egeler RM, Schilham MW, Lankester AC (2012) Intact interferon signaling in peripheral blood leukocytes of high-grade osteosarcoma patients. Cancer Immunol Immunother 61(6):941–947. doi:10.1007/s00262-012-1232-6
21. Delgado D, Webster DE, DeSantes KB, Durkin ET, Shaaban AF (2010) KIR receptor-ligand incompatibility predicts killing of osteosarcoma cell lines by allogeneic NK cells. Pediatr Blood Cancer 55(7):1300–1305. doi:10.1002/pbc.22665
22. Tsukahara T, Kawaguchi S, Torigoe T, Asanuma H, Nakazawa E, Shimozawa K, Nabeta Y, Kimura S, Kaya M, Nagoya S, Wada T, Yamashita T, Sato N (2006) Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. Cancer Sci 97(12):1374–1380. doi:10.1111/j.1349-7006.2006.00317.x, CAS317 [pii]
23. Chong AS, Boussy IA, Jiang XL, Lamas M, Graf LH Jr (1994) CD54/ICAM-1 is a costimulator of NK cell-mediated cytotoxicity. Cell Immunol 157(1):92–105. doi:10.1006/cimm.1994.1208, S0008-8749(84)71208-1 [pii]
24. Tarozzi A, Mariani E, Facchini A (1995) In vitro cytolytic activity of human NK cells against osteosarcoma cell lines. Boll Soc Ital Biol Sper 71(7–8):221–226
25. Mariani E, Tarozzi A, Meneghetti A, Cattini L, Facchini A (1998) TNF-alpha but not IL-1 and IL-6 modifies the susceptibility of human osteosarcoma cells to NK lysis. Int J Oncol 13(2):349–353
26. Mariani E, Tarozzi A, Meneghetti A, Cattini L, Facchini A (1997) Human osteosarcoma cell susceptibility to natural killer cell lysis depends on CD54 and increases after TNF alpha incubation. FEBS Lett 406(1–2):83–88
27. Meneghetti A, Mariani E, Santi S, Riccio M, Cattini L, Paoletti S, Facchini A (1999) NK binding capacity and lytic activity depend on the expression of ICAM-1 on target bone tumours. Int J Oncol 15(5):909–914
28. Zamai L, Zauli G, Bavelloni A, Marmiroli S, Cataldi A, Weber G, Vitale M (1995) Tiazofurin induces a down-modulation of ICAM-1 expression on K562 target cells impairing NK adhesion and killing. Cell Immunol 164(1):100–104. doi:10.1006/cimm.1995.1147, S0008-8749(85)71147-1 [pii]
29. Xiao P, Xue L, Che LH, Peng JJ, Wu HX, Li Y, Qiao H (2008) Expression and roles of MICA in human osteosarcoma. Histopathology 52(5):640–642. doi:10.1111/j.1365-2559.2008.02989.x, HIS2989 [pii]

