BIOLOGY-DRIVEN TARGETED THERAPY OF PEDIATRIC SOFT-TISSUE AND BONE TUMORS: CURRENT OPPORTUNITIES AND FUTURE CHALLENGES

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BIOLOGY-DRIVEN TARGETED THERAPY OF PEDIATRIC SOFT-TISSUE AND BONE TUMORS: CURRENT OPPORTUNITIES AND FUTURE CHALLENGES

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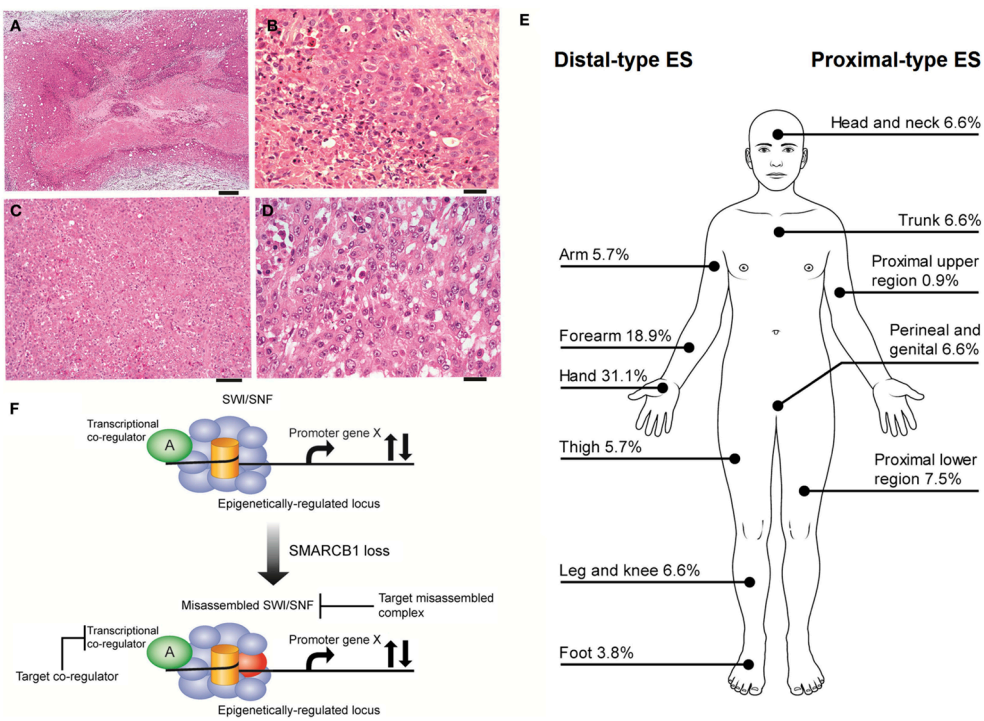

Figure source: Noujaim J, Thway K, Bajwa Z, Bajwa A, Maki RG, Jones RL and Keller C (2015) Epithelioid sarcoma: opportunities for biology-driven targeted therapy. Front. Oncol. 5:186. doi: 10.3389/fonc.2015.00186

Recent advances in the understanding of the biological basis of pediatric soft-tissue and bone tumors, especially owing to the advent of "omics" technologies, have led to an exponential increase in the current knowledge on the genetic and cellular patho-mechanisms that drive these diseases. This offers the unprecedented opportunity to develop and implement targeted therapies such as monoclonal antibodies, small molecules, oncolytic viruses, and immunotherapies in standard and/or personalized treatment regimens. However, to date only a few examples document a successful translation of discoveries from the bench to the bedside. Recent international expert congresses further emphasize the urgent need for a more rapid and especially more successful translational process.

Hence, we strongly believe that a Frontiers Research Topic aiming at this aspect would fit just in time and be relevant for a broad readership.

This Frontiers Research Topic intended to provide a platform for active and interdisciplinary discussion, to summarize current state-of-the-art knowledge on all basic research and translational aspects in pediatric soft-tissue and bone tumors, and to offer new perspectives on how to further promote and accelerate the translational process. It comprises high-quality original articles and timely reviews.

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EDITORIAL
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Editorial: Biology-Driven Targeted Therapy of Pediatric Soft-Tissue and Bone Tumors: Current Opportunities and Future Challenges

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The Editorial on the Research Topic

Biology-Driven Targeted Therapy of Pediatric Soft-Tissue and Bone Tumors: Current Opportunities and Future Challenges

Sarcomas constitute a large and diverse group of malignant neoplasias and the predominant group of non-CNS-related solid tumors in childhood and adolescence (1). Recent advances in the understanding of the genetic and biological basis of pediatric soft tissue and bone tumors, especially owing to the advent of "omics" technologies, have led to an exponential increase in the current knowledge on the genetic and cellular pathomechanisms that drive these diseases (2).

This offers the unprecedented opportunity to develop and implement targeted therapies, such as monoclonal antibodies, small molecules, and immunotherapies in standard and/or personalized treatment regimens (2). However, to date, only a few examples document a successful translation of discoveries from the bench to the bedside, which significantly improved patient outcome while having little adverse effects (3, 4). Recent reviews (5) and international expert congresses further emphasize the urgent need for a more rapid and especially more successful translational process (6–8) (Kovar et al., Schäfer et al.).

This *Frontiers Research Topic* entitled "*Biology-driven targeted therapy of pediatric soft-tissue and bone tumors: current opportunities and future challenges*" was dedicated to this aspect and provided a transdisciplinary forum for researchers working at the interfaces between basic cell biology, tumorigenesis, and personalized medicine. Many excellent researchers have contributed to this topic now covering the most common but also rare sarcoma entities of this age group, such as osteosarcoma, Ewing sarcoma, rhabdomyosarcoma, and epithelioid sarcoma.

In order to accelerate the development of novel targeted therapeutics, suitable genetically engineered animal models and xenograft models are required. In this regard, Geier et al. discuss preclinical human tumor xenograft models of pediatric sarcomas that may be used practically to identify novel agents and how "omics" approaches may be implemented for identification of novel biomarkers, which can discriminate sensitive and resistant tumors to these agents. Since the ultimate goal of anticancer therapy is to kill cancer cells, it is important to assess in detail the modes of cell death in preclinical models. Rello-Varona et al. survey different modes of cell death and propose standards of how to adequately assess them, which is especially important in such a heterogeneous group of tumor entities such as human sarcomas.

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Osteosarcoma is the most common pediatric bone cancer (1). Sampson et al. discuss current knowledge on the role of micro RNAs (miRNAs) and their target genes in osteosarcoma and evaluate their potential use as therapeutic agents. They also summarize the efficacy of inhibition of oncogenic miRNAs or expression of tumor suppressor miRNAs in preclinical models of osteosarcoma (Sampson et al.).

Ewing sarcoma is the second most common pediatric bone-associated sarcoma (1). Since its discovery in 1921 by James Ewing, the precise histogenesis of Ewing sarcoma remains enigmatic (9). Despite this histogenetic uncertainty, Ewing sarcoma is genetically well characterized by the presence of pathognomonic *EWSR1-ETS* gene fusions (usually *EWSR1-FLI1*) (10), which drive this disease by acting as oncogenic transcription factors (10). In their review, Cidre-Aranaz and Alonso assess five major EWSR1-FLI1 target genes, their signaling pathways, and shed light on how these pathways could be exploited therapeutically. Indeed, earlier work showed that some EWSR1-FLI1 target genes are very specifically expressed in Ewing sarcoma relative to normal tissues (11), thus possibly constituting attractive targets for (immuno)-therapeutic intervention. In accordance, subsequent experiments showed that IL2 transgenic Ewing sarcoma cells elicit tumor-specific T and NK cell responses *in vitro* and *in vivo* (12). In a subsequent study, Reuter et al. now investigate the role of immunostimulation by OX40 ligand (also known as CD252 or tumor necrosis factor ligand family member 4) transgenic Ewing sarcoma cells. The authors found that OX40L expression in Ewing sarcoma cells enhanced immune stimulation, suggesting that the OX40/OX40L pathways should be considered in the design of immunotherapies against Ewing sarcoma (Reuter et al.). However, immunotherapeutic advances are not limited to Ewing sarcoma. Roberts et al. provide exiting new insights in immunotherapy of Ewing sarcoma and other pediatric sarcomas and point out the concept of integrating antibody-based and cell-based immunotherapy into an overall treatment strategy of sarcoma.

An innovative alternative approach for treatment of Ewing sarcoma is targeting the tumor's micro-environment, instead of targeting the tumor cells directly. In fact, bone lesions from primary or metastatic Ewing sarcoma are characterized by extensive bone remodeling and osteolysis. Redini and Heymann expand on this important aspect and propose targeting the bone tumor micro-environment in Ewing sarcoma using osteoclast inhibitors, such as bisphosphonates and antagonists of receptor activator of NF-kappa B ligand (RANKL). In addition to this, Deel et al. summarize the known molecular alterations within the Hippo pathway in sarcomas and highlight how several pharmacologic compounds have shown activity in modulating Hippo components, providing proof-of-principle that Hippo signaling may be harnessed for therapeutic application in sarcomas.

Rhabdomyosarcomas are the most common soft tissue tumors of childhood (1) and constitute a heterogeneous group of cancers with myogenic differentiation featuring diverse cytogenetic and mutational aberrations (7). Hettmer et al. describe two disease-relevant mouse myogenic tumor models driven either by oncogenic *Kras* in *p16p19*^{null} or by a mutant *Smoothened* allele. In line with this, Ridzewski et al. explore the therapeutic value of four *Smoothened* inhibitors in four different rhabdomyosarcoma cell lines. They found that some inhibitors induced strong proapoptotic and antiproliferative effects in some rhabdomyosarcoma cell lines, while others paradoxically induced cellular proliferation at certain concentrations (Ridzewski et al.). Because of this heterogeneous response, the authors propose to conduct pretesting of *Smoothened* inhibitors in patient-derived short-term rhabdomyosarcoma cultures or patient-derived xenograft mouse models before applying these drugs to patients (Ridzewski et al.). In an accompanying article, Schott et al. provide evidence that oncogenic *RAS* mutants confer resistance of RMS13 rhabdomyosarcoma cells to oxidative stress-induced ferroptotic cell death, which has important implications for the development of targeted therapies for rhabdomyosarcoma and which might at least partially explain heterogeneous responses on drug treatment depending on the *RAS* mutation status.

Noujaim et al. summarize clinically relevant biomarkers (e.g., SMARCB1, CA125, dysadherin, and others) with respect to targeted therapeutic opportunities for epithelioid sarcoma, which is a soft tissue sarcoma of children and young adults for which the preferred treatment for localized disease is wide surgical resection. However, current treatment regimens for epithelioid sarcoma still lack systematic coherence, and medical management is to a great extent undefined, which is why especially for patients with regional and distal metastases, the development of targeted therapies is greatly desired (Noujaim et al.). Noujaim et al. also examine the role of EGFR, mTOR, and polykinase inhibitors (e.g., sunitinib) in the management of local and disseminated disease. The authors propose to build a consortium of pharmaceutical, academic, and non-profit collaborators so that a roadmap can be developed toward effective biology-driven therapies of epithelioid sarcoma (Noujaim et al.). We believe that this *Frontiers Research Topic* has provided an excellent platform on which such consortia can be built on.

We anticipate that the data presented in the aforementioned original and review articles will be of great value for the scientific community to ultimately improve patient care and outcome. The success of this *Frontiers Research Topic* would not have been possible without the outstanding contribution of excellent scientists that served either as peer reviewers or additional guest associate editors.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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EWS/FLI1 target genes and therapeutic opportunities in Ewing sarcoma

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Ewing sarcoma is an aggressive bone malignancy that affect children and young adults. Ewing sarcoma is the second most common primary bone malignancy in pediatric patients. Although significant progress has been made in the treatment of Ewing sarcoma since it was first described in the 1920s, in the last decade survival rates have remained unacceptably invariable, thus pointing to the need for new approaches centered in the molecular basis of the disease. Ewing sarcoma driving mutation, *EWS–FLI1*, which results from a chromosomal translocation, encodes an aberrant transcription factor. Since its first characterization in 1990s, many molecular targets have been described to be regulated by this chimeric transcription factor. Their contribution to orchestrate Ewing sarcoma phenotype has been reported over the last decades. In this work, we will focus on the description of a selection of EWS/FLI1 targets, their functional role, and their potential clinical relevance. We will also discuss their role in other types of cancer as well as the need for further studies to be performed in order to achieve a broader understanding of their particular contribution to Ewing sarcoma development.

Keywords: Ewing sarcoma, EWS/FLI1, DAX-1, GLI1, FOXO1, FOXM1, CCK, LOX

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Introduction

Ewing sarcoma is a rare tumor that arises mainly in the bones of children and adolescents. Despite the improvements in treatment achieved during the last decades, survival rates have remained unacceptably low, even in patients with localized disease, since a great proportion of Ewing sarcoma tumors are refractory to conventional treatment and relapses are frequent (1). In addition, approximately 25% of cases present disseminated disease at diagnosis, which have a very poor prognosis (2). Thus, there is an urgent need for new targeted therapies that may offer a higher efficiency and less adverse effects than the conventional chemo/radiotherapies that are used nowadays.

In this sense, understanding the molecular basis of Ewing sarcoma pathogenesis provides key information that may help to design new targeted biological therapies. Ewing sarcomas are characterized by chromosomal translocations that fuse the *EWSR1* gene to some members of the ETS family of transcription factors (3), being FLI1 the most frequently implicated [t(11;22)(q24;q12)] (4). The EWS/FLI1 fusion protein is an aberrant transcription factor that is essential for Ewing tumor development, since it regulates the expression of multiple target genes and governs the oncogenic processes that lead to malignant transformation of a yet undefined cancer precursor cell. Provided that the oncogenic properties of EWS/FLI1 rely on its capability to induce or repress specific target genes, these target genes can likewise offer interesting opportunities to identify new targeted therapies.

In the past years, an important effort to identify EWS/FLI1 genes functionally relevant for Ewing sarcoma pathogenesis has been carried out. As a consequence, many genes that play an important role in Ewing sarcoma have been identified (5–17). This has revealed some key molecular pathways involved in Ewing pathogenesis, and more importantly it has provided new molecular targets.

A comprehensive discussion of all EWS/FLI1 target genes identified to date and their implications in targeted therapy is beyond the scope of this review. Thus, here we have focused on a selection of six EWS/FLI1 target genes that, in our opinion, can represent attractive opportunities for future studies that may lead to discovering new therapeutic approaches. This selection takes into account the presence of significant data – in Ewing or in other systems – regarding potential therapeutic applications. Four genes encode for transcriptional regulators while the other two encode for secreted proteins.

Transcriptional Regulators

DAX-1 (NR0B1)

DAX-1 is a gene that belongs to the super family of nuclear receptors (official name *NR0B1*, standing for Nuclear Receptor Subfamily 0, Group B, Member 1). Nuclear receptors are transcription factors that undergo activation upon binding of small ligands such as retinoic acid or steroids. However, there is no known ligand for DAX-1, and thus we refer to it as an orphan nuclear receptor. Germline mutations in this gene are the cause of dosage-sensitive sex reversal (DSS) in XY individuals and adrenal hypoplasia congenital (AHC), which is characterized by adrenal insufficiency, and hypogonadotropic hypogonadism in males. DAX-1 is a master regulator of steroidogenesis that negatively regulates the steroidogenic factor 1 (SF1), an important transcriptional activator of genes involved in steroid hormone production (18, 19). In addition, DAX-1 plays an important role in several biological processes such as osteoblast differentiation (20), ion homeostasis and transport, lipid transport, or skeletal development (21) among others. More recently, DAX-1 has been involved in the maintenance of mouse embryonic stem cell pluripotency through regulation of stem cell genes like *Oct-3/4* (22–24).

Given that DAX-1 function is mainly linked to steroidogenesis, it was surprising to find this gene associated to Ewing sarcoma, a tumor with no known relationship with steroidogenic tissues. Gene expression profiles performed in two heterologous cell models ectopically expressing EWS/FLI1 (HEK293 and HeLa cells) demonstrated that DAX-1 was specifically induced by EWS/FLI1, but not by wildtype FLI1 (25). In addition, it was shown that DAX-1 was highly expressed in Ewing sarcoma cell lines and tumors, while it was not expressed in other pediatric tumors such as rhabdomyosarcoma or neuroblastoma. Finally, DAX-1 expression was demonstrated to depend on EWS/FLI1 expression in the A673 Ewing sarcoma cell line upon EWS/FLI1 knockdown. An independent study showed similar findings, confirming that DAX-1 is a target of the EWS/FLI1 oncoprotein (26).

Several functional studies have demonstrated that DAX-1 plays a critical role in Ewing sarcoma pathogenesis: DAX-1 knockdown impairs Ewing sarcoma cell proliferation, G1 cell arrest induction, inhibits anchorage independent growth of colonies in soft agar, and drastically inhibits growth of xenotransplanted tumor cells in immunodeficient mice (9, 25, 26). These results are highly consistent since they were obtained in independent laboratories, using several Ewing sarcoma cell lines (TC71, EWS502, and A673) and different gene knockdown technologies (i.e., transient retrovirus infection or inducible expression of EWS/FLI1 shRNAs). Interestingly, characterization of the gene expression profile regulated by DAX-1 in Ewing sarcoma cell lines has also provided interesting findings regarding the function of DAX-1 in Ewing sarcoma. These studies showed that a significant percentage of the genes regulated by EWS/FLI1 in Ewing sarcoma cells are also under the control of DAX-1, reinforcing the importance of DAX-1 in Ewing sarcoma pathogenesis. In fact, two independent works demonstrated that EWS/FLI1 and DAX-1 transcriptional profiles share a significant number of genes, suggesting that DAX-1 not only contributes to the EWS/FLI1 transcriptional signature but also that there is a hierarchy controlled by EWS/FLI1 and in which some genes, such as *DAX-1*, can play a more prominent role (9, 27). The study of the mechanism through which EWS/FLI1 upregulates DAX-1 expression in Ewing sarcoma cells revealed an unexpected finding: EWS/FLI1 directly interacts with *DAX-1* promoter through binding to a GGAA-rich sequence (9, 28). This motif resulted to be a polymorphic microsatellite located in the *DAX-1* promoter. It has been demonstrated that EWS/FLI1 binds similar sequences located in the promoters of other EWS/FLI1 target genes, indicating that this mechanism of gene transcriptional activation is frequently used by EWS/FLI1 to regulate the expression of some oncogenic genes (28) [i.e., *Caveolin-1* (*CAV1*) (7), *glutathione S-transferase M4* (*GSTM4*) (29), *FCGRT* (*Fc fragment of IgG, receptor, transporter, alpha*), *FVT1/KDSR* (*3-ketodihydrosphingosine reductase*)or *ABHD6* (*Abhydrolase Domain-Containing Protein*) (30)]. The fact that *DAX-1* expression is regulated through a polymorphic repeat of the GGAA motif raised the question if the number of repeats could be somehow linked to the level of *DAX-1* expression and, as a consequence, to the malignant phenotype of Ewing sarcoma. Several biochemical studies demonstrated a relationship between the number of GGAA repeats and the degree of promoter activation, indicating that it was necessary a minimum of nine repeats to obtain a response to EWS/FLI1 (30). However, the attempts to establish a relationship between the length of the microsatellite located in *DAX-1* promoter and the clinical prognosis have raised contradictory results. For instance, GGAA microsatellites were longer in African populations, which are known to have a lower incidence of Ewing sarcoma but a worse overall survival when compared to European populations (31, 32). Conversely, in another study based on 112 patients, the length of the DAX-1 microsatellite showed no influence on clinical outcomes (33).

Taking into account all these results, *DAX-1* can be considered as one of the most relevant EWS/FLI1 gene targets. The fact that DAX-1 expression results essential for EWS/FLI1-mediated oncogenesis opens the possibility, at least in theory, to consider *DAX-1* targeting as an attractive therapeutic approach in Ewing sarcoma. As a consequence, a more profound understanding of the functions that DAX-1 exerts in Ewing sarcoma and the

molecular mechanism involved in them can provide new clues on how to interfere with its expression or function in this cancer (34).

DAX-1 is located in the nucleus of Ewing sarcoma cells, where it presumably interacts with other transcription factors and cofactors to regulate downstream target genes that are important for oncogenesis. Interestingly, a combination of biochemical and gene expression profile experiments leads to the observation that EWS/FLI1 and DAX-1 interact physically. Specifically, it was found that both the amino- and carboxyl-termini of DAX-1 interacted with EWS/FLI1 (27). This result opens the attractive possibility that interfering EWS/FLI1-DAX-1 interaction could lead to new therapeutic opportunities. To go forward in this line of work, it would be necessary to finely map the regions involved in this interaction in order to design small molecules with the ability to block it. Since DAX-1 and EWS/FLI1 interaction could be necessary for full EWS/FLI1-mediated oncogenesis, disturbing it could be therapeutically valuable.

DAX-1 has been shown to interact in different cellular contexts with a variety of transcriptional regulators, mainly corepressors. For example, DAX-1 interacts with Alien corepressor through its silencing domain and this interaction has been shown to be important for AHC pathogenesis (35). DAX-1 has also been shown to interact directly with the androgen receptor, NR3C4, inhibiting its activation (36) and other partners such as NR5A1 (37) and ESRRγ (38). To date, a systematic analysis of the protein–protein interactions in which DAX-1 is involved in Ewing sarcoma cells and the role that these interactions can play in Ewing sarcoma pathogenesis has not been carried out. Experiments focused on identifying and characterizing these interactions could provide clues for designing synthetic drugs to target them. On the other hand, it has been shown that DAX-1 C-terminal domain contains a potent transcriptional repressor domain that, when altered by mutations in AHC patients, impairs its nuclear localization, and therefore its transcriptional activity (39), suggesting that there is a potential field for developing drugs to modulate DAX-1 subcellular localization and consequently its function.

As with any new drug, the possible side effects of a new therapeutic approach must be also taken into consideration. For instance, prolonged DAX-1 blockage may lead to disequilibrium in steroid hormones production, which could lead to Cushing-like syndrome (40). These hypothetical complications, compared with the severity of Ewing sarcoma itself, would be perfectly assumable. One theoretical advantage of using therapeutic approaches targeting *DAX-1* is that this gene is only expressed in a limited number of tissues, mainly in adrenal gland and testis, and probably DAX-1 targeting will only affect these organs. In summary, although there are currently no drugs able to target DAX-1 and block its function, studies intended to understand its structure, its mechanism of interaction with other transcriptional (co)factors, and the identification of other protein–protein interactions in the Ewing sarcoma context could provide new insights to design new therapeutic molecules (**Figure 1**).

GLI1

GLI1 (*Glioma-Associated Oncogene Homolog 1)* is a transcription factor belonging to the Kruper family of zinc finger proteins. GLI1 is a component of the canonical Hedgehog pathway: extracellular Sonic Hedgehog (Shh) binds to the PTCH receptor causing the liberation of Smooth (SMO) from the PTCH-SMO complex. Subsequently, activated SMO releases GLI1 from the complex that it forms with Suppressor of Fused (SUFU), which permits GLI1 nuclear translocation where it regulates gene transcription of genes involved in normal cell growth and differentiation such as the embryonic pattern formation (41). Although this pathway is mainly active during embryogenesis, it remains active in some adult tissues, where it is involved in homeostasis and stem-cell maintenance (42, 43).

Zwerner et al. described an association between EWS/FLI1 and GLI1 in Ewing sarcoma cells. They showed that NIH3T3 cells expressing *EWS/FLI1* presented the expected malignant phenotype concomitantly with augmented levels of GLI1 (44). Moreover, when GLI1 expression was knocked-down by RNA interference, the transformed phenotype was reduced (demonstrated by a decrease in the anchorage independent growth) indicating that GLI1 plays an important role in the maintenance of the malignant phenotype induced by EWS/FLI1. Interestingly, SUFU overexpression, which is expected to inhibit GLI1, also produced similar effects in NIH3T3 cells. In TC32 Ewing sarcoma cells, EWS/FLI1 knocking down using RNA interference produced a reduction in *GLI1* expression levels. Also, ChIP studies demonstrated that GLI1 is a direct target of EWS/FLI1 (45). Moreover, when a shRNA against GLI1 was used in the Ewing sarcoma cell line TC32, the transformed phenotype was inhibited (measured by reduction in anchorage independent growth) (44). Interestingly, and in contrast with what it is usually observed in other types of cancer, GLI1 deregulation in Ewing sarcoma is independent of Shh since its activation did not produce phenotypic changes nor did a pharmacological blockage of SMO using cyclopamine (an inhibitor of Shh signaling by direct binding to SMO) (45).

Subsequently Joo et al. (46) showed that Ewing primary tumors expressed high levels of GLI1. These authors also confirmed using RNAi that the expression of GLI1 in Ewing sarcoma cells (TC71) is dependent on EWS/FLI1 and that GLI1 expression was relevant for the maintenance of the transformed phenotype. Strikingly, re-analysis of gene expression profiles showed that genes that were traditionally thought to be transcriptionally modulated by EWS/FLI1, such as *NKX2.2*, *Patched (PTCH)* or *GAS1*, were indeed dependent on GLI1 expression, meaning that the gene expression network regulated by EWS/FLI1 holds a hierarchy in which GLI1 has a prominent role.

Deregulation of the Shh–GLI1 pathway has been showed to lead to tumorigenesis and aggressive phenotypes (progression, metastasis and therapeutic resistance) of numerous cancer types such as basal cell carcinomas (47), colorectal carcinoma (48), breast cancer (49), and bone and soft tissue sarcomas (50).

Given the importance of Shh–GLI1 pathway in cancer, some therapeutic approaches, focused on the blocking of this pathway, have been developed over the years. One of these strategies consisted in searching for small molecule inhibitors of the pathway. Thus, Shh–GLI1 pathway inhibitors, such as cyclopamine, have been successfully tested in some cancer types such as medulloblastoma (51), pancreatic adenocarcinoma (52), small-cell lung cancer (SCLC) (53), gastric adenocarcinomas (54), and esophageal cancer (55).

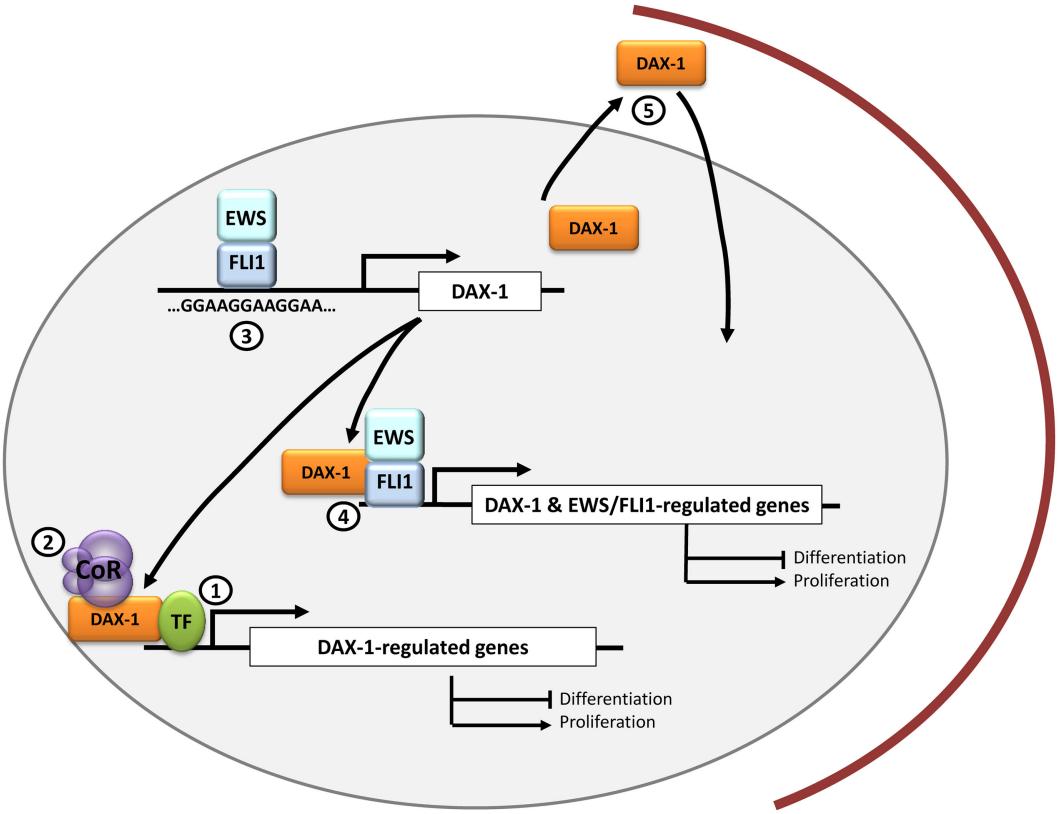

FIGURE 1 | DAX-1 and therapeutic opportunities in Ewing sarcoma. DAX-1 expression is upregulated by EWS/FLI1 in Ewing sarcoma cells through a direct interaction with a polymorphic GGAA microsatellite located in the *DAX-1* promoter. Since DAX-1 expression is essential for EWS/FLI1-mediated oncogenesis, it is still necessary to ascertain if it interacts with other transcription factors (1) and/or co-repressors (2) in the nucleus of Ewing sarcoma cells. This could open new therapeutic approaches for designing molecules to target these interactions. Potential therapeutic targets may be molecules that prevent EWS/FLI1 binding to the GGAA-rich motifs in *DAX-1* promoter (3) or drugs directed toward the EWS/FLI1-DAX-1 interaction, whose concurrence could be necessary to regulate the expression of certain genes (4). Finally, DAX-1 C-terminal domain can impair DAX-1 nuclear localization when altered so it could be potentially targeted to modulate its subcellular localization and thus its function (5).

The fact that GLI1 expression is constitutively induced by EWS/FLI1 in Ewing sarcoma suggests that drugs acting upstream GLI1 will be ineffective in blocking this pathway in this cancer. In agreement with this, cyclopamine treatment of Ewing sarcoma cells would have no effect on the malignant characteristics of Ewing sarcoma cells. For this reason, efforts should be directed toward developing and studying drugs targeting GLI1 expression or function directly. In this sense, arsenic trioxide (ATO), an old drug recently reintroduced in the repertoire of anticancer drugs, has been found to inhibit cell growth by targeting GLI proteins (56). In the specific case of Ewing sarcoma, ATO presented cytotoxicity in cell lines with upregulated GLI1 expression (TC-71, SKES and A4573), and curbed xenograft growth performed with TC-71 cells (57). ATO was also found to inhibit Ewing cells (RDES and A673) migration and invasiveness, thus implying that it could also have a therapeutic effect on metastasis (58). Of note, ATO has already been tested in combination with other chemotherapeutic drugs (etoposide and paclitaxel) in a preliminary study that included Ewing sarcoma and metastatic osteosarcoma patients where tumor growth was controlled in 75% of cases (59). However, since Ewing sarcoma is mainly a pediatric cancer, it is necessary to further investigate its effects and to be prudent when designing clinical studies given the roles of the Shh–GLI1 pathway in development.

Finally, it has been described a correlation between GLI1 expression levels and/or prognosis and recurrence in some cancer types. For instance, in a study comprising 25 clinical samples of colorectal carcinoma, Shh expression was found upregulated and, interestingly, when GLI1 expression was analyzed by *in situ* hybridization, it was mainly found in the malignant crypts of adenocarcinomas (48). Also, it has been described a positive correlation between GLI1 expression and tumor grade and/or lymph node status, pointing to a role of GLI1 in metastasis. Some examples are breast cancer, where high GLI1 expression measured in a TMA containing 204 tumor samples was associated with poor prognosis and progressive stages of disease (49) or bone and soft tissue sarcomas, where higher GLI1 expression correlated with more aggressive outcomes (50). In the specific case of Ewing sarcoma, these studies remain to be performed, especially considering that a deeper knowledge on this field could lead to a more efficient patient stratification that could help improve treatment.

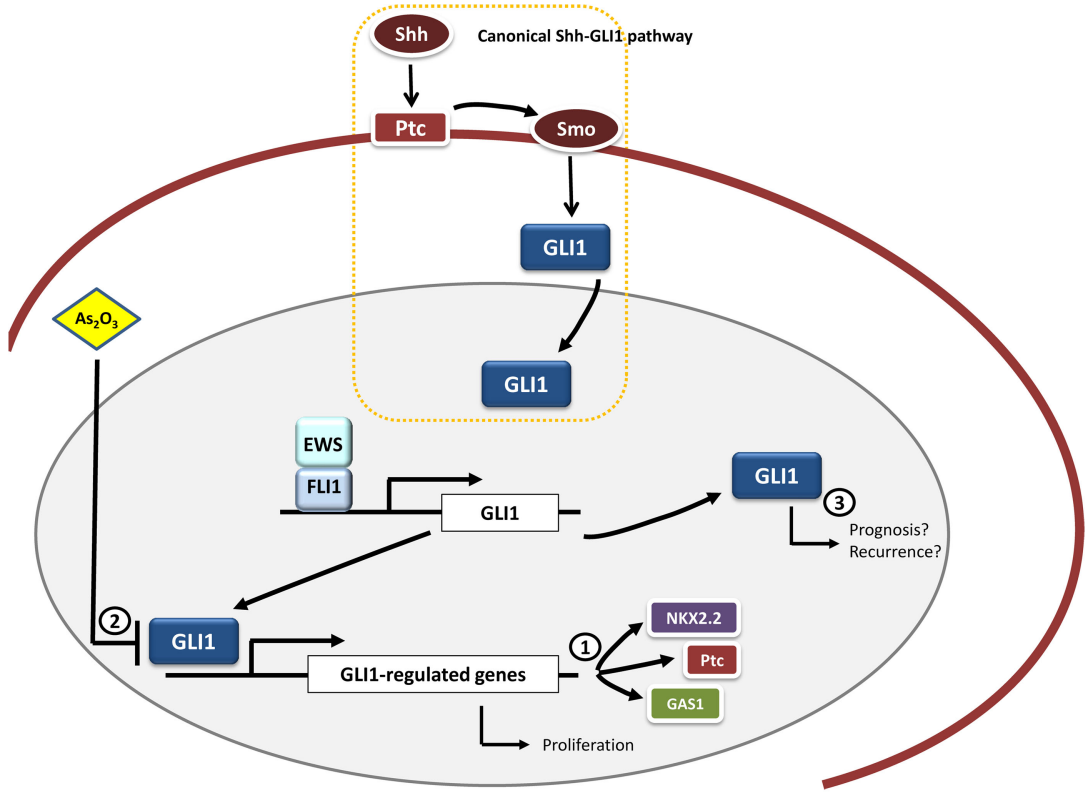

FIGURE 2 | GLI1 and therapeutic opportunities in Ewing sarcoma. *GLI1* is an upregulated direct target gene of EWS/FLI1 in Ewing sarcoma cells. Functional studies have demonstrated that GLI1 expression is relevant for the maintenance of the transformed phenotype in this system. Moreover, some genes transcriptionally modulated by EWS/FLI1 depend on GLI1 expression, including *NKX2.2, PATCH*, and *GAS1* (1). Therapeutic opportunities may include the use of molecules capable of inhibiting GLI1-mediated transcription, such as arsenic trioxide (As_2O_3) (2). Also it may be interesting to ascertain the possible link between GLI1 expression pattern and prognosis in Ewing sarcoma given that this correlation between GLI1 expression and bad prognosis exists in other tumor types (breast cancer and bone and soft tissue sarcomas) (3).

There is still an urgent need for further functional studies that can ascertain the exact role of this pathway in Ewing sarcoma development and progression. These studies could help to synthesize new compounds or small molecules that could target GLI1 with better efficacies either alone or in combination with normal chemotherapeutic treatments (**Figure 2**).

Forkhead Box (FOX) of Transcription Factors

Forkhead box proteins are an extensive family of transcriptional regulators that share a common DNA binding domain (the forkhead domain). There are 19 subgroups (FOXA to FOXS) organized on the basis of sequence homology inside and outside the forkhead domain. FOX proteins regulate gene networks that are involved in cell cycle progression, proliferation, differentiation, metabolism, senescence, survival, or apoptosis (60). Thus, it is not strange that these transcription factors have been shown to have roles in cancer. Interestingly, some members of this family have been shown to act as tumor suppressor genes, while others have been shown to be pro-oncogenic. Examples of both of these opposed functions have been identified in Ewing sarcoma.

The FOXO subgroup (consisting of FOXO1, FOXO3A, FOXO4, and FOXO6) are key negative regulators of cell proliferation and survival. They induce cell cycle arrest at G1 (61) and apoptosis and DNA repair (62). They are thus considered bona fide tumor suppressors. For example, in prostate cancer, *FOXO1* is found transcriptionally downregulated and the induction of its expression in prostate cancer cells inhibits cell proliferation and survival (63). In addition, FOXO1 has been also shown to regulate other hallmarks of cancer such as angiogenesis. Thus, FOXO1 loss of function increases blood vessel formation and promotes endothelial cell proliferation and migration (64, 65).

FOXOs transcriptional activity is regulated by changes in their cellular localization, which is mediated by protein kinases such as the serum/glucocorticoid kinase (SGK) and the protein kinase B (AKT) [reviewed in Ref. (66)]. These transcription factors can also undergo different post-translational modifications that regulate their activity including deacetylation mediated by Sirt1 and ubiquitination mediated by Skp2 and Mdm2 (67).

EWS/FLI1 binds to the FOXO1 promoter and represses its expression in Ewing sarcoma cells (68). In accordance with this, FOXO1 is expressed at lower levels in primary Ewing sarcoma as compared to other tissues (16). Induction of FOXO1 in two Ewing sarcoma cells (A673 and SKNMC) resulted in impaired cell proliferation and reduced soft agar colony formation capability, confirming that FOXO1 is a tumor suppressor in Ewing

sarcoma and that its inhibition is important for Ewing sarcoma growth. Interestingly, EWS/FLI1 also indirectly regulates the subcellular localization of FOXO1 and thus controls its transcriptional activity. CDK2- (which is upregulated by EWS/FLI1 and acts as a negative regulator of FOXO1 transcriptional activity) and AKT-mediated phosphorylation of FOXO1 cooperate to block its transport to the nucleus thus inhibiting its transcriptional activity. These findings demonstrate that EWS/FLI1 blocks FOXO1 activity at several different levels in Ewing sarcoma cells.

Since FOXO1 acts as a tumor suppressor in Ewing sarcoma, a valuable therapeutic approach can consist in the reactivation of FOXO1 activity. In this regard, methylseleninic acid (MSA), a chemical compound previously shown to reactivate FOXO1 in prostate cancer, was tested in Ewing sarcoma cells (69). Treatment of Ewing sarcoma cells with MSA induced FOXO1 expression in a concentration-dependent manner, which correlated with apoptotic-mediated cell death. This effect was mediated at least in part by FOXO1, since the knockdown of endogenously induced FOXO1 significantly reduced the apoptotic effect of MSA. Notably, administration of MSA in an orthotopic mouse xenotransplantation model significantly reduced tumor growth, suggesting that MSA could be a potential therapeutic approach in Ewing sarcoma. However, it should be taken into account that high concentrations of selenium are usually associated with intoxication, which can make this approach problematic. This means that any potential application of MSA should use effective, low doses, which in combination with conventional chemotherapeutic drugs can reach the desired anti-tumoral effects. Particularly, MSA has already been proved to synergize well with some chemotherapeutic drugs that are frequently used in Ewing sarcoma, such as etoposide or doxorubicin (70) (**Figure 3**). Since reactivation of FOXO1 in Ewing sarcoma cells has shown to be effective both *in vitro* and *in vivo*, more studies are necessary to understand the mechanism involved in the regulation of FOXO1 expression and its transcriptional activity in order to identify new therapeutic targets.

FOXM1 is another member of the FOX family of transcription factors that contrary to FOXO displays a pro-oncogenic role in cancer. In fact, FOXM1 is one of the most commonly overexpressed genes in solid tumors (71). Initially, FOXM1 was described as a proliferation-specific mammalian transcription factor, expressed in proliferating cells but not in quiescent or terminally differentiated cells. In addition to this, and over the years, FOXM1 has also been implicated in cell migration, invasion, angiogenesis, metastasis, or oxidative stress (72).