30. Zhu S, Denman CJ, Cobanoglu ZS, Kiany S, Lau CC, Gottschalk SM, Hughes DPM, Kleinerman ES, Lee DA (2013) The narrow-spectum HDAC inhibitor entinostat enhances NKG2D expression without NK cell toxicity, leading to enhanced recognition of sarcoma. Pharmacol Res (In Press)
31. Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, Campana D (2010) Cytotoxicity of activated natural killer cells against pediatric solid tumors. Clin Cancer Res 16(15):3901–3909. doi:10.1158/1078-0432.CCR-10-0735, 1078-0432.CCR-10-0735 [pii]
32. Pahl JH, Ruslan SE, Buddingh EP, Santos SJ, Szuhai K, Serra M, Gelderblom H, Hogendoorn PC, Egeler RM, Schilham MW, Lankester AC (2012) Anti-EGFR antibody cetuximab enhances the cytolytic activity of natural killer cells toward osteosarcoma. Clin Cancer Res 18(2):432–441. doi:10.1158/1078-0432.CCR-11-2277
33. Waldhauer I, Steinle A (2008) NK cells and cancer immunosurveillance. Oncogene 27(45):5932–5943. doi:10.1038/onc.2008.267
34. Lu SM, Xiao P, Xue L, Che LH, Yang P, Li Y, Qiao H (2008) Prevalent expression of MHC class I chain-related molecule A in human osteosarcoma. Neoplasma 55(3):266–272
35. Lee JA, Ko Y, Kim DH, Lim JS, Kong CB, Cho WH, Jeon DG, Lee SY, Koh JS (2012) Epidermal growth factor receptor: is it a feasible target for the treatment of osteosarcoma? Cancer Res Treat 44(3):202–209. doi:10.4143/crt.2012.44.3.202
36. Pahl JH, Ruslan SE, Kwappenberg KM, van Ostaijen-Ten Dam MM, van Tol MJ, Lankester AC, Schilham MW (2013) Antibody-dependent cell lysis by NK cells is preserved after sarcoma-induced inhibition of NK cell cytotoxicity. Cancer Immunol Immunother 62(7):1235–1247. doi:10.1007/s00262-013-1406-x
37. Mariani E, Meneghetti A, Tarozzi A, Cattini L, Facchini A (2000) Interleukin-12 induces efficient lysis of natural killer-sensitive and natural killer-resistant human osteosarcoma cells: the synergistic effect of interleukin-2. Scand J Immunol 51(6):618–625, sji737 [pii]
38. Guma SR, Lee DA, Yu L, Gordon N, Hughes D, Stewart J, Wang WL, Kleinerman ES (2013) Natural killer cell therapy and aerosol interleukin-2 for the treatment of osteosarcoma lung metastasis. Pediatr Blood Cancer. doi:10.1002/pbc.24801
39. Liebau C, Merk H, Schmidt S, Roesel C, Karreman C, Prisack JB, Bojar H, Baltzer AW (2002) Interleukin-12 and interleukin-18 change ICAM-I expression, and enhance natural killer cell mediated cytolysis of human osteosarcoma cells. Cytokines Cell Mol Ther 7(4):135–142
40. Mills L, Huang G, Worth LL (2005) The role of interferon gamma and NK cells in the eradication of pulmonary osteosarcoma metastases by IL-12. AACR Meeting Abstracts 2005(1):1413c
41. Pelham JM, Gray JD, Flannery GR, Pimm MV, Baldwin RW (1983) Interferon-alpha conjugation to human osteogenic sarcoma monoclonal antibody 791 T/36. Cancer Immunol Immunother 15(3):210–216
42. Flannery GR, Pelham JM, Gray JD, Baldwin RW (1984) Immunomodulation: NK cells activated by interferon-conjugated monoclonal antibody against human osteosarcoma. Eur J Cancer Clin Oncol 20(6):791–798
43. Honorati MC, Neri S, Cattini L, Facchini A (2003) IL-17 enhances the susceptibility of U-2 OS osteosarcoma cells to NK cell lysis. Clin Exp Immunol 133(3):344–349
44. Gordon N, Koshkina NV, Jia SF, Khanna C, Mendoza A, Worth LL, Kleinerman ES (2007) Corruption of the Fas pathway delays the pulmonary clearance of murine osteosarcoma cells, enhances their metastatic potential, and reduces the effect of aerosol gemcitabine. Clin Cancer Res 13(15 Pt 1):4503–4510. doi:10.1158/1078-0432.CCR-07-0313
45. Kinoshita H, Yoshikawa H, Shiiki K, Hamada Y, Nakajima Y, Tasaka K (2000) Cisplatin (CDDP) sensitizes human osteosarcoma cell to Fas/CD95-mediated apoptosis by down-regulating FLIP-L expression. Int J Cancer 88(6):986–991
46. Hayashi T, Hideshima T, Akiyama M, Podar K, Yasui H, Raje N, Kumar S, Chauhan D, Treon SP, Richardson P, Anderson KC (2005) Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. Br J Haematol 128(2):192–203. doi:10.1111/j.1365-2141.2004.05286.x, BJH5286 [pii]