Christensen et al. showed that EWS/FLI1 upregulated the levels of FOXM1 in four Ewing sarcoma cell lines, although the mechanism appeared to be indirect (17). In agreement with this, FOXM1 is expressed at high levels in Ewing sarcoma cell lines and primary tumors. In order to characterize the relevance of FOXM1 in Ewing sarcoma pathogenesis, the authors performed FOXM1

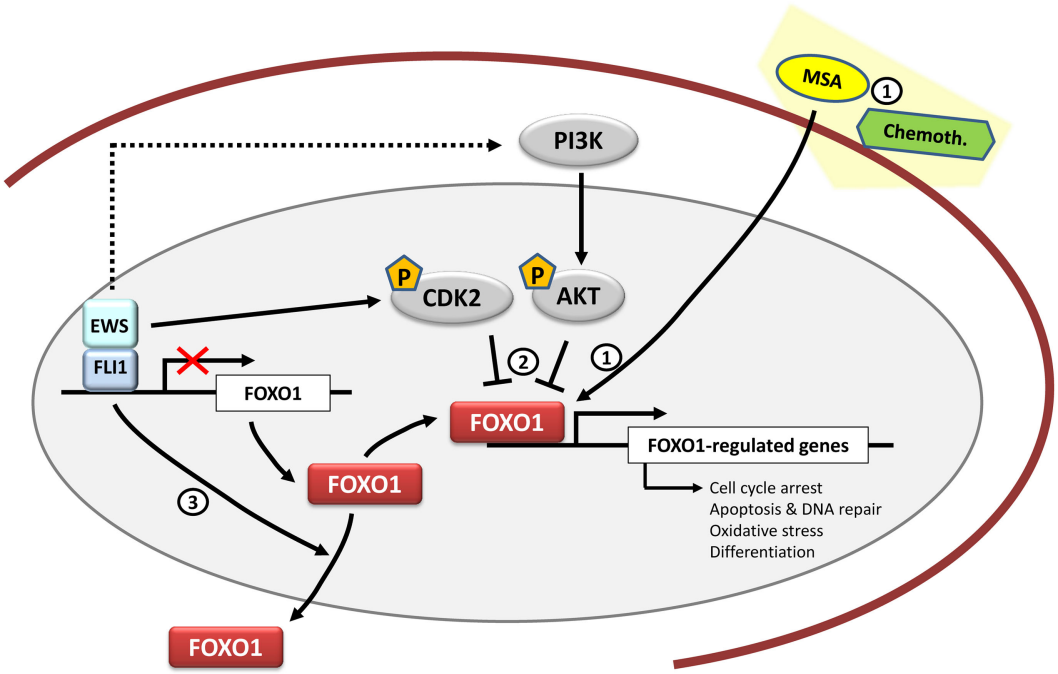

FIGURE 3 | FOXO1 and therapeutic opportunities in Ewing sarcoma. *FOXO1* is a direct target gene of EWS/FLI1 and its expression is repressed by EWS/FLI1 in Ewing sarcoma cells. Functional studies have shown that *FOXO1* acts as a tumor suppressor in the Ewing sarcoma cell context. Therapeutically, Methane Sulfonic Acid (MSA) may be used as a potential treatment in synergy with other chemotherapeutic agents such as doxorubicin or etoposide. However, its mechanism of action in Ewing sarcoma is still unknown (1). Also there is still a need to clarify the FOXO1 activities mediated by kinases such as CDK2 and AKT (2) together with the regulation of its subcellular localization (3), and to determine if they may be mediated by EWS/FLI1.

knockdown experiments demonstrating that FOXM1 downregulation correlates with a significant reduction in anchorage independent growth.

Interestingly, pharmacological approaches addressed to reduce FOXM1 levels have also been tested in Ewing sarcoma cells with notable results. Thiostrepton, a thiazole antibiotic, has been shown to act as a proteosomal inhibitor (73) and also to physically interact with FOXM1 consequently inhibiting FOXM1 binding to target promoters (74). FOXM1 expression was inhibited by treatment with thiostrepton, which paralleled with an increase in apoptosis in a variety of Ewing sarcoma cell lines (17). Thiostrepton was also shown to inhibit tumor growth in mouse xenograft models (75). Strikingly, in this work, thiostrepton was able to concomitantly inhibit the expression of EWS/FLI1 both at mRNA and protein levels in three Ewing cell lines and in tumors derived from thiostrepton-treated mouse xenograft models (75). Although the mechanism by which thiostrepton promotes EWS/FLI1 downregulation was not characterized, these results suggest that this drug may show greater efficacy in Ewing sarcoma tumors in comparison to other tumors.

As stated above, FOXM1 is frequently overexpressed in cancer and takes part in each hallmark of cancer. Consequently it has been argued that targeting FOXM1 could provide an opportunity to treat cancer. It has also been proposed that FOXM1 could be the "Achilles heel" of cancer (76). Taken together, these findings suggest that targeting FOXM1 may be also an opportunity for Ewing sarcoma treatment (**Figure 4**).

Secreted Proteins

Cholecystokinin

Cholecystokinin (CCK) is a neuropeptide that displays a diversity of functions in the organism. It was originally discovered in the gastrointestinal tract, where it mainly regulates pancreatic secretion of digestive enzymes. In addition, CCK is one of the most abundant and widely distributed neuropeptides in the brain, where it modulates intrinsic neuronal excitability and synaptic transmission. CCK is secreted as a prohormone (proCCK) that subsequently undergoes post-translational processing (tyrosine sulfatation, endoproteolytic cleavage, basic residue removal, and C-terminal amidation), resulting in the production of CCK biologically active forms, mainly CCK8 (77).

More than two decades ago, CCK was found to be specifically expressed in a group of human cancer cell lines that included Ewing sarcoma, neuroepithelioma and leiomyosarcomas, as opposed to other tumor cell lines derived from osteogenic sarcomas, rhabdomyosarcoma, melanoma, and SCLC (78). Subsequent studies carried out in tumor specimens confirmed that CCK expression was high in the majority of Ewing sarcomas, whereas in other tumors, CCK-positive cases ranged from 50% in leiomyosarcomas to 0% in medulloblastomas, central primitive neuroectodermal tumors (PNET), neuroblastomas, and rhabdomyosarcomas (79). In agreement with this, a later study demonstrated the presence of proCCK in the supernatant of Ewing sarcoma cell lines in culture, indicating that CCK is actively

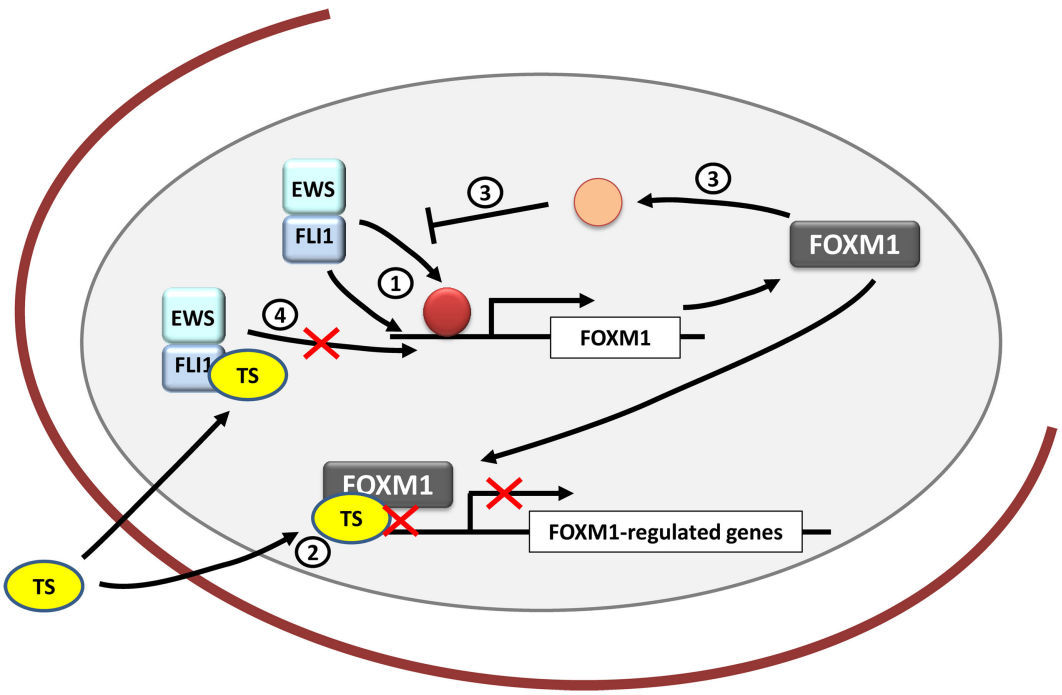

FIGURE 4 | FOXM1 and therapeutic opportunities in Ewing sarcoma. *FOXM1* is upregulated by EWS/FLI1 in Ewing sarcoma cells, although it is unknown whether its regulation is direct or indirect (1). Thiostrepton (TS) blocks FOXM1 in Ewing sarcoma cells decreasing their neoplastic features (2). However, the exact mechanism underlying these effects remains unexplained. In addition, FOXM1 has been shown to be capable of inhibiting EWS/FLI1 probably by an indirect mechanism that still needs to be clarified (3). Also TS has been proved to inhibit EWS/FLI1 expression in Ewing cells, although the exact mechanism is still unknown (4).

secreted by Ewing sarcoma cells (80). Interestingly, these authors found high concentrations of proCCK in the plasma of Ewing sarcoma patients but not in patients with other pediatric tumors such as osteosarcoma, neuroblastoma, nephroblastoma, rhabdomyosarcoma or synovial sarcoma. Interestingly, the levels of proCCK in plasma correlated with tumor size and recurrence. In addition, proCCK levels in plasma decreased after chemotherapeutic treatment, concurrently with a reduction in tumor size and in one patient, proCCK levels increased again correlating with tumor recurrence. All together, these results consistently demonstrate that CCK is expressed and secreted at high levels in Ewing sarcoma.

The first data demonstrating a relationship between *CCK* expression and EWS/FLI1 came from studies performed in heterologous systems: ectopic expression of EWS/FLI1 in the RD rhabdomyosarcoma cell line and in HeLa cells (81) upregulated *CCK* mRNA levels. This relationship between EWS/FLI1 and CCK was confirmed in Ewing sarcoma cells. Thus, EWS/FLI1 knockdown in A673 and SK-PN-DW Ewing sarcoma cell lines downregulated CCK mRNA levels, demonstrating that *CCK* expression is dependent on EWS/FLI1. Whether *CCK* is a direct or indirect target of EWS/FLI1 is a question that yet remains to be determined (8). Regarding the functional relevance of CCK in Ewing sarcoma, it was shown that downregulation of *CCK* using a shRNA inducible system, inhibited cell proliferation *in vitro* and tumor growth *in vivo*. In addition, CCK-rich culture media or exogenous CCK-8 was able to stimulate Ewing sarcoma cell proliferation *in vitro,* suggesting that CCK is an autocrine growth factor in Ewing sarcoma cells (8, 82). Unfortunately, to date no studies have been carried out to decipher the mechanisms that underlie this effect in Ewing sarcoma.

The fact that *CCK* is highly expressed in Ewing sarcoma and the observation that it can act as an autocrine growth factor *in vivo* suggest that blocking this autocrine loop, for example, using CCK receptor antagonists, could be of therapeutic interest. CCK and gastrin (a closely related hormone) share two G-protein coupled receptors, named CCKAR and CCKBR that trigger numerous pathways that transmit the mitogenic signal to the nucleus to promote cell proliferation. Whereas CCKA receptors are specific for CCK, CCKB receptors can bind CCK and gastrin with high affinity. Expression of CCK receptors in Ewing sarcoma has been scantly studied with contradictory results. Schaer and Reubi reported a lack of CCK receptors expression in a collection of 11 Ewing sarcoma tumors using autoradiography and ^{32}P-labeled CCK-8 as a probe (79). However, more recently it was demonstrated the existence of both CCKA and B receptors mRNA in two Ewing sarcoma cell lines (A673 and SK-PN-DW) and a cohort of ten primary tumors (8).

Treatment of Ewing sarcoma cell lines with devazepide, a specific CCKAR antagonist derived from the benzodiazepine family, induced apoptosis *in vitro* and significantly reduced the tumor growth in a mouse xenograft model (83). However, these effects were observed with IC_{50} values 10,000-fold higher that those necessary to efficiently block the binding of CCK to its CCKA receptor. In addition, one specific antagonist of the CCKB receptor (L365 260) had no effect on Ewing sarcoma cell proliferation or viability (83). These results suggest that in Ewing sarcoma cells there could be an alternative mechanism of action that could involve CCK receptors other than the standard ones, and open the possibility that cell proliferation induced by CCK in Ewing sarcoma cell lines could also be mediated through a yet unknown mechanism.

Regardless of the possibility to block CCK-induced proliferation with specific antagonists, the expression of CCK receptors in tumors can itself be therapeutically useful. In this sense, a model of metastatic medullary thyroid cancer has been successfully used to evaluate the diagnostic and therapeutic potential of radiolabeled gastrin directed to target CCKB receptor-expressing tumors *in vivo* (84). Using this approach, a collection of radiolabeled peptides derived from gastrin and cholecystokinin families showed anti-tumoral activity in xenograft models of medullary thyroid cancer (85) [also extensively reviewed in Ref. (86)]. This means that radiolabeled CCK or other compounds with high affinity for CCK receptors could be useful for diagnosis (i.e., imaging) and perhaps also for the treatment of Ewing sarcoma.

In summary, although high levels of CCK in Ewing sarcoma tumors were described more than two decades ago, research in this field has been scattered during the last years, and many questions remain unresolved. For example, it is not clear enough what type of CCK receptors are expressed in Ewing sarcoma tumors or the mechanism and intracellular signaling pathways involved in CCK-mediated cell proliferation. Any progress in this regard would help to develop molecules capable of interfering with this autocrine loop (**Figure 5**).

LOX

Lysyl oxidase (LOX) (protein lysine-6-oxidase; EC 1.4.3.13) is a member of a family of lysyl oxidases that share the enzyme catalytic domain. This family includes LOX and the LOX-like proteins LOXL1 to 4. These enzymes catalyze lysine-derived covalent crosslinking of collagen and elastin and therefore their function is key for maintaining the structural integrity of the extracellular matrix [extensively reviewed in (87–90)]. LOX is synthesized as a 50-KDa inactive proenzyme (preLOX), which is secreted to the extracellular environment where it is proteolytically processed into a functional 32-KDa LOX enzyme and an 18-KDa propeptide (LOX-PP). Together with the critical role that LOX plays in maintaining the properties of the connective tissues, it has been also shown to play important roles in cancer.

The first evidence of a relationship between LOX and cancer comes from experiments designed to identify genes involved in Ras-mediated transformation of NIH-3T3 mouse fibroblasts (91). Several functional experiments demonstrated that *LOX* had properties that are characteristic of a suppressor gene. Thus, *LOX* antisense cDNA was able to retransform *H-ras*-transformed revertants (92) and confer tumorigenic features to normal rat kidney fibroblasts (NRK-49F) (93).

Since LOX is proteolytically processed into a fragment containing the lysyl oxidase enzymatic activity and an N-terminal propeptide (LOX-PP), experiments were conducted to determine in which of these fragments resided the tumor suppressor activity. Thus, Palamakumbura et al. described for the first time that recombinant LOX-PP was able to inhibit neoplastic transformation features in Ras-transformed mouse fibroblasts such as

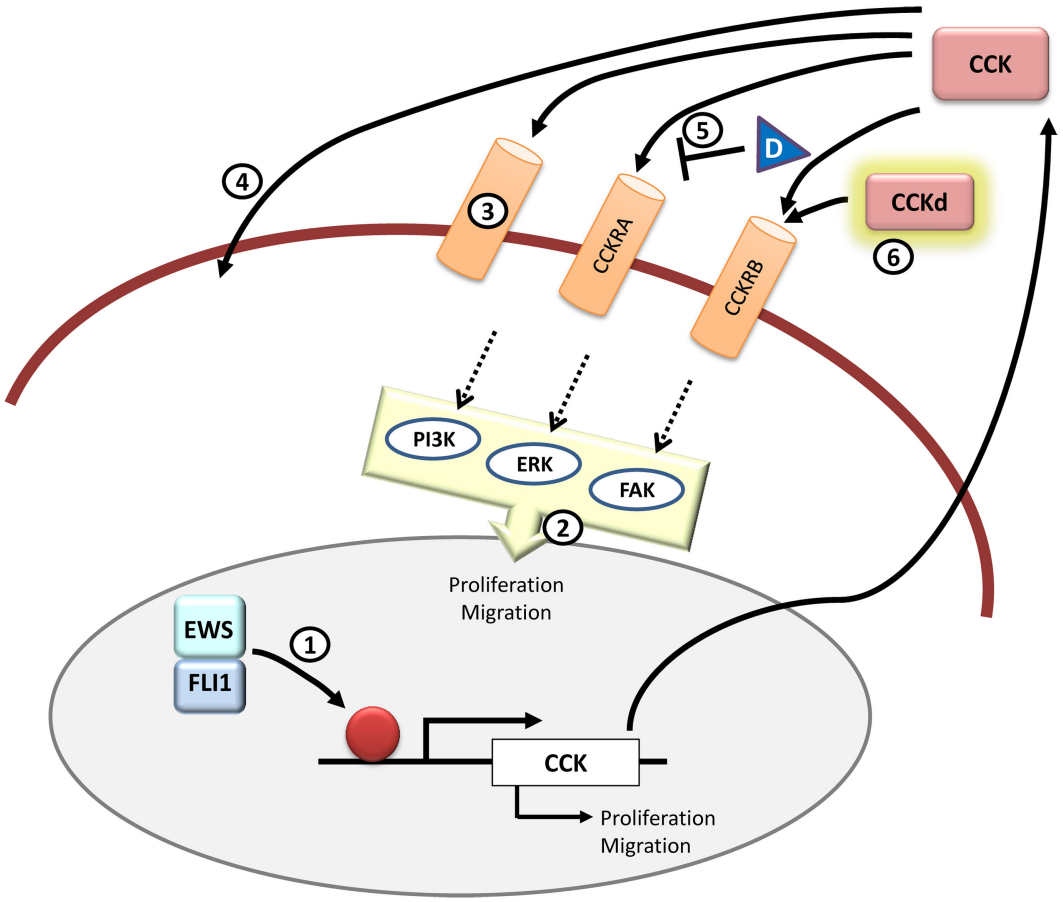

FIGURE 5 | CCK and therapeutic opportunities in Ewing sarcoma. *CCK* is an EWS/FLI1 target gene and its expression is elevated in Ewing sarcoma cells. Functionally, inhibition of CCK expression impairs growth and migration in Ewing sarcoma cells. It still remains to be addressed if *CCK* is a direct EWS/FLI1 target or not (1) and which is the exact signaling pathway that takes place once CCK binds to its receptors in the cells (2). Also it is still unknown whether CCK binds exclusively to its canonical CCKRs or if there are other receptors (3) or even if it can enter directly into the cell through some yet unknown mechanism (4). Therapeutically, it may be interesting to further test receptor antagonists other than devazepide (D) that could interfere with CCK binding to its receptors in Ewing sarcoma cells (5). Also, from a diagnosis point of view, it could be useful to test radiolabeled CCK derivatives (CCKd) to be used in imaging technologies (6). All in all, more studies are needed to define the principal components and pathways that are involved in the CCK-autocrine loop.

growth in anchorage independent conditions and Ras-dependent induction of *NFκB* (94). Currently, numerous studies support that the tumor suppressor activity of LOX resides in the 18-kDa propeptide fragment LOX-PP and not in the lysyl oxidase enzyme.

In agreement with its tumor suppressor activity, LOX expression has been reported to be downregulated in many different types of human cancer, such as fibrosarcoma, rhabdomyosarcoma, and melanoma cells (95), lung (96), pancreatic cancer (97), prostate (98), and colorectal cancers (99), which means that LOX expression levels negatively correlate with malignant transformations. By contrast, LOX expression has been also reported to be increased in a number of human cancers [i.e., breast and colon carcinomas (100, 101)] particularly in the metastatic and more aggressive forms of the disease. Interestingly, in these cases, metastatic and invasive properties have been related to the lysyl oxidase activity of LOX, rather than to LOX-PP (100, 101).

The anti-tumor activity of LOX-PP has been demonstrated in various types of tumor cells although the mechanism underlying the tumor suppressor activity of LOX-PP still needs to be clarified. Data obtained until now indicate that LOX-PP can act at different levels, and that the pathways and functions affected can depend of the cancer or cell model studied. For example, in Her-2/neu-transformed NF639 breast cancer cells, ectopic expression of LOX-PP interferes with fibronectin-stimulated tyrosine phosphorylation of cellular proteins involved in integrin signaling, inactivating the focal adhesion kinase (FAK), and consequently diminishes the migratory response (102). In other breast cancer cells driven by Her-2/neu (ERBB2), LOX-PP expression suppressed AKT, ERK, and NFκB activation, as well as cell migration, growth in soft agar and tumor formation in nude mice (103). Moreover, in cells derived from prostate cancer (DU145 and PC-3), LOX-PP blocks FGF-2 binding to the cell, inhibiting MAPK/ERK and PI3K/Akt pathways and blocking serum-stimulated DNA synthesis and cell proliferation (104). On the other hand, LOX-PP decreased the levels of NF-κB and cyclin D1 in Her-2/neu-transformed NF639 breast cancer cells and MIA PaCa-2 pancreatic cancer cells, together with a reduction in migration and growth in soft agar (105). Finally, in PANC-1

pancreatic cancer cells, LOX-PP also impaired AKT and ERK activity and growth in soft agar and cell migration (97).

Recently, a connection between LOX and Ewing sarcoma pathogenesis has been also demonstrated. Thus, EWS/FLI1 knockdown in Ewing sarcoma cells induces the expression of *LOX* indicating that this gene is strongly repressed by EWS/FLI1 in these cells (15). An independent study showed that *LOX* is a direct target of EWS/FLI1 by using ChIP assays (106). In agreement with this, LOX expression was found to be low or undetectable in a group of Ewing sarcoma cell lines and primary tumors (15). Since these data suggested that LOX could act as a tumor suppressor in Ewing sarcoma, functional studies were carried out. Thus, ectopic expression of LOX-PP in the A673 Ewing sarcoma cell line reduced cell proliferation, cell migration, anchorage independent growth, and impaired tumor growth *in vivo*, indicating that it had tumor suppressor activities in this cell, in line with what was observed in other tumors. By contrast, the mature LOX enzyme displayed the opposite effects. Interestingly, when full-length LOX, including LOX enzyme and LOX-PP activities was expressed in A673 cells, the anti-tumor effects prevailed (15). Altogether, these studies indicate that LOX plays an important role in Ewing pathogenesis by acting as a tumor suppressor gene.

The mechanisms involved in LOX-PP-mediated suppression in Ewing sarcoma have only been partially studied. In one study, ectopic expression of LOX-PP showed to impair ERK signaling pathway, whereas the PI3K/AKT pathway remained unaffected (15). Interestingly, in this work, an analysis of the gene expression profile induced by LOX-PP expression in the A673 Ewing cell line showed that a significant proportion of the genes affected belonged to pathways involved in cell proliferation and cell cycle control. Given the impact that LOX-PP expression has on tumorigenesis, it is necessary to extend these studies in order to characterize in more detail the pathways that may be affected by the exposition of Ewing sarcoma cells to LOX-PP, and particularly to determine which specific growth factor pathways could be affected by LOX-PP.

Other interesting aspect that remains to be determined is the identification of the proteins that interact with LOX-PP in Ewing sarcoma cells. In other cell types, LOX-PP has been shown to interact with a number of proteins such as Hsp70, c-Raf or CIN85 (107), so it would be interesting to identify and characterize LOX-PP partners in the specific Ewing sarcoma cell context and to elucidate their role in LOX-PP mediated tumor suppression.

The fact that LOX-PP acts as a tumor suppressor gene in cancer, and specifically in Ewing sarcoma, invites to assess the therapeutic value of LOX-PP. The easiest strategy is to evaluate the effect of the administration of LOX-PP on tumor cells. Thus, recombinant LOX-PP has been used to ascertain its therapeutic potential in several cancer types both *in vitro* and *in vivo* (94, 97, 102–105, 108, 109). In all cases, exogenous LOX-PP reduced tumor cells growth, supporting the therapeutic usefulness of this strategy. Interestingly, in one study, the combination of LOX-PP with the chemotherapeutic agent doxorubicin in breast and pancreatic cancer cells *in vitro* showed an enhanced cytotoxic effect of doxorubicin when the cells were first sensitize by incubation with LOX-PP (105). These results mean that even if LOX-PP is not capable of inducing complete cell death by itself, it could potentially sensitize cancer cells to standard therapies thus allowing to lower the doses and adverse side effects associated to conventional chemotherapy and radiotherapy. At the moment, there are no data about the effect of exogenous administration of LOX-PP, alone or in combination with chemotherapeutic drugs, on Ewing sarcoma cells. These preclinical studies are therefore needed to test if this strategy can represent a promising line of research in order to find new therapeutic approaches to treat Ewing sarcoma patients.

Since *LOX* expression, and thus LOX-PP, is downregulated in Ewing sarcoma cells by EWS/FLI1 (15, 106), other therapeutic approach could be the induction of LOX expression, and thus LOX-PP, in these cells. In this line, it has been proposed that EWS/FLI1 binds to *LOX* promoter and downregulates *LOX* expression by recruiting the NuRD transcriptional repressor complex containing the HDACs and LSD1 associated proteins. Interestingly, the use of HDACs inhibitors (vorinostat/SAHA) or LSD1 inhibitors (HCI-2509) induced an increase in the levels of LOX mRNA in A673 Ewing sarcoma cells, which suggest that the anti-tumor effect of these drugs could be mediated, at least in part, by the upregulation of *LOX* (106). However, induction of *LOX* expression to achieve an increased production of anti-tumorigenic LOX-PP in Ewing sarcoma cells may not be as beneficial as expected: while induction of *LOX* expression would cause an increase in LOX-PP, it also would produce an increase in the production of the LOX mature enzyme, which has been showed to be pro-oncogenic in Ewing sarcoma cells and other tumors (15, 100, 101).

Other opportunities for therapeutic interventions could be derived from the identification and characterization of LOX-PP interactions with other proteins, mainly intracellular proteins involved in cell signaling and regulation of tumorigenic processes. Biochemical studies have shown that LOX-PP is an intrinsically disordered protein (110). These proteins are expected to participate in signaling processes due to their capability to adopt interconverting structures and to interact with their partners, and have been proposed to be potential drug targets (111). Thus, characterization of the exact motifs that are involved in LOX-PP interactions can open the door to the identification of targetable proteins and the design of small molecules capable to reproduce the effect of LOX-PP.

In summary, LOX, and more specifically LOX-PP, has been showed to have anti-tumorigenic properties, which could be exploited to treat cancer cells. Regarding Ewing sarcoma, it is yet more than necessary to characterize the pathways involved in LOX-PP mediated tumor-suppression, in particular the identification of the protein interactions that mediate this response, in order to identify key factors that could provide new therapeutic targets (**Figure 6**).

Conclusion

Ewing sarcoma is driven by EWS/FLI1, which is a protein generated by a tumor-specific aberrant translocation. Although it may seem like a perfect target for therapeutic applications, directed therapies toward it have failed to reach the clinic (112). For this reason, the identification of EWS–FLI target genes and their role in tumor signaling networks have been addressed in the last years, and some excellent reviews have assessed this topic (4, 113, 114).

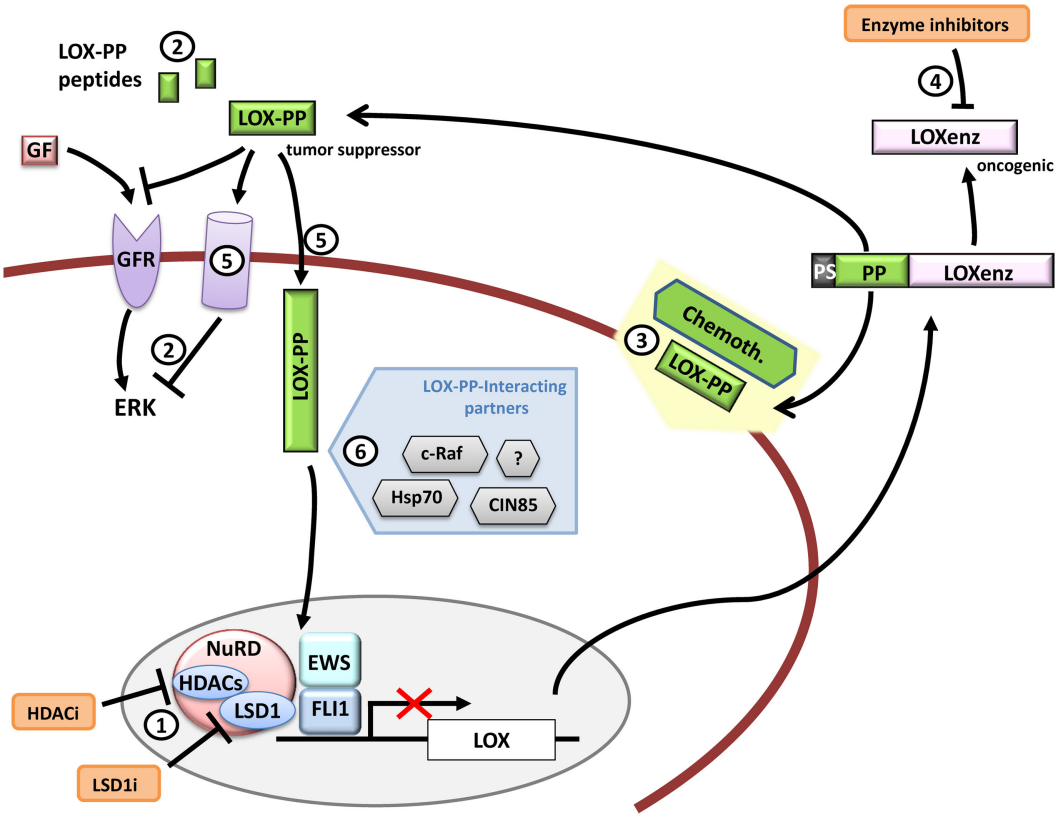

FIGURE 6 | LOX and therapeutic opportunities in Ewing sarcoma. *LOX* expression is repressed by EWS/FLI1 in Ewing sarcoma cells. Functional studies have demonstrated that LOX acts as a tumor suppressor gene in Ewing sarcoma and that its activity resides in its propeptide domain (LOX-PP). Therapeutic opportunities could include for example (1) *LOX* de-repression by targeting repression complexes that interact with EWS/FLI1 at the *LOX* promoter, (2) administration of LOX-PP or LOX-PP active derived peptides to block ERK signaling alone or in combination with traditional chemotherapy (3) or blocking the LOXenz fraction activity (4). The mechanisms through which LOX-PP exerts its anti-tumor activity are largely unknown, especially in Ewing sarcoma. For instance, it is currently unknown if LOX-PP specific receptors (5) (intracellular or transmembrane) are necessary to produce its anti-tumor activities or if the different LOX-PP-interacting proteins may interfere with its activity in Ewing sarcoma (6). Any advance in these aspects could provide new clues to design new therapeutic tools.

This review is focused on the EWS/FLI1 downstream regulatory network, particularly on EWS/FLI1 up- and down-regulated target genes on which the study of potential targeted therapies could be of clinical interest. Also, we stated some current questions regarding pathways and unknown mechanisms underlying the functional effects of these genes in Ewing sarcoma that still remain unresolved and could help find key clues for the future studies of this disease. There are plenty of mechanisms regarding EWS/FLI1 target genes that are still unknown and a deeper knowledge on them could potentially lead to the development of more specific and less toxic therapies in Ewing sarcoma.

Author Contributions

FC-A and JA wrote the manuscript and designed the figures. JA corrected and supervised the manuscript.

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REVIEW
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MicroRNAs and potential targets in osteosarcoma: review

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Osteosarcoma is the most common bone cancer in children and young adults. Surgery and multi-agent chemotherapy are the standard treatment regimens for this disease. New therapies are being investigated to improve overall survival in patients. Molecular targets that actively modulate cell processes, such as cell-cycle control, cell proliferation, metabolism, and apoptosis, have been studied, but it remains a challenge to develop novel, effective-targeted therapies to treat this heterogeneous and complex disease. MicroRNAs (miRNAs) are small non-coding RNAs that play critical roles in regulating cell processes including growth, development, and disease. miRNAs function as oncogenes or tumor suppressors to regulate gene and protein expression. Several studies have demonstrated the involvement of miRNAs in the pathogenesis of osteosarcoma with the potential for development in disease diagnostics and therapeutics. In this review, we discuss the current knowledge on the role of miRNAs and their target genes and evaluate their potential use as therapeutic agents in osteosarcoma. We also summarize the efficacy of inhibition of oncogenic miRNAs or expression of tumor suppressor miRNAs in preclinical models of osteosarcoma. Recent progress on systemic delivery as well as current applications for miRNAs as therapeutic agents has seen the advancement of miR-34a in clinical trials for adult patients with non-resectable primary liver cancer or metastatic cancer with liver involvement. We suggest a global approach to the understanding of the pathogenesis of osteosarcoma may identify candidate miRNAs as promising biomarkers for this rare disease.

Keywords: osteosarcoma, microRNA, mutations, cell proliferation, apoptosis

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Introduction

Osteosarcoma (OS) is an aggressive bone cancer that affects children and adolescents. Approximately 60% of cases are pediatric patients between 10 and 20 years of age (1). Several studies suggest that OS arises from primitive mesenchymal bone-forming cells that undergo aberrant alterations in the differentiation program. This results in a heterogenic cancer, with complex etiology, characterized by vast genomic instability, highly abnormal karyotypes, and multiple genomic aberrations with copy number gains and losses occurring at multiple chromosomes (2, 3). Patients with certain rare and inherited syndromes, such as Li–Fraumeni syndrome, hereditary retinoblastoma, Rothmund–Thomson syndrome, Bloom syndrome, and Werner syndrome have a higher incidence of OS (4). Treatment involves standard chemotherapy administered before and after surgery, and may be followed by radiation, which achieves a 5-year survival rate of 60–70% of patients. However, the

survival of patients with locally advanced or metastatic tumors at diagnosis, and recurrent disease remains low (~20%), and the median survival time for these patients is only 23 months (5). Current clinical trials of cytotoxic chemotherapy and targeted agents may achieve an objective response in a subset of patients with OS, but have not increased overall survival in the recent treatment era. Further, constitutive and acquired resistance to these therapies remains a clinical challenge. Therefore, a global understanding of the underlying factors of tumor biology will assist in the identification of diagnostic and prognostic markers and therapeutic targets for the management of patients with OS.

MicroRNAs (miRNAs) belong to the group of small, non-coding, regulatory RNA molecules, ranging between 18 and 25 nucleotides in length (6). They recognize and bind specific target mRNAs by complete or partial base-pairing mostly at the 3′-untranslated region (UTR) of the target genes to post-transcriptionally regulate gene expression. Since their discovery nearly 20 years ago, bioinformatics and biological studies have identified >1,000 miRNAs that regulate possibly 50% of human genes. Each miRNA likely controls hundreds of gene transcripts (7). The sequences of miRNAs are highly conserved among distantly related organisms, indicating their participation in essential biological processes as development, cellular differentiation, metabolism, proliferation, and apoptosis. Moreover, they regulate biological systems as stemness, immunity, and cancer. Studies show that more than 50% of miRNA genes are located at fragile chromosomal sites and in proximity to regions of deletion or amplification that are altered in human cancer, implicating a direct involvement for miRNAs in tumorigenesis (8). Families of miRNAs that share similar "seed" sequences or are located in close proximity on a single genomic locus may be co-expressed to form miRNA regulatory networks in particular physiological or pathological contexts. miRNAs that are underexpressed in cancers are tumor suppressors (loss of miRNA contributes to the malignant phenotype), while highly expressed miRNAs function as oncogenes (gain of miRNA contributes to the malignant phenotype). These expression changes control many genes involved in cell proliferation or apoptosis. Therefore, expression profiles of miRNAs may be applied as biomarkers for cancer diagnosis.

The first study on miRNA expression in OS, published by Gao et al. (9), identified 182 miRNAs from a human OS cell line, indicating that miRNAs may contribute to the pathogenesis of OS. Recently, whole genome analysis of DNA copy number, mRNA gene expression, and miRNA transcript profiling performed in seven OS patient tumors identified a signature of 38 differentially expressed miRNAs in OS tumors compared to normal osteoblasts (10). Of these, expression levels of 28 miRNAs were down-regulated and 10 were upregulated ≥10-fold in tumors versus osteoblasts, providing likely candidates for further investigation. Other miRNA profiling studies have shown altered expression of several distinct miRNAs in OS tumors including miR-135b, miR-150, miR-542-5p, and miR-652 that were validated in a separate group of tumors (11). The analysis of common insertion site (CIS)-associated genes identified three miRNAs (miR-181, miR-17-5p, and miR-26a-5p) as significant upstream regulators in human OS (12). Also, aberrant expression of individual miRNAs is well-recognized to play a role in the initiation and progression of various cancers. The oncogenic miR-17–92 cluster is overexpressed in several types of cancer and promotes cell proliferation (13). In contrast, downregulation of the miR-15/16 family increases expression of anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and myeloid leukemia cell differentiation protein (Mcl-1) (14) and loss of let-7a facilitates amplification of the c-myelocytomatosis virus (*MYC*) oncogene to promote B-cell tumorigenesis (15). Characterization of these miRNAs that display altered expression in OS may provide distinct miRNAs or miRNA signatures related to particular molecular patterns associated with this disease.

Unlike many other types of cancer, there are no traditional biomarkers for OS. The presence of metastatic disease and histologic response assessed following adjuvant chemotherapy (i.e., the extent of necrosis) are the only generally accepted predictors of event-free survival (16). The identification of new diagnostic miRNA biomarkers has the potential to complement existing risk prediction models and could eventually have a prognostic value in this disease. miRNA expression signatures are undergoing clinical investigation in pediatric patients with central nervous system (CNS) tumors (NCT01595126, NCT01556178), CNS tumors along with leukemia and lymphoma (NCT01541800), acute myeloid leukemia (AML) (NCT01229124), and neurofibromatosis Type 1 (NF-1) (NCT01595139). Also, the molecular analysis of solid tumors (MAST) clinical trial (NCT01050296) is designed to prospectively characterize the molecular, cellular, and genetic properties of primary and metastatic solid tumors in patients including OS. These studies present a novel opportunity to investigate the expression of miRNAs in the blood, body fluids, and tissue of patients as an early predictor of cancer as well as a marker of response to therapy. Of note, one Phase 1 clinical trial conducted by MiRNA Therapeutics Inc. is evaluating miR-34 as an miRNA replacement therapy in patients with non-resectable primary liver cancer, with liver metastasis from other cancers, and a cohort of patients with hematological malignancies (NCT01829971).

The various genomic and molecular alterations, which are linked to the development and progression of OS are well established. These include germline mutations, gene amplifications and deletions, overexpression and activation of receptor tyrosine kinases (RTKs), enhanced cell proliferation, resistance to apoptosis, metastasis, drug resistance genes, and miRNAs [reviewed in Ref. (17); and available at http://osteosarcoma-db.uni-muenster.de]. These alterations mediate changes that affect the expression and function of several genes and gene regulatory networks. miRNA profiling and computational analyses have identified associations between miRNAs and many gene and gene products linked to these aberrant factors. This review discusses some of the prominent pathological factors of OS that may be regulated by miRNAs and highlights miRNAs that are validated in preclinical OS models.

miRNAs in the Pathogenesis of OS

Germline Mutations

Osteosarcoma is characterized by complex, unbalanced karyotypes, and the pattern of abnormalities varies among patients. Numerical and structural chromosome abnormalities are

detected in the majority of OS tumors (58%) (3, 17–19). Common numerical chromosomal abnormalities are polyploidy, caused by errors in mitosis, aneuploidy, germline mutations, deletions, duplications, and unbalanced translocations. These include gain of chromosome 1, loss of chromosomes 9, 10, 13, and/or 17, partial or complete loss of the long arm of chromosome 6 and ring chromosomes (7%) (19, 20). Characteristic reciprocal translocations are absent in OS and rearrangements of chromosomes 11, 19, and 20 are frequent structural abnormalities (21). Two of the most prominent genes that harbor germline mutations are the retinoblastoma tumor suppressor gene (*RB1*) and the *TP53* tumor suppressor gene (2). These genes are important for mitotic checkpoints and are thought to be the underlying cause of chromosomal instabilities. Most OS tumors contain inactivation of both the retinoblastoma (Rb) and p53 pathways.

RB1

The retinoblastoma protein (pRb) was the first described tumor suppressor. pRb1 is a checkpoint protein that binds the E2F family of transcription factors and inhibits cell-cycle progression. The activation of cyclin-dependent kinases (CDK) and cyclins by mitogenic signals phosphorylates pRb, which dissociates from E2F transcription factors. This leads to the activation of E2F target genes to facilitate the G1/S transition and S-phase progression (22). Thus, loss of function of the *RB1* gene drives tumorigenesis in many adult and pediatric cancers including OS (23, 24), retinoblastoma (25), medulloblastoma, supratentorial primitive neuroectodermal tumor (sPNET) (26), and acute lymphoblastic leukemia (ALL) (27). Gene mutations occur in 20–40% of patients with sporadic OS (28) and loss of heterozygosity (LOH) at 13q, the site of location of the *RB1* gene occurs in approximately 70% of OS cases (29). Germline mutations along with genetic loss or deletions of *RB1* are associated with inactivation of pRb. These are considered as high risk factors for the development of OS and are linked to poor outcome (29).

An miRNA signature consisting of miR-9-5p, miR-138, and miR-214 was predicted to target mRNA genes that encode proteins involved in pRb-signaling in OS (30). However, the experimental validation of these miRNA:mRNA interactions has not been conducted. Other miRNAs including miR-449a, miR-449b, and the miR-17–92 locus have been described in the regulation of Rb/E2F (E2F transcription factor) pathway in many cancers (31). Mechanistically, miR-449a and miR-449b were direct transcriptional targets of the E2F transcription factor 1 (E2F1) and negative modulators of pRb phosphorylation by inhibition of (cyclin-dependent kinase 6) *CDK6* and cell division cycle 25A (*CDC25A*) (32). This was consistent with the previously identified regulation of CDK/pRb/E2F1 through an autoregulatory feedback mechanism by miR-449a (33). Interestingly, miR-449a was expressed in low levels in OS cell lines and tumors and directly targeted the binding site within the 3′-UTR of the *BCL2* mRNA (34). In a separate study, restoration of miR-29a in osteoblastic cells using miRNA mimetics was shown to repress *BCL2* mRNA and induce E2F3 and its transcriptional target, E2F1 (35). Collectively, these findings support a pro-apoptotic and tumor suppressor role for miR-29a that may participate with miR-449 to regulate the Rb/E2F signaling network in OS.

miR-17–92 is a well-studied polycistronic miRNA cluster in cancer. The miR-17–92 locus contains 15 miRNAs that form four "seed" families, miR-17, miR-18, miR-19, and miR-92. Many of these are reported to be amplified in diffuse B-cell lymphoma, lung, breast, and pancreatic cancers, as well as OS (13, 36, 37). Direct target genes of miR-17–92 include *E2F1*, phosphatase and tensin homolog (*PTEN*), and *p21* (13). Two members of the miR-17–92 cluster (miR-17-5p and miR-18a) were among five highly expressed oncogenic miRNAs in several OS cell lines that were suggested to be predictive of poor disease prognosis (36). In the Sleeping Beauty (SB) transposon-based forward genetic screen, miR-17-5p was one of the three enriched upstream regulators identified in an OS mouse model (12). Future studies to establish the function of miR-17–92 in OS are warranted.

TP53

The human p53 gene encodes the p53 tumor suppressor protein that plays a crucial role in maintaining genomic stability. The p53 tumor suppressor functions as a transcription factor that regulates the expression of various genes that are involved in cell-cycle arrest, DNA repair, and apoptosis. In normal, unstressed cells, mouse double minute 2 (MDM2) binds and inhibits p53 function to allow p53 degradation via the ubiquitin/proteasome pathway. In stressed cells, p53 is stabilized, and p53 transcriptional activity is promoted. Alterations in the *TP53* gene are associated with functional inactivation of p53 and less favorable prognosis in OS (38). Genetic abnormalities of p53, such as allelic loss (75–80%), gene rearrangements (10–20%), or point mutations (20–30%) are reported in 50% of OS patients (39). The *MDM* gene is also amplified in 16% of OS patients and is associated with aggressive disease (40). Patients with the Li–Fraumeni syndrome with a germline mutation of *TP53* are predisposed to developing OS.

Studies involving various cancer types have validated several miRNAs that are components of the signaling cascades that regulate p53 expression. miR-125b, miR-504, miR-25, and miR-30d are reported to directly bind the p53 mRNA and negatively regulate p53 expression (41). In addition, miR-34a, miR-192, miR-194, miR-215, miR-605, and miR-29 regulate upstream components and indirectly activate p53 (41). Of these, miR-34a is a key tumor suppressor that regulates numerous genes that are involved in DNA damage and repair. A positive feedback loop mediated by p53 target sites in the miR-34a promoter also controls transactivation of miR-34a by p53 (42, 43). Mutations in *TP53*, functional inhibition of p53, and hypermethylation of the miR-34a promoter are all associated with the loss of miR-34a expression in tumors (44). In a p53-expressing OS cell line, DNA damage-induced miR-34a expression was dependent on p53, which in turn led to the induction of cell-cycle arrest, promotion of apoptosis, and DNA repair (45). This was not observed with p53-deficient OS cells, illustrating that miR-34a was a downstream target of p53. Restoration of miR-34a with miRNA mimics in OS cells repressed p53 and runt-related transcription factor 2 (RUNX2) and suppressed tumor cell growth (46). Notably, miR-34a was demonstrated to be a target of C/EBPα CCAAT/enhancer-binding protein-alpha (C/EBPα, encoded by the gene *CEBPA*) during granulopoiesis, and low expression in leukemic blasts with CEBPA mutations

elevated levels of E2F3 and its transcriptional target, E2F1 (47). A clinical study of the role of miR-34a and miR-194 in pediatric patients with AML with mutated CEBPA was recently concluded by the Children's Oncology Group and the results of this study are pending (NCT01057199).

p53 can transcriptionally induce miR-192 and miR-215 in several types of cancer (48). These miRNAs were markedly downregulated in OS (49), and transactivation of miR-192 and miR-215 by p53 was also reported in OS cell lines (48). The ratio of expression levels of miR-192 and miR-215 has been proposed to differentiate p53-negative and p53-expressing OS patient tumors (49). In addition, miR-215 has been implicated in p53-mediated chemoresistance to methotrexate and the thymidaylate synthase inhibitor, Tomudex, in OS cell lines. Resistance was mediated through repression of the denticleless protein homolog (DTL), a cell-cycle-regulated nuclear, and centrosome protein (50). Together, these findings support miR-34a, miR-192, and miR-215 as candidates for novel biomarkers of prognosis and drug response in OS.

Gene Amplifications

Several genetic deletions and amplification are considered predisposing conditions to OS. Genomic amplifications (homogeneously staining regions) occur in approximately 30% of OS cases (20), which are associated with the action of oncogenes, such as apurinic/apyrimidinic exonuclease 1 (*APEX1*), cellular homolog of avian *MYC*, RecQ protein-like 4 (*RECQL4*), *CDK4*, *MDM2*, *RUNX2*, and vascular endothelial growth factor A (*VEGFA*) (51). Many of these amplified genes are involved in cellular proliferation, survival, and angiogenesis of OS. Amplification of the 12q13 chromosomal region (containing *MDM2* and *CDK4*) or *INK4A* deletion at location 9p21 can affect both the p53 and pRb pathways, and may sometimes occur simultaneously with *RB* or *TP53* alterations (14). Of these amplified genes, the miRNAs targeting *MYC* and *RUNX2* have been well-characterized in OS.

MYC

The *MYC* oncogene encodes a transcription factor that regulates genes that control cell growth and cell-cycle progression (52). Genetic and epigenetic alterations associated with constitutive c-Myc activation promote oncogenesis in a variety of cancers (52). The *MYC* locus is amplified in ~30% of OS tumors (53), and c-Myc protein is overexpressed in the majority of OS cases (54). Thus, dysregulation of c-Myc is an important component of OS pathogenesis. The c-Myc transcription factor globally silences several miRNAs either by inhibition of tumor suppressor miRNAs (including miR-15a/16-1, miR-34 family, miR-23), or activation of oncogenic miRNAs (e.g., miR-17–92). In addition c-Myc forms a feedback regulatory loop involving direct or indirect repression of let-7, a well-recognized tumor suppressor miRNA, through the RNA-binding protein, LIN28 (15, 55). Thayanithy et al. (56) demonstrated significant decreases in expression levels of miRNAs at the 14q32 locus (miR-369-3p, miR-544, miR-134, and miR-382) in OS cell lines and tumors compared to normal bone tissues. This correlated with c-Myc overexpression and enrichment of the miR-17–92 cluster. In addition, miR-135b (57) and miR-33b (58) were demonstrated to directly repress c-Myc in OS cells and restoration inhibited cell proliferation, migration, and invasion. Interestingly, the expression of let-7 family members was attenuated in OS cell lines (59, 60). This was predicted to affect the regulation of oncogenes that influence cell-cycle progression and apoptosis. Since let-7 targets multiple oncogenes including *MYC, RAS, CCND, BCL2* (61), this miRNA may be an interesting candidate for future investigation in this disease.

RUNX2

The *RUNX2* gene located on chromosome 6p12–p21 is frequently amplified in OS and is associated with tumor growth (20). This gene encodes a transcription factor that is necessary for both osteoblast differentiation and chondrocyte maturation. RUNX2 is linked to many human cancers including breast, prostate, and bone cancer and also cancer metastasis in bone (62). High expression levels often correlate with poor response to chemotherapy (63, 64). The RUNX2 protein has been shown to directly interact with p53 and pRb transcription factors (58), but the precise function of RUNX2 in OS pathogenesis is unclear. An inverse correlation of expression between miR-23a and *RUNX2* mRNA levels in OS cells and tumors was demonstrated by He et al. (65). An association between miR-23a and *RUNX2* and chemokine (C–X–C motif) ligand 12 (*CXCL12*) mRNA was demonstrated as enrichment of miR-23a suppressed transcriptional activity of RUNX2 and inhibited proliferation in OS cells and xenograft tumors (65). These studies indicate a tumor suppressor function for miR-23a in this disease. In addition, miR-103a was reported to play a role in the regulation of osteoblast differentiation by directly targeting the 3′-UTR of *RUNX2* mRNA to inhibit matrix mineralization and bone formation (66). Other miRNAs, miR-135 and miR-203, were identified to modulate RUNX2 in breast cancer cells (67). Inhibition of RUNX2 interacting proteins by miRNAs also affected RUNX2 stability and transactivation potential. Protein expression levels of RUNX2 and the co-transcription factor, SATB2, were regulated by miR-205 and overexpression of the special AT-rich sequence-binding protein 2 (SATB2) activated RUNX2 and reversed the inhibitory effects of miR-205 on osteoblastic differentiation (68). These studies provide more comprehensive details on the involvement of miRNAs involved in osteoblast regulation and OS.

Receptor Tyrosine Kinase Activation

Aberrant activation of RTKs and their ligands promote malignant progression in OS. These RTKs include epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-1R), vascular endothelial cell growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and mesenchymal–epithelial transition factor (c-Met). RTK activation via gene mutations, gene amplifications, protein overexpression, and/or ligand-dependent autocrine/paracrine loops has been demonstrated in patient primary tumors, cell lines, and xenograft tumors. This is generally associated with cell proliferation, cell survival and metastasis, and overall poor prognosis. In the past decade, several monoclonal antibodies and small molecule inhibitors targeted against RTKs have been evaluated in pediatric patients with solid tumors including OS (69). These agents were well-tolerated but showed limited single-agent activity in

patients. Moreover, resistance to these therapies due to cross-talk between receptors resulted in the activation of compensatory RTK cell survival signaling to facilitate tumor progression (15). A more detailed understanding of biological mechanisms of drug response and resistance will assist in addressing the challenges of RTK inhibition. Recent experimental studies have investigated the role of several tumor suppressor miRNAs in the regulation of *IGF-1R* and *MET* in OS.

IGF-1R

IGF-1R is a transmembrane receptor that is activated by IGF-1 and IGF-2 ligands and mediates signaling involved in processes, such as cell proliferation, migration, and differentiation (70). High expression levels of IGF-1R, IGF-1, and IGF-2 have been demonstrated in many cancers including breast, prostate, colon, and pediatric cancer (71, 72). IGF-1R is overexpressed in ~45% of OS patients (72). In 2013, Chen et al. demonstrated that miR-16 expression levels were low in OS cell lines and inversely correlated with *IGF-1R* mRNA levels. miR-16 is a member of the mir-15/16 family that functions as a tumor suppressor in a variety of cancers. These miRNAs target *BCL2* and numerous genes involved in the G1/S transition, such as cyclin D1 (*CCND1*), cyclin D3 (*CCND3*), cyclin E1 (*CCNE1*), and *CDK6* (14). The restoration of miR-16 in OS cells suppressed proliferation by inhibition of IGF-1R and the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway. These findings were significant since MAPK activation is associated with the induction of proliferative and anti-apoptotic signaling in OS (73) and in resistance mechanisms to targeted therapies (74). Han et al. (75) demonstrated that miR-194 directly targeted *CDH2* and *IGF-1R* mRNA to suppress OS cell proliferation and metastasis *in vitro* and *in vivo*. A tumor suppressor role of miR-194 was also described for gastric cancer (76) and lung cancer (77), though bone morphogenetic protein 1 (*BMP1*) and the cyclin-dependent kinase inhibitor *p27(kip1)* were the targets of miR-194 in these cancers. Furthermore, expression of miR-133b correlated negatively with IGF-1R, Bcl2-like 2 (Bcl2L2), Mcl-1, and c-Met protein levels in OS cells (78). This suggests the potential of miR-133b to function as a master regulator of critical genes, which control cell survival in OS. These insights into the miRNA-mediated regulation of IGF-1R provide new details of biological mechanisms of response and resistance to IGF-1R inhibition in preclinical models of OS.

MET

The *MET* oncogene encodes the receptor for the hepatocyte growth factor (HGF), a cytokine that stimulates invasive growth of normal and neoplastic cells. The c-Met receptor is overexpressed in a variety of human malignancies, including sarcomas, and particularly in chordoma (94.4%), chondrosarcoma (54.2%), and OS (23.3%), determined in 122 cases of malignant bone tumors (79). Activation of c-Met increases phosphatidylinositol-3-kinase (PI3K)/Akt, Src, c-Jun N-terminal kinase, signal transducer and activator of transcription 3 (STAT3) and MAPK pathway signaling, and is implicated in acquired resistance to EGFR and angiogenesis inhibitors (80, 81). Two miRNAs that were found to directly target *MET* mRNA in OS were miR-34a (82) and miR-199a-3p (83). This was consistent with previous reports that c-Met is one of the common targets for the miR-34 family (42). The overexpression of c-Met in tumors of p53-deficient mice and in Li–Fraumeni patients (84) suggests that miR-34/p53/c-Met may form a gene regulatory network that cooperatively controls tumor progression in OS. Restoration of either miR-34a or miR-199a-3p with respective miRNA mimics in OS cell lines achieved a reduction of cell migration and invasion. In addition, the repression of miR-199a-3p was associated with inhibition of mechanistic target of rapamycin (mTOR) as well as STAT3. In an independent investigation, miR-199a-3p and let-7a were evaluated in OS cells using lipid-modified dextran-based polymeric nanoparticles as a delivery system (85). Studies of effective delivery methods for miRNAs are relevant to achieve optimal miRNA enrichment or gene silencing in OS cells and tumors. These studies demonstrated that a lipid-modified dextran-based polymeric nanoparticle platform may be an effective non-viral carrier for efficient and effective miRNA delivery *in vivo*.

Cell Proliferation

The PI3K/Akt and MAPK pathways are two of the most frequently activated signal transduction pathways associated with OS. They contribute to disease initiation and development, uncontrolled cell proliferation, tumor cell invasion and metastasis, cell-cycle progression, inhibition of apoptosis, angiogenesis, and chemoresistance. The PI3K/Akt pathway is activated by the binding of ligands to respective RTKs (including IGF-1R, c-Met, and EGFR). Downstream signals activate targets involved in cell survival and inactivate pro-apoptotic proteins. The MAPK pathway is also activated by IGF-1R and EGFR signals, which may lead to cross-talk with the PI3K/Akt pathway. Aberrant activation of the MAPK pathway is often linked to lung metastasis (86) and drug resistance in OS (74). The influence of miRNAs on components of these pathways has been studied in OS pathology.

PI3K/Akt

The PTEN protein functions as a tumor suppressor that negatively regulates activation of the Akt pathway to inhibit cell proliferation (87). Gene deletions at the *PTEN* locus account for loss of PTEN function in 15–33% of OS patients (88). Consequently, loss of PTEN is associated with tumor progression. *PTEN* mRNA was directly targeted by miR-221 in OS cell lines and high miR-221 levels were shown to correlate with low *PTEN* mRNA and protein expression (89). Enrichment with miR-221 mimics induced OS cell survival while attenuation with antimiRs induced apoptosis, demonstrating that this is an oncogenic miRNA in OS. Notably, inhibition of miR-221 enhanced sensitivity to cisplatin (89), indicating the involvement of miR-221 in drug resistance mechanisms. Other miRNAs including miR-92a and members of the miR-17 and miR-130/301 families were found to show an inverse correlation in expression levels with *PTEN* mRNA expression levels in OS tumors (37) to provide additional candidates for investigation. Of these, inhibition of miR-17 resulted in increased *PTEN* mRNA in OS, which was associated with suppression of tumor growth and metastasis (90).

Another critical component in the PI3K/Akt pathway is the mTOR protein kinase. The mTOR complex consists of mTOR complex-1 (mTORC1), which regulates cellular proliferation,

and mTOR complex-2 (mTORC2), which phosphorylates and activates Akt. Direct repression of mTOR by the miR-101 tumor suppressor was reported in OS (91), and inhibition of cell proliferation and apoptosis were mediated through suppression of mTOR. Growth inhibition by miR-101 was also shown in hepatocellular carcinoma cells (92), and miR-101 was also found to contribute to cisplatin-induced apoptosis. Thus, these miR-221/miR-17/PTEN and miR-101/mTOR interactions provide new insight into the role of miRNAs as potential regulators of aberrant PI3K/Akt pathway signaling in OS. Further, the identification of miRNAs associated with resistance to cisplatin is promising for the development of biomarkers of chemoresistance in this disease.

MAPK

Epidermal growth factor receptor signaling with activation of the MAPK pathway occurs in 49% of OS cases and is linked to metastatic disease (93). Ras/Raf is upstream of MAPK/ERK kinase (MEK). Aberrant activation of the Ras/Raf/MAPK pathway is known to be specifically associated with OS initiation, progression, and outcome. A direct interaction between miR-217 and KRas was recently reported to participate in MAPK activation in OS (94). In a study evaluating miR-143 in OS tumors, a direct association of elevated EGFR phosphorylation and matrix metalloprotease-9 (MMP-9) levels and low miR-143 expression was reported (95). The MMP family of proteolytic enzymes facilitates tumor cell invasion and metastasis through degradation of various components of the extracellular matrix (96). MMP-9 degrades collagen type IV, the major component of the basement membrane and overexpression is associated with tumor metastasis (96). In this study, EGF promoted activation of EGFR and induced MMP-9 to enhance the ability of OS cells to metastasize. Significantly, miR-143 was reported to inhibit EGFR signaling-dependent OS cell invasion.

Apoptosis

Apoptosis is a critical event in embryonic development and in maintenance of tissue homeostasis of multicellular organisms. The activation of anti-apoptotic signals facilitates uncontrolled cell growth in cancer cells. The major apoptotic pathways include both the intrinsic and extrinsic pathways. The intrinsic pathway is mediated by mitochondrial components and triggered by intracellular stimuli, such as DNA damage, cytotoxic agents, growth factor suppression, and/or oxidative stress. The extrinsic pathway is initiated by the binding of death ligands, Fas ligand (FasL), tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and TNF-like weak inducer of apoptosis (TWEAK) to the TNF receptor (TNFR) superfamily of death receptors (97). Other key proteins in tumor cells that control apoptosis include the tumor suppressor p53 and PI3K/Akt activation that regulate downstream substrates that trigger or prevent apoptosis, respectively (97). miRNAs directly targeting components of the basic apoptotic pathways in OS have been identified.

Intrinsic Apoptotic Pathway

Low levels of miR-133a in OS cell lines and tissues were found to correlate with tumor progression and poor prognosis (98). Restoration of miR-133a inhibited cell proliferation and induced apoptosis in OS cell lines. A similar tumor suppressor role for miR-133a was reported in colorectal cancer (99). The regulatory mechanism of miR-133a involved direct targeting and suppression of B-cell lymphoma-extra large (Bcl-xL) and Mcl-1 proteins. Both Bcl-xL and Mcl-1 are anti-apoptotic proteins that are highly expressed in OS and promote cell survival (100). The miR-29 family comprises three isoforms arranged in two clusters, miR-29b-1/miR-29a located on chromosome 7 and miR-29b-2/miR-29c on chromosome 1, and these miRNAs have been identified as tumor suppressors in chronic lymphatic leukemia (CLL), AML, lung cancer, and breast cancer (101). Low levels of miR-29a were observed in OS, which when restored, induced apoptosis leading to the silencing of *BCL2* and *MCL1* and enrichment of the tumor suppressors *E2F1* and *E2F3* (35). miR-29 is currently being clinically evaluated as a biomarker for primary measure outcome of histone deacetylase inhibitor, AR-42, in adult and pediatric AML patients (NCT01798901). A clinical trial to evaluate the molecular mechanism and clinical significance of the interaction between Twist1 and other epithelial-to-mesenchymal regulators through the miR-29 family is also underway (NCT01927354). Another study investigating the role of miR-29b in patients with oral squamous cell carcinoma has been proposed (NCT02009852). Collectively, these data support a tumor suppressor function of miR-29 and suggest that the use of synthetic miR-29 oligonucleotides or agents increasing miR-29 expression can be incorporated in the study of expression changes of critical genes in OS.

Extrinsic Apoptotic Pathway

Fas ligand (FasL or CD95L) is a type-II transmembrane protein that belongs to the TNF family (97). FasL binds the Fas receptor (Fas, also called apoptosis antigen 1, Apo1, or cluster of differentiation 95, CD95) and induces apoptosis. Low Fas expression in OS tumor cells was associated with disease development and progression. Huang et al. demonstrated that miR-20a encoded by the miR-17–92 cluster attenuated *FAS* levels and regulated Fas-mediated apoptosis in OS cells (102). Another miRNA, miR-106a, which was downregulated in OS cell lines and tumors, was associated with regulation of *FAS* (103). miR-106a is part of a miRNA cluster (miR-17, miR-18a, miR-92a, and miR-106b), and cross-talk between miRNAs was suggested to mediate *FAS* repression. Interestingly, the pro-apoptotic gene BH3-only (*BIM*) was suppressed by miR-17 only (103) to support the regulation of Fas-mediated apoptosis by this miRNA cluster in OS cells.

Metastasis

In general, OS patients with lung metastasis have a poor prognosis. The overall survival rate is low (~25%) for those patients who present with metastases (~20% of all cases) (104). The process of metastasis involves dissemination of cells from the primary tumor, invasion of the extracellular matrix, and proliferation of cells at distant sites (105). A number of factors, including Rho-associated coiled-coil kinase 1 (ROCK1), MMPs, and c-Fos (the cellular homolog of v-fos), are involved in tumor metastasis. Recent studies have identified several miRNAs that directly target the mRNAs encoding these proteins. The identification of miRNAs associated with metastatic disease holds promise as

circulating miRNA biomarkers assessing disease characteristics that may be detected in serum and plasma of patients.

Rho-Associated Coiled-Coil Kinase 1

The ROCK1 protein serine/threonine kinase is a downstream effector of the small GTPase RhoA, and is a regulator of the actomyosin cytoskeleton. The RhoA/ROCK pathway participates in the process of tumorigenesis in numerous types of cancer. ROCK1 promotes contractile force generation and is involved in cell motility, metastasis, and angiogenesis in cancer cells (106). Several miRNA:*ROCK1* mRNA associations have been described. Generally, *ROCK1*-associated miRNAs, miR-340 (107), miR-335 (108), miR-145 (109), and miR-144 (110), were weakly expressed in OS cell lines and tissues and correlated inversely to ROCK1 overexpression. In addition, low expression levels of these miRNAs were associated with OS progression and metastasis through mechanisms involving ROCK1 to provide initial evidence that supports these miRNAs as predictors of poor prognosis in OS.

MMP-13

Matrix metalloproteases are produced either by tumor cells or stromal cells. Overexpression of MMP proteins is an important predictive factor for relapse or nodal metastasis of many carcinomas (111, 112). MMP-13 expression is common in lung metastasis (31). Recent evidence shows the emerging roles of miRNAs in direct repression of MMP through inhibition of gene transcription or (113) inhibition of *MMP* RNA levels (114). Osaki et al. showed that the expression of miR-143 was significantly downregulated in comparison to expression levels in parental (HOS) cell line and subclone (143B) human OS cell line, which shows lung metastasis in a mouse model (31). This finding correlated with MMP-13 upregulation, which implicated MMP-13 as a downstream mediator of miR-143 function in OS metastasis.

FOS

FBJ murine osteosarcoma viral oncogene homolog (*FOS*) is the transforming gene identified originally in the FBi and FBR murine sarcoma viruses (115). The c-Fos protein is part of a heterodimeric complex with JUN and is a major component of the Activator Protein-1 (AP-1) transcription factor complex. AP-1 regulates cell growth, differentiation, transformation, and bone metabolism (116). c-Fos is overexpressed in 61% of OS tumors compared to benign and normal tissue (117). It is also enriched in high-grade lesions and in patients with metastases (42%). c-Fos overexpression in transgenic mice was associated with OS development, suggesting a potential role in tumor initiation (118). The correlation between low expression of miR-181b/miR-21 signaling and *FOS* upregulation was made in malignant gliomas (119). miR-181b modulated *FOS* expression by directly targeting the binding site within the 3′-UTR. mir-221 was a predicted gene target of FOS (10), but the integrated analyses of *FOS* mRNA and regulatory miRNAs have not been experimentally studied in OS.

Drug-Resistant Genes

Drug resistance is often mediated through the activation of several molecular pathways that inhibit apoptosis and promote cell survival, to compensate for the effects of chemotherapy and targeted inhibition. In addition, increased DNA damage repair and ejection of the drug from the cell by drug efflux pumps reduce the efficacy of many cytotoxic agents. Experimental studies have demonstrated that the altered expression of specific miRNAs that regulate these cellular processes leads to drug resistance in different cancers. In OS cells, overexpression of miR-221 and miR-101 caused cisplatin resistance, mediated through the PTEN/Akt pathway (89, 92), while conversely, increased miR-217 expression levels were associated with reduction in KRas and enhanced sensitivity to quercetin and/or cisplatin (50). In the study conducted by Song et al., miR-215 was shown to induce G2 arrest in OS and colon cancer cells leading to chemoresistance to methotrexate and Tomudex (50).

MDR1

An underlying cause of multi-drug resistance (MDR) in OS is the overexpression of one or more of the ATP-binding cassette (ABC) transporters. Many cytotoxic agents are substrates for the *MDR1* (*ABCB1*) gene, resulting in overexpression of P-glycoprotein (P-gp), a 170–190 kDa transmembrane glycoprotein that belongs to the ABC superfamily (120). *MDR* (in particular, *ABCB1*, *ABCG2*, and *ABCC* family members) mediates the efflux of many cytotoxic agents in OS to decrease drug efficacy (120). Zhu et al. (121) have demonstrated that *MDR1*/P-gp expression in human cancer cells was regulated by high levels of miR-27a and miR-451 expression. miR-27a/miR-27a* is a miRNA pair derived from a single precursor. In this study, the sensitivity to and intracellular accumulation of cytotoxic drugs that were transported by P-gp were enhanced by treatment with antagomirs against miR-27a, demonstrating a role in *MDR1*-mediated chemoresistance in OS. miR-27a/miR-27a* was also shown to promote pulmonary OS metastases formation (122) to suggest this miRNA functions as an oncogene in this disease. In contrast, in head and neck squamous cell carcinoma, miR-27a* was demonstrated as a tumor suppressor by targeting the EGFR signaling axis (123) to illustrate fundamental differences in miRNA expression between OS and other types of cancer.

Challenges and Future Perspectives

Recent studies have generated a vast amount of DNA sequencing and genomic data that have provided tremendous insight into the molecular pathology of OS (4). Several genetic and epigenetic alterations in OS have been established that are linked to the development, proliferation, and survival of tumor cells (17, 19). The mapping of human miRNA genes has also identified specific miRNAs in OS that modulate gene expression and cellular processes. This has provided new insight into the complex genetic mechanisms of OS tumorigenesis. Many of these miRNA genes are located in cancer-associated genomic regions or in fragile sites (8, 18) and are reportedly associated with the development, progression, and metastasis of OS tumors (represented in **Figure 1** and summarized in **Table 1**). miRNAs are intriguing molecules, as the expression patterns appear to be tissue and cancer-type specific, and the small size is amenable to development for clinical applications. Of interest, circulating miRNAs from tumor cells that are detected in the blood of

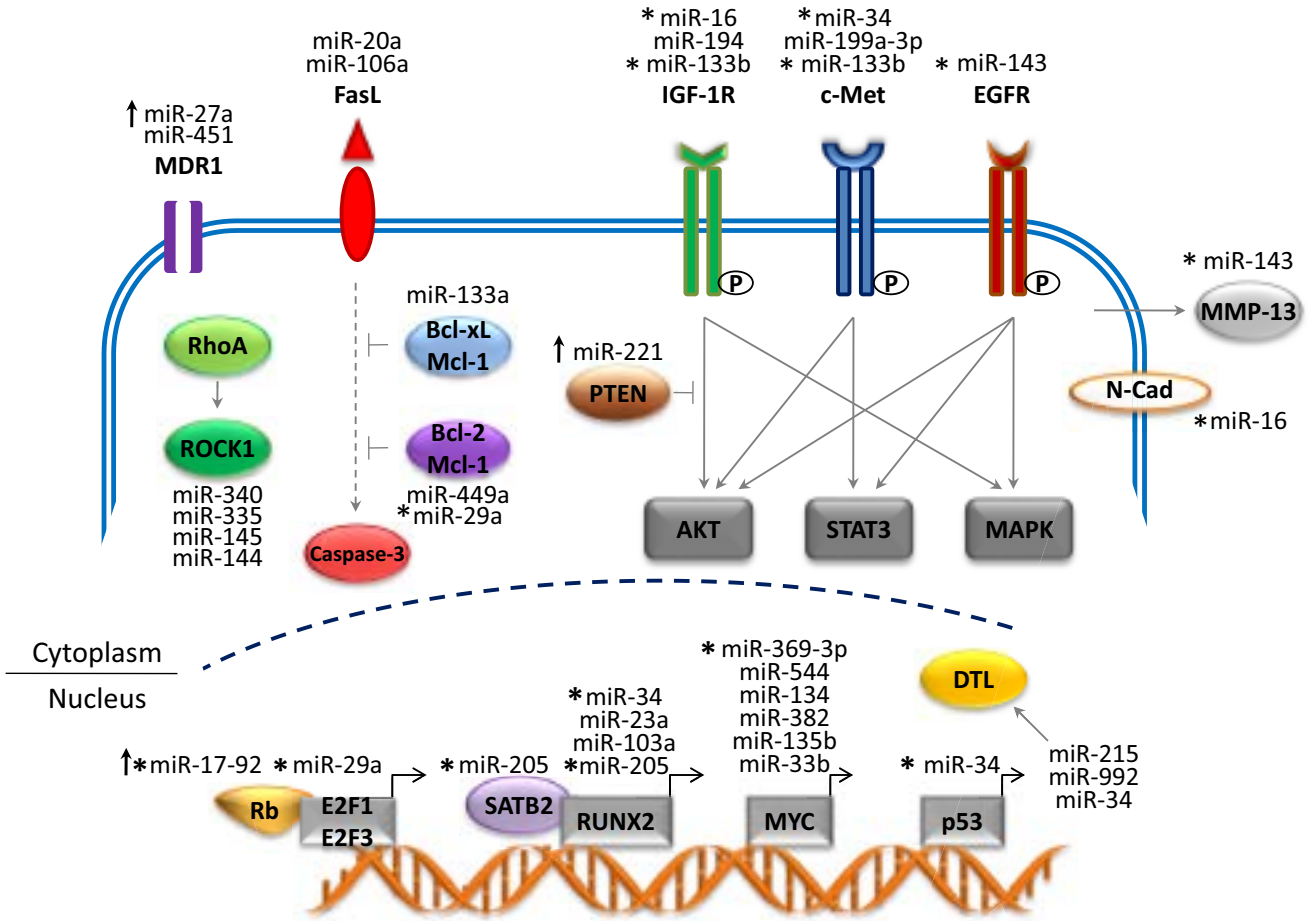

FIGURE 1 | Network of miRNAs and target genes in OS. The figure depicts altered miRNA genes that play a critical role in the development and progression of OS. The majority of miRNAs are downregulated (tumor suppressors) and target genes are overexpressed (oncogenes). Upregulated miRNAs (oncogenes) are depicted by upward arrows and target genes are repressed (tumor suppressors). Abbreviations: MDR1, multi-drug resistance 1; FasL, Fas ligand; IGF-1R, insulin-like growth factor 1 receptor; EGFR, epidermal growth factor receptor; ROCK1, Rho-associated coiled-coil kinase 1; Bcl2, B-cell lymphoma-2; Bcl-xL, B-cell lymphoma-extra large; Mcl-1, myeloid leukemia cell differentiation protein; PTEN, phosphatase and tensin homolog; MMP-13, matrix metalloprotease-13; N-Cad, N-Cadherin; SATB2, special AT-rich sequence-binding protein 2; RUNX2, runt-related transcription factor 2; DTL, denticleless protein homolog). Solid gray arrows represent activated signaling pathway; solid blunt lines represent inhibition of signals; dotted gray lines represent indirect signaling pathways.

patients with cancer present a novel opportunity to use miRNAs as an early predictor of cancer as well as a marker of response to therapy.

Research characterizing distinct OS-associated miRNAs is still in its infancy. The dysregulation of miRNAs in OS is likely influenced by a variety of factors, which are only starting to be understood. Since OS is a disease that is marked by genetic abnormalities including mutations, single-nucleotide polymorphisms (SNPs), and gene amplifications, it is expected that these alterations may also affect miRNA function. Mutations in the miRNA recognition sites of target mRNAs may affect miRNA binding, resulting in escape from regulation by a specific miRNA. Gene mutations and sequence variation mutations affecting miRNA sequences can potentially affect either processing and/or expression of mature miRNAs to prevent recognition of mRNA targets. However, at present, there is no clear association between mutations and SNPs identified in miRNA precursors in tumors, and cancer cell lines. These are not generally attributed to tumorigenesis and do not alter the secondary structure or function of the mature miRNAs (125, 126). Only few studies of this type have been conducted in OS specifically. Further screening for genetic variants in miRNA genes warrants investigation to determine whether genetic aberrations in miRNAs are integrated into the known cytogenetic abnormalities observed in OS.

Many of the miRNAs discussed in this review are preliminary findings based on *in vitro* studies using cell lines derived from OS tumors and are not fully validated *in vivo* or in functional studies. The robust confirmation of individual or miRNA signatures in preclinical disease models is important for potential applications to cancer treatment. Reduced levels of mature miRNAs in tumors may be a consequence of true absence (lack of inheritance), secondary loss (genetic loss, epigenetic silencing), or defects in biogenesis pathways or transcriptional repression. Quantitative

TABLE 1 | Prominent clinopathological factors associated with OS and regulatory miRNAs that are validated in preclinical OS models.

OS-associated factor	Target gene/pathway	miRNA	Altered protein(s)	miRNA function	Reference
Germline mutation	*RB1*	miR-17–92	Not verified in OS	Oncogene	(36, 37)
	TP53	miR-34	p53	[a]TS	(45)
		[b]miR-192	p53/RUNX2	TS	(46)
		[b]miR-215	p53	TS	(48)
Gene amplification	*MYC*	[b]miR-369-3p	c-Myc	TS	(56)
		[b]miR-544			
		[b]miR-134			
		[b]miR-382			
		miR-135b	c-Myc	TS	(57)
		miR-33b	c-Myc	TS	(58)
	RUNX2	miR-23a	RUNX2/CXCL12	TS	(65)
		miR-205	RUNX2/SATB2		(66)
Receptor tyrosine kinase activation	*IGF-1R*	miR-16	IGF-1R	TS	(124)
	MET	miR-194	IGF-1R/N-Cadherin	TS	(75)
		miR-133b	IGF-1R/Bcl2L2/Mcl-1/c-Met	TS	(78)
		miR-34	c-Met	TS	(82)
		miR-199a-3p	c-Met	TS	(83)
Cell proliferation	PI3K/Akt	miR-221	PTEN	Oncogene	(89)
		miR-17	PTEN	Oncogene	(37)
	MAPK	miR-143	pEGFR	TS	(95)
Apoptosis	Intrinsic pathway	miR-133a	Bcl-xL/Mcl-1	TS	(100)
		miR-29	Bcl2/Mcl-1/MMP	TS	(35)
	Extrinsic pathway	miR-20a	FasL	TS	(102)
		miR-106a	FasL	TS	(103)
		miR-17	BIM	TS	(103)
Metastasis	*ROCK1*	miR-340	ROCK1	TS	(107)
		miR-335	ROCK1	TS	(108)
	MMP-13	miR-145	ROCK1	TS	(109)
		miR-144	ROCK1	TS	(110)
	FAS	miR-143	MMP-13	TS	(31)
		miR-20a	FasL	TS	(102)
Drug resistance	*MDR1*	miR-27a	P-gp	Oncogene	(121)

[a]*TS, tumor suppressor.*
[b]*miRNA signature.*

real-time reverse transcription polymerase chain reaction (qRT-PCR) and oligonucleotide microarray (microchip) analysis are the most common methods for measuring miRNA levels, but there is currently no standardized technique for evaluation of miRNA expression, which is critical for clinical translation. In addition, miRNA potential targets can be predicted using computational algorithms, such as TargetScan (127) and microRNA.org (128). By computer predictions and stable isotope labeling with amino acids in cell culture (SILAC), a single miRNA has multiple targets and is capable of inhibiting the translation of hundreds of proteins (129). These are valuable tools for the integrated and functional analyses of miRNA and mRNA targets, and miRNA gene networks, which are also essential for understanding global miRNA roles in OS tumors.

Importantly, miRNA targets are tissue specific and the regulatory roles are in particular physiological or pathological contexts. Approximately 60% of mRNAs have evolutionarily conserved sequences that are predicted to bind miRNAs (130). Thus, the expression of target genes may be controlled by several different miRNAs, and cross-talk between miRNA networks may affect an individual miRNA-based effect. Consequently, an individual miRNA may have oncogenic or tumor suppressor properties in different cell types. Finally, further research to develop strategies for effective and safe miRNA delivery systems is needed. Localized delivery or the use of polyethylene glycol (PEG) in PEGylated liposomes, lipidoids, and biodegradable polymers are being tested [reviewed in Ref. (131)]. Improvements in delivery formulations will reduce the risk of hepatotoxicity, organ failure, and death in preclinical mouse models (131). The design of miRNA precursor mimics (e.g., short hairpin RNAs) or true pre-miRNAs may also minimize toxic side effects while retaining targeted functions. Nonetheless, these small molecules have greatly enhanced our knowledge of the molecular mechanisms that regulate gene expression in OS, and it is hoped miRNAs will be successfully developed to improve the current management of OS.