47. Fujii H, Trudeau JD, Teachey DT, Fish JD, Grupp SA, Schultz KR, Reid GS (2007) In vivo control of acute lymphoblastic leukemia by immunostimulatory CpG oligonucleotides. Blood 109(5):2008–2013. doi:10.1182/blood-2006-02-002055, blood-2006-02-002055 [pii]
48. Brandau S, Riemensberger J, Jacobsen M, Kemp D, Zhao W, Zhao X, Jocham D, Ratliff TL, Bohle A (2001) NK cells are essential for effective BCG immunotherapy. Int J Cancer 92(5):697–702. doi:10.1002/1097-0215(20010601)92:5, <697::AID-IJC1245>3.0.CO;2-Z [pii]
49. Yamanegi K, Yamane J, Kobayashi K, Kato-Kogoe N, Ohyama H, Nakasho K, Yamada N, Hata M, Nishioka T, Fukunaga S, Futani H, Okamura H, Terada N (2010) Sodium valproate, a histone deacetylase inhibitor, augments the expression of cell-surface NKG2D ligands, MICA/B, without increasing their soluble forms to enhance susceptibility of human osteosarcoma cells to NK cell-mediated cytotoxicity. Oncol Rep 24(6):1621–1627
50. Yamanegi K, Yamane J, Kobayashi K, Kato-Kogoe N, Ohyama H, Nakasho K, Yamada N, Hata M, Fukunaga S, Futani H, Okamura H, Terada N (2012) Valproic acid cooperates with hydralazine to augment the susceptibility of human osteosarcoma cells to Fas- and NK cell-mediated cell death. Int J Oncol 41(1):83–91. doi:10.3892/ijo.2012.1438
51. Ogbomo H, Michaelis M, Kreuter J, Doerr HW, Cinatl J Jr (2007) Histone deacetylase inhibitors suppress natural killer cell cytolytic activity. FEBS Lett 581(7):1317–1322
52. Kopp LM, Ray A, Denman CJ, Senyukov VS, Somanchi SS, Zhu S, Lee DA (2013) Decitabine has a biphasic effect on natural killer cell viability, phenotype, and function under proliferative conditions. Mol Immunol 54(3–4):296–301. doi:10.1016/j.molimm.2012.12.012
53. Rao-Bindal K, Zhou Z, Kleinerman ES (2012) MS-275 sensitizes osteosarcoma cells to Fas ligand-induced cell death by increasing the localization of Fas in membrane lipid rafts. Cell Death Dis 3:e369. doi:10.1038/cddis.2012.101
54. Rao-Bindal K, Koshkina NV, Stewart J, Kleinerman ES (2013) The histone deacetylase inhibitor, MS-275 (Entinostat), downregulates c-FLIP, sensitizes osteosarcoma cells to FasL, and induces the regression of osteosarcoma lung metastases. Curr Cancer Drug Targets 13(4):411–422
55. Berg SL, Cairo MS, Russell H, Ayello J, Ingle AM, Lau H, Chen N, Adamson PC, Blaney SM (2011) Safety, pharmacokinetics, and immunomodulatory effects of lenalidomide in children and adolescents with relapsed/refractory solid tumors or myelodysplastic syndrome: a Children's Oncology Group Phase I Consortium report. J Clin Oncol 29(3):316–323. doi:10.1200/JCO.2010.30.8387
56. Liu Y, Wu HW, Sheard MA, Sposto R, Somanchi SS, Cooper LJ, Lee DA, Seeger RC (2013) Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. Clin Cancer Res 19(8):2132–2143. doi:10.1158/1078-0432.CCR-12-1243
57. Wu L, Parton A, Lu L, Adams M, Schafer P, Bartlett JB (2011) Lenalidomide enhances antibody-dependent cellular cytotoxicity of solid tumor cells in vitro: influence of host immune and tumor markers. Cancer Immunol Immunother 60(1):61–73. doi:10.1007/s00262-010-0919-9
58. Talmadge JE, Schneider M, Collins M, Phillips H, Herberman RB, Wiltrout RH (1985) Augmentation of NK cell activity in tissue specific sites by liposomes incorporating MTP-PE. J Immunol 135(2):1477–1483
59. Kubista B, Trieb K, Blahovec H, Kotz R, Micksche M (2002) Hyperthermia increases the susceptibility of chondro- and osteosarcoma cells to natural killer cell-mediated lysis. Anticancer Res 22(2A):789–792
60. Yamada N, Yamanegi K, Ohyama H, Hata M, Nakasho K, Futani H, Okamura H, Terada N (2012) Hypoxia downregulates the expression of cell surface MICA without increasing soluble MICA in osteosarcoma cells in a HIF-1alpha-dependent manner. Int J Oncol 41(6):2005–2012. doi:10.3892/ijo.2012.1630
61. Balsamo M, Manzini C, Pietra G, Raggi F, Blengio F, Mingari MC, Varesio L, Moretta L, Bosco MC, Vitale M (2013) Hypoxia downregulates the expression of activating receptors

involved in NK-cell-mediated target cell killing without affecting ADCC. Eur J Immunol 43(10):2756–2764. doi:10.1002/eji.201343448
62. Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, Singh H, Hurton L, Maiti SN, Huls MH, Champlin RE, Cooper LJ, Lee DA (2012) Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One 7(1):e30264. doi:10.1371/journal.pone.0030264
63. Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, Suttorp M, Seifried E, Ottmann OG, Bug G (2013) Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy. doi:10.1016/j.jcyt.2013.06.017
64. Chang YH, Connolly J, Shimasaki N, Mimura K, Kono K, Campana D (2013) A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells. Cancer Res 73(6):1777–1786. doi:10.1158/0008-5472.CAN-12-3558

ERRATUM TO

Current Advances in Osteosarcoma

Eugenie S. Kleinerman

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7,

DOI 10.1007/978-3-319-04843-7_20

The Publisher regrets that the title of the Preface reads incorrectly in the print and online versions of this title. The correct title of the Preface is:

Oseosarcoma: The State of Affairs Dictates a Change.
What Do We Know?
Can We Apply These Discoveries and Alter Clinical Research
Practices to Achieve Success?

Also in the Preface, the title and the correct name of the editor's endowed chair are cut off at the end of the Preface. These should read as follows:

Eugenie S. Kleinerman, M.D.
Professor and Head, Division of Pediatrics
Professor, Department of Cancer Biology
The Mary V. and John A. Reilly Distinguished Chair
University of Texas M. D. Anderson Cancer Center

The online version of the original book can be found at
http://dx.doi.org/10.1007/978-3-319-04843-7

Index

E.S. Kleinerman (ed.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 804, DOI 10.1007/978-3-319-04843-7,