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A review: molecular aberrations within Hippo signaling in bone and soft-tissue sarcomas

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The Hippo signaling pathway is an evolutionarily conserved developmental network vital for the regulation of organ size, tissue homeostasis, repair and regeneration, and cell fate. The Hippo pathway has also been shown to have tumor suppressor properties. Hippo transduction involves a series of kinases and scaffolding proteins that are intricately connected to proteins in developmental cascades and in the tissue microenvironment. This network governs the downstream Hippo transcriptional co-activators, YAP and TAZ, which bind to and activate the output of TEADs, as well as other transcription factors responsible for cellular proliferation, self-renewal, differentiation, and survival. Surprisingly, there are few oncogenic mutations within the core components of the Hippo pathway. Instead, dysregulated Hippo signaling is a versatile accomplice to commonly mutated cancer pathways. For example, YAP and TAZ can be activated by oncogenic signaling from other pathways, or serve as co-activators for classical oncogenes. Emerging evidence suggests that Hippo signaling couples cell density and cytoskeletal structural changes to morphogenic signals and conveys a mesenchymal phenotype. While much of Hippo biology has been described in epithelial cell systems, it is clear that dysregulated Hippo signaling also contributes to malignancies of mesenchymal origin. This review will summarize the known molecular alterations within the Hippo pathway in sarcomas and highlight how several pharmacologic compounds have shown activity in modulating Hippo components, providing proof-of-principle that Hippo signaling may be harnessed for therapeutic application in sarcomas.

Keywords: Hippo, sarcoma, osteosarcoma, Ewing sarcoma, rhabdomyosarcoma, mesenchymal, targeted therapy, pediatric cancers

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Introduction

Overview of Pediatric Sarcomas

Sarcomas account for ~1% of all malignancies, but occur with higher frequency in children compared to adults, comprising ~15% of all childhood malignancies (1). The mainstay of treatment includes combining primary tumor control with surgery and/or radiation and systemic chemotherapy. While survival rates for localized sarcomas have improved to >70%, children with metastatic or recurrent disease continue to have dismal outcomes (2, 3).

Malignant bone and soft-tissue sarcomas arise in connective tissues (including bone, fat, muscle, blood vessels, deep skin tissues, nerves, and cartilage) and represent a histologically and molecularly heterogeneous group of tumors. Although the precise cell of origin of most of these tumors is not known, sarcomas are thought to develop as a result of genetic alterations in mesenchymal progenitor cells. While older adult patients often develop sarcomas with complex genetic karyotypes, there are relatively few genetic mutations driving tumorigenesis for the majority of childhood sarcomas, with the exception of some characteristic chromosomal translocations. In cases where the underlying molecular pathogenesis has been identified, this has not translated into improvements in survival rates for those patients with advanced or aggressive tumors, as many of the molecular drivers have not been able to pharmacologically modulated (2, 3). Discovering therapeutically targetable proteins that may be collaborating with such tumorigenic drivers is a promising new frontier for molecular oncology.

Overview of Hippo Signaling

The delineation of the Hippo pathway began in 2003 with identification of the *Drosophila hippo* gene. Hippo loss-of-function phenotypes were described concurrently by the Pan and Hariharan laboratories while screening for genes that negatively regulate tissue growth (4, 5). Subsequent studies unveiled Hippo signaling as an evolutionarily conserved cascade consisting of adaptor proteins and inhibitory kinases that regulate Yorkie, a pro-growth transcriptional regulator (6–8). Hippo signaling is highly conserved between *Drosophila* and mammals, and homologous pathway components across species are well described (9, 10). For this review, focus will be on mammalian Hippo signaling.

As shown in **Figure 1**, the mammalian Hippo pathway relays plasma membrane and cytoplasmic signals into the nucleus, where it regulates the expression of a diverse group of target genes that control essential cellular processes, including proliferation, differentiation, and apoptosis. Canonical Hippo transduction involves serine/threonine kinases mammalian STE20-like protein kinase 1/2 (MST1/2, which are homologs of *Drosophila* Hippo) (4, 5, 11, 12) and large tumor suppressor homolog 1/2 (LATS1/2) (7, 13, 14), which, in conjunction with adaptor proteins Salvador homolog 1 (SAV1) (12) and Mob kinase activator 1 (MOB1) (15), phosphorylate and inhibit the transcriptional co-activators Yes-associated protein 1 (YAP, a homolog of Yorkie) and transcriptional co-activator with PDZ-binding motif (TAZ) [also known as WW domain-containing transcription regulator 1, WWTR1] (16). The Hippo pathway is "ON" when MST1/2 and LATS1/2 kinases are active. Through an interaction between the PPxY (PY) motifs of LATS1/2 and the WW domains of YAP and TAZ, activated LATS1/2 lead to phosphorylation of YAP and TAZ, which results in YAP/TAZ cytoplasmic retention and β-TRCP (β-transducin repeat-containing E3 ubiquitin protein ligase)-dependent proteasomal degradation (9, 10). When Hippo signaling is inactive or "OFF", YAP and TAZ are localized to the nucleus, where they serve as transcriptional co-activators for TEA domain-containing sequence-specific transcription factors (TEADs) (17–21) as well as other transcription factors (16).

Regulation of the Hippo Pathway

Much of our understanding of Hippo regulation comes from studies performed in epithelial tissue. In this context, the transcriptional activities of YAP and TAZ are regulated by four interconnected inputs: (1) plasma membrane proteins, which complex with YAP and TAZ directly to sequester them at cell–cell junctions; (2) upstream adaptor proteins, which activate core Hippo kinases to ultimately phosphorylate and repress YAP and TAZ; (3) regulatory cross-talk from other signaling pathways;

Abbreviations: AMOT, angiomotin; APC, adenomatous polyposis coli; ARMS, alveolar rhabdomyosarcoma; β-TRCP, β-transducin repeat-containing E3 ubiquitin protein ligase; BMI-1, B-lymphoma Mo-MLV insertion region 1 homolog; BMP2, bone morphogenetic protein 2; BRAF, v-raf murine sarcoma viral oncogene homolog B; cAMP, adenylyl cyclase pathway; CD44, CD44 antigen; CDKN2A, cyclin-dependent kinase inhibitor 2A; ChIP-Seq, chromatin immunoprecipitation followed by high-throughput DNA sequencing; CRB, crumbs complex proteins; CTGF, connective tissue growth factor; dLats, large tumor suppressor (or warts); DMBA, 9,10-dimethyl-1,2-benzanthracene; DVL, disheveled; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EHE, epithelioid hemangioendothelioma; ERK, extracellular signal-regulated kinases; ERMS, embryonal rhabdomyosarcoma; EWS, Ewing sarcoma; FAT4, FAT tumor suppressor homolog 4; FOXM1, transcription factor forkhead box M1; GEMM, genetically engineered mouse model; GPCRs, G protein-coupled receptors; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; hTERT, telomerase reverse transcriptase; ID2, inhibitor of DNA binding 2; KIBRA, kidney and brain protein; LATS1/2, large tumor suppressor homolog 1/2; LLGL1, lethal giant larvae homolog 1; MAPK, mitogen-activated protein kinase; MARK1, MAP/microtubule affinity-regulating kinase 1; MCAT, muscle CAT elements; MDM2, mouse double minute 2; MEF2, myocyte enhancer factor 2; MEK, MAPK kinase; MMP-9, matrix metallopeptidase 9; MOB1, Mob kinase activator 1; MRFs, myogenic regulatory factor family; MSC, mesenchymal stem cell; MST1/2, serine/threonine kinases mammalian STE20-like protein kinase 1/2; mTOR, mechanistic target of rapamycin; MYCN, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog; Myf5, myogenic factor 5; MyHC, myosin heavy chain; MyoD, myogenic differentiation 1; NF2, neurofibromin 2 (or Merlin); NRSTS, non-rhabdomyosarcoma soft-tissue sarcoma; NSCLC, non-small cell lung cancer; OCT4, octamer-binding transcription factor-4; OS, osteosarcoma; p16INK4A, prototypic INK4 protein; PCNA, proliferating cell nuclear antigen; PDE, phosphodiesterase; PKA, protein kinase A; PKN1, protein kinase N1; PP1A, protein phosphatase 1, catalytic subunit, alpha isozyme; PP2A, protein phosphatase 2, regulatory subunit B, delta1; PPARγ, peroxisome proliferator-activated receptor gamma; RAF, v-raf murine sarcoma viral oncogene homolog; RAS, rat sarcoma viral oncogene homolog; RASSFs, Ras-association domain-containing family of proteins; RB1, retinoblastoma 1; RHO, rhodopsin; RMS, rhabdomyosarcoma; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; RUNX2, runt-related transcription factor 2; SARAH domain, Salvador-Rassf-Hpo binding domain; SAV1, salvador homolog 1; SMADs, mothers against decapentaplegic proteins; Snai1/2, snail family zinc finger 1/2; SOX2, SRY (sex determining region Y)-box 2; STS, soft-tissue sarcoma; TAO-1, thousand and one amino acid protein 1; TAZ, transcriptional co-activator with PDZ-binding motif (or WWTR1); TCGA, The Cancer Genome Atlas; TEADs, TEA domain-containing sequence-specific transcription factors; TGFβ, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TP53, tumor protein p53; TP73, tumor protein p73; Twist1, twist family bHLH transcription factor 1; UVB, ultraviolet radiation B; VGLL3, vestigial-like 3; WNT, wingless-type MMTV integration site family; YAP, yes-associated protein 1.

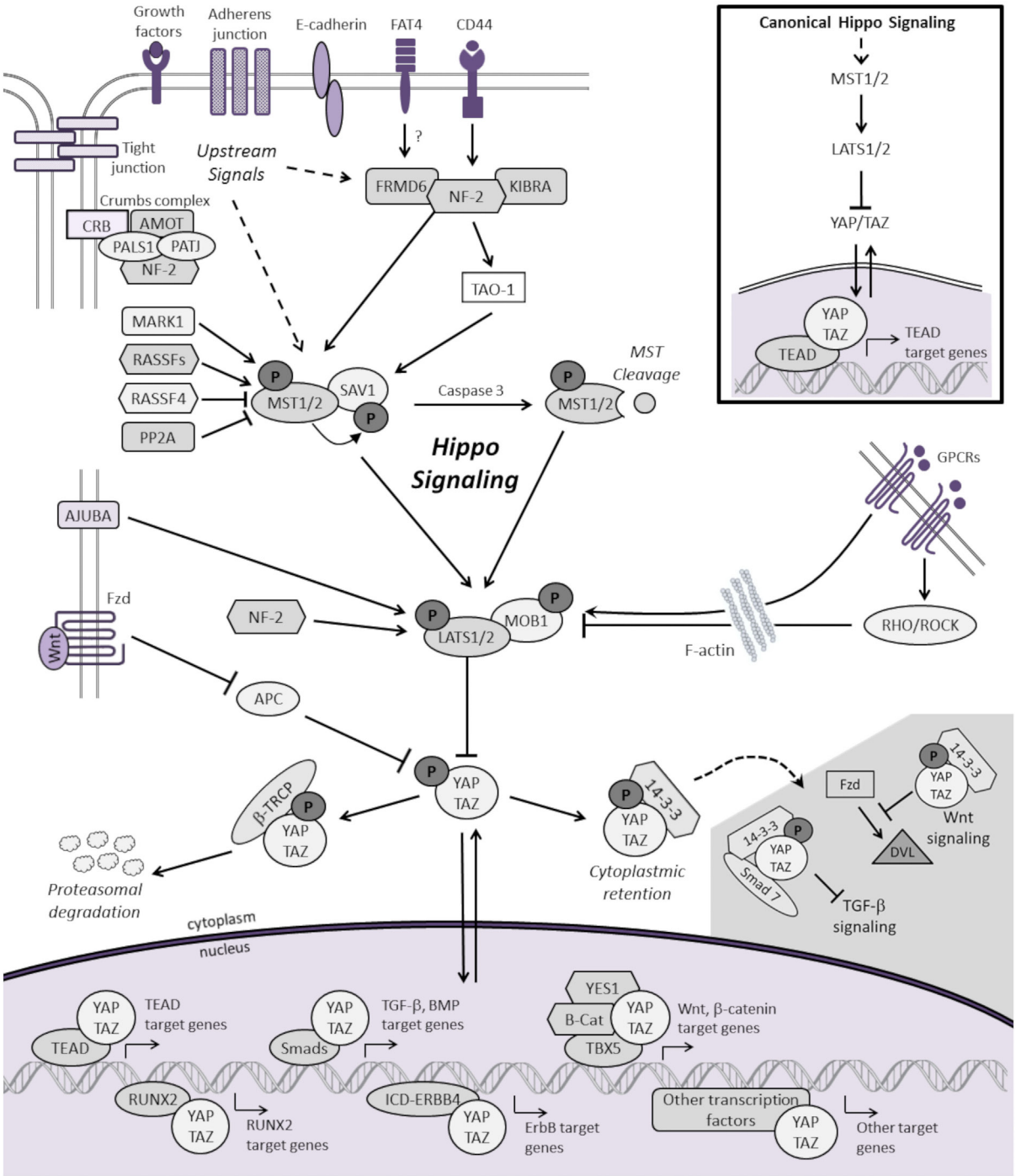

FIGURE 1 | Schematic representation of the mammalian Hippo signaling cascade. Canonical Hippo transduction involves MST1/2 and LATS1/2 kinases, which, in conjunction with SAV1 and MOB1, phosphorylate, and inhibit the transcriptional co-activators YAP and TAZ. Regulation of YAP and TAZ are governed by plasma membrane proteins, cytoskeletal adaptor proteins, regulatory cross-talk from other signaling pathways, and intrinsic and extrinsic mechanical cues with the actin cytoskeleton. For simplicity, not all the known protein–protein interactions and regulators of Hippo signaling are represented. When Hippo signaling is "OFF", YAP/TAZ translocate to the nucleus to serve as transcriptional co-activators for TEADs as well as other transcription factors (only a few of which are represented here) involved in cellular proliferation, differentiation, self-renewal, and apoptosis. See text for additional details.

and (4) intrinsic and extrinsic mechanical forces within the cell, which exert local control over YAP and TAZ localization. An overview of Hippo regulation is summarized below. For more detail, see the review by Grusche and colleagues (22), as well as three recent proteomic analyses that identified key protein–protein interactions with Hippo kinases, and YAP and TAZ within the global signaling network (23–25).

Regulation Through Plasma Membrane Proteins

Growth control is signaled through plasma membrane proteins to upstream Hippo proteins, often in response to increased cell density. The Crumbs polarity complex, other polarity proteins, and adherens junctions, which all modulate each other, contribute inputs to various Hippo components (22, 26). E-cadherin and the junction-associated Ajuba protein family modulate MST and LATS kinases, respectively. The Crumbs complex involves transmembrane proteins that recruit scaffold proteins that localize to apical junctions and mediate cell polarity (27, 28). G-protein-coupled receptor (GPCR) ligands have been identified as regulators of Hippo signaling (29). Depending on the coupled G-protein, LATS1/2 kinases can either be activated or inhibited. YAP and TAZ directly influence the GPCR transcriptional activity, as YAP/TAZ are required for the expression of many GPCR-mediated target genes (29). The transmembrane hyaluronate receptor CD44 interacts with neurofibromin 2 (NF2, also known as Merlin) and other scaffold proteins to recruit LATS to the cell membrane, where it is phosphorylated (9, 30–32). Finally, the atypical cadherin protein Fat (*Drosophila*) is required for localization of Expanded (FRMD6 in mammals) to apical junctions, which results in activation of Hippo (MST1/2) (33). In avian cells, FAT4 has been shown to inhibit *YAP1*-mediated neuroprogenitor cell proliferation and differentiation (34).

Regulation Through Upstream Intracellular Adaptor Proteins

The core Hippo pathway is controlled by a complex upstream regulatory network. MST and LATS kinase activity are regulated by several upstream proteins, including Ras-association domain-containing family proteins (RASSFs1-10) (35, 36), kidney and brain protein (KIBRA) (37–39), thousand and one amino acid protein 1 (TAO-1) (40), MAP/microtubule affinity-regulating kinase 1 (MARK1) (41), and NF2. Via their interaction through a homologous SARAH (SAlvador–RAssf–Hpo) binding domain, RASSFs and SAV1 regulate MST activity (42). MST1/2 complexes with SAV1 to directly phosphorylate LATS1/2. MST1/2 bound to SAV1 can also bind to and phosphorylate MOB1, which binds LATS1/2 to promote autophosphorylation. While a growing inventory of functional interactions between upstream proteins and Hippo kinases are well described, the degree to which their binding is dependent on tissue type or cellular context, as well as their reliance on canonical Hippo signaling, requires further investigation. Several of the aforementioned proteins can also directly alter YAP activity in a manner independent of MST and/or LATS kinases (31, 43).

The Hippo pathway plays a major role in arbitrating cell contact inhibition, cell proliferation, and promoting apoptosis (44). As cells increase in confluence, the tumor suppressor NF2 localizes near cell junctions to activate Hippo signaling (45, 46). YAP suppression has been shown to rescue the hyperproliferative phenotypes caused by NF2 inactivation in both mesothelioma (47) and meningioma (48). Furthermore, overexpression of a dominant-negative TEAD suppressed the tumor growth resulting from liver-specific NF2 deletion in mice (49). A negative feedback loop between YAP/TAZ and LATS2 has also been described. YAP and TAZ stimulation and TEAD binding induces LATS2 expression, both directly and by inducing NF2 (50). In addition, YAP and TAZ may negatively regulate each other. For example, Taz accumulates in the livers of Yap knockout mice, while either *in vitro* suppression or overexpression of Yap results in inverse changes to Taz protein expression (50).

Regulation Through Cross-Talk with Other Pathways

Cell status and function, as well as overall tissue and organismal growth, is governed by an integrated network of morphogenic signals. Hippo transduction is proving to be a hub for such integration (51–53). Although studies are needed to clarify intra-pathway cross-talk in sarcomas, many of these pathways have been individually implicated in sarcomagenesis. YAP and TAZ are well recognized as co-activators for transcription factors of numerous signaling cascades. The specific ways in which signaling networks synergize or antagonize Hippo to coordinate biologic activity is only beginning to be understood. We highlight a few examples of regulatory cross-talk and refer to the studies referenced in **Table 1** for additional details.

One example is illustrated by the relationship between the WNT and Hippo pathways. WNT activity is critical in myogenesis (54) and osteogenesis (55), and has recently been shown to be important in sarcomagenesis as well (56, 57). Rosenbluh et al. performed genome-scale loss-of-functions screens on 85 cancer cell lines (including osteosarcoma) and determined that WNT-active cancers are dependent upon β-catenin forming a complex

TABLE 1 | Pathway cross-talk with Hippo signaling.

Pathway cross-talk	Reference
Developmental pathways	
Wnt/β-catenin	(67–70)
TGF-β	(60, 61, 71–74)
Notch	(67, 75–77)
Hedgehog	(78–80)
MAP kinase related	
MAPK/Erk	(81–83)
GPCRs	(29, 84, 85)
SAPK/JNK	(86, 87)
ErbB tyrosine kinases	(88)
PI3K/mTOR/Akt	(41, 89–91)
Jak/Stat	(92, 93)
Ras	(94–96)
Sox2	(97, 98)
MMP family	(99)
Mevalonate pathway	(100, 101)
Cellular metabolism	(102, 103)
Epigenetic modification	(104)
Cell cycle/CDK1	(105)

with YAP and the transcription factor TBX5 to promote transcription of anti-apoptotic genes that are essential for cancer cell transformation and survival (58). This relationship was validated in a β-catenin-derived orthotopic colon cancer murine model, where Yap was required for tumor formation (58). In another study using murine cardiac muscle, knockdown of Hippo components Sav1, Mst1/2, or Lats2 results in increased Yap activity and cardiomyocyte proliferation with phenotypic cardiomegaly. Gene profiling from these mice reveal an elevated WNT signature, and the phenotypic effects could be offset by conditional loss of one β-catenin allele (59).

TGFβ and Hippo signaling also collaborate to direct cell behavior. YAP and TAZ associate with SMADs to promote transcription of TGFβ and BMP target genes (60–62). TGFβ signaling alters YAP/TAZ expression to drive mesenchymal stem cell (MSC) fate. For example, treatment of MSCs with BMP2 leads to increased TAZ expression and enhanced interaction with RUNX2 to promote osteoblast differentiation (63). Notch and Hippo signaling provide another example of coordinated cross-talk. Notch has been shown to be a driver of both bone and soft-tissue sarcomas (64–66). While no studies have examined the interplay of Notch and Hippo in sarcomas, overexpression of *Yap1* in mouse intestinal epithelia stimulates Notch signaling and the expansion of undifferentiated progenitor cells. However, treatment with γ-secretase inhibitors to block Notch signaling prevents the intestinal dysplasia caused by YAP (67). Together, these insights provide a deeper appreciation for the complex molecular circuitry that regulates Hippo activity in cell biology and malignancy.

Cytoskeletal Regulation Through Mechanical Influences

To sustain proper function, from facilitating organ development during embryogenesis to maintain homeostasis postnatally, cells must perceive their microenvironment and respond appropriately to stimuli. In addition to transmitting biochemical signals, cells also extract information from mechanical cues. Mechanotransduction is the ability to perceive and translate physical stimuli [elasticity of the extracellular matrix (ECM) and forces exerted by cell–cell or cell–matrix interactions] into biochemical signals on a cellular level. Cells adapt to changes in tension through rapid cytoskeletal remodeling (106–108). YAP and TAZ have emerged as dynamic factors linking remodeling to nuclear transcriptional outputs that control cell behavior. Thus, by modulating YAP/TAZ activity, mechanical stimuli can direct cell fate and guide stem cell maintenance, proliferation, and differentiation (107, 109–111). For example, in *Drosophila*, the tension modulated within the cytoskeleton causes proportionate changes in wing growth through an Ajuba-Warts (homolog of LATS) complex (112).

In situations of high mechanical stress and low cell confluence, YAP and TAZ are transcriptionally active, resulting in proliferation and tissue growth. However, with increasing cell contact, adhesion molecules stimulate LATS activity, resulting in YAP/TAZ phosphorylation and nuclear exclusion (44). Both F-actin polymerization and stress fiber formation lead to the nuclear localization and activation of YAP/TAZ, whereas disrupting F-actin inhibits YAP/TAZ transcriptional activity (113–116). As shown in **Figure 2**, ECM stiffness and cell shape/spreading can also regulate YAP/TAZ localization by regulating the activity of Rho-GTPases and the formation of stress fibers and actin bundles (106, 110, 113). In MSCs, YAP and TAZ act as both sensors of mechanotransduction and mediators of cellular responses to mechanical signals (117, 118). YAP and TAZ remain inactive in the cytoplasm and direct MSCs to differentiate into adipocytes when human MSCs are exposed to low ECM stiffness, are cultured on a soft matrix, or are manipulated into a small round shape. However, YAP and TAZ are active in the nucleus and MSCs differentiate into osteoblasts when they are subjected to high ECM stiffness, are grown on a stiff matrix, or are stretched and manipulated into a "spread-out" morphology (119, 120). This mechanical control over YAP/TAZ activity supersedes density cues from cell–cell or cell–matrix contact (113, 115).

Interestingly, manipulation of YAP/TAZ expression can overrule mechanical influences to direct differentiation. When YAP/TAZ is suppressed, MSCs grown on a stiff ECM will undergo adipogenic differentiation. However, when activated YAP is overexpressed, MSCs grown on a soft ECM will undergo osteogenic differentiation (113). Knockdown of LATS1/2 has almost no effect on YAP/TAZ regulation by mechanical cues, and LATS-insensitive TAZ still responds to mechanical cues (113). Therefore, cellular mechanical stress can directly impact proliferation and tissue growth through YAP/TAZ, independent from Hippo signaling. Together, these studies emphasize the importance of cytoskeletal regulation of YAP and TAZ transcriptional activity, and demonstrate that YAP and TAZ are required for mechanical signals to direct MSC fate.

Summary of Hippo Regulation

In summary, while the mechanistic and functional interactions between Hippo signaling and other regulatory pathways and cellular processes are not entirely understood, it is apparent that Hippo transduction links cell density and cell contact cues to morphogenic signals that regulate cell behavior. During development and tissue regeneration, the tumor suppressor function of Hippo signaling serves to offset the proliferative effects of other pathways. However, during malignant transformation, Hippo transduction is suppressed as cells evade contact inhibition, allowing the downstream effectors, YAP and TAZ, to co-activate TEADs as well as other transcription factors, to promote pro-proliferative and anti-apoptotic properties.

Hippo Signaling in Mesenchymal Stem Cell Fate

While the precise cellular origin for most sarcomas remains uncertain, they are presumed to arise from mesenchymal precursors that fail to undergo terminal differentiation. These precursors have stem-like characteristics, including high proliferative and self-renewal potential. Therefore, insight into MSC regulation, lineage commitment, and differentiation (121), may shed light on sarcoma biology. As shown in **Figure 2C**, sarcoma subtypes are histologically described by the features of their presumed mesenchymal lineage. Summarized below are the known roles

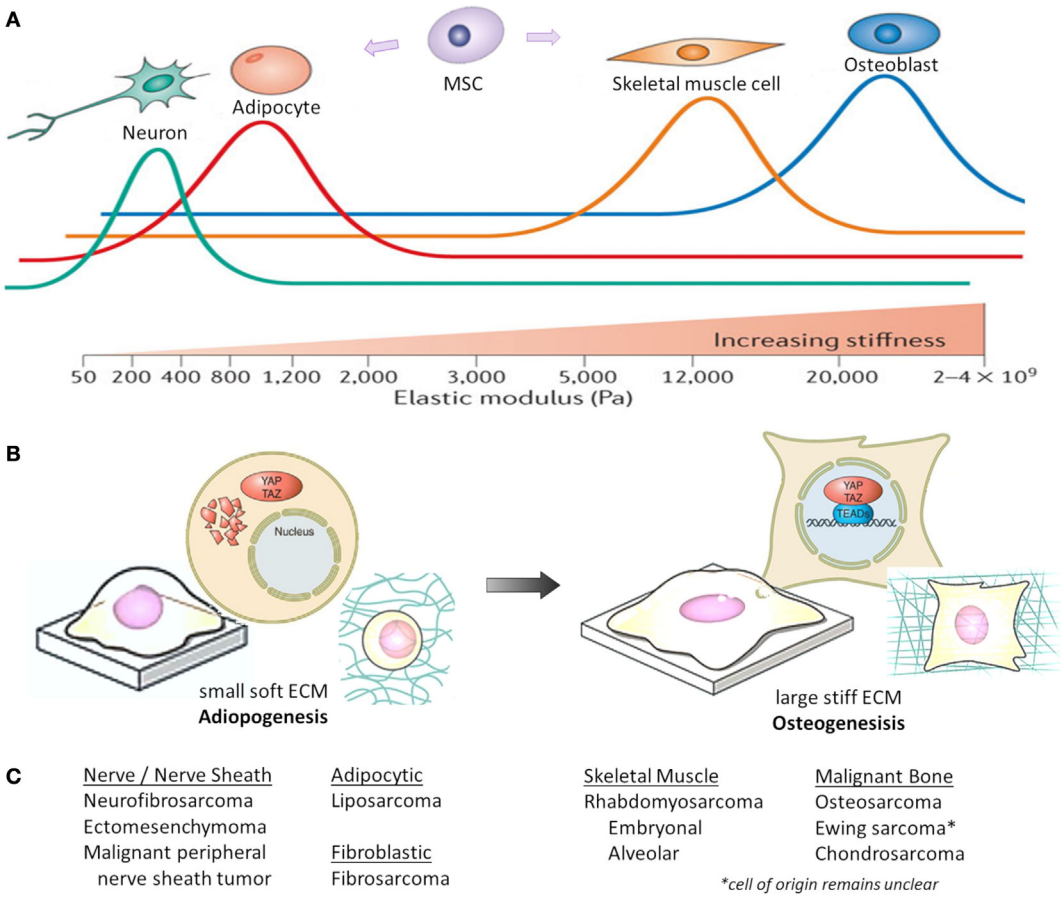

FIGURE 2 | Mechanical and physical influences on MSC cell fate. Cell geometry and ECM stiffness regulate MSC lineage commitment into neurons, adipocytes, skeletal muscle cells, or osteoblasts. **(A)** Increasing ECM stiffness *in vitro* (by increasing type I collagen concentration and crosslinking) compromises tissue organization, inhibits apoptosis and lumen formation, and destabilizes adherens junctions. Through modeling different ECM elasticities *in vitro*, MSCs differentiate into the varying lineages at elasticities that recapitulate the physiological ECM stiffness of their corresponding natural niche (shown as colored lines, with peaks indicating maximal differentiation). Pa, Pascal. **(B)** When MSCs are either cultured on a soft matrix or are manipulated into a small round shape, YAP/TAZ remain inhibited in the cytoplasm and MSCs differentiate into adipocytes. However, when MSCs are either grown on a stiff matrix or stretched and manipulated into a "spread-out" morphology, YAP/TAZ localize to the nucleus as MSCs differentiate into osteoblasts. **(C)** Corresponding histologic sarcoma subtype [2013 WHO classification (230)], which may reflect varying lineage differentiation from mesenchymal progenitor cells. This represents only a theoretical link between mechanotransduction influencing mesenchymal progenitors and sarcoma, and not all sarcoma subtypes are represented. Figures **(A,B)** are modified with permission from Halder et al. (108) and Piccolo et al. (117).

of Hippo signaling in modulating normal bone (osteogenic), fat (adipogenic), and muscle (myogenic) development, which are the origins of the most common sarcomas. YAP/TAZ are also critical mediators of cancer stem cell biology, a topic reviewed by others (122).

Hippo Signaling in Osteogenic Differentiation

Osteogenic differentiation is coordinated by the transcription factor, RUNX2, and a host of co-regulators (123), which activate the expression of osteoblast-specific genes, including osteocalcin (63, 124, 125). Through direct binding of the TAZ WW domain to the PY motif on RUNX2, TAZ has been identified as a transcriptional co-activator of RUNX2. Expression of an active TAZ mutant enhances RUNX2-driven gene expression two to threefold (63, 126), while knockdown of TAZ in MSCs inhibits osteogenesis when the cells are cultured under conditions favoring osteoblast differentiation (63). Transgenic mice with osteoblast-specific overexpression of Taz have significantly higher whole body bone mineral density, increased bone formation, and higher expression of RUNX2, osteocalcin, ALP, and osterix (127). TAZ-mediated osteogenesis may also occur downstream of the WNT pathway, since WNT3A can cause PP1A-mediated TAZ dephosphorylation, leading to TAZ nuclear localization and induction of osteogenic differentiation (68).

While the role of TAZ in supporting osteogenesis is clear, the role of YAP is more complex. When an activated YAP mutant was overexpressed in MSCs, osteogenic differentiation was promoted over adipogenic differentiation, even under conditions favoring the latter (113). However, YAP can also act as repressor of RUNX2 when it is regulated by non-canonical pathways (128). For example, when Src/Yes tyrosine signaling is inhibited, Yap tyrosine phosphorylation is blocked, Yap dissociates from RUNX2, and

osteocalcin is induced (128, 129). Last, there is evidence that YAP is a direct target of SOX2, a transcription factor important for MSC cell fate; in situations of high SOX2 or YAP expression, osteogenesis is blocked, while depletion of either SOX2 or YAP enhances osteogenesis (98).

In addition to YAP/TAZ, there is evidence that upstream scaffold proteins influence osteogenesis. *Rassf2* knockout mice develop bone-remodeling defects, and *in vitro* studies show that ablation of RASSF2 suppresses osteoblastogenesis while promoting osteoclastogenesis (130).

Hippo Signaling in Adipogenic Differentiation

A key transcription factor orchestrating adipogenesis is peroxisome proliferator-activated receptor gamma (PPARγ), which contains a PY motif for binding the WW domains on YAP and TAZ (63). In this context, binding of TAZ has an inhibitory role, suppressing transcriptional activity. When cultured under conditions that promote adipogenic differentiation, knockdown of TAZ permits MSCs to differentiate toward this lineage (63). Similarly, treatment with the small molecule KR62980 (a ligand for PPARγ that antagonizes adipocyte differentiation) does so by promoting TAZ nuclear localization and enhanced interaction between TAZ and PPARγ (131).

Recent work has shed light on the role of YAP in adipogenesis. Similar to osteogenesis, YAP is downstream of SOX2. However, YAP levels must be fine-tuned; both over or under-expression of YAP inhibits adipogenesis. Mechanistically, YAP induces the Wnt antagonist Dkk1 to diminish osteogenic signaling in favor of adipogenesis. In addition to YAP and TAZ, upstream Hippo regulators have been implicated. The Hippo adaptor protein SAV1 contains WW domains that can interact with the PY motif within PPARγ (132). MST1/2 stimulated SAV1 to bind PPARγ, which stabilizes and increases PPARγ levels, ultimately leading to adipogenic differentiation. In addition, knockdown of MST1/2 or SAV1 results in the inhibition of adipogenesis (132), though it is not known whether this effect is through canonical Hippo transduction or an alternate pathway.

Hippo Signaling in Myogenic Differentiation

Myogenic differentiation is driven by the myogenic regulatory factor family [MRFs: MyoD, myogenin, MRF4, and myogenic factor 5 (Myf5)]) (133–135) in coordination with myocyte-specific MEF2 enhancer factors (136, 137). In murine C2C12 skeletal muscle myoblasts, YAP supports an undifferentiated phenotype and promotes myoblast proliferation (138–140). Upon differentiation, nuclear YAP is translocated to the cytoplasm, with a 20-fold increase in YAP phosphorylation. Overexpression of YAP S127A, a mutant that cannot be phosphorylated at the LATS-regulated site, impedes myotube formation, and alters the expression of MRFs (139). Activation of YAP causes upregulation of Myf5, which promotes myoblast proliferation. Activated YAP also leads to downregulation of MyoD and MEF2, which are important in cell-cycle exit and differentiation, as well as upregulation of inhibitors of MyoD and MEF2, such as ID2, Twist1, and Snai1/2 (133, 138). In activated satellite cells, which are resident stem cells of skeletal muscle, high YAP activity prevents differentiation and promotes proliferation (138, 140). YAP suppression dramatically reduces satellite cell-derived myoblast proliferation (140). Additionally, muscle CAT (MCAT) elements, which are TEAD-binding sites, are found in the promoters of genes that are selectively expressed in terminally differentiated skeletal muscle (140, 141).

Interestingly, while YAP inhibits myogenic differentiation, some studies suggest TAZ may enhance myogenesis. TAZ physically binds MyoD to enhance binding to the myogenin gene promoter to activate MyoD-dependent gene transcription (142, 143). Ectopic overexpression of TAZ in C2C12 myoblasts results in accelerated myofiber formation, whereas TAZ loss lessened myogenic differentiation (142).

Evidence of upstream Hippo pathway regulators in muscle differentiation is limited. However, MST was found to have a pro-differentiation role during an investigation of caspase 3 in myogenesis (144). While caspases are classically known for their role in apoptosis, non-apoptotic functions have been reported. This appears to be the case in myogenesis, as caspase 3 was robustly activated in differentiating myoblasts without inducing apoptosis. Caspase 3-deficient myoblasts or C2C12 cells treated with caspase inhibitors are less able to differentiate, in part due to caspase 3-mediated regulation of MST1. Additionally, MST1 is a substrate for caspase 3, and cleaved MST1 was enriched in myoblasts undergoing differentiation. In caspase 3-deficient myoblasts, introduction of the cleaved MST1 induced myogenic differentiation, proving a link between these two pathways. However, MST1 activation must be tightly controlled, as MST1 activation in wild-type myoblasts ultimately led to cell death (144). While this study suggests a role for MST1 in myogenic differentiation, connections between MST1 activation by caspase 3 and the canonical Hippo pathway in muscle remain to be determined.

The Molecular Basis for Hippo Signaling in Sarcomas

Sarcomas comprise a group of clinically and histologically diverse tumors of mesenchymal origin. They can develop anywhere in the body, with about half arising in bone and half in soft tissues. In children and adolescents, osteosarcoma (OS) and Ewing sarcoma (EWS) are the two most common malignant bone sarcomas, while rhabdomyosarcoma (RMS) and non-rhabdomyosarcoma soft-tissue sarcomas (NRSTSs) are the major classes of malignant soft-tissue sarcomas (145).

As reviewed earlier, Hippo signaling is essential for proper organ growth, amplification of tissue-specific progenitor cells during tissue regeneration, and cellular proliferation (10, 146). In 2007, Dong and colleagues generated a liver-specific conditional *Yap1* transgenic mouse model that develops hepatocellular carcinoma (10). This led to the understanding that YAP is important in cancer and identified Hippo signaling as a tumor suppressor pathway in mammals. In other genetically engineered mouse models (GEMMs), mutations or altered expression of Hippo pathway genes gives rise to sarcomas, substantiating Hippo pathway deregulation in sarcomagenesis (138, 147–149). The next section will review the molecular basis of dysregulated Hippo signaling in bone and soft-tissue sarcomas. Each subsection will highlight the pro-tumorigenic role of YAP/TAZ, with subsequent cataloging of other Hippo pathway member involvement. **Table 2** summarizes these alterations.

Hippo Signaling in Osteosarcoma

Osteosarcoma is the most common primary malignancy of bone, with a 5-year overall survival of 60–70% (150). Given its decreased radiosensitivity compared to other sarcomas, surgical resection with chemotherapy is the mainstay of treatment. OS tumors are characterized by complex genomic rearrangements as well as copy number variations (151, 152). Mutations or loss-of-function of tumor suppressors *RB1* and *TP53* are two of the most common genetic alterations and are reported in ~50 and ~30% of tumors, respectively (151). Aberrations in Hippo signaling are proving to be important in the biology of OS.

YAP

Human tissue microarray analyses have revealed high YAP1 protein expression in OS compared to surrounding non-cancerous tissue, and expression correlates with staging (153). These findings corroborate other studies which showed high YAP1 expression in 78% of human OS samples and an increase in Hippo pathway target genes (80, 97, 154). Nuclear localization of Yap was found in Kios-5 murine OS cell lines, and Yap (and Taz, to a lesser extent) protein expression was also increased. *In vitro* suppression of Yap was associated with decreased cell proliferation and invasion, as well as decreased expression of Runx2, CyclinD1, and MMP-9. Decreased tumor growth was observed with *in vivo* Yap suppression in murine xenografts (155), as well as transgenic mouse models (80).

The mechanism of YAP upregulation in OS is complex but appears to be due in part to the stem cell transcription factor SOX2. In murine OS cell lines, Sox2 was found to directly repress the Hippo pathway activators, Nf2 and Kibra, leading to increased YAP. When grown as osteospheres, where stem cells are enriched, YAP expression was higher (and Nf2 lower) compared to adherent cells. In cells depleted of Sox2, either Yap overexpression or Nf2 suppression restored osteosphere formation. Conversely, suppressing Yap or overexpressing Nf2 promoted osteogenic differentiation and prevented osteosphere formation. The differentiated phenotype of OS cells induced by Nf2 could be overcome by either overexpressing wild-type or constitutively active mutant Yap, but not mutant Yap with a deficient TEAD-binding site. This regulation of Yap by Sox2 occurs through canonical Hippo signaling, as suppression of either Mst1/2 or Lats1/2 abolished Nf2-induced osteogenic differentiation as well as changes in Yap expression and function (97).

YAP can also be upregulated by Hedgehog (Hh) pathway activation. Malignant OS occurs with high penetrance in *Ptch1*$^{c/+}$;*p53*$^{+/-}$;HOC-Cre mutant mice, in which Hh signaling is partially upregulated in a p53 heterozygous background. Resultant tumors have high Yap1 expression, which is significantly reduced with Hh inhibition, and suppression of Yap1 blocks tumor progression. This same study showed that the Hh-Yap axis may regulate the expression of H19, a maternally imprinted long non-coding RNA implicated in tumorigenesis (80).

RASSFs

Two RASSFs (RASSF5 and RASSF10) have been implicated as tumor suppressors in OS. Similar to other RASSF family members, *RASSF5* and *RASSF10* are seen downregulated in human tumors (including OS) by CpG island promoter hypermethylation (156). In a human tissue microarray representing 45 OS samples, RASSF5 was significantly downregulated and expression negatively correlated with distant metastasis (157). In human U2OS cells, *in vitro* suppression of RASSF5 conveyed resistance to TNF-α-induced apoptosis, which is thought to occur through interaction and inactivation of the pro-apoptotic function of MST1 (158). Conversely, overexpression of RASSF5 in human OS cell lines decreases cell proliferation, increases apoptosis, and inhibits invasion.

NF2

In humans, germline or somatic mutations in one allele of *NF2* result in the disease neurofibromatosis type 2, which is associated with schwannomas, meningiomas, and ependymomas. However, mice heterozygous for *Nf2* develop a variety of malignant tumors at high frequency, including OS (63%). Somatic mutations of the wild-type *Nf2* allele were found in almost all of these tumors, implying that loss of heterozygosity of *Nf2* may be required for sarcomagenesis (147).

CD44

CD44 is a cell-surface glycoprotein that transmits extracellular signals to the ERK, AKT, and Hippo pathways (82, 159). CD44 was found to be suppressed by NF2, leading to decreased migration and invasion in OS cell lines *in vitro*, although an enhanced OS malignant phenotype was observed with knockdown of *CD44* in mice xenografts (160). Others have shown that NF2 mediates contact growth inhibition through ECM signals by complexing with CD44 (32).

MOB1

In vitro overexpression of MOB1A impairs cellular proliferation, while suppression of MOB1A leads to aberrant mitosis (15). In double-mutant mice lacking both *Mob1A* and *Mob1B*, complete loss of both alleles (*Mob1A*$^{\Delta/\Delta}$*1B*$^{tr/tr}$, null mutation of *Mob1A*, gene trap of *Mob1B*) is embryonically lethal. However, double-mutant mice retaining one allele of either (*Mob1A*$^{\Delta/+}$*1B*$^{tr/tr}$ or *Mob1A*$^{\Delta/\Delta}$*1B*$^{tr/+}$) survive and spontaneously develop tumors with 100% penetrance within 70 weeks. Extraskeletal OS arose in 24% (9/37) of mice, while benign exostosis occurred in 92% (34/37). All the tumors examined from either single heterozygote (*Mob1A*$^{\Delta/+}$*1B*$^{tr/tr}$ or *Mob1A*$^{\Delta/\Delta}$*1B*$^{tr/+}$) group revealed loss of the wild-type *Mob1* allele, suggesting loss of heterozygosity may be necessary for tumor growth (148).

Hippo Signaling in Ewing Sarcoma

Ewing sarcoma is the second most common malignant bone tumor in children and young adults. Although the 5-year overall survival is about 70%, 30–40% of patients either present with metastatic disease or develop recurrence, where outcomes are worse (161). EWS is characterized by a t(11;22) chromosomal translocation, which generates a fusion gene encoding the EWS-FLI1 chimeric protein that is thought to be the predominant driver of EWS tumorigenesis (162). The molecular basis for dysregulated Hippo signaling in EWS is beginning to be studied, as summarized below.

TABLE 2 | Proposed involvement of Hippo pathway components in sarcoma biology.

Sarcoma type	Component	Summary of proposed pathologic role	Reference
Osteosarcoma	YAP	*YAP1* expression is elevated and correlates with tumor staging and an increase in Hippo target genes Suppression of YAP promotes differentiation, and decreases cell proliferation and tumor growth YAP is a direct target of SOX2 in osteoprogenitors and *YAP1* expression is altered by SOX2 abundance OS transgenic mice with upregulated Hedgehog signaling display high *YAP1* expression The long non-coding RNA H19 is aberrantly induced by *YAP1* overexpression	(80, 153, 155)
	RASSFs	*RASSF5* is downregulated in human OS tumors and expression negatively correlates with metastasis *In vitro* overexpression of RASSF5 leads to decreased cell proliferation and invasion *RASSF10* promoter is epigenetically silenced through hypermethylation	(156–158)
	NF2	*NF2* expression is decreased and NF2 is shown to be a direct target of SOX2 in osteoprogenitors 63% of $Nf2^{+/-}$ mice develop OS. Increased penetrance and decreased latency and survival with $Nf2^{+/-}p53^{+/-}$ mice. Both groups show loss of wild-type *Nf2* allele	(97, 147, 160, 198, 199)
	MOB1	24% of $Mob1A^{\Delta/+}1B^{tr/tr}$ or $Mob1A^{\Delta/\Delta}1B^{tr/+}$ mice develop extraskeletal OS in 25–70 weeks	(148)
Ewing sarcoma	YAP	*In vitro* YAP suppression decreases proliferation in EWS cells BMI-1 stabilization of YAP is proposed to be a means for EWS cells to overcome contact-inhibition	(163)
	RASSFs	Hypermethylation of *RASSF1A* and *RASSF2* occurs at high frequency and correlates with worse outcomes	(165, 166)
ERMS	YAP	*YAP1* is elevated in human tumors and correlates with increased proliferation and clinical outcomes Copy number gains of the *YAP1* locus are reported YAP suppression results in decreased proliferation and increased differentiation *Myf5-* or *Myod1-hYap1 S127A* mice generate ERMS tumors within 4–8 weeks after *Yap1 S127A* expression 100% of *Pax7-hYap1 S127A* mice generate ERMS-like tumors within 10–11 weeks after injury	(138, 178)
ARMS	YAP	*YAP1* expression is increased in human tumor samples *In vitro* suppression of YAP results in decreased proliferation and increased senescence	(138, 178)
	RASSF4	*RASSF4* is a PAX3-FOXO1 target gene Overexpression of RASSF4 promotes cell proliferation *In vitro* loss of RASSF4 leads to decreased cell growth	(178)
NRSTS	YAP	STSs display gene amplification and overexpression of *YAP1* with increased TEAD-associated genes YAP complexes with TEAD and the cell cycle transcription factor FOXM1 to support STS tumorigenesis	(186, 191)
	RASSF1A	*RASSF1A* hypermethylation is reported in ~20% of adult STSs and correlates with clinical outcomes	(189)
	MST1/2	Hypermethylation of *MST1* and *MST2* occurs in 37 and 20% of STS, respectively	(187, 200)
	LATS1/2	Hypermethylation of *LATS1* is associated with poorer prognosis and shorter survival times in human STS	(149, 188, 189)
		60% of $Lats1^{-/-}$ mice die *in utero* but 14.3% of surviving female $Lats1^{-/-}$ mice develop fibrosarcomas by 4–10 months. Adding carcinogen exposure decreases latency and increases penetrance to 83%	
Fibrosarcoma	MOB1	22% of $Mob1A^{\Delta/+}1B^{tr/tr}$ or $Mob1A^{\Delta/\Delta}1B^{tr/+}$ mice develop fibrosarcoma in 25–70 weeks	(148)
	NF2	7% of $Nf2^{+/-}$ mice develop fibrosarcoma. 32% of $Nf2^{+/-}p53^{+/-}$ mice develop fibrosarcoma	(147)
EHE	TAZ-CAMTA1 YAP-TFE3	TAZ-CAMTA1 and YAP1-TFE3 fusion proteins are pathognomonic findings in EHE tumor samples	(193–195)

YAP

YAP suppression in human EWS cell lines decreases proliferation and anchorage-independent colony formation (163). A relationship between YAP and BMI-1, a Polycomb complex protein involved in chromatin remodeling (164), has been proposed. In studies examining the effect of cell density in cultured EWS cells, loss of BMI-1 had no effect in low-density, while it caused cell-cycle arrest and death under conditions of confluence. These findings may be due in part to the role of BMI-1 in stabilizing YAP expression and activity, and may serve

as a means for BMI-1-driven EWS cells to overcome contact inhibition (163).

RASSFs

Hypermethylation of the promoter regions of *RASSF1A* and *RASSF2* has been described in EWS and is correlated with worse clinical outcome (165, 166). One study of 55 human EWS tumors reported methylation rates for *RASSF1A* and *RASSF2* of ~52 and ~42%, respectively (165). In *in vitro* studies, overexpression of either RASSF1A or RASSF2 in EWS cells reduced their ability to form colonies (165). In a separate study, methylation of *RASSF1A* was observed in 75% (3/4) of EWS cell lines and 68% (21/31) of human tumors (166), though these studies are contradicted by other reports that did not demonstrate increased *RASSF1A* hypermethylation (167, 168). The EWS-FLI1 fusion protein has recently been shown to provoke widespread epigenetic changes, including altered DNA methylation, although it is not known whether there is a direct effect on *RASSF* expression (168, 169).

Hippo Signaling in Rhabdomyosarcoma

Rhabdomyosarcomas are soft-tissue sarcomas and account for approximately 8% of all pediatric solid tumors (170). The two major histological subtypes are termed embryonal (ERMS) and alveolar (ARMS) rhabdomyosarcoma. ERMS, which is more common, typically arises in the head and neck or retroperitoneum of younger children and conveys a better prognosis (localized tumors have >70% 5-year overall survival) (171, 172). ERMS tumors demonstrate numerous chromosomal aberrations, including genomic amplifications, loss of heterozygosity of specific chromosomal regions, frequent chromosomal gains in 2, 8, 12, and 13, and loss-of-imprinting (171–174). ARMS make up about 25–30% of cases and usually arise in the extremities or trunk and occur more frequently in adolescents. ARMS is characterized by recurrent chromosomal translocations, principally t(2;13) and t(1;13), which result in the expression of PAX3-FOXO1 and PAX7-FOXO1 fusion proteins, respectively (175). These aberrant chimeric proteins are oncogenic transcription factors that confer a poor prognosis (5-year overall survival <15% for metastatic or recurrent tumors) (173, 175–177). Interestingly, fusion-negative histologic ARMS have a cytogenetic and molecular profile similar to ERMS, and correspondingly similar clinical behavior (177).

YAP

YAP protein is upregulated in both ERMS and ARMS tumors (138, 178). In ERMS and fusion-negative ARMS, this is due in part to increased *YAP1* locus copy number. The importance of YAP in ERMS was confirmed by the remarkable finding that expression of YAP S127A is sufficient for ERMS tumorigenesis in a GEMM (138). This finding was particularly surprising given prior work showing YAP1 S127A expression in adult mouse muscle caused atrophy (179). Similar to this study, limb stiffness and gait defects were the initial phenotypes observed in Myf5/MyoD-YAP1 S127A mice (138). However, analysis of their muscle beds found that within the muscle damage were sites of active muscle regeneration and expansion of mononucleated cells. These were confirmed to be ERMS lesions, as they stained positive for ERMS histological markers. Tumor cells from these mice were transplantable, leading to secondary ERMS tumors with short latency. Given the high proportion of mononucleated cells in the primary tumor, Tremblay and colleagues hypothesized that satellite cells could serve as an ERMS cell of origin in this model. While expression of YAP1 S127A in the Pax7 (satellite) cell lineage did not induce ERMS formation, YAP1 S127A did transform satellite cells in the context of muscle injury. This suggests that hyperactive YAP signaling in activated satellite cells has transformative properties.

Using this GEMM model, hyperactive YAP signaling in ERMS tumors was found to induce a myogenic differentiation block. When YAP S127A expression was reduced, tumors rapidly regressed, and tumor cells spontaneously expressed markers of terminal muscle differentiation. Similarly, endogenous YAP suppression in ERMS RD cell xenografts caused myogenic differentiation (138). These findings are in line with earlier work implicating a role for YAP signaling in regulating myogenic differentiation. In proliferating C2C12 and satellite cells, YAP levels are high and localized in the nucleus. Upon differentiation stimulus, YAP mRNA expression is reduced and YAP becomes cytoplasmic (139, 140). This suggests an important role for YAP signaling in maintaining a high proliferative and anti-differentiation state. Similarly, YAP S127A can block C2C12 and satellite cell *in vitro* differentiation. This differentiation block is believed to be due to transcriptional changes induced by YAP-TEAD, particularly through upregulation of pro-proliferative genes and repression of MYOD1 and MEF2 regulation of terminal differentiation genes (138).

Additional studies have supported a role for YAP in RMS. A subset of ERMS tumors harbor mutations in the *PKN1* gene (encoding a kinase of the protein kinase C superfamily), which imparts a gene expression signature associated with activated YAP (180). In ARMS cells, *in vitro* genetic suppression of YAP induces growth arrest and senescence (178).

RASSF4

A role for the Hippo pathway in ARMS began with the identification of *RASSF4* as a PAX3-FOXO1 target gene (178). Using transcriptional profiling studies, PAX3-FOXO1-expressing myoblasts were found to upregulate *RASSF4* expression. Further, PAX3-FOXO1-positive ARMS cell lines and human tumors had elevated *RASSF4* levels, and high *RASSF4* expression was associated with worse RMS clinical prognosis. Loss-of-function studies demonstrated that RASSF4 was promoting cell proliferation and senescence evasion in ARMS cells. These RASSF4 functions were due to inhibition of MST1 signaling to MOB1. While no changes in signaling to LATS1 were observed, RASSF4-deficient ARMS cells did express lower levels of YAP1 protein. However, cells expressing a hyperactive YAP1 (YAP S127A) could not reverse the phenotypes associated with RASSF4 loss, suggesting an indirect connection between RASSF4 and YAP signaling (178). Altogether, these studies suggest that suppression of MST1-MOB1 signaling is an important oncogenic function of RASSF4 in ARMS.

TEAD-NCOA2 Fusions

NCOA2 is a transcriptional co-activator for steroid and nuclear hormone receptors. Fusion of *TEAD* to *NCOA2* was found in

tumor tissue removed from a 4-week-old child with spindle cell RMS (181), a rare variant of ERMS (182). While *NCOA2* gene rearrangements with other gene partners are seen in high frequency in congenital spindle cell RMS and mesenchymal chondrosarcomas (181, 183), the clinical and molecular significance of TEAD as a binding partner in this case is not known.

Hippo Signaling in Non-Rhabdomyosarcoma Soft-Tissue Sarcomas

Non-rhabdomyosarcoma soft-tissue sarcomas comprise the fifth most common group of solid tumors in children, accounting for 8–9% of childhood malignancies. These are histologically heterogeneous tumors that share some biologic characteristics. Surgical resection results in remission for about 80% of patients presenting with localized disease, though survival for those with unresected or metastatic disease remains poor (184). Many NRSTS, particularly those common in children, are characterized by disease-defining chromosomal translocations. Examples include synovial sarcoma t(X;18) and alveolar soft part sarcoma t(X;17), which result in the SYT-SSX and ASPL-TFE3 oncogenic fusion proteins, respectively (145). Other NRSTSs that are more common in adults, such as leiomyosarcoma or undifferentiated sarcoma, display multiple complex karyotypic abnormalities with frequent mutations in the TP53 and RB tumor suppressor pathways (185).

YAP

Nuclear staining for YAP is increased in a subset of human STS samples, compared to corresponding normal connective tissue (186). KRAS-based [$LSLKras^{G12D/+}$;$Tp53^{fl/fl}$ (KP)] GEMMs were used to further investigate the role of YAP in STS. *Yap* suppression in allograft tumors generated from KP cells results in decreased cell proliferation and tumor growth, and treatment with verteporfin to block the YAP–TEAD interaction decreased transcription of *Yap1* target genes. Many of the downregulated mRNAs in this model were noted to also be targets of *Foxm1*, a transcription factor involved in cell-cycle progression. FOXM1 is ordinarily inhibited by direct interaction with members of the TP53 and RB tumor suppressor pathways, and it is often overexpressed in malignancies where these tumor suppressor functions have been lost (186). FOXM1 expression was found to be increased in a variety of human sarcoma samples. In xenograft studies, FOXM1 suppression inhibited sarcoma growth. Co-immunoprecipitation and ChIP-seq experiments reveal that FOXM1 physically associates with a YAP/TEAD complex (186). YAP suppression in human sarcoma cell lines resulted in decreased proliferation and decreased FOXM1 expression, suggesting a novel role for YAP in co-activating FOXM1-mediated transcription in STS.

MST1/2

Hypermethylation of *MST1* and *MST2* promoters occurs in 37 and 20% of all STS (including RMS), respectively (187). In leiomyosarcoma samples, hypermethylation of *RASSF1A* and *MST2* were mutually exclusive, implying a common signaling pathway may exist for both genes. Surprisingly, methylation of the *MST1* promoter appears to correlate with a decreased risk of tumor-related mortality (187), albeit from a retrospective cohort with a small sample size.

LATS

Reduced *LATS* gene expression was observed in 14% (7/50) of human adult STS tumors (188). These findings correlate with subtype, as three of four myxoid liposarcomas, three of seven leiomyosarcomas, and one of nine malignant fibrous histiocytomas showed reduced or no expression of LATS1. In one of those samples, an allelic loss of the *LATS1* locus in chromosome 6q23-25.1, resulting from a missense point mutation, was observed. The other six samples showed aberrant hypermethylation of the putative *LATS1* promoter (188), corroborating another study showing hypermethylation of the *LATS1* promoter in 7% (3/43) of human STS samples (187). Hypermethylation of *LATS1* in STSs is associated with a worse prognosis and shorter survival times (189). It is not known whether epigenetic regulation of Hippo pathway kinases alters the expression of YAP and TAZ.

In transgenic mouse models, most mice (60/101) homozygous for a null mutation in *Lats1* died *in utero* or within post-natal day 1. However, ~14% of surviving female $Lats1^{-/-}$ mice developed large NRSTS by 4–10 months of age consistent with fibrosarcomas. After exposure to the carcinogen DMBA and repeated exposure to UVB, 83% (10/12) of $Lats1^{-/-}$ mice developed STSs, whereas no wild-type or heterozygous $Lats1^{+/-}$ mice developed tumors (149).

RASSF1A

Epigenetic silencing of *RASSF1A* via hypermethylation of its promoter occurs in 20% (17/84) of adult STSs (189). (This study included six cases of RMS, which did not reveal *RASSF1A* hypermethylation.) *RASSF1A* silencing was especially common in leiomyosarcomas, and overall was associated with an increase in tumor-related death.

VGLL3

Like YAP, VGLL3 is a TEAD co-activator and has been identified as an inhibitor of terminal adipogenic differentiation, suggesting that it has a core role in mesenchymal cell fate (190). In a study of 404 adult STSs, recurrent amplifications of chromosomes 11q22 and 3p12, which contain genes for *YAP1* and Vestigial-like 3 (*VGLL3*), respectively, were identified in 10% of cases. Genomic amplification corresponded to overexpression of *YAP1* and *VGLL3* at the message level, and an increase in TEAD-associated genes. *In vitro* suppression of *YAP1* or *VGLL3* decreased cell proliferation and in the case of *VGLL3*, decreased migration (191). In a smaller study, analysis of eight NRSTS tumors identified 3p11-12 as a commonly amplified region of a ring chromosome 3 that was associated with high expression of *VGLL3* (192).

TAZ-CAMTA1 and YAP-TFE3 Fusions

Fusions between the *WWTR1* (gene name for TAZ protein) and *CAMTA1* genes were first noted in a NRSTS subtype termed epithelioid hemangioendothelioma (EHE) (193). EHEs are vascular sarcomas that can develop in bone, soft tissue, or visceral organs, and they demonstrate a clinical behavior intermediate

between benign hemangiomas and high-grade angiosarcomas. Sequencing of two tumors identified the t(1;3) translocation between *WWTR1* and *CAMTA1*, and showed the fusion product to be under transcriptional control of the TAZ promoter. A larger study investigating 17 EHE tumors confirmed the translocation in all samples. The translocation and resulting transcript were not seen in epithelioid hemangioma and epithelioid angiosarcoma, morphologic mimics of EHE (194).

Subsequently, a *YAP1-TFE3* fusion product was identified in nine EHE samples that were morphologically different from the *WWTR1-CAMTA1* fusion-positive tumors (195). These findings were corroborated by two additional studies, the largest of which included 35 tumors and used a combination of IHC, FISH, and RT-PCR to validate *WWTR1-CAMTA1* fusion events in 33 cases and YAP1-TFE3 protein in two cases (196, 197). The oncogenic role of these signature fusions in EHE, or the role of Hippo signaling in vascular sarcomas, has not yet been established.

Targeting Hippo Signaling for Therapy

Recognition of the importance of Hippo signaling in malignancy has led to preclinical studies aimed at targeting components of this pathway for anti-cancer therapy. Modeled genetic manipulation of Hippo components exhibit profound effects on tumorigenicity, which provides optimism that modulators of Hippo components could be effective in patients. Indeed, the Hippo cascade involves many protein–protein interactions that could serve as novel targets. For details on each potential modulator, see recent reviews in Ref. (201, 202).

Small Molecule Modulators of the Hippo Pathway

As listed in **Table 3** and highlighted in **Figure 3**, several pharmacologic compounds, that directly or indirectly modulate Hippo pathway activity, have been identified. However, a number of important challenges exist. First, while kinases are often excellent targets for small molecule inhibitors, the majority of kinases in the Hippo pathway are tumor suppressors, and restoring lost tumor suppressive function is not easily achieved. Moreover, and as highlighted here, aberrant hyperactivity of oncogenic YAP and TAZ is often seen in malignancy as a result of mutations in proteins from other signaling networks, even in the presence of intact upstream Hippo kinase activity. However, small molecules aimed at increasing YAP/TAZ phosphorylation-induced nuclear export and proteosomal degradation could be effective at reducing their activity.

As such, inhibiting the activity of YAP/TAZ is the most obvious and presumably the most potent anti-cancer approach. Three porphyrin-related compounds were identified as top hits in a small molecule library screen of potential modulators for inhibiting the transcriptional activity of YAP *in vitro*. Verteporfin is a photosensitizer used clinically to treat patients with macular degeneration (203). Verteporfin binding to YAP alters YAP conformation to prevent it from binding to TEAD transcription factors. *In vivo* experiments in murine systems show verteporfin inhibits YAP-induced liver overgrowth by decreasing cell proliferation (49). *In vitro* treatment of retinoblastoma cells with verteporfin caused decreased cell proliferation and down-regulation of the pluripotency marker OCT4 (204). Other small molecule inhibitors, such

TABLE 3 | Pharmacologic modulators of the Hippo pathway.

Key	Compound	Mechanism	References
A	Fostriecin derivative	Inhibits PP2A	(210)
B	FTY720	Activates PP2A	(211)
C	9E1	Inhibits MST1 activity	(212)
D	C19	Activates MST/LATS	(213)
E	TM-25659	Modulates TAZ localization	(214)
F	Pyrrolidone 1	14-3-3 protein stabilizer	(215)
G	Verteporfin	Inhibits YAP-TEAD interaction	(49)
	Cyclic YAP-like peptide	Inhibits YAP-TEAD interaction	(205)
	VGLL4-like peptide	Inhibits YAP-TEAD interaction	(216)
	ABT-263, TW37	Inhibit BCL-xL (a YAP target)	(208, 217)
H	Dasatinib	Inhibits β-catenin-YAP-TBX5 complex	(58)
I	Epinephrine	Activates LATS through GPCRs	(29, 218)
	Dobutamine	Causes YAP phosphorylation	(219)
J	Phenoxodiol	SPHK1 inhibitor	(220, 221)
	BrP-LPA	LPA analog that blocks LPA receptors	(222)
	Thrombin	Acts on PARS to activate YAP	(223)
K	LT3015 Sphingomab	Monoclonal antibodies to LPA, S1P	(224–226)
L	Ibudilast	Inhibits PDE	(218, 227, 228)
M	Statins	HMG-CoA reductase inhibitors	(100, 101)
N	Y27632	RHO/ROCK inhibitors	(113, 116, 229)
	HA1077		
	Botulinum toxin C3		
O	Blebbistatin	F-actin destabilizers	(113, 115, 116)
	Cytochalasin D		(114–116)
	Latrunculin A/B		(113, 115, 116)
	ML7		(115)
P	WNT (or other pathway) modulators		(see Regulation Through Cross-Talk with Other Pathways and Hippo Modulation to Augment Other Pathway-Directed Therapies)

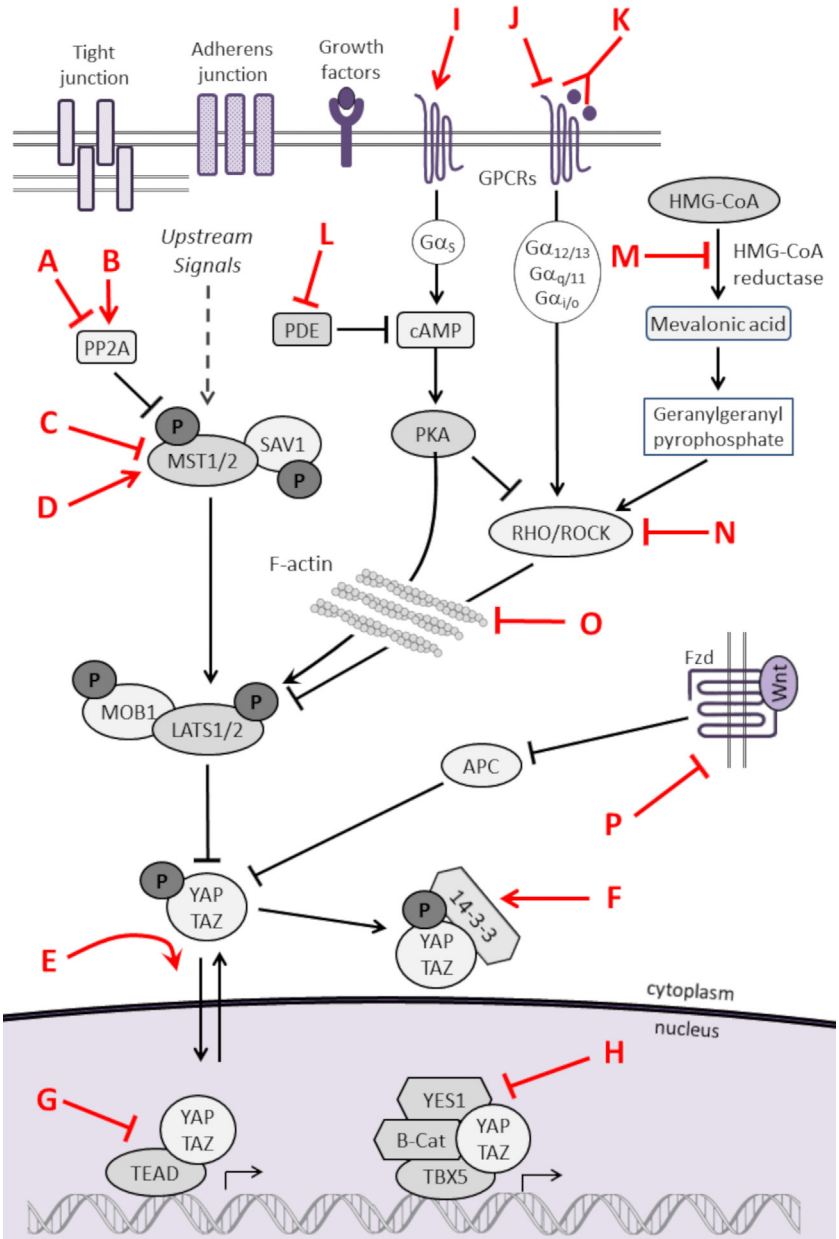

FIGURE 3 | Pharmacologic modulators of the Hippo pathway. The Hippo cascade involves many protein–protein interactions that could serve as novel targets, and numerous pharmacologic compounds either directly or indirectly modulate Hippo activity. Some of the compounds activate Hippo components and others have an inhibitory role. While not all referenced studies have proven that modulation of upstream regulators result in concomitant changes in YAP or TAZ activity, these provide proof of principal that targeting Hippo signaling could be harnessed as a novel strategy to treat sarcomas. This is not an inclusive list, and other compounds are known to modulate Hippo components. Figure is modified with permission from Park et al. (202). Letters in Red correspond to the letters in the Key in **Table 3**.

as cyclic YAP-like peptides and TM-25659, have been developed to interfere with YAP/TAZ–TEAD interactions (205).

Another challenge is that the Hippo pathway is ubiquitously expressed and thus, systemic treatment may cause detrimental side effects. This is particularly important in the pediatric population, where normal growth and development in most tissues likely rely on intact Hippo signaling. Similarly, GPCRs, although relatively accessible to inhibition, have broad physiological functions. However, intestine-specific conditional *Yap1* knockout mice develop normally (206), implying that in some instances, YAP/TAZ may be dispensable for tissue development. YAP and TAZ are responsive to tissue-specific regulatory elements, presenting a theoretical possibility of targeting Hippo signaling in specific cells or tissues.

Hippo Modulation to Augment Other Pathway-Directed Therapies

Evidence suggests Hippo-directed therapies may synergize with other targeted modulators. By serving as a parallel means of cancer cell survival, YAP promotes resistance to RAF and MEK inhibitors in *BRAF*/*RAS*-mutated tumors. YAP overexpression was observed in tumors harboring a *BRAF* mutation from patients with melanoma or NSCLC, and YAP expression levels inversely correlated to the patients' initial response to RAF and MEK inhibition. Furthermore, YAP suppression enhanced MEK inhibition in murine xenografts of human NSCLC, melanoma, and pancreatic adenocarcinoma with *BRAF* or *KRAS* mutations (207, 208). Similarly, YAP upregulation of EGFR through a YAP–TEAD complex at the *EGFR* promoter has been shown to partly explain the reduced translational impact of EGFR inhibitors in cancer. Inhibition of the YAP–TEAD interaction using verteporfin results in decreased EGFR expression and enhanced chemosensitivity to 5-fluorouracil and EGFR inhibitors in mouse xenografts of esophageal cancer (209). Finally, mTOR inhibition with rapamycin results in decreased TAZ expression in hepatocellular carcinoma (90).

Conclusion

The Hippo signaling pathway is an evolutionarily conserved tumor suppressor network important not only for proper cell, tissue and organ development, homeostasis, and repair, but it is also found dysregulated in many human cancers. While much of the early investigation on Hippo signaling in cancer was performed in epithelial malignancies, dysregulation of the Hippo pathway also occurs in sarcomas, cancers of mesenchymal origin. In a range of bone and soft-tissue sarcomas, Hippo signaling is commonly thwarted by upregulation of YAP or TAZ. However, genetic and epigenetic dysregulation of upstream core Hippo pathway members, and adaptor proteins has been noted. The role of Hippo signaling in mechanotransduction in both normal and cancerous mesenchymal cell behavior and fate provides additional insight into sarcoma biology. Further studies will be needed to clarify the underlying mechanisms of Hippo pathway dysregulation in specific sarcoma subtypes, providing a foundation upon which to develop successful therapeutic interventions.

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The importance of being dead: cell death mechanisms assessment in anti-sarcoma therapy

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Dedication:
To the memory of Alba Pérez.

Cell death can occur through different mechanisms, defined by their nature and physiological implications. Correct assessment of cell death is crucial for cancer therapy success. Sarcomas are a large and diverse group of neoplasias from mesenchymal origin. Among cell death types, apoptosis is by far the most studied in sarcomas. Albeit very promising in other fields, regulated necrosis and other cell death circumstances (as so-called "autophagic cell death" or "mitotic catastrophe") have not been yet properly addressed in sarcomas. Cell death is usually quantified in sarcomas by unspecific assays and in most cases the precise sequence of events remains poorly characterized. In this review, our main objective is to put into context the most recent sarcoma cell death findings in the more general landscape of different cell death modalities.

Keywords: cell death mechanisms, sarcoma, translocation-bearing sarcomas, apoptosis, necrosis, autophagic cell death, mitotic catastrophe

INTRODUCTION

FACTS

- Sarcomas are a highly heterogeneous group of mesenchymal tumors.
- Among cell death mechanisms, only apoptosis has been extensively studied in sarcomas.
- Fusion proteins, actors of translocation-derived sarcomagenesis, play an anti-apoptotic role in sarcomas.
- Proper and deeper assessment of cell death in sarcomas is mandatory.

CHALLENGES

- Can we improve the current therapeutic protocols in sarcomas through a better knowledge of cell death mechanisms?
- Can we assess more accurately the sequence of events of every type of cell death?
- Which are the key molecules that determine tumor cell death after therapy?
- Do translocation-bearing sarcomas have specific weaknesses in their cell death signaling networks?

Cancer therapies are aimed to induce the specific destruction of tumor cells without compromising patient health. This makes cell death mechanisms a central point of any therapeutic approach (1, 2). However, no every death is equally desirable in terms of therapy (3). The need of theoretical arrangement in the field has become evident during the past years. Our knowledge on cell death mechanisms has increased enormously and

Abbreviations: AIF, apoptosis inducing factor; Akt, protein kinase B; Apaf-1, apoptotic protease activating factor 1; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-x_L, B-cell lymphoma-extra large; BubR1, mitotic checkpoint serine/threonine-protein kinase BUB1 beta protein; CD133, prominin-1; CD99, cluster of differentiation 99 protein; cFLIP, cellular FLICE inhibitory protein; Chk1, checkpoint kinase 1; c-Myc, cellular avian myelocytomatosis viral oncogene homolog; DAPI, 4′,6-diamidino-2-phenylindole; DRAL, downregulated in rhabdomyosarcoma LIM protein; ERG, protein encoded by erythroblast transformation-specific related gene; ERK, extracellular signal-regulated protein kinase; EWS, Ewing sarcoma RNA-binding protein; FasL, fas ligand protein; FLI1, Friend leukemia integration 1 transcription factor; FOXO1, forkhead box protein O1; HDAC1, histone deacetylase 1; IGF-1R, insulin-like growth factor 1 receptor; IHQ, immunohistochemistry; JAK, Janus kinase; MC, mitotic catastrophe; MDM2, mouse double minute 2 homolog protein; MEK, mitogen-activated protein kinase kinase; miRNA, micro RNA; MLKL, mixed lineage kinase domain-like protein; mTOR, mammalian target of rapamycin protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells protein; NGFR, low-affinity nerve growth factor receptor; Noxa, Phorbol-12-myristate-13-acetate-induced protein 1; *oct4*, octamer-binding transcription factor 4; p21, cyclin-dependent kinase inhibitor 1; PARP, poly (ADP-ribose) polymerase; PAX, protein encoded by paired box gene; PD, progression disease; PDGFR, platelet-derived growth factor receptor; PET-CT, positron emission tomography; PI, propidium iodide; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; Plk1, polo-like kinase 1; PM, plasma membrane; PR, partial response; pRb, retinoblastoma protein; puma, p53 upregulated modulator of apoptosis; RAF, *raf* proto-oncogene serine/threonine-protein kinase; RANK, receptor activator of nuclear factor κB; ROS, reactive oxygen species; *sox2*, sex determining region Y-box 2; SSX, protein encoded by synovial sarcoma X breakpoint gene; STS, soft tissue sarcoma; SYT, protein encoded by synovial sarcoma translocation on chromosome 18 gene; TNFR, tumor necrosis factor receptor; TRAIL, TNF-related apoptosis-inducing ligand; VEGFR-2, vascular endothelial growth factor receptor 2.

the available methodology has become more and more sophisticated. Therefore, a clear nomenclature based on reliable markers has been proposed (1, 4). Additionally, the growing number of cell death participants have been organized in clear hierarchic frameworks (5).

Sarcomas are a rare and heterogeneous group (more than 50 different clinical and molecular entities) of malignant tumors with mesenchymal origin. Molecular biology of sarcomas has remained elusive until recently, and a better knowledge remains as an unmet need (6). New drugs against potential targets in tumor cells with a crucial role in their metabolism or pro-survival fitness could improve the prognosis of these patients. Indeed, the relatively high rate of therapeutic failure and tumor relapse demands a better assessment of cell death induction. But scientific efforts in this discipline are historically undermined by the relative low investments and isolated work (7).

The scientific landscape involving cell death mechanisms in sarcomas can be improved. The majority of articles included in the present review focused on apoptosis (mostly) and necrosis, whose morphological characters (**Figure 1**) and signaling players (**Figure 2**) are better described. Many studies about cell death in sarcomas just describe the occurrence of cell death without a proper characterization of the sequence-of-events leading to a particular form of death. The aim of the present review is to help sarcoma researchers to face new knowledge on cell death mechanisms in order to routinely include it in their assessments.

CELL DEATH MECHANISMS

APOPTOSIS

Apoptosis involves a cellular controlled demolition process. Signaling cascades are finely orchestrated and secured, to ensure its perfect onset only when it is required (8). Caspases are the major actors in cellular demolition; once triggered, caspases can cross-activate each other and thus amplify the apoptotic signal (8). Apoptosis is by far the most studied form of cell death in sarcoma research. Nevertheless, researchers either employ uninformative methods about the form of death (i.e., Trypan Blue assay), or the mechanisms leading to such death are not always fully analyzed. Apoptosis recognition is easy by simple morphological features visible under the microscope: nuclear condensation and fragmentation, blebbing etc. (**Figure 1**). Other techniques (immunofluorescence or western blotting of cleaved caspases and/or caspase substrates, etc.) can be used to monitor specific mediators and executors of the process (9–11). Based on their biochemical features, we can describe two major pathways in apoptotic signaling: the intrinsic or mitochondrial pathway and the death receptor pathway (**Figure 2**).

Mitochondrial apoptosis

The "intrinsic pathway" is defined by the role of the mitochondria as encounter point of most of its initiators and mediators. The Bcl-2 family of proteins controls this pathway by regulating the formation of a pore in the mitochondrial outer membrane (12). Several signaling pathways converge in the regulation of Bcl-2 proteins, from DNA-damage sensor system to organelle stress and malfunction or growth factor signaling (**Figure 2**) (13, 14). In order to demonstrate that a drug or physiological input induces apoptosis through the mitochondrial pathway, exogenous overexpression of anti-apoptotic Bcl-2 family members can be performed; this should either prevent cell death or switch the mechanism to necrosis.

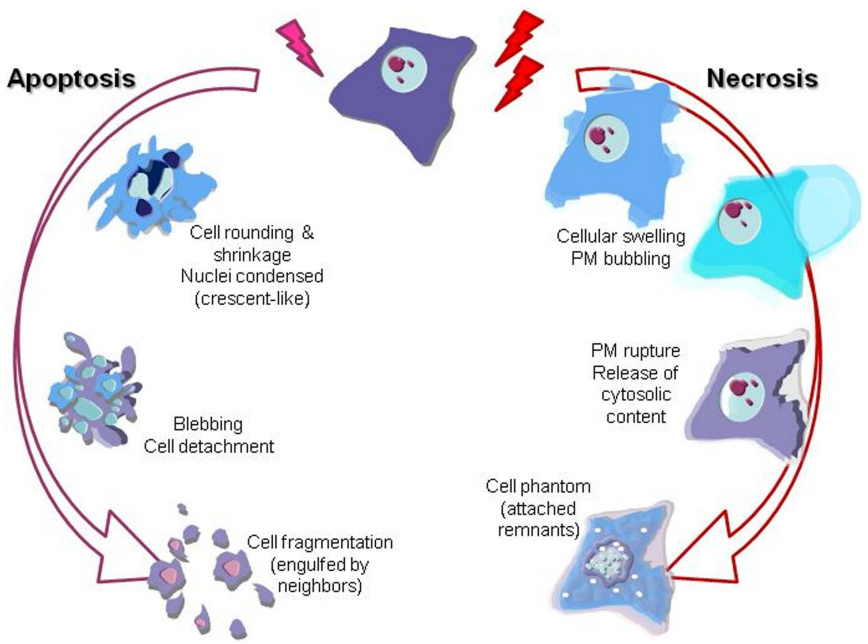

FIGURE 1 | Graphical illustration of the most prominent morphological features of apoptotic and necrotic cell death mechanisms. Nuclei changes (karyorrhexis), cytoplasm shrinkage, and blebbing are the most evident descriptors of apoptosis. On the other hand, necrosis is clearly recognizable by cell swelling (loss of osmotic barrier) leading to the plasma membrane (PM) breakage and final release of the inner soluble content and nuclei karyolysis.

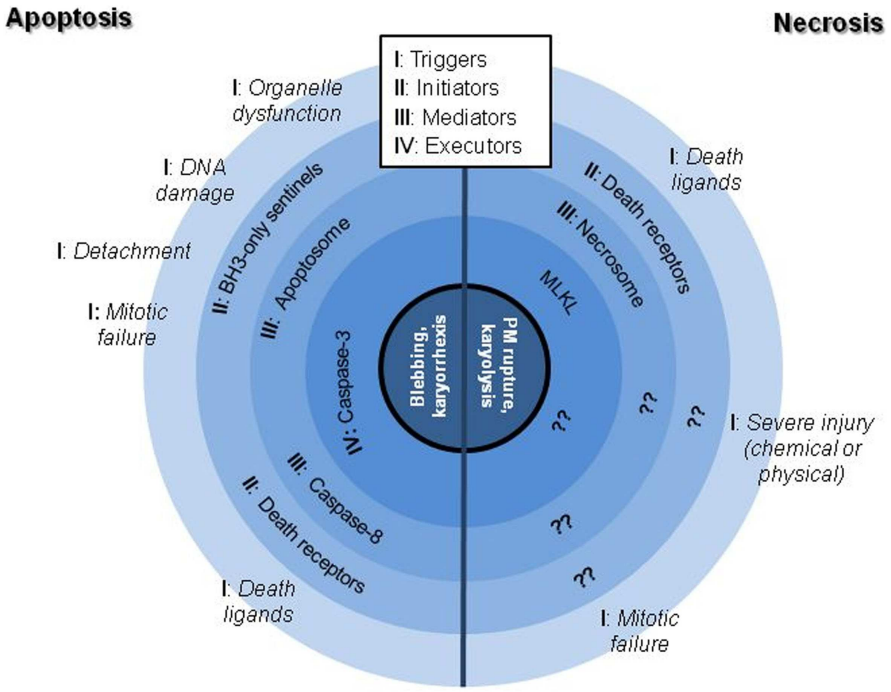

FIGURE 2 | Schematic representation of the better characterized signaling hubs of apoptotic and necrotic cell death mechanisms. Note that necrotic processes are substantially worse described than apoptotic ones, being still controversial if the execution phase is protein-driven or result of a massive metabolic failure.

Some sarcomas rely on the presence of specific aberrant fusion proteins, generated after chromosomal rearrangements. Deregulation of gene expression in sarcomas driven by these chimeric oncoproteins can occur at different levels (epigenetic silencing, transcription activity, messenger processing, etc.) affecting every cellular process, including apoptosis (**Figure 3**). In the case of Ewing Sarcoma (ES), the fusion proteins EWS-FLI1 or EWS-ERG have an inhibitory effect on part of the apoptotic machinery (15, 16). This effect is mediated by direct or indirect interactions with several signaling pathways modulating apoptosis repression and inducing sustained growth (17–20). Alveolar rhabdomyosarcoma (aRMS) is also dependent on fusion proteins involving different PAX proteins with FOXO1, which also targets different signaling networks in order to ensure evasion of apoptosis (21, 22). SYT-SSX chimera proteins are present in the majority of synovial sarcoma tumors. They are involved in resistance to pro-apoptotic stimuli by modulating the levels and the activity of key apoptotic players of the Bcl-2 family of proteins (23). Furthermore, certain translocation-bearing sarcomas are also characterized by failure to complete tissue differentiation (i.e., RMS to skeletal muscle, liposarcoma to adipocytes) in a process mediated by their specific fusion protein and linked to the inhibition of apoptosis induction (24–26). Several recent studies have linked miRNAs status with apoptosis regulation in chromosome translocation-bearing sarcomas. Hence, mitochondrial apoptotic resistance in ES correlates with miR-125b upregulation through p53 and Bak (27) but overexpression of miR-206 in RMS promotes proliferation arrest and some sort of cell death (28). Overexpression of miR-145 and miR-451 in liposarcoma cell lines decreases cellular proliferation, impairs cell cycle progression, and boosts cell death (29), whereas overexpression of miR-26a-2 has the opposite effects (30).

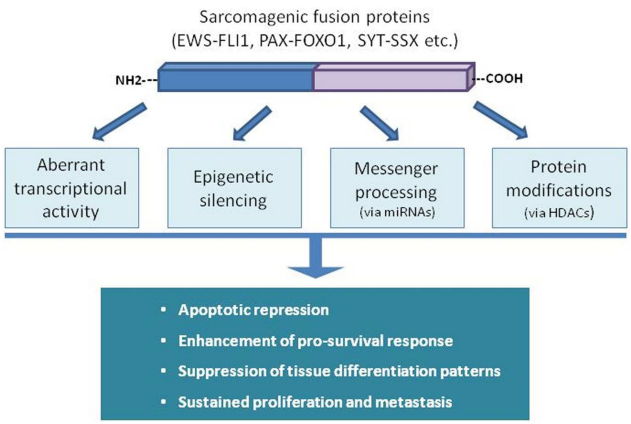

FIGURE 3 | Fusion proteins in sarcomas disturb the natural physiological balance between pro-survival and death signaling inputs through different ways. The panoply of mechanisms and cellular targets disturbed demonstrates the powerful tumorigenic effect of a single event of genomic rearrangement.

The levels and status of key pro- and anti-apoptotic proteins are also crucial for understanding the differential sensitivity of cells toward apoptosis. Most ES cells have both the p53 downstream

pathway and the DNA-damage signaling pathway functionally intact. The resistance of some ES cell lines to p53-induced apoptosis has been linked to a high Bcl-2/Bax ratio and low levels of Apaf-1 (31). However, the influence of fusion proteins inactivates p53 by deacetylation at Lys-382 driven by both EWS-FLI1 and HDAC1 (32), meaning that re-expression or re-activation of p53 could be a good strategy against these tumors. Similar phenomena occur in other fusion-positive sarcomas and accordingly, histone deacetylase inhibitors have been successfully tested as apoptotic inducers in different sarcoma types (33, 34). p53 re-activator agents as Nutlin-3 and/or PRIMA-1 are able to induce apoptosis successfully through Noxa, Puma, or p21 upregulation in both mutant and wild-type p53 sarcoma cell lines (35–37). Among downstream p53 targets p21, c-Myc, Bax, MDM2, DRAL, Bcl-2, and Bcl-x_L have been suggested as key apoptotic regulators in different sarcoma models (38–43). Plasma membrane-anchored growth receptors such as NGFR or IGF-1R have an anti-apoptotic role (44, 45). In contrast, distinct behaviors have been suggested for the closely related receptors PDGFR α and β (46). Thus, PDGFR α favors cellular stemness and PDGFR β promotes angiogenesis in the tumor stroma. Hepatocyte-growth-factor activator inhibitors (HAI-1 and HAI-2) act as tumor suppressors leading to apoptosis and necrosis in leiomyosarcoma (47). Also, inhibition of endogenous tyrosine kinase B (TrkB) signaling suppresses cell proliferation and increases apoptosis in cultured leiomyosarcoma cells (48). In this context, tyrosine kinase inhibitors like Sorafenib induce apoptosis on many leiomyosarcoma or synovial sarcoma cell lines by inhibiting the RAF/MEK/ERK signaling pathway, among others (49, 50). Apoptotic cascades induced by other kinase inhibitors like JAK1 and 2 have been analyzed in detail in RMS and ES cells. These inhibitors lead to the alteration of the balance between the pro-apoptotic Bax and the anti-apoptotic proteins Bcl-2 and Bcl-x_L, the release of cytochrome *c*, and the activation of caspase-9, -8, and -3 (51, 52).

Many other different strategies have been used in sarcomas to induce mitochondrial apoptosis. Betulinic acid is able to target the mitochondria in ES, promoting the permeabilization of the outer membrane resulting in the release, from the mitochondria to the cytosol, of soluble factors such as AIF and cytochrome *c*, who ultimately leads to caspase activation (53). Direct targeting of mitochondrial physiology was also explored in RMS with photodynamic therapy (54) and ROS-generation agents (55). Proteasome inhibitors as Bortezomib generate a major stress in the cell machinery, triggering a number of different reactions, many of them aimed to induce apoptosis. Bortezomib has been successfully employed in different pre-clinical models (56, 57). Heat shock proteins are among the most important actors against protein stress in cells. Accordingly, Hsp-90 antagonists had been shown to induce transient growth arrest and apoptosis in RMS cells (58). Likewise, some metabolic disruptors like 2-deoxyglucose, Lovastatin, and Catechins have been successfully tested as promoters of mitochondrial apoptosis by unbalancing the equilibrium of Bcl-2 family of proteins (59–61). Furthermore, down-regulation of inhibitor of apoptosis proteins (IAPs) also leads to apoptosis, identified by PARP cleavage, in pediatric sarcomas (62).

To keep their correct physiology, cells rely in their interaction with neighbors and microenvironment, meaning that detachment is a major apoptotic trigger. The process of detachment-induced apoptosis is termed anoikis (4). The lack of attachment activates signals from the plasma membrane, mostly by integrins and the focal adhesion kinase (FAK) that regulate the BH3-only proteins through the mitochondrial commitment to cell suicide (**Figure 2**) (63). Cell culture in non-adherent conditions, like soft-agar, is the better way to study this process. Suppression of anoikis cell death is considered an important hallmark of transformed cells and thus, a pre-metastatic key process (64).

Anoikis resistance in sarcomas has been described to be associated with integrins, Bcl-2 and caspase-8, CD99 isoforms, RANK, and ERK (65–68). ES cells survival in non-adherent conditions is mediated by E-cadherin dependent spheroid formation, avoiding apoptotic triggering by means of the PI3K/Akt pathway (69). Scotlandi *et al.* demonstrated the relevance of IGF-1R in the anoikis-resistant ES cell line TC71. Impairment of IGF-1R signaling (by neutralizing antibodies or siRNAs expression) led to a lower survival in anchorage-independent growth conditions and a decrease on metastatic ability (70). In synovial sarcoma, the increased IGF-2 synthesis protects cells from anoikis and is required for tumor formation *in vivo* (71). Another trans-membrane growth factor receptor, the ErbB4 Tyrosine kinase, gets phosphorylated in ES spheroids and its expression is linked to anoikis avoidance, metastatic disease, and bad outcome (72). In RMS, spheroids obtained after cell culture enrichment express stem cell gene markers such as *oct4, pax3, sox2, c-myc,* and *nanog.* It was also found that CD133 was upregulated in these spheres, conferring cells higher resistance to Cisplatin and Chlorambucil *in vivo* (73). In osteosarcoma (OS) cells, anoikis can be induced by zoledronic acid, DNA methylation inhibitors as decitabine or cyclooxygenase-2 inhibitors via PI3K/Akt pathway inhibiting β-catenin, TrkB, and E-cadherin (74–76).

Several of the aforementioned reports present indeed interesting data for a number of plausible targets concerning mitochondrial apoptosis. However, it is worth noting that in most of these cases, apoptotic analyses rely only in AnnexinV (AnnV) tests or caspase-3 activation kits, being uninformative about the precise processes involved. Although extended in the community, when the end-points of AnnV-PI tests are not carefully selected, this could lead to the misidentification of late apoptotic and necrotic cells; similarly, caspase-3 is a common final step in apoptotic cell death that does not imply a single precise activation pathway (**Figure 2**) (11).

The death receptor pathway

Caspase-8 is the most characteristic mediator of the "death receptor pathway" (**Figure 2**). In this case, the triggers of the apoptotic process are extracellular signals (mostly from the TNF family) and the initiators and mediators encounter not in the mitochondrial outer membrane but rather close to the plasma membrane (77). Besides direct stimulation of cell death, death receptors can also induce specific protein synthesis by means of the NF-κB pathway that balances and even counteracts the apoptotic signaling (78).

TRAIL is a death ligand that has been studied in several sarcomas for therapeutic purposes (79–81). TRAIL-induced apoptosis is regulated by other receptors and downstream effectors including

cFLIP and the Bcl-2 family (82–84). The TRAIL receptor, death receptor 5, has been identified as a mediator of chemically induced apoptosis in RMS, synovial sarcoma and leiomyosarcoma, activating several apoptosis triggers (85–87). TNFα and FasL receptors play also a significant role in the survival/apoptotic balance with p21 as critical mediator of the anti-apoptotic effect of TNFα-induced NF-κB (88, 89). Bad, a pro-apoptotic member of the Bcl-2 gene family, has been linked to FasL induced apoptosis in ES (90). Activation of death receptors could be combined with other challenges like doxorubicin, interleukin-12, or immunotoxins (91–93). Some other TNF receptor-related proteins, like NGFR, have been proposed to be crucial in specific sarcomas (94). Thus, there is still a need for a better understanding of the role of the other cell death receptors in sarcomas.

Besides the death receptors themselves, the best strategy to enhance extrinsic apoptosis is repressing NF-κB activation. This rationale has been employed with success against ES and synovial sarcoma (95, 96). Sensitization to apoptosis has also been achieved by re-expressing caspase-8 through demethylation or gene transfer (97).

NECROSIS

Necrosis, in contrast to apoptosis, has been viewed classically as a form of accidental death brought about by injury to the cell by pathogens or toxins. Despite the extended pre-judice, necrosis is more than a mere accidental death (5). Loss of plasma membrane integrity, the "cellular explosion", is the major morphological feature and characteristic element of necrosis (**Figure 1**) (9, 98). Non-accidental or "regulated" necrosis has attracted a growing interest in the scientific community in the last years. Necroptosis is the best known phenotype in this group. It is induced by either the activation of death receptors or specific injuries that are followed by the recruitment of the so-called necrosome of which the principal participants are the receptor-interacting protein kinases (RIPK1 and RIPK3), which finally activate the executor MLKL (**Figure 2**) (99).

Necroptosis is just starting to be studied in sarcomas. It can be distinguished from apoptosis by its distinct morphology and the inability of caspase inhibitors to prevent it (10, 11). In an OS model, RIPK1-mediated necroptosis was confirmed as the main cell death mechanism involved in Shikonin therapy, as only Necrostatin-1 (an inhibitor of RIPK1) was able to induce treatment reversion (100). Basit *et al.* found that Obatoclax (a Bcl-2 inhibitor) treatment in RMS cell lines promoted necroptosis rather than autophagic cell death, being autophagy only a necessary event required for the necrosome assembly (101). So, it becomes clear that there is still a big room for improvement in the accurate characterization of regulated necrosis responses in anti-sarcoma therapy.

OTHER SCENARIOS FOR THE CELL DEATH DRAMA

The long-standing dichotomy apoptosis-necrosis is in part nothing but a classification artifact. Many times the exact nature of the mechanism triggered relies simply on the intensity of the injury or on the available energy (102). Furthermore, in the cell death landscape, there are other "circumstances" worth of some additional explanation.

A classical example of "double-edged sword" is autophagy, sometimes included as a cell death mechanism, although it usually proceeds as a pro-survival process. Autophagy targets apoptotic-signaling mitochondria for isolation and degradation, thus interrupting the apoptotic outcome. Several proteins cross-link autophagy and apoptosis signaling pathways, being mTOR one of the most studied (103). As a process impacting the energy availability, autophagy also dialogs with necrotic signaling and some reports point to a close relationship with necroptotic triggering (101, 102). Again, it seems to be a question of threshold. In many cases, an excessive autophagy can lead to cell death but this death follows a mixed pattern with parallel apoptotic or necrotic phenotypes. Only when inhibition of autophagy can impede cell death and the final phenotype is considered non-apoptotic cell death, we can classify it as "autophagic cell death" (4, 102). Among the different techniques available, autophagy can be better followed by microscopy assessment of autophagosome formation (11, 104).

To our knowledge, except for some interesting report showing autophagic triggering of necroptosis in RMS (101), no instances of true autophagic cell death have been described in sarcomas yet. Indeed, its role in cancer therapy is still controversial (102). In ES and OS, the protective role of autophagy was insufficient to block apoptotic cell death when triggered by either the intrinsic or the death receptor pathways (105, 106). Autophagy has also been described to be actively removing micronuclei in OS cells, generating an interesting connection with the stabilization of cells recovering from failures during mitosis (107).

Mitotic catastrophe (MC), previously classified as a form of cell death, constitutes a crossroad that could drive cells to die with either apoptotic or necrotic features, go into senescence, or even survive (108). Again, the precise features of the final death phenotype depend on cell context and energy availability (108, 109). The clearest triggers of MC are the dysfunctions of the mitotic spindle. Those dangers are monitored by specific checkpoint proteins determining the final outcome. Thus, cells evading the mitotic arrest have an increase in chromosome instability (110). MC can be easily followed by means of microscopy observation, usually aided with fluorescent markers, video-microscopy, and cell fate imaging analysis.

Proper metaphase arrangement is required for mitosis and is a key process monitored by several checkpoint regulators (**Figure 2**). BubR1, involved in the mitotic spindle checkpoint, has been shown to be necessary for survival in some RMS cell lines and its knockdown promoted growth suppression and "mitotic catastrophe" but the final outcome was not elucidated (111). Plk1 is another major component of MC signaling: siRNA inhibition of Plk1 killed RMS cells and the chemical inhibitor BI 2536 induced G_2/M arrest and cell death in OS cell lines (112, 113). Inhibitors of Aurora kinases block the formation of the cleavage furrow, disrupting cytokinesis, and killing leiomyosarcoma and synovial sarcoma cells (114, 115). Chk1 blockade with CEP-3891 caused an abrogation of the S and G_2 checkpoints after ionizing radiation, giving rise to nuclear fragmentation as a consequence of defective chromosome segregation and promoting cell death (116). Many active drugs tested in sarcoma cells have been described to disrupt normal cell cycle. Those compounds range from small molecules or plant derivatives, to cell cycle kinase inhibitors, viral proteins etc. Several studies showed

Table 1 | Summary of already published clinical trials that evaluate target therapies in sarcomas, classified regarding the mechanism of action.

	Mechanism of action	Drugs	Trial (reference)	Study population	Benefits	Common severetoxicities
Apoptosis	**PARP inhibitors**	Olaparib	Phase II (127)	Recurrent/metastatic adult ES (failure to prior CH), $n = 12$ patients	NO responses SD: 4 patients, TTP: 5.7 weeks	No significant toxicities
	Heat shock protein inhibitors	Retaspimycin (Hsp-90 INH)	Phase I (128)	Metastatic and/or unresectable STS, $n = 54$ patients	PR: 2 patients (proof of clinical activity)	Grade 3–4: Fatigue Nausea and vomiting Headache Artharalgia
	Proteaseome inhibitor	Bortezomib	Phase II (129)	Metastatic OS, ES, RMS, and STS with no prior treatment for advanced disease, $n = 25$ patients	Lack of benefit (trial prematurely closed)	Grade 3–4: Neuropathy Asthenia Myalgias
	MDM2 inhibitor	RG7112	Proof of mechanism study (130)	WDLS or DDLS with MDM2 amplification receive RG7112 prior to surgery, $n = 20$ patients	SD: 14 patients, IHQ: activation of p53 pathway	Grade 3–4 Neutropenia Thrombocytopenia
			Phase I (131)	Phase I trial with extension cohort for sarcoma patients, $n = 30$ (sarcoma patients)	Metabolic responses (PET-CT) IHQ: activation of p53 (MDM2-independent)	Grade 3–4 Cytopenias
	PI3K-AKT-mTOR pathway inhibitors	Ridaforolimus (mTOR INH)	Phase II (132)	Pre-treated advanced bone and STS, $n = 212$ patients	RR: 1.9%, clinical benefit: 28.8%	Grade 3–4 Fatigue Stomatitis Hypertriglyceridemia Anemia Thrombocytopenia
			Phase III (133)	Advanced bone and STS with clinical benefit to previous CH were randomized to maintenance Ridaforolimus or Placebo, $n = 711$ patients	Improvement in PFS (17.7 weeks with Ridaforolimus vs. 14.6 weeks with Placebo, HR: 0.72, p: 0.001)	Similar to previous study
		Everolimus (mTOR INH)	Phase II (134)	Pre-treated advanced bone and STS, $n = 41$ patients	Poor clinical activity	Grade 3–4 Hyperglicemia Stomatitis Pain Asthenia
	Anti-angiogenic therapy	Sorafenib (VEGFR2, VEGFR3, PDGFR, and c-Kit INH)	Phase II (135)	Pre-treated advanced STS, $n = 101$ patients	RR: 14.5%, SD: 32.9% (leiomyosarcoma better PFS)	Grade 3–4 Fatigue Diarrhea Hand–foot Syndrome Nausea and vomiting
		Pazopanib (VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, and c-Kit INH)	Phase III (136)	Pre-treated non-adipocytic STS randomized to PAZOPANIB vs. PLACEBO, $n = 369$ patients	Improvement in PFS (4.6 months with PAZOPANIB vs. 1.6 months with Placebo, HR: 0.31, $p < 0.0001$)	Grade 3–4 Asthenia Hypertension Anorexia Alteration of transaminases
Mitotic catastrophe	**CDK inhibitors**	Palbociclib (CDK4 and CDK6 INH)	Phase II (137)	WDLS or DDLS with CDK4 amplification and pRb expression	66% of patients free of PD at 12 weeks	Grade 3–4 Anemia Neutropenia Thrombocytopenia

CH: chemotherapy, DDLS: dedifferentiated liposarcoma, HR: hazard ratio, INH: inhibitor, MPNST: malignant peripheral nerve sheath tumor, PFS: progression-free survival, RR: response rate, SD: stabilization disease, STS: soft-tissues sarcoma, TTP: time to progression, WDLS: well-differentiated liposarcoma.

Table 2 | Summary of clinical trials that are ongoing and evaluate target therapies in sarcomas, classified regarding the mechanism of action.

		Ongoing trials specific for sarcomas	Status www.clinicaltrials.gov	Identifier www.clinicaltrials.gov
Apoptosis	**PARP inhibitors**	ESP1/SARC025 global collaboration: a Phase I study of a combination of the PARP inhibitor, niraparib, and temozolomide in patients with previously treated, incurable Ewing sarcoma	Ongoing, but not recruiting	NCT02044120
		Olaparib in adults with recurrent/metastatic Ewing's sarcoma.	Ongoing, but not recruiting.	NCT01583543
	Heat shock protein inhibitor	A trial of ganetespib Plus sirolimus: phase 1 includes multiple sarcoma subtypes and Phase 2 MPNST	Ongoing, but not recruiting	NCT02008877
	PI3K-AKT-mTOR pathway inhibitors	Phase II study of everolimus in children and adolescents with refractory or relapsed osteosarcoma	Recruiting	NCT01216826
		Phase II open label, non-randomized study of Sorafenib and everolimus in relapsed and non-resectable osteosarcoma (SERIO)	Ongoing, but not recruiting	NCT01804374
		Study of everolimus with bevacizumab to treat refractory malignant peripheral nerve sheath tumors	Ongoing, but not recruiting	NCT01661283
		Phase II study of everolimus in children and adolescents with refractory or relapsed rhabdomyosarcoma and other soft tissue sarcomas	Recruiting	NCT01216839
	Anti-angiogenic therapy	Sorafenib tosylate, combination chemotherapy, radiation therapy, and surgery in treating patients with high-risk stage IIB–IV soft tissue sarcoma	Recruiting	NCT02050919
		Pazopanib hydrochloride followed by chemotherapy and surgery in treating patients with soft tissue sarcoma	Recruiting	NCT01446809
		Activity and tolerability of pazopanib in advanced and/or metastatic liposarcoma. a phase ii clinical trial	Recruiting	NCT01692496
		Study of pazopanib in the treatment of osteosarcoma metastatic to the lung	Recruiting	NCT01759303
		Study of pre-operative therapy with pazopanib (votrient®) to treat high-risk soft tissue sarcoma (NOPASS)	Recruiting	NCT01543802
Mitotic catastrophe	**Aurora-kinase inhibitors**	Alisertib in treating patients with advanced or metastatic sarcoma	Recruiting	NCT01653028
	CDK inhibitors	PD0332991 in patients with advanced or metastatic liposarcoma	Recruiting	NCT01209598

cell cycle arrest and changes in the levels of MC mediators as Survivin. For example, Keyomarsi's group showed that combined therapy with doxorubicin and roscovitine in synovial sarcoma and leiomyosarcoma induced a synergistic increase in autophagy in addition to a marked arrest in G_2/M (117). Links between MC and autophagy have also been commented previously for OS (107). In any case, it would be desirable to perform an exhaustive mitotic study or cell fate analysis together with the proper assessment of the nature of cell cycle blockade (metaphase arrest, G_2 stop, or even senescence).

CELL DEATH MECHANISMS IN ANTI-SARCOMA CLINICAL TRIALS

New targeted therapies linked to key cell death mechanisms are continuously being developed (118). Preferred to cytostatic alternatives, cell death induction is the goal of the vast majority of cancer treatments. And among the known mechanisms, apoptosis is the center of therapeutic developments (118). As a non-inflammatory mechanism, apoptosis is traditionally considered cleaner than necrosis, but its exact relevance in overall therapeutic success is uncertain. Necrosis, due to its pro-inflammatory nature, has been regarded as a back door for metastatic cells to escape from the primary tumor (3, 119). But, depending on the circumstances, necrosis could be effective enough to induce tumor clearance (120). Conversely, a particular apoptotic phenotype with the ability to trigger immune response against cancer cells has been described (119). Moreover, classic chemotherapeutic agents are shown to induce apoptosis by interfering with the normal cell division processes and this could lead to the triggering of MC (108, 109, 121). Induction of MC *vs.* direct apoptosis triggering depends of the effective drug concentration within the cells and thus, could be different among the tumor mass (122). MC drives most of

the cells to major death mechanisms but opens the gates for the appearance of new stable karyotypes translating into perhaps new resistant cancer clones (108, 123, 124).

The treatment of advanced sarcomas is based on classic chemotherapeutic agents: anthracyclines and ifosfamide as first option and, after progression, other agents like gemcitabine in combination with docetaxel (or Dacarbazine) and trabectedin. The benefit of chemotherapy is well-known, but limited, because a high percentage of patients die due to the disease in approximately 1 year from diagnosis (125, 126).

In the past years, several sarcoma-focused clinical trials have evaluated the activity in monotherapy of novel drugs with known connections to a particular cell death mechanism (**Table 1**). So far, only two phase III trials have been reported, reflecting that targeted therapies have been mostly developed in recent years and remain in a pre-clinical stage (127–137). The first trial was focused on the mTOR signaling pathway, which links apoptosis with autophagy (102, 103). The study evaluated the role of ridaforolimus as maintenance therapy after clinical benefit to chemotherapy (133). The other trial analyzed the activity of Pazopanib (a multitargeted kinase inhibitor) in pre-treated soft-tissue sarcoma patients (136).

It is easily noticeable that many of the targets mentioned above have still not reached the clinical trial stage in sarcomas. Further research should be aimed to fill that gap by a better description of the pre-clinical effects in terms of quantity and quality (type, characterization, assessment of resistant phenotypes, etc.) of the induced cell death. A summary of the ongoing clinical trials in sarcomas are included in **Table 2**.

CONCLUDING REMARKS

As often happens with research on rare diseases, sarcoma research suffers from funding shortage and delayed implementation of technical advances. But there is also an urgent need to improve current therapeutic modalities in sarcomas and reduce their burden. Additionally, due to their heterogeneity, sarcoma models are very difficult to compare among them. Those constrains define sarcoma research today. Cell death induction is the basis of cancer therapy, but we are still far from understanding the mechanisms of cell death signaling in sarcomas. The relatively low attention paid to particular phenomena like autophagy or MC, with crucial roles in therapy success, is symptomatic that we need to get back to the laboratory benches and improve our methods (3, 118, 124). We abuse too often of indirect tests, easy to read-out in flow cytometers, or high-content analyzers. And perhaps, we rely too much in bibliographic data, not looking for the actual connections between our treatments and the specific cell death trigger.

Sarcoma research needs the implementation of a better determination of cell death mechanisms. The definition of the nature of cell death is not a vain effort as the differences in mechanisms could have tremendous consequences in terms of chemo-resistance or in immunogenic potential (108, 119, 123, 124). We need to dedicate more time to define cell death circumstances, but sometimes it seems that this attention only happens when researchers are faced with unusual/specific cell death signals (death receptors, MC, necroptosis etc.) while relying in the bulk caspase-3 or AnnV-PI kits for the rest of the occasions.

The extra work we are proposing is neither difficult nor exhausting, as it requires only to spend a little time looking "what" actually happens to our cells (and "when"). Cell death is evident to the trained eye by merely observing the cells in the cell culture room's inverted microscope (**Figure 1**). Then, there are enough valuable tests, clear and easy to perform, for the major cell death pathways (138). Performed in the correct set of end-points a simple DAPI staining would serve to determine whether we are facing apoptosis, necrosis, or MC (10, 11). Therefore, we encourage researchers to perform those tests and include their results in their publications prior to embark themselves into more complex analysis about the intimacy of cell physiology. Let's concentrate on describing better "what" is happening before moving on solving "how" it is happening.

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REVIEW
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Preclinical childhood sarcoma models: drug efficacy biomarker identification and validation

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Over the past 35 years, cure rates for pediatric cancers have increased dramatically. However, it is clear that further dose intensification using cytotoxic agents or radiation therapy is not possible without enhancing morbidity and long-term effects. Consequently, novel, less genotoxic, agents are being sought to complement existing treatments. Here, we discuss preclinical human tumor xenograft models of pediatric cancers that may be used practically to identify novel agents for soft tissue and bone sarcomas, and "omics" approaches to identifying biomarkers that may identify sensitive and resistant tumors to these agents.

Keywords: human tumor xenografts, drug sensitivity, expression profiling, copy number variation, preclinical pharmacology, bioinformatics, biomarkers, drug efficacy

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Drug Development for Pediatric Cancer

Over the past 35 years, cure rates for children with hematologic and solid tumors have risen dramatically. For acute lymphoblastic leukemia the 5-year event-free survival (EFS) is 85–90%, whereas one-half to two-thirds of children with Ewing Sarcoma, rhabdomyosarcoma, or osteosarcoma (OS) are surviving disease-free for prolonged periods after aggressive treatment with surgery, radiation, and multiagent chemotherapy. For the remaining patients, it has been possible to slow progression of disease with use of intensified therapy, but cure has remained elusive. Furthermore, dose intensification/compression and introduction of new agents continues to decrease cancer mortality in children (1), although the limits of cytotoxic therapy may be close to maximal. More problematic is that these therapeutic modalities are associated with significant mortality and often long-term debilitating sequellae (2). The overriding problem is treatment failure due to the development of drug resistance. Whether this results from selection of a pre-existing clone, or through therapy-induced mutation remains to be extensively explored. A second major problem is the limited repertoire of active antineoplastic agents, targeted for childhood cancers, making it difficult to develop effective therapy for resistant tumor subtypes, even when they are identified early in the clinical course. As with recent advances in the management of adult cancers, the development of novel therapies for childhood solid tumors will require a more complete understanding of the biologic characteristics that confer the malignant phenotype that can be used to guide the integration of cytotoxic and molecularly targeted therapies most likely to confer clinical benefit.

Developing new therapies for childhood solid tumors presents certain constraints that are seldom encountered with the neoplastic diseases of adults. Childhood tumors are rare; hence, the numbers of children with a particular diagnosis restrict large-scale drug evaluation or randomized clinical trials. For example, relatively few agents receive testing in children, and from 1980 to 2003 only a single agent (teniposide) was labeled for use in children compared

to more than 50 anti-cancer agents approved for use in adult oncology; furthermore, <15% of anti-cancer drugs approved for use in adult indications have labeling for children (3). As most drug-screening strategies focus on the selection of new anti-cancer agents with specific activity against adult neoplastic diseases (e.g., colon, lung, breast, etc.), agents with specific activity against childhood malignancies might not be identified.

A further restriction on drug development is that many "common" cancers of childhood respond to drugs of established efficacy, resulting in cure of a substantial number of patients. This ethically precludes the use of "experimental" agents at diagnosis. However, over the last decade, survival rates for patients with disseminated tumors at diagnosis have improved only slightly, if at all. This lack of progress is attributed, in part, to the slow rate at which most novel anti-cancer agents enter the clinical setting and the failure to optimally integrate laboratory and clinical efforts in a manner most likely to generate new therapeutic approaches with a high probability of success.

As heavily pretreated patients are most often the population recruited for Phase II trials, failure to identify a potentially useful agent could result from assessment against multi-drug resistant tumors. Thus, as we have demonstrated, an agent that shows marginal or no activity against recurrent tumors resistant to one or more drugs may have clear efficacy in advanced but previously untreated disease (4). Model systems by which such agents, or combinations of agents, can be identified, and their use optimized, are presented in this chapter. These models offer a unique resource for the development of new therapies for pediatric cancers, and offer the potential to identify biomarkers that may at some point allow patient stratification.

Tumor Xenograft Models

Selecting Models Based Upon Gene Expression

To address some of the issues mentioned above, the NCI funded the Pediatric Preclinical Testing Program (PPTP), a consortium of groups with pediatric preclinical cancer models that could screen potential new agents and drug combinations (5, 6). Selection of suitable models for the PPTP screen involved solicitation of pediatric xenograft and cell line models from laboratories in the U.S and elsewhere. Initial screening, using cDNA array technology (7), compared 95 models with 112 patient samples representing similar histologies. Tumor models that most closely clustered with the patient samples representing the same histology were selected. A second screen (Affymetrix U133 plus 2 arrays. CEL files available at: http://gccri.uthscsa.edu/pptp) further refined the models that were included in the final screening program (8). Sixty models representing most solid tumors and acute lymphoblastic leukemia were selected for primary and secondary screens. Of these 72% are from direct patient tumor transplants into mice (patient-derived xenografts, PDX), and 48% are from tumors at diagnosis. Twenty-seven cell lines were also characterized, and demographic data for all models are available at http://gccri.uthscsa.edu/pptp.

Fidelity of DNA Copy Number Aberrations

Single nucleotide polymorphism (SNP) analysis demonstrated similar gains and losses of DNA copy number in model tumors as reported for the respective histotype (8), and revealed non-random events that also were highly correlated with tumor type (8). All models were DNA fingerprinted using short tandem repeat (STR) assays, and profiles filed as a reference for determining fidelity of lines during passage. More recently, each model has been characterized using the Agilent's SurePrint G3 Gene Expression microarray platform where four replicate tumors approximately 200–300 mm^3 per tumor line were used to create a more robust expression profile dataset. Exome sequencing has been completed for approximately 90 cell line and xenograft models. Thus, it is now possible to test the sensitivity of a particular model based upon an "actionable" mutation (9, 10).

Long Non-Coding RNAs

The Agilent Sureprint G3 Gene Expression version 1 array is able to measure 34,809 unique mRNA variables, which is far more than previous Affymetrix platforms that currently dominate the vast collection of arrays found in the Gene Expression Omnibus (GEO). A novel feature of this particular array is the measurement of long-intergenic non-coding RNAs (lincRNA). The lincRNAs provide an additional transcriptomic perspective that is valuable in understanding tumor biology (11) and may explain variation in response to drug treatment. In our analysis of pediatric solid tumors, we observed that lincRNA expression is able to discriminate cancer populations as accurately as protein coding gene expression. Such an observation is interesting and points to the relevance of lincRNA in studying malignant disease. Notwithstanding this interesting yet isolated molecular view, the real power of cancer genomic data lies in the ability to integrate different levels of molecular evidence to elucidate novel insights about cancer biology (12, 13).

Establishing an *In Vivo* Screen

Response Criteria

One of the reasons that preclinical models have generally failed to predict clinical utility of agents is the different criteria for assessing activity in the model compared to the clinic. For example, inhibition of tumor growth rate by 80% in the laboratory is regarded as biologically significant, whereas a similar effect in a patient is classified as progressive disease. For the PPTP screen, response criteria were "modeled" after clinical response criteria, and that an active agent should cause objective tumor regression. These criteria were based upon several preclinical studies that related regressions in mice to responses of agents in phase I clinical trials. Notably regression of rhabdomyosarcoma xenografts to melphalan, topotecan, irinotecan, and camptothecin combinations (14–17), as well as neuroblastoma xenografts (16, 18), correlated with activity in clinical trials (4, 19–22). Using these criteria to define activity, known clinically effective agents could be identified. Similarly, criteria for acute lymphoblastic leukemia models were developed that identify known clinically identified active agents (23). Preclinical models of medulloblastoma accurately predicted the clinical activity of topotecan (24). Models

of Wilms tumor (nephroblastoma) also identified known active drugs (cyclophosphamide, vincristine) using these criteria as did Ewing sarcoma models (cyclophosphamide, cisplatin). Validation of other models is ongoing through a series of clinical trials being conducted through the Children's Oncology Group (COG). The PPTP developed response criteria that resemble clinical response criteria, fully recognizing that both cytostatic as well as cytotoxic agents would be evaluated (6). Each tumor within a treatment group is given a score dependent on the response [progressive disease 1 (PD1)] where there is <50% growth inhibition scores 0, whereas maintained complete response (25) scores 10. The group score is the median. This allows large datasets to be reduced to a "Heat Map" format, as shown in **Figure 1A** for standard cytotoxic agents screened against sarcoma models. The heat map format allows comparison of multiple drugs and shows that the objective response rate (ORR) for "known" actives (vincristine, cyclophosphamide, cisplatin, and topotecan) is approximately 40%. **Figure 1B** shows a schematic of the median tumor response for each response classification.

Evaluation of Standard Cytotoxic Agents

All solid tumor testing to date in the PPTP used subcutaneous models, whereas for acute lymphoblastic leukemias (ALL) disseminated models were used. This review will focus only on the responses of sarcomas. One way to validate preclinical models ("model" is defined as a panel of tumors having the same pathologic diagnosis) is to ascertain whether the model identifies agents of known utility against the disease in children. Standard agents such as vincristine showed activity (i.e., induced tumor regressions ≥50%) in RMS models but no activity against EWS xenografts. Cyclophosphamide showed activity in all three tumor types, whereas cisplatin was active in some EWS and RMS models. Topotecan also demonstrated activity against EWS and RMS models, with disease stabilization in two OS models. Thus, the models identify agents with known single agent activity in these pathologies. Overall, sarcoma models showed marked sensitivity to anti-mitotic agents with an ORR of 34.7% when tested in mice at the maximum tolerated dose/schedule (MTD). Temozolomide, used in combination treatment of relapse sarcoma, showed broad-spectrum activity when tested at the MTD in mice. By contrast, a dose level in mice giving systemic exposure on the high side of that achievable in humans (66 mg/kg, **Figure 1**) showed activity only against Rh28 RMS that is deficient in MGMT required for repair of O^6G adducts (26, 27).

The testing of experimental cytotoxic drugs against the OS, EWS, and RMS panels is presented in **Figure 2** in "Heat Map" format (6). For eribulin (28) and abraxane (29), plasma exposures to these drugs in mice, at the doses tested, appear relevant for human exposure, whereas exposures to docetaxel and cabazitaxel substantially exceed those attainable in humans. As shown above, the models are responsive to anti-mitotic agents, perhaps reflecting a high proliferative fraction in xenograft models. By contrast, the tubulin-binding agent, BAL101553, showed no significant antitumor activity against sarcoma models. Hence, tumor sensitivity is not necessarily a consequence of increased proliferation.

The alkylating agent PR-104, a pre- pro-drug activated under hypoxia and by the aldoketo reductase AKR3C3 (30, 31), showed significant broad-spectrum activity when tested at the mouse maximum tolerated dose/schedule (MTD). However, at dose levels in mice that approximate human drug exposure, PR-104 was not active against solid tumor xenograft models. The non-camptothecin topoisomerase I inhibitor, GENZ644282, was active against SK-NEP-1 Ewing sarcoma, whereas topotecan was not. Other cytotoxic agents having novel mechanisms of action [aplidin, KPT-330 (selinexor, a CRM1/XPO1 inhibitor),

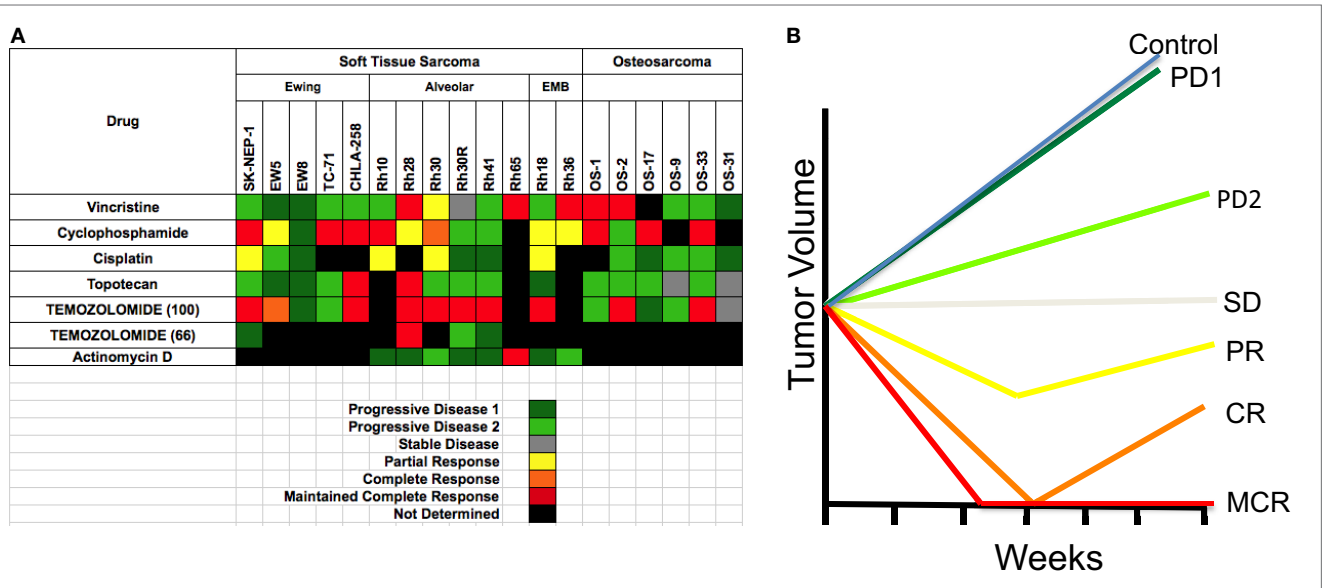

FIGURE 1 | (A) Heat map representation of the standard cytotoxic drugs screened by the PPT. Xenograft tumor models are shown at the top, grouped by histotype. Agents tested are shown in the left column. **(B)** The graph shows a representation of tumor responses, and the designation of the response.

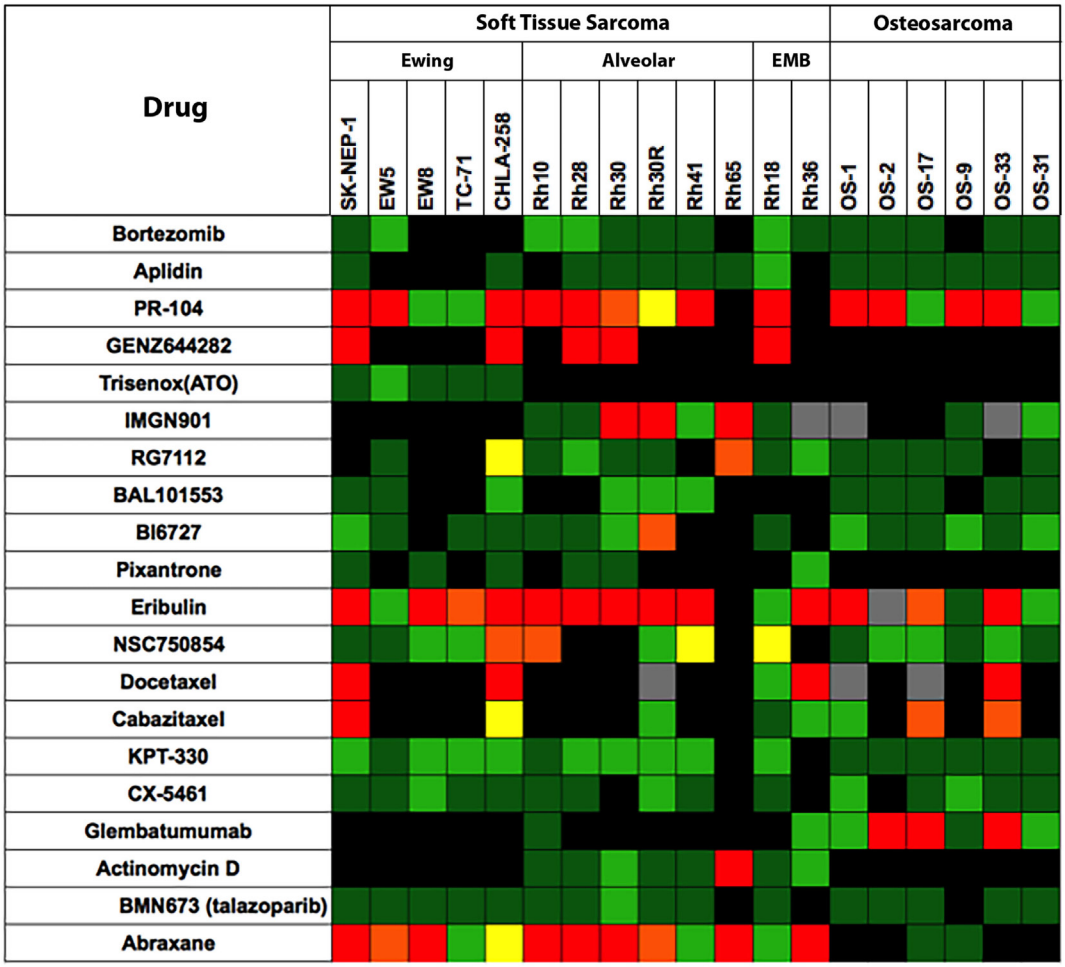

FIGURE 2 | Efficacy testing results for 20 cytotoxic agents tested against sarcoma models by the PPTP. Color codes are as for **Figure 1**.

CX-5461 (RNA pol I inhibitor)] and the PARP1 inhibitor, BMN-673, showed little or no antitumor activity against sarcoma models.

Evaluation of Signaling Inhibitors

Shown in **Figure 3** are testing results for 25 "signaling" inhibitors. These include classical inhibitors of the IGF–PI3K–TOR pathway including antibodies and drugs targeting IGF-1R (19D12, IMC-A12, BMS-754807), and small molecule drugs that selectively inhibit PI3K (XL-147), AKT (MK-2206), TOR (rapamycin, AZD8055, INK128), MEK (AZD6244) as well as multikinase inhibitors (sorafenib, SU11248, cabozantinib), and inhibitors of mitotic kinases (MLN8237, BI6727). In this dataset, there are 357 tumor/drug evaluations. The ORR was 5.6% (20/357 tests). Of these, inhibitors of mitotic kinases [PLK1 (BI6727), Aurora kinase (MLN8237)], and the kinesin inhibitor (GSK923295A) showed the greatest activity, consistent with the activity of other "non-signaling" anti-mitotic drugs (vincristine, eribulin). Excluding the responses to mitotic inhibitors in the "signaling" drug set, the ORR was a dismal 2.4% (9/291 tests).

Critical Evaluation of PPTP Models

The PPTP used exclusively xenograft models, hence these preclinical studies are useful for identifying agents that work predominantly via direct action on tumor cells. Xenograft models are, by definition, not suitable for evaluating immune-regulators, and the stromal elements are mouse. Despite these obvious limitations, these sarcoma xenografts identify each of the cytotoxic drugs known to be active, and have identified novel agents and combinations that have advanced to clinical evaluation through COG. The ORR to signaling inhibitors is disappointingly low (2.4%), which is of concern. However, there is reason to consider that these results are going to be representative of the clinical activity of signaling agents when given individually. For example, notable exceptions are the response to selumetinib (MEK inhibitor) in an astrocytoma with a $BRAF^{V600E}$ mutation (9, 32), the complete response to dasatinib in the Ph^{+} ALL-4 xenograft (33), expected

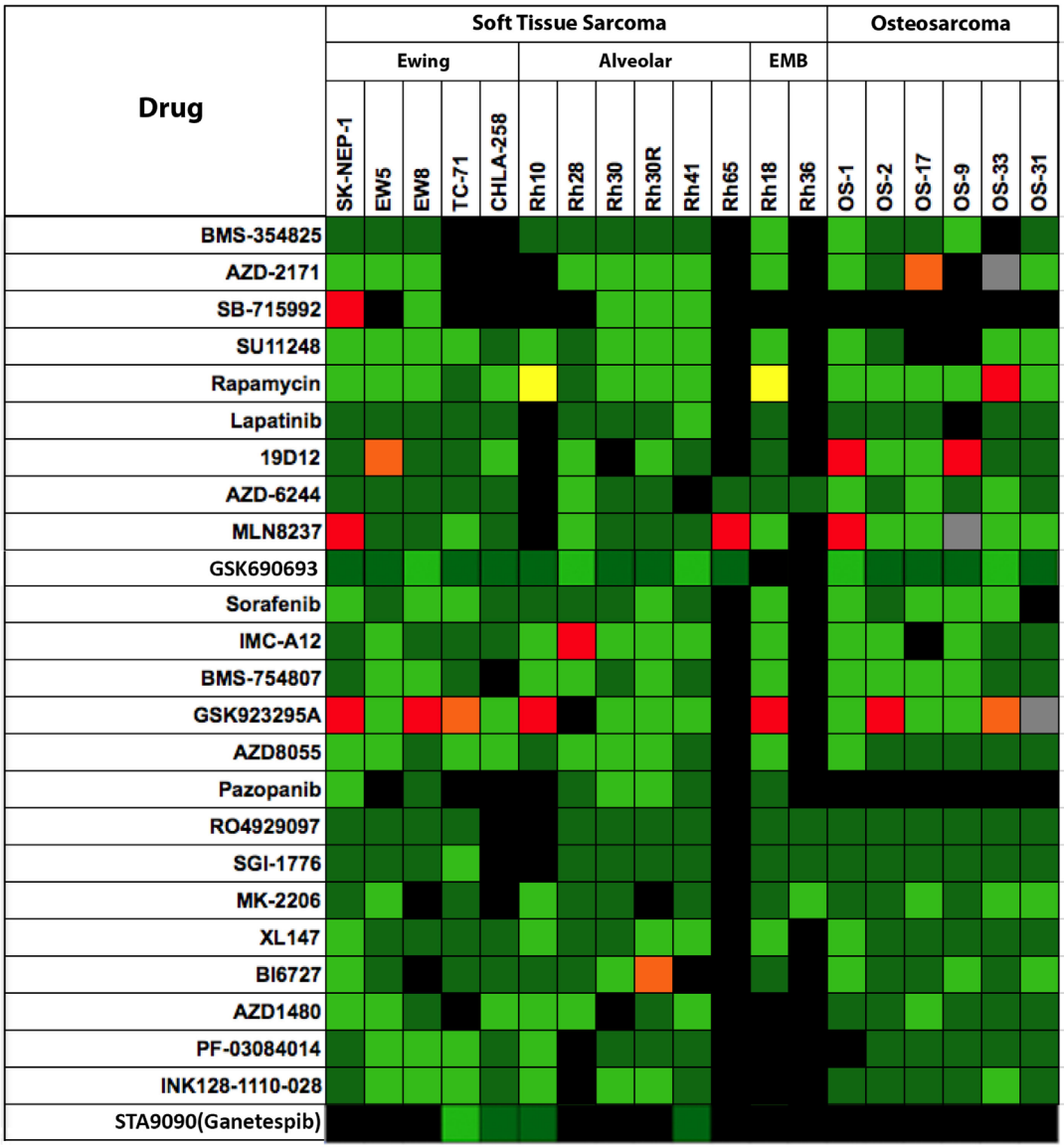

FIGURE 3 | Efficacy testing results for 25 signaling inhibitors tested against sarcoma models by the PPTP. Color codes are as for **Figure 1**.

based on the preclinical and clinical activity for dasatinib against Bcr-Abl expressing leukemias and responses in Ewing sarcoma and other sarcomas to IGF-1 receptor targeting antibodies (34, 35). Although PPTP did not test crizotinib, ALK-mutant or ALK-amplified neuroblastoma xenografts included in the PPTP neuroblastoma panel were responsive to this agent (36, 37). These results suggest that subcutaneous xenografts can indeed identify both cytotoxic drugs and signaling inhibitors that have clinical utility against the appropriate cancers in children, and hence are an appropriate primary screening tool. However, if these preclinical results are relevant to clinical responses, it is clear that developing agents of this class will yield a very low response rate, and that matching inhibitor to patient tumor characteristics will be required.

From the PPTP experience, the major factor that prevents accurate translation of preclinical data to the clinic is the difference in drug exposures in mice compared to those achieved in children (38). If differential host tolerance is normalized, then the predictive value of the preclinical data appears to be good. Obviously, there will be exceptions. For example, drug access to brain may limit the use of a drug shown to be effective against brain tumors when grown subcutaneously in mice. However, secondary orthotopic models can relatively easily identify these "false positive" results.

Another issue is the site of growth – heterotopic (subcutaneous) or orthotopic? Clearly, the subcutaneous sarcoma models identify known active agents, and accurately predict for clinical activity (melphalan, camptothecins, etc.), thus fulfill the basic function

as set out by the PPTP. Whether drug activity dramatically differs in orthotopic models requires rigorous experimentation, and use of endpoints that can be equated between subcutaneous and orthotopic models. One problem in comparing heterotopic and orthotopic models is that tumor volume at the start of therapy is often significantly smaller in orthotopic models, hence these tend to be more sensitive by virtue of size (drug access?).

Mining for Biomarkers of Drug Response

Expression Profiling

As noted by the NCI-EORTC Working Group on Cancer Diagnostics, the number of markers that have emerged as clinically useful is very small. One of the problems has been small datasets, and initial promising results have not been validated in larger trials (39, 40). Fully realizing the limitation of relatively few preclinical models (~50) and within a tumor type very few models (5–10), thus, at best, our correlations derived from expression data and response data are hypothesis generating. Gene expression profiles have been established for both cell line panels and the xenograft models, as well as SNP profiles. Thus, potentially, sensitivity *in vitro* can be correlated with either expression patterns or DNA copy number variation (CNV). Such profiles could then be tested for predictive value for response against the *in vivo* cancer models. Alternatively, expression or CNV profiles that correlate with sensitivity or resistance to an agent in the animal models may predict those patients who may benefit from this treatment. However, although data may be obtained on almost 50 models, it is best to consider, at this time, such data as hypothesis generating. For the analyses presented, we have used data from all models, and not just from soft tissue and bony sarcomas, as there are too few models for which data are available.

As was illustrated by Lander, the greatest challenge to revealing the fruit of nature by omic technology is in our ability to succinctly probe and dissect millions of read outs within the global scope of a sparse random realization (22). In general, the dimensionality digestion of genome-wide mRNA is complex in two-sample experiments and becomes even more so when considering large cohorts of diverse samples. For example, in preclinical drug evaluation, the biological diversity of samples within sensitive or resistant xenografts is likely heterogeneous and not sampled uniformly.

$$\theta(x, y) = \frac{\mu(x) - \mu(y)}{\sigma(x) + \sigma(y)} \tag{1.1}$$

$$y = \beta_0 + \sum_{i=1}^{p} x_i \beta_i + \in \tag{1.2}$$

$$E(y) = \hat{\beta}_0 + \sum_{i=1}^{p} \hat{\beta}_i x_i \tag{1.3}$$

Equation Set 1: Measures of mRNA Association

Classically, the mRNA difference between two classes is evaluated by the so-called signal-to-noise or simply stated as the difference between means relative to the variances, see equation 1.1, where x is the log distribution of class A mRNA and y is the log distribution of class B mRNA, respectively. In this scenario, two classes are statistically different if the mean separation is large relative to the variance within each class, which is typically assessed by permuting the class labels several times to estimate an empirical probability of observing the realized statistic (24) or more elegantly by bootstrapping if sample size permits. Such an approach is useful when considering treatment-condition effects or lineage differences in biological experiments. However, when considering a diverse set of tumors whose preclinical drug outcome does not necessarily follow lineage trends, there is a lack of statistical difference between classes after compensating for multiple hypothesis testing. Additionally, a class label is likely not perfect to discriminate and to guide biomarker discovery unless the drug would tailor to specific cancer disease characteristics. Furthermore, on a genome-wide scale we have found the mapping between mRNA and drug sensitivity to be problematic unless a continuous random variable is considered.

In cancer cell sensitivity modeling with microarrays, the linear relationship between basal mRNA measurements and drug sensitivity is a simplistic analytical approach to generate new hypotheses about a drug's chemical biology (41–43). From a statistical perspective, the case of linearity is argued because microarray model inputs and sensitivity outputs are typically normally distributed and those examples that do not follow a normal trend can be discarded as outliers. Whether or not our variables are specifically tied to the pharmacodynamic action is an afterthought. Rather, large-scale microarray data mining is able to identify a set of concerted changes that are associated with drug sensitivity. The dissection of the molecular pattern with regard to drug sensitivity is not possible unless additional experiments are performed; for example, RNA interference or preclinical xenograft validation. As an alternative to experimentation, the molecular pattern or "hits" discovered are queried against public databases that integrate several molecular data levels to attest whether or not the pattern is associated with, for example, survival, or a specific cancer population. Moreover, any approach in machine learning or predictive inference involves training and validation using statistically independent realizations of a given process. Cross-validation, a statistical technique to estimate prediction error, is absolutely necessary when selecting biomarkers but may still reveal poor predictors because such few samples are available or the underlying data are not representative. However, the coupling of cellular screening with preclinical xenograft studies may provide a reliable platform to identify robust biomarkers or de-prioritize the significance of cellular biomarkers. Those molecular features that are predictive in both model systems are likely indicative of sensitivity.

The dependent variable choice can vary by drug but usually involves the relative half maximal inhibitory concentration (rIC_{50}) *in vitro* or relative tumor regression *in vivo*. In order to estimate linear coefficients between rIC_{50} and mRNA, we use a high-dimensional method introduced by Zou and Hastie coined the elastic net (44). The elastic net is a regression optimization that considers all probable model fits efficiently, which performs variable or model selection in a continuous rather than

one-model-at-a-time discrete manner; those variables not influential in predicting y have linear coefficients, i.e., β's, set to zero.

$$\min_{\beta_0,\beta}\left[\frac{1}{2n}\sum_{i=1}^{n}\left(y_i-\beta_0-x_i\beta\right)+\lambda P_\alpha(\beta)\right] \quad (2.1)$$

$$P_\alpha(\beta)=\sum_{j=1}^{p}\left(\frac{(1-\alpha)}{2}\beta_j^2+\alpha\left|\beta_j\right|\right) \quad (2.2)$$

Equation Set 2: Elastic Net Regression

The objective function and criteria for guiding the process are shown in equation set 2 and is easily executed using a software implementation provided by the Matlab® Statistics Toolbox. As a custom pre-processing step, only genes with a significant univariate correlation are considered initial inputs to the elastic net algorithm. The genes identified by univariate correlation are an associated subset of all possible genomic correlates and are dictated by an arbitrary local type I error rate that vastly underestimates the realized type I error. Whether or not we incur false positives is of no concern, as these will be removed by the elastic net regression. The α parameter, shown in equation 2.2, is able to pool several correlated features and eliminate those that are not informative. The α that results in the lowest mean squared error, based on 10-fold cross-validation, is selected as the best model and hence most predictive gene network. A critical and sometimes overlooked step in predictive model building is the correct utilization of cross-validation, as illustrated well by Hastie, Tibshirani, and Friedman (45). This includes any initial gene selection steps being in the cross-validated estimate of prediction error. In our approach, we pre-process the gene list by removing any genes that are not significantly correlated and this step is included in the cross-validated error estimate for different α values. On the other hand, our global pre-processing steps that exclude any information about our target function are performed prior to any modeling, which include z-score transformation of inputs and outputs as well as removal of training samples whose output is not consistent with a normal probability curve.

Example, Anti-Mitotic Drugs for Biomarker Application in PPTP

The drugs, MLN8237 (alisertib) (46, 47) and BI6726 (volasertib) (48), are both somewhat effective anti-mitotic targeted therapies evaluated by the PPTP that inhibit Aurora kinase A (AURKA) and Polo-like kinase 1, respectively. The cellular sensitivity of these kinase inhibitors is quite striking and showed cell growth inhibition across most pediatric cell lines screened. The drugs, eribulin and vincristine, are both highly effective agents that target microtubule dynamics in general. These two drugs were shown to be very active in the PPTP xenograft panel. Vincristine is a "known" active agent being used in many "standard-of-care" protocols, whereas eribulin has just entered phase I testing in children as a cancer therapeutic. Both drugs were potent cytotoxics *in vitro* with a median rIC_{50} concentration of 0.224 and 0.2 nM, respectively. These drugs, in the examples that follow, show a range of predictability between *in vitro* and *in vivo* systems. Additionally, we are able to hypothesize global predictors of agents that target microtubule dynamics by comparing signatures (47, 49, 50).

In these examples, we are able to show whether or not *in vitro* drug sensitivity models are valid by applying receiver-operating characteristic (ROC) curve analysis to known xenograft outcomes. For these analyses, we used a binary system dividing responses into disease progression [progress disease (PD)] or progression-free disease that included objective regression and stable disease (MCR, CR, PR, SD), and model predictions. As we noted before, the *in vitro* prediction is a continuous random variable that summarizes expected rIC_{50}, *y*, given changes in mRNA, *x*. That is, a single xenograft has a composite score defined by the linear combination of mRNA features derived *in vitro*. In general, discriminatory power is defined as the trade off between sensitivity and specificity, respectively. A ROC curve measures the discriminatory power of a score when applying different score thresholds rather than measure performance at a single arbitrary cut off, i.e., positive predicted values are sensitive while negative predicted values are resistant, and is reported overall as the area under the ROC curve (AUC); for more detail, see Ref. (51).

Vincristine

To "calibrate" the PPTP tumor panels, we evaluated the standard chemotherapeutic agent, vincristine, an agent included in the backbone of most treatment regimens for solid tumors and acute lymphoblastic leukemia. Vincristine binds to tubulin dimers, the subunits of microtubules, inhibiting assembly of microtubule structures. Disruption of the microtubules prevents formation of the mitotic spindle required to segregate chromosomes and arrests mitosis in metaphase. Although the basis for selectivity for tumor vs. normal cells is not fully understood, vincristine is a component of most curative therapies used for treatment of pediatric cancers, although the proportion of patients who benefit from vincristine may be 30–50%. Thus, identifying biomarkers for response may assist in identifying patients whose tumors would be sensitive to this drug. As shown in **Figure 1**, vincristine was evaluated against five Ewing sarcomas (SK-NEP-1, EW5, EW8, TC-71, and CHLA258), six alveolar rhabdomyosarcomas (Rh10, Rh28, Rh30, Rh30R, Rh41, and Rh65), two embryonal rhabdomyosarcomas (Rh18 and Rh36), and six OSs (OS-1, OS-2, OS-9, OS-17, OS-31, and OS-33). Objective regressions were observed in four rhabdomyosarcoma models and two OS models. Additional regressions were observed in Wilms tumor, and all eight ALL models (not shown).

Limited single agent data on vincristine in OS are available from the 1960s (19, 20). Several subsequent single arm and randomized trials combining vincristine with other conventional agents failed to clearly demonstrate a role for vincristine in neoadjuvant chemotherapy. There have been few recent clinical trials of microtubule-targeted therapies in OS (reviewed in (52)). In an Italian pediatric solid tumor phase 2 study, a response to vinorelbine was observed in one of five patients with OSs (53). However, OS is not generally considered to be sensitive to anti-mitotic agents. As the proliferative fraction of xenografts is greater than that in the patient tumors, it is probable that anti-mitotic agents show as more active in the models that they are in the clinic.

The elastic net regression algorithm selected 188 mRNA variables, 35 of which were lincRNAs, based upon the log rIC_{50} of 22 PPTP cell lines. The *in vitro* linear model with these 188 mRNA inputs predicted 44 solid tumor xenograft outcomes (26 PD, 1 SD, 3 PR, 2 CR, and 12 MCR) very well with an area under the curve of 0.88. According to Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com), MAPK9, MARK2, NEFL, PVRL3, and SHC1 biomarkers are involved in microtubule dynamics. Interestingly, MARK2, a sensitive correlate, is important for microtubule stability (41) and has been shown to slow microtubule growth upon *in vitro* knockdown (54). Potentially, tumor cells that are rich with MARK2 indicate that they are more reliant on efficient microtubule dynamics to proliferate, and hence, are more reliably targeted by vincristine.

Of note, our analysis did not identify ABCB1 as a significant predictor, whereas there is an extensive literature that attests to vincristine being transported out of cells via this efflux pump. A primary caveat to our analysis approach is that we initially filter out genomic correlates at an arbitrarily chosen local type I error rate. Additionally, the linear regression approach dictates that the best predictors will be normally distributed; as this will produce the lowest mean squared error, given that a linear model is essentially predicting the expected value. In total, there were 1,604 possible genomic correlates when deriving our linear regression model. ABCB1 was not even considered because it was weakly correlated relative to other genomic correlates, and hence, did not pass our local type I error threshold. However, upon visual inspection of ABCB1 DNA copy number and mRNA across the panel of cells and xenografts tested, we see that a pattern does exist but is non-linear and ABCB1 mRNA is, in general, not normally distributed. This particular pattern is a good example of how a linear regression approach, robust as it may be, will overlook "interesting" dimensions whose activity is limited to only a subset of samples.

Vincristine is an established drug that is usually combined with actinomycin D, doxorubicin, or cyclophosphamide and has demonstrated success in pediatric cancer patients. Our signature may perhaps identify patients that have an increased likelihood of responding to vincristine treatment alone. Furthermore, the excellent validation performance and significance of discovered biomarkers prioritize this signature for additional validation and potential for clinical utilization as a companion diagnostic marker when treating with vincristine alone.

Eribulin

Eribulin is probably the most active agent evaluated in the PPTP screen, causing tumor regressions of 18 of 35 (51%) of solid tumor models and all eight acute lymphoblastic leukemia models, **Figure 4** (28). Of note, drug exposures in mice causing regressions of tumors appear similar to patient exposures reported from adult clinical trials. Eribulin is a fully synthetic macrocyclic ketone analog of halichondrin B, a natural product derived from the marine sponge Halichondria okadai (55, 56). Halichondrin B and eribulin are capable of inducing irreversible mitotic blockade and apoptosis by inhibiting microtubule dynamic instability (57). Dynamic instability applies to the growth and shortening of microtubules required for mitosis. Eribulin inhibits microtubule growth by binding with high affinity at the plus ends (58). The mechanism of inhibition of microtubule dynamic instability by eribulin is distinctive from that of other tubulin-binding antimitotic agents in that eribulin suppresses the growth parameters at microtubule plus ends without affecting microtubule shortening parameters (58, 59).

Analysis of the eribulin data with approximately equal numbers of responding and non-responding solid tumor xenograft models, thus provided an interesting test of the value of the "omics" database. The elastic net regression algorithm selected 139 mRNA variables, 36 of which were lincRNAs, based upon the log rIC_{50} of 22 PPTP cell lines. The *in vitro* linear model with these 139 mRNA inputs predicted 25 solid tumor xenograft outcomes (8 PD, 2 SD, 1 PR, 4 CR, and 10 MCR,) quite well with an area under the ROC curve of 0.7. According to IPA, ATXN2, BBS10, DLG4, EFNB2, KIF18A, NUSAP1, and PTPRM biomarkers are involved in microtubule dynamics. Interestingly, NUSAP1, a sensitive correlate, is reportedly involved in several cellular processes relevant to eribulin mechanism that covers segregation of sister chromatids, condensation of mitotic chromosomes, mitosis, bundling of microtubules, and aberration of mitotic spindle (60) as well as morphology of mitotic spindle (61). KIF18A, another sensitive correlate, is also quite interesting. Kinesin family member 18A is reportedly involved in alignment and congression of chromosomes (62) as well as de-polymerization of microtubules (63). Another noteworthy biomarker is ABCB1, a protein that encodes a drug transporter MDR1b (also known as P-glycoprotein). ABCB1 transports a variety of hydrophobic drugs, including eribulin (64). Furthermore, the decent validation performance and significant relevance of discovered biomarkers prioritizes this signature for additional validation and potential clinical utilization as a companion diagnostic marker in the treatment of pediatric cancer patients.

Alisertib (MLN8237): An Inhibitor of Aurora Kinase A (AURKA)

The Aurora serine/threonine protein kinases are a family of three kinases (Aurora A–C) with different tissue and temporal expression profiles. These enzymes play key roles in mitosis and meiosis, defects in which can lead to abnormal mitotic events and induction of programed cell death (apoptosis) (65). AURKA is essential, as is highlighted by the fact that genetically engineered null mice are embryonic lethal (dying at the blastocyst stage) (66). AURKA activity is also required for centrosome duplication and separation, microtubule-kinetochore attachment, spindle checkpoint, cytokinesis (67, 68), the G2/M transition (69), and phosphorylation of Polo-like kinase 1 (70). Furthermore, AURKA has been implicated as an oncogenic driver in human cancers (71). AURKA has been found to be over-expressed in cancer cells and the AURKA gene locus is amplified in selected adult tumors (72). When tested by the PPTP at the maximum tolerated dose/schedule (MTD), alisertib exhibited good activity, notably against neuroblastoma and ALL models (46), **Figure 5**.

Analysis of this dataset using the elastic net regression algorithm selected 69 mRNA variables, 24 of which were lincRNAs, based upon the log rIC_{50} of 22 PPTP cell lines. Despite a strong training validation, the *in vitro* linear model with these 69 mRNA inputs

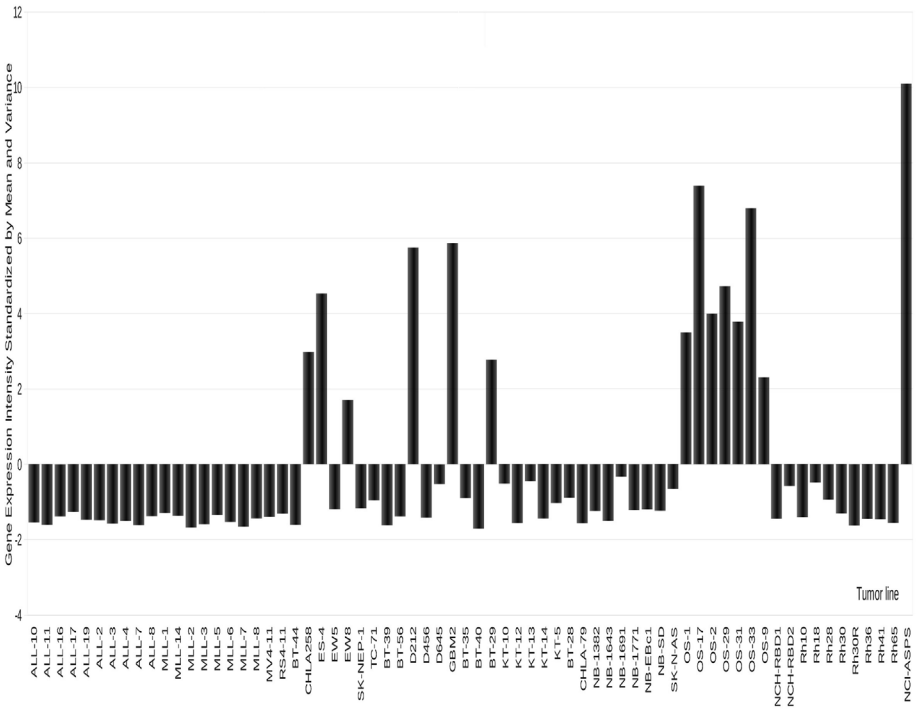

FIGURE 4 | Expression of GPMNB in PPTP xenografts. High-level expression is detected in osteosarcoma, and some Ewing sarcoma models as well as in two glioblastoma xenografts.

predicted 39 xenograft outcomes (20 PD, 4 SD, 1 PR, 4 CR, and 10 MCR) poorly with an area under the curve of 0.48 or practically random discrimination. According to IPA, there were no biomarkers that had a documented interaction with the drugs target, AURKA. Furthermore, the poor validation performance and insignificance of discovered biomarkers with respect to the molecular target de-prioritizes any additional validation or clinical utilization of this signature. In this example and given the data at-hand, the spectrum of cellular sensitivity observed is not translatable to preclinical xenograft models with respect to messenger-RNA.

AURKA Copy Number

In contrast to expression profiling, gene copy number analysis for AURKA appears to support an inverse relationship between AURKA expression and sensitivity. Increased copy number was present 14 of the solid tumors. Loss of copy number was detected in seven solid tumors and one leukemia model. Furthermore, the correlation between gene expression variation and CNV was strong, placing this locus in the top 1.6% of all genes tested. While there is no absolute relationship between CNV and tumor sensitivity, of the 14 solid tumors with increased copy number, there were only two that showed sensitivity to alisertib. By contrast, five of the eight models demonstrating decreased copy number were sensitive models to alisertib (46). It is of note that at drug exposures achieved in patients, only the most sensitive preclinical models (ALL) are likely to respond to treatment. However, several rhabdoid tumor models were relatively sensitive to alisertib, and responses were observed in several patients with CNS rhabdoid tumors (73).

Volasertib (BI6727): An Inhibitor of Polo-Like Kinase 1 (PLK1)

In vitro volasertib demonstrated cytotoxic activity (median rIC_{50} value of 14.1 nM, range 6.0–135 nM), and at the MTD-induced significant differences in EFS in 19 of 32 (59%) of the evaluable solid tumor xenografts and in two of four of the evaluable ALL xenografts. Objective responses (CR's) were observed for 4 of 32 solid tumors (two neuroblastoma, one glioblastoma, and one rhabdomyosarcoma) and one of four ALL xenografts (48). Volasertib is a dihydropteridinone (Bl 6727) that targets the Polo-like kinase (Plk) family of proteins in an ATP-competitive manner at low nanomolar concentrations and thereby induces mitotic arrest and apoptosis (74). Plk1 is a serine/threonine-specific kinase that regulates multiple steps in mitosis and that is essential for progression through mitosis (75). Numerous lines of evidence suggest that Plk1 is oncogenic through driving cell cycle progression, and overexpression of the gene transforms NIH 3T3 cells (76). Plk1 is highly expressed in multiple cancers (75, 77, 78), and in some malignancies expression of Plk1 may be prognostic (77). Plk1 is over-expressed in several childhood cancers and cell lines. RNA interference and small molecule inhibitor screens suggest that Plk1 may be a relevant therapeutic target in a variety of pediatric malignancies including neuroblastoma, rhabdomyosarcoma, and OS (79–81).

From the *in vitro* sensitivity dataset, the elastic net regression algorithm selected 121 mRNA variables, 17 of which were lincRNAs, based upon the log rIC_{50} of 22 PPTP cell lines. The *in vitro* linear model with these 121 mRNA inputs predicted 36 xenograft

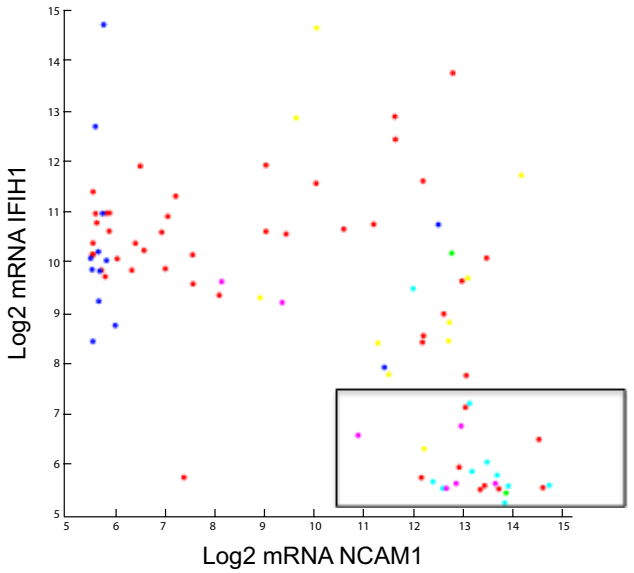

FIGURE 5 | Log2 Agilent mRNA pattern between NCAM1 and IFIH1. Seneca Valley Virus (NTX-010) sensitivity as defined by PPTP is overlaid. The boxed area shows 24 of 26 cell lines and xenografts that were sensitive to NTX-010.

outcomes (28 PD, 1 SD, 1 PR, and 6 CR) quite well with an area under the curve of 0.79. According to IPA, PKMYT1, DNHD1, KAT7, DDX39B, RASGRF1, and MAD2L1 biomarkers reportedly have interactions with the drug target, PLK1. Specifically, KAT7 (82), DNHD1 (83), DDX39B (84), and RASGRF1 (85) are known to have protein–protein interactions with PLK1 while mutant PLK1 (51–356 AA deletion) increases MAD2L1 protein localization to kinetochores from misguided chromosomes of metaphase cells (86) and PLK1 protein increases inhibition of active PKMYT1 (87) as well as increase phosphorylation of a PKMYT1 protein fragment (88). MAD2L1 and PKMYT1, both negatively correlated with rIC_{50}, may point to PLK1 targets over-expressed when PLK1 is mutated. Interestingly, PKMYT1 is a protein kinase that plays an important role in mitosis by decreasing activation of CDK1 (89, 90) while increasing phosphorylation of CDK1 (89–91). The elevated PKMYT1 mRNA in sensitive cells is possibly indicating a cellular compensation for over active mitotic phase of the cell cycle due to mutated PLK1, and hence, these cell populations are ideal targets for PLK1 inhibition by BI6727. Furthermore, the good validation performance and significant relevance of discovered biomarkers prioritize this signature for additional validation. Recently, PLK1 was reported to phosphorylate PAX3-FOXO1 in alveolar rhabdomyosarcoma, and inhibition triggered tumor regressions (92).

Glembatumumab Vedotin

Glembatumumab vedotin is an antibody-drug conjugate (ADC) that combines an anti-GPNMB antibody with the anti-mitotic agent monomethyl auristatin E (vedotin) (93). When internalized, vedotin is released and results in cell cycle arrest and cell death (94). Glembatumumab vedotin showed *in vitro* cytotoxicity that was related to GPNMB expression, and it induced complete regressions in GPNMB-expressing melanoma and breast cancer xenografts (93, 95, 96).

The transmembrane glycoprotein NMB (GPNMB or osteoactivin), is primarily expressed in intracellular compartments (e.g., lysosomes and melanosomes) in non-malignant cell such as melanocytes, osteoclasts, and osteoblasts (97–99). GPNMB is also expressed on monocytes and dendritic cells, and its expression on the latter has been proposed to play a role in the inhibition of T-cell activation by antigen-presenting cells (APC) (100–102). Membrane GPNMP is over-expressed in hepatocellular carcinoma (103), breast cancer (95, 104), glioblastoma (105), and melanoma (93, 98), making it a reasonable candidate for targeted therapeutics. As shown in **Figure 4**, GPNMB is expressed highly in several OS xenografts [and also in one alveolar soft part sarcoma (ASPS) examined]. In a limited screen using models with high-level expression glembatumumab vedotin demonstrated intermediate to high activity in five of six OS xenografts, with a maintained complete response in three of the lines (52). In each of the lines that demonstrated a maintained complete response to glembatumumab vedotin (OS-2, OS-17, and OS-33), there is 2^+ to 3^+ staining for GPNMB by immunohistochemistry, although the percentage of cells positive is as low as 5% of tumor cells for one line. These observations support the position that while GPNMB expression may be necessary for tumor regression to glembatumumab vedotin treatment, it is not sufficient for response to this agent (52). The value of the expression data is further emphasized by searching publically available databases. For example, the single ASPS xenograft model expressed very high levels of GPNMB. Reference to limited patient data available, confirms high-level expression in all samples, suggesting that GPNMB-directed therapy may be valuable. However, it is recognized that ASPS is a slow-growing indolent tumor (as is the xenograft), hence whether an anti-mitotic "warhead" on glembatumumab would be effective would have to be explored in preclinical models.

Seneca Valley Virus (NTX-010)

One of the agents evaluated through the PPTP was the replication competent picornavirus, Seneca Valley Virus (NTX-010) (106). NTX-010 is a newly discovered, naturally occurring picornavirus being developed as an oncolytic virus for human cancers. In a cell line screen of NTX-010, approximately half of cancer cells with one or more neuroendocrine properties were permissive and allowed selective infection (107). Notably, the most sensitive cell line, IMR-32, was derived from a childhood neuroblastoma. By contrast, only 3 of 80 non-endocrine cells were permissive to virus replication. The majority of non-permissive cancer cell lines do not bind and/or internalize NTX-010, suggesting that binding and entry through a productive internalization pathway is the primary determinant of viral tropism for neuroendocrine tumor cells. Neuroblastoma, Ewing sarcoma, as well as medulloblastoma and alveolar rhabdomyosarcoma demonstrate neuroendocrine markers. *In vitro* NTX-010 demonstrated a marked cytotoxic effect in a subset of the cell lines from the neuroblastoma, Ewing sarcoma, and rhabdomyosarcoma panels. *In vivo* the most consistent activity was observed for the rhabdomyosarcoma and the neuroblastoma panels, with all four of the alveolar

rhabdomyosarcoma xenografts and four of five neuroblastoma xenografts achieving CR or maintained CR (106).

An overlooked aspect of our analytical approach is normality of rIC_{50}. As mentioned previously, linear correlation and regression methods require that the response variable, rIC_{50}, be normally distributed. NTX-010 is the only agent considered herein that exhibits a non-normal rIC_{50} profile. On a natural scale, the rIC_{50} profile appears to be discrete while on a logarithmic scale we observe normality for sensitive lines, i.e., any cell growth inhibition within dose range, whereas resistant lines, i.e., no inhibition at maximum dose, are saturated at the highest dose tested. Furthermore, measures of linear correlation in this context are likely highlighting differential sensitivity within sensitive population but are likely informative nonetheless.

The elastic net regression algorithm selected only 29 mRNA variables, two being lincRNAs, based upon the log rIC_{50} of 22 PPTP cell lines. The *in vitro* linear model did well when discriminating 22 xenograft outcomes (10 PD, 2 PR, 4 CR, and 6 MCR) given an area under the curve of 0.71. A notable mRNA feature is IFIH1 or interferon induced with helicase domain 1. IFIH1 is a picornavirus surveillance protein in innate antiviral response (108, 109). We speculate that a low level of IFIH1 is a marker of permissive replication in tumor cells. Taken together, high-level expression of CD56 (NCAM1) and low expression of IFIH1 accurately identifies 24 of 26 cell lines and xenografts as being sensitive to NTX-010 (106), as shown in the boxed area of **Figure 5**.

We further interrogated both *in vitro* and *in vivo* data to determine if other IFIH1-like factors are associated with sensitivity. A genome-wide unpaired *t*-test assuming that unequal population variances was computed between responders and non-responders where responders were sensitive cells or xenografts with maintained complete response (25) and non-responders were resistant cells or xenograft with progressive disease 1 (PD1); multiple hypothesis testing was corrected by Storey *q*-value (110) and all computational analyses were performed with Matlab Bioinformatics and Statistics toolboxes. As biologists and also from a practical statistical perspective, we search to see if discovered gene changes are enriched in a meaningful biological category. The hypergeometric probability distribution is appropriate to calculate the chance of observing category overlap at random and is utilized in, for example, the Broad Institute Molecular Signature Database (111). An insightful method to then prioritize categories is to integrate domain knowledge by scoring sets according to gene change consistency with literature findings and is heavily utilized in, for example, IPA.

Overall, we detected 692 Agilent mRNA variables when controlling a false discovery rate of 5%, i.e., Storey *q*-value <0.05. From IPA, we were able to infer by right-tailed Fisher's exact test that discovered differential mRNA is predictive of several interesting functional categories related to virus attenuation as well as detecting highly elevated NCAM1, a receptor already speculated to be involved in NTX-010 cell entry (106). Notable categories of decreased activity in responders are infiltration by APC, antiviral response of cells, natural killer (NK) cell homeostasis, and activation of NK cells while a notable category of increased activity in responders is viral replication (vesicular stomatitis virus, replication of RNA virus, Murine herpesvirus 4).

The landscape of gene–gene correlations genome-wide that exists naturally either due to evolutionary redundancy or other factors is problematic when searching for mRNA correlates that are global and not confined to whatever cell lines happen to be in the training set. Interestingly, a NTX-010 lincRNA correlate (chr1:213453777–213480277; hg19) was upstream of RPS6KC1 and a gene–gene mRNA correlation was significant between these two. This observation points to the inherit difficulty of modeling basal mRNA and drug response. In this particular example, we can infer from genomic proximity that this non-coding mRNA feature is likely acting as a promoter of RPS6KC1. RPS6KC1, a candidate oncogene in endometrial cancer (112), is a meaningful drug–gene correlation given observations that NTX-010 tends to show response in neuroendocrine tumors (113). By establishing this "link" we were drawn to a significant drug–gene correlation that was de-prioritized by the elastic net regression algorithm. However, for the vast majority of proteins that are modified epigenetically or in distant trans interactions, such direct hypotheses are not easily formulated.

Bioinformatics Tool Development and Availability

As new cancer genomic datasets come online, there is a need to rapidly develop tools, portal interfaces, and standards of analysis that robustly turn multiple sourced molecular data into an insightful axis of molecular relationships. The basic cancer dataset is a matrix of samples and genes with entries corresponding to a molecular readout such as gene expression or DNA copy number. A standard set of statistical methods adopted in the bioinformatics community for analyzing such a matrix are hierarchical cluster analysis (114), gene set enrichment analysis (115), sample randomization statistics (114–116), regression analysis (41, 42, 44), and dimensionality reduction methods (117–119). Additionally, most software tools for analyzing cancer genomic data (120–123) are made publicly available at no cost to non-profits with the caveat that there is no free lunch; prospective users typically agree to terms of conditions that include limited liability on the part of the tool creator.

Critical Evaluation of Bioinformatics Analysis of PPTP Data

The obvious limitation of the bioinformatics analyses presented here is the relatively small sample size used to identify correlates. We have derived sensitivity data and, based upon expression profile differences between cell lines, have attempted to predict sensitivity to drugs of xenograft models. *In vitro*, cell lines from different tumor types (including leukemias) have been used, thus potentially biasing analyses to profiles exhibited by leukemia cells that tend to be more sensitive to many of the agents tested. To make correlations between *in vitro* sensitivity and *in vivo* models, we have used only the solid and brain tumors, and have excluded the leukemia models, as these have very different expression profiles (8). Thus, it is likely that analyses may be biased when there is a preponderance of one type of tumor in the sensitive or resistant cohort. Additional weaknesses

include a failure to integrate exome mutation analysis, and changes in expression profiles subsequent to drug treatment (i.e., dynamic profiling). Despite these obvious weaknesses, the analyses do focus on specific genes/pathways that can be tested prospectively.

Future Directions

Within the PPTP consortium, approximately 150 patient-derived xenograft models have been established. Most have been characterized by expression profiling and exome sequencing, hence a valuable omics database has been created against which new agents can be profiled. However, it is clear that to accurately represent molecular subtypes of different cancers additional models need to be established. Several novel agents identified in the PPTP screen are in phase I/II testing for treatment of childhood cancer. For sarcomas, the models identify some anti-mitotic agents as being highly active. Whether this reflects an increased rate of proliferation in models compared to patient tumor, or is revealing the Achilles Heel of these cancers, is open to debate. The activity of signaling inhibitors against the xenograft models has been somewhat disappointing, but this may reflect the lack of activity in human cancers overall. Certainly, in models with "actionable" mutations, specific inhibitors show impressive activity. However, it is clear that development of this type of targeted therapeutic must differ from the paradigm used for developing cytotoxic agents.

As was mentioned previously, the real power of cancer genomics data lies in the ability to integrate multiple molecular data sources. Open web portals that provide access to publicly available multi-source cancer genomic data, largely from the Tumor Cancer Genome Atlas (TCGA), are advancing our understanding of cancer genomes (124) and their susceptibility to anti-cancer agents. Literally within a click or two an investigator can begin to hypothesize how their gene of interest or empirical pathway is active in specific cancer patient populations or associated with cancer cell drug sensitivity or resistance. Here, we have discussed the value and limitations of deriving relationships between *in vitro* cell line sensitivity and *in vivo* responsiveness to several agents. Potentially, identification of synergistic combinations *in vitro* can be tested in xenograft models to develop rational combination therapies. The examples were chosen to illustrate the value and limitations of this approach. Further refinement and validation of such "signatures" are required, possibly using a further test set of xenografts, or through modulation of genes by RNA interference approaches. Ultimately, it will be important to determine whether such approaches are relevant to patient responses to single agents or to complex therapeutic regimens.

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Distinct malignant behaviors of mouse myogenic tumors induced by different oncogenetic lesions

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Rhabdomyosarcomas (RMS) are heterogeneous cancers with myogenic differentiation features. The cytogenetic and mutational aberrations in RMS are diverse. This study examined differences in the malignant behavior of two genetically distinct and disease-relevant mouse myogenic tumor models. *Kras; p1619^{null}* myogenic tumors, initiated by expression of oncogenic *Kras* in *p16p19^{null}* mouse satellite cells, were metastatic to the lungs of the majority of tumor-bearing animals and repopulated tumors in seven of nine secondary recipients. In contrast, *SmoM2* tumors, initiated by ubiquitous expression of a mutant Smoothened allele, did not metastasize and repopulated tumors in 2 of 18 recipients only. In summary, genetically distinct myogenic tumors in mice exhibit marked differences in malignant behavior.

Keywords: rhabdomyosarcoma, myogenic differentiation, metastasis, transplantation

INTRODUCTION

Rhabdomyosarcomas (RMS) are heterogeneous cancers with myogenic differentiation (1). Fusion-positive RMS tumors carry exclusive chromosomal translocations at t(2;13)(q35;q14) or t(1;13)(p36;q14) and exhibit aggressive clinical behavior (2, 3). The remaining, fusion-negative spectrum of human RMS comprises a diverse group of tumors with frequent RAS pathway activation (4, 5) and variable mutations, including loss of heterozygosity at the *PTCH1* locus (6, 7) in a subset of fusion-negative RMS. PTCH1 serves as a Hedgehog (Hh) receptor, and loss of PTCH1 function results in de-repression of downstream Hh pathway signaling. The contributions of RMS-relevant oncogenic pathways, including RAS and Hh signaling, to myogenic tumor formation were previously tested in mice (8, 9). This report highlights the distinct phenotypes of two mouse myogenic tumor models – those initiated by combined *Cdkn2a* (*p16p19*) disruption and *Kras* expression in transplanted mouse muscle satellite cells (10) and those arising in the skeletal muscle of mice with activated Hh signaling due to expression of a mutant, constitutively active smoothened *(SmoM2)* allele (11, 12). We demonstrate significant differences in tumor-repopulating activity and prevalence of lung metastases between *Kras*-driven and Hh-driven myogenic tumors in mice. These observations reveal marked differences in malignant behavior between genetically distinct mouse myogenic tumors, suggesting that an understanding of the distinct oncogenetic underpinnings of tumors on the fusion-negative RMS spectrum may be informative for clinical prognosis and treatment.

MATERIALS AND METHODS

MICE

R26-SmoM2 (mixed genetic background including 129/Sv and Swiss Webster as main components) (11), CAGGS-CreER (11), and NOD.CB17-Prkdc^{scid}/J (NOD.SCID) mice were purchased from The Jackson Laboratory. *p16p19^{null}* mice (B6.129 background) were obtained from the NIH/Mouse Models of Human Cancer Consortium. Mice were bred and maintained at the Joslin Diabetes Center Animal Facility. All animal experiments were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee.

SARCOMA INDUCTION

Kras; p16p19^{null} myogenic tumors were initiated by fluorescence-activated cell sorting of *p16p19^{null}* satellite cells, followed by lentiviral transduction to introduce oncogenic *Kras(G12v)* and implantation in the gastrocnemius muscles of NOD.SCID mice as previously described (10). *R26-SmoM2*;*CAGGS-CreER* were injected with Tamoxifen (1 mg/40 g) on postnatal day 10 to activate expression of CRE recombinase and SMOM2. *R26-SmoM2*;*CAGGS-CreER* spontaneously developed multifocal skeletal muscle tumors (*SmoM2* tumors) as previously described (11, 12).

HISTOPATHOLOGY

Tumor tissue was dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin. Standard H&E stained sections were prepared. Staining for Actin (Dako, M0635, 1:200), Desmin (Dako, M0760, 1:50), and Ki67 Ki67 (Vector Labs, VP-K451, 1:250) was performed as previously described (10).

LUNG METASTASES

Tumor-bearing mice were monitored at least twice weekly for health problems, and were sacrificed once tumors reached a volume of 1 cm^3 or were ill. Lungs were dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin. Standard

H&E stained sections were prepared and evaluated for the presence of metastases by Roderick T. Bronson.

TUMOR TRANSPLANTATION

Tumors were harvested, digested in DMEM + 0.2% collagenase type II (Invitrogen) + 0.05% dispase (Invitrogen) for 90 min at 37°C in a shaking waterbath, triturated to disrupt the remaining tumor pieces, and filtered through a 70 mm cell strainer. Red blood cells were lysed from tumor cell preparations by 3 min incubation in 0.15 M ammonium chloride, 0.01 M potassium bicarbonate solution on ice. Defined numbers of tumor cells were resuspended in 10–15 ml of HBSS with 2% FBS and injected into the gastrocnemius muscles of 1- to 3-month-old, anesthetized NOD.SCID mice using a transdermally inserted dental needle attached to a Hamilton syringe via polyethylene tubing. Recipient muscles were preinjured 24 h before cell implantation by injection of 25 ml of a 0.03 mg/ml solution of cardiotoxin (from *Naja mossambica*, Sigma) in order to enhance cell engraftment. Mice were screened once weekly for the development of tumors at the injection sites.

STATISTICS

Differences between *Kras; p16p19^{null}* and *SmoM2* mouse myogenic tumors were evaluated by *T*-test (Ki67 indices), Fisher's Exact test (prevalence of lung metastases), and Kaplan–Meier analysis (tumor-repopulating activity).

RESULTS

Kras; p16p19^{null} AND *SmoM2* MOUSE TUMORS EXHIBIT A MYOGENIC TUMOR PHENOTYPE

Kras; p16p19^{null} mouse myogenic tumors were induced by intramuscular implantation of *Kras(G12v)*-expressing *p16p19^{null}* muscle satellite cells (10). In contrast, SmoM2 mouse myogenic tumors were initiated by ubiquitous activation of a mutant, constitutively active smoothened *(SmoM2)* allele in *R26-SmoM2*;*CAGGS-CreER* mice (11, 12). The phenotypes of *Kras; p16p19^{null}* and *SmoM2* myogenic tumors were previously described (10–12). In brief, *Kras; p16p19^{null}* tumors contained bundles of cells with large, atypical nuclei, frequent mitotic figures, and occasional multinucleated giant cells. Subsets of cells (<50% of all tumor cells) expressed terminal muscle differentiation markers such as desmin and actin (**Figure 1A**), and the proliferative index as evidenced by the percentage of Ki67-expressing nuclei was 41.6 ± 12.5% (range 30.5–59.3%; four tumors evaluated) (**Table 1**). *SmoM2* tumors contained many multinucleated, elongated cells with abundant cytoplasm interspersed with small round cells. *SmoM2* tumors lacked cellular atypia and diffusely expressed desmin and actin in many tumor cells (more than 75% of all tumor cells; **Figure 1B**). As previously reported (12), the Ki67 index of *SmoM2* tumors was 19.1 ± 15.9% (range 3.4–41.8%; six tumors evaluated) and lower than that observed in *Kras; p16p19^{null}* tumors ($p = 0.05$; **Table 1**).

Kras; p16p19^{null} AND *SmoM2* MOUSE MYOGENIC TUMORS HAVE DIFFERENT METASTATIC POTENTIAL

The lung is the primary organ affected by distant sarcoma metastases in humans. To assess the metastatic potential of *Kras; p16p19^{null}* and *SmoM2* tumors, random lung sections obtained from tumor-bearing animals were screened for the presence of metastases. Six of seven mice with *Kras; p16p19^{null}* myogenic tumors were found to have lung metastases at the time of death (mice were sacrificed 17–28 days after detection of palpable tumors) (**Figure 2**). In contrast, 0 of 8 mice with SmoM2 myogenic tumors had lung metastases at the time of death (mice were sacrificed at 38–55 days of age and 5–21 days after detection of palpable tumors). The prevalence of lung metastases in *Kras; p16p19^{null}* and SmoM2 myogenic tumor-bearing mice was significantly different ($p = 0.001$).

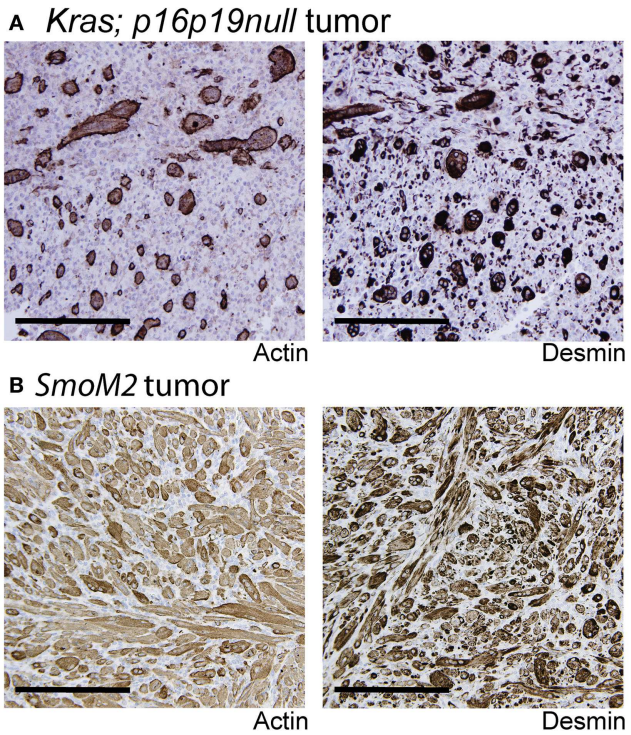

FIGURE 1 | Terminal myogenic differentiation in *Kras; p16p19^{null}* and *SmoM2* mouse tumors. (A) Subsets of *Kras; p16p19^{null}* tumors cells express terminal muscle differentiation markers, actin and desmin. **(B)** The majority of SmoM2 tumor cells express actin and desmin. Images were taken at 20× (scale bars indicate 100 μm).

Kras; p16p19^{null} AND *SmoM2* MOUSE MYOGENIC TUMORS DIFFER IN TUMOR-REPOPULATING ACTIVITY

Most malignant tumors contain cells that have the capacity to repopulate secondary tumors when transplanted into a susceptible secondary environment, and this assay has been used as a test of the malignancy of distinct tumors and tumor cell subsets (13). To evaluate the tumor-repopulating activity of *Kras; p16p19^{null}* and *SmoM2* mouse myogenic tumors, viable tumor cells were transplanted into the cardiotoxin-pre-injured gastrocnemius muscles of NOD.SCID mice. The *Kras; p16p19^{null}* tumor cell pool contains approximately 70% GFP+ cells and 30% GFP− cells (10). Because tumor-repopulating activity in *Kras; p16p19^{null}* tumors resides within the Kras-expressing, GFP+ subset of tumor cells descended from virally infected satellite cells (Figure S1 in Supplementary

Table 1 | Differences in the malignant behavior of *Kras; p16p19^{null}* and *SmoM2* mouse tumors.

	***Kras; p16p19^{null}* tumors**	***SmoM2* tumors**
Terminal muscle differentiation	Actin/desmin expression in <50% of tumor cells	Actin/desmin expression in >75% of tumor cells
Ki67 index ($p = 0.05$)	$41.6 \pm 12.5\%$	$19.1 \pm 15.9\%$
Metastases ($p = 0.001$)	7 of 9 mice with lung metastases	0 of 10 mice with lung metastases
Transplantation ($p < 0.001$)	7 of 9 transplanted mice developed tumors (50 cells injected)	2 of 10 transplanted mice developed secondary tumors (100–150 k cells injected)

Kras; p16p19^{null} and SmoM2 mouse myogenic tumors exhibit profound differences in tumor-repopulating activity and metastatic behavior.

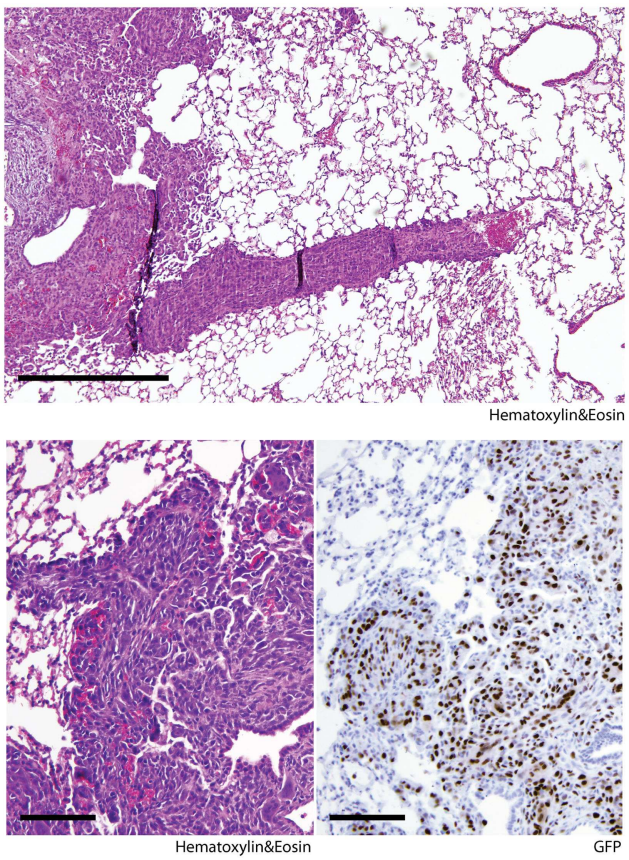

FIGURE 2 | *Kras; p16p19^{null}* mouse myogenic tumors metastasize to the lungs of tumor-bearing animals. Random lung sections from *Kras; p16p19^{null}* tumor-bearing mice show metastases. Tumor cells invade lung capillaries (top panel). Similar to primary tumors arising from GFP+ *Kras*-expressing; *p16p19^{null}* satellite cells, lung metastases are GFP+ (bottom right panel). Images were taken at 10× and 20× (scale bars indicate 100 μm)

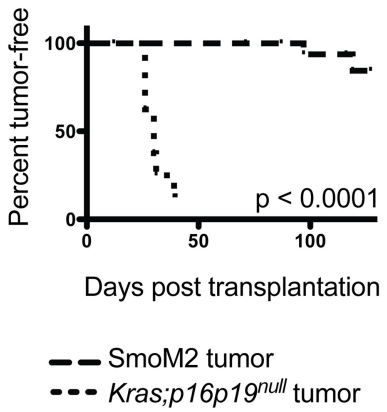

FIGURE 3 | *Kras; p16p19^{null}* tumor cells repopulate tumors in secondary recipients more effectively than *SmoM2* mouse tumor cells. Pi^{-}Ca^{+}GFP^{+} *Kras; p16p19^{null}* tumor cells were sorted independently from two primary tumors and injected into the cardiotoxin-pre-injured gastrocnemius muscles of NOD.SCID mice (50 cells per injection). Pi-Ca+ *SmoM2* tumor cells were sorted independently from four primary tumors and injected into the cardiotoxin-pre-injured gastrocnemius muscles of NOD.SCID mice (100,000–150,000 cells per injection). Recipient mice were monitored for the occurrence of secondary tumors at the injection site for up to 4 months.

Material), *Kras; p16p19^{null}* tumor cells were sorted for transplantation from two *Kras; p16p19^{null}* primary tumors as GFP+, Pi−, Calcein+ cells. Seven of nine mice injected with only 50 GFP+, Pi−, Calcein+ *Kras; p16p19^{null}* tumor cells developed secondary tumors at the injection site 26–39 days after tumor cell injection. For *SmoM2* tumors, viable tumor cells were sorted as PI− Calcein+ cells from primary tumors obtained from four mice. Surprisingly, despite significantly higher numbers of cells transplanted (100,000 to 150,000 PI−, Calcein+ SmoM2 tumor cells per recipient), only 2 of 18 recipient mice developed secondary tumors, which were detected 71 and 127 days after cell injection. These experiments indicate marked differences in tumor-repopulating activity of *Kras; p16p19^{null}* and *SmoM2* tumors ($p < 0.001$, **Figure 3**), in terms of both the frequency of tumor-repopulating cells and the latency of secondary tumor formation.

DISCUSSION

Our findings highlight differences in the malignant phenotype and behavior of mouse myogenic tumors driven by activation of distinct RMS-relevant oncogenic pathways. *Kras; p1619^{null}* myogenic tumors were metastatic to the lungs of the majority of tumor-bearing animals and contained high tumor-repopulating activity. In contrast, *SmoM2* tumors did not metastasize and were substantially less effective in repopulating tumors in secondary recipients. These observations indicate that genetically distinct myogenic tumors in mice display marked differences in their malignant behavior.

The two model systems described in this study were induced by different experimental methods. *SmoM2* tumors originated from Cre-mediated activation of a conditionally expressed transgene. *Kras; p16p19^{null}* mouse tumors, on the other hand, were initiated by viral transduction and intramuscular implantation of target satellite cells. We note that *Kras; Tp53$^{-/-}$* mouse myogenic tumors (14, 15), induced by Cre-mediated activation of oncogenic hits instead of viral transduction, exhibit a phenotype that closely resembles the *Kras; p16p19^{null}* mouse tumors described here. For example, *Kras; p16p19^{null}* share their propensity to metastasize to the lungs of tumor-bearing animals with *Kras; Tp53$^{-/-}$* mouse tumors (14). Nevertheless, it is possible that differences in the tumor induction strategy (such as off-target effects of viral transduction) could contribute to the observed differences in malignant behavior between *SmoM2* and *Kras; p16p19^{null}* mouse myogenic tumors.

Similar to mouse myogenic tumors, human fusion-negative RMS comprises a group of tumors with clear differences in histology, myogenic differentiation state, oncogenic pathway activation, and genetic background. In recent years, subsets of human RMS tumors that exhibit a combination of specific genetic and phenotypic characteristics were distinguished. For example, a subset of human fusion-negative RMS with spindle cell/sclerosing histology was recently found to exhibit diffuse MyoD expression, carry frequent somatic MyoD mutations, and portend a poor prognosis (16, 17). Also, children with *TP53* germline mutations are predisposed to develop anaplastic RMS at a young age (18), and germline mutations in *DICER1* were linked to a genetic susceptibility to develop RMS of the genitourinary tract (19). Future extended (epi-)genotype/phenotype correlations might pinpoint clinically/biologically distinct subgroups of human fusion-negative RMS and identify biomarkers to facilitate prognostication and/or stratification of therapy.

AUTHOR CONTRIBUTIONS

SH, RB, and AW conceived experiments, analyzed data, wrote, and approved of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2015.00050/abstract

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ORIGINAL RESEARCH
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Oncogenic RAS mutants confer resistance of RMS13 rhabdomyosarcoma cells to oxidative stress-induced ferroptotic cell death

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Recent genomic studies revealed a high rate of recurrent mutations in the RAS pathway in primary rhabdomyosarcoma (RMS) samples. In the present study, we therefore investigated how oncogenic RAS mutants impinge on the regulation of cell death of RMS13 cells. Here, we report that ectopic expression of NRAS12V, KRAS12V, or HRAS12V protects RMS13 cells from oxidative stress-induced cell death. RMS13 cells engineered to express NRAS12V, KRAS12V, or HRAS12V were significantly less susceptible to loss of cell viability upon treatment with several oxidative stress inducers including the thioredoxin reductase inhibitor Auranofin, the glutathione (GSH) peroxidase 4 inhibitor RSL3 or Erastin, an inhibitor of the cysteine/glutamate amino acid transporter system x_c^- that blocks GSH synthesis. Notably, addition of Ferrostatin-1 confers protection against Erastin- or RSL3-induced cytotoxicity, indicating that these compounds trigger ferroptosis, an iron-dependent form of programed cell death. Furthermore, RMS13 cells overexpressing oncogenic RAS mutants are significantly protected against the dual PI3K/mTOR inhibitor PI103, whereas they are similarly sensitive to DNA-damaging drugs such as Doxorubicin or Etoposide. This suggests that oncogenic RAS selectively modulates cell death pathways triggered by cytotoxic stimuli in RMS13 cells. In conclusion, our discovery of an increased resistance to oxidative stress imposed by oncogenic RAS mutants in RMS13 cells has important implications for the development of targeted therapies for RMS.

Keywords: rhabdomyosarcoma, RAS, cell death, apoptosis, ROS

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Introduction

Rhabdomyosarcoma is the most common soft-tissue sarcoma in childhood and adolescence and can be divided into two major histopathologies, i.e., alveolar (ARMS) and embryonal (ERMS) (1, 2). Recent data obtained from two next-generation sequencing (NGS) studies revealed that RMS harbor a high rate of recurrent mutations in the RAS pathway (3, 4).Whole-genome and whole-exome sequencing of 147 tumor/normal pairs showed recurrent alterations in the RAS genes predominantly in the ERMS subtype, i.e., NRAS in 11.7%, KRAS in 6.4%, and HRAS in 4.3% of cases (4). In an independent study, genomic analysis of 13 primary RMS samples and matched normal tissue revealed that the most common cancer consensus gene mutations in RMS were in the RAS

pathway, including mutations in NRAS, KRAS, and HRAS (3). In this study, 75% (6/8) of high-risk ERMS tumors harbored RAS pathway mutations and these mutations were significantly associated with risk-group assignment (3). Additional studies documented activation of the RAS pathway by oncogenic mutations in HRAS, KRAS, or NRAS in RMS, i.e., in 42% [12/26] of RMS (5) and in 35% (5/14), 22% (7/31) (6, 7), and 11.7% (8) of ERMS tumors.

RAS proteins constitute key components of cellular signaling pathways originating from cell surface receptors (9). Oncogenic RAS proteins control a complex molecular network including cell survival as well as cell death pathways (9). Also, oncogenic RAS has been implicated in regulating the sensitivity of cancer cells to oxidative stress (10). Depending on the cellular context, e.g., on the sensitivity toward apoptotic stimuli and the status of RAS effector pathways, oncogenic RAS proteins may exert antiapoptotic and proapoptotic functions (9).

Despite the documented relevance of oncogenic RAS to drive tumorigenesis of RMS, little is yet known about the impact on cell death and survival signaling pathways. In the present study, we therefore investigated the role of oncogenic RAS genes in the control of cell death of RMS.

Materials and Methods

Cell Culture and Chemicals

RMS13 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium (Life Technologies, Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mM glutamine, and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). PI3K/mTOR inhibitor PI103 (11) was purchased from Merck Millipore (Darmstadt, Germany), RSL3 from InterBIOScreen Ltd. (Moscow, Russia), Auranofin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All chemicals were purchased from Sigma (Deisenhofen, Germany) unless indicated otherwise.

Transduction

For overexpression of RAS mutants, RMS13 cells were transduced with *pMSCV-puro* vector containing oncogenic RAS mutants (i.e., NRAS12V, KRAS12V, or HRAS12V; respective vectors were sequenced to verify the identity of the individual mutant RAS) or empty vector using the packaging cell line Platinum-E. Stable cell lines were selected with puromycin.

Determination of Cell Viability, Cell Density, Cell Count, Colony Formation, Apoptosis, and Cell Death

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Cell density was assessed by crystal violet staining (0.75% crystal violet, 50% ethanol, 0.25% NaCl, 1.57% formaldehyde). Crystal violet dye was resolubilized in 1% sodium dodecyl sulfate (SDS) and absorbance at 550 nM was measured by microplate reader (Infinite M200, Tecan Group Ltd., Maennedorf, Switzerland). Cell counts were determined by CASY cell counter (OLS

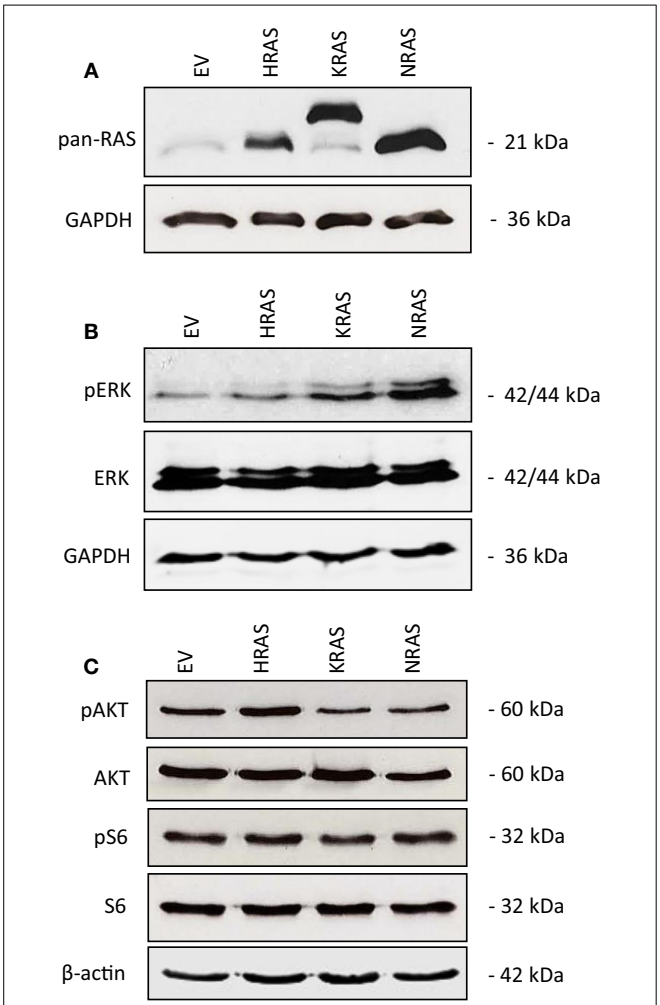

FIGURE 1 | Effects of oncogenic *RAS* genes on RAS/MEK/ERK and PI3K/AKT/mTOR signaling of RMS13 cells. RMS13 cells expressing empty vector (EV), HRAS12V, KRAS12V, or NRAS12V were analyzed for RAS protein expression using a pan-RAS antibody **(A)**, for expression and phosphorylation of ERK **(B)**, and for expression and phosphorylation of AKT and S6 ribosomal protein **(C)** by Western blotting. Representative blots are shown.

OMNI Life Science, Bremen, Germany). Apoptosis was determined by analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (FACSCanto II, BD Biosciences, Heidelberg, Germany), as described previously (12). Cell death was assessed by measuring loss of plasma membrane integrity by PI-emitted fluorescence and flow cytometry. For colony assay, cells were seeded as single cells (200 cells/well) in six-well plates and cultured for 10 days before colonies were stained with crystal violet (Roth, Karlsruhe, Germany) and counted.

Western Blot Analysis

Western blot analysis was performed as described previously (12) using the following antibodies: mouse anti-AKT (BD Biosciences), rabbit anti-pAKT, rabbit anti-p4E-BP1, rabbit anti-4E-BP1, rabbit anti-pS6, mouse anti-S6, rabbit anti-pERK, rabbit anti-ERK, rabbit anti-pan-RAS (Cell Signaling, Beverly, MA, USA). Mouse anti-GAPDH (HyTest, Turku, Finland) or

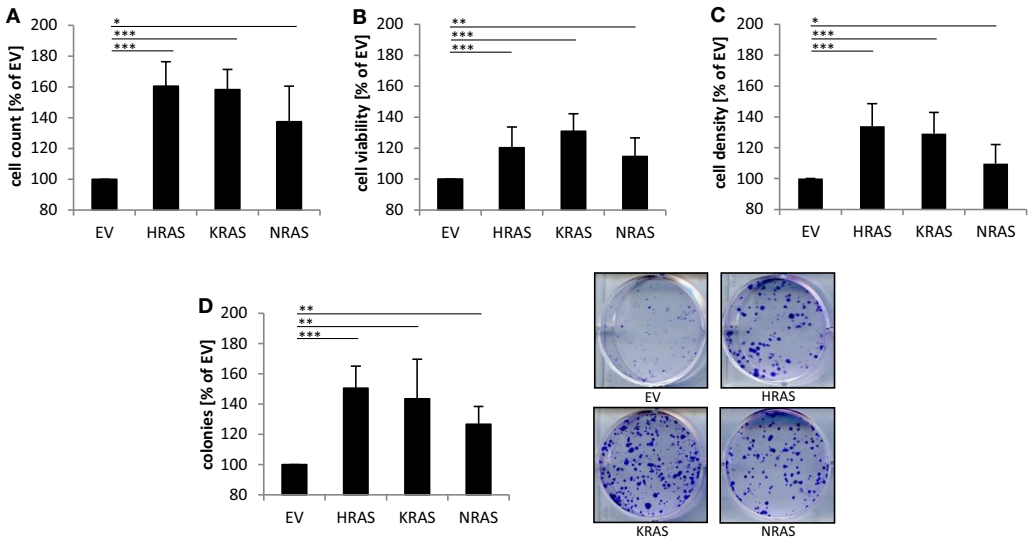

FIGURE 2 | Effects of oncogenic *RAS* genes on cell numbers and clonogenic growth of RMS13 cells. RMS13 cells expressing EV, HRAS12V, KRAS12V, or NRAS12V were incubated for 48 h and analyzed for cell counts **(A)**, cell viability using MTT assay **(B)**, and cell density using crystal violet assay **(C)**; results are expressed as percentage of cells expressing EV. Clonogenic survival was assessed by colony formation assay at day 10 **(D)**. The number of colonies was counted after crystal violet staining and is expressed as percentage of cells expressing EV [**(D)**, left panel]; representative images are shown [**(D)**, right panel]. Mean + SD of three independent experiments performed in triplicate are shown; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

mouse anti-β-Actin (Sigma) were used as loading controls. Goat anti-mouse IgG, donkey anti-goat IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc.), and goat anti-mouse IgG1 or goat anti-mouse IgG2b (Southern Biotech, Birmingham, AL, USA) conjugated to horseradish peroxidase were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Also, donkey anti-mouse IgG or donkey anti-rabbit (LI-COR Biotechnology, Bad Homburg, Germany) labeled with IRDye infrared dyes were used for detection. Representative blots of at least two independent experiments are shown.

Statistical Analysis

Statistical significance was assessed by Student's *t*-test (two-tailed distribution, two-sample, unequal variance).

Results

Effects of Oncogenic *RAS* Genes on RAS/MEK/ERK and PI3K/AKT/mTOR Signaling of RMS13 Cells

To investigate the impact of oncogenic mutant variants of RAS in RMS, we ectopically expressed *NRAS12V*, *KRAS12V*, or *HRAS12V* in the RMS cell line RMS13 that harbors wild-type RAS. Ectopic expression of mutant *RAS* genes was confirmed by Western blot analysis using a pan-RAS antibody (**Figure 1A**). To determine whether overexpression of mutant *RAS* genes affects activation of RAS/MEK/ERK and/or PI3K/AKT/mTOR pathways, we assessed in parallel the phosphorylation status of key components of these pathways. Overexpression of mutant *RAS* genes resulted in increased phosphorylation of ERK or AKT (**Figures 1B,C**; Figure S1 in Supplementary Material), indicating that overexpression of mutant *RAS* genes results in increased activation of downstream signaling pathways.

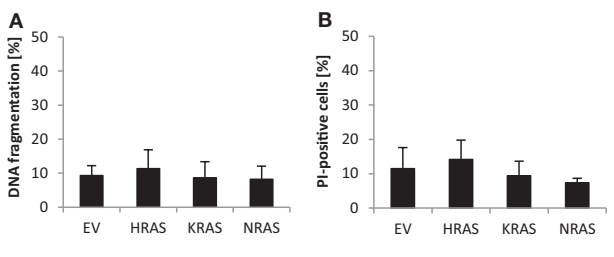

FIGURE 3 | Effects of oncogenic *RAS* genes on spontaneous cell death of RMS13 cells. RMS13 cells expressing EV, HRAS12V, KRAS12V, or NRAS12V were incubated for 48 h. Apoptosis was determined by analysis of DNA fragmentation of PI-stained nuclei **(A)**, and cell death was determined by PI staining **(B)** using flow cytometry. Mean + SD of three independent experiments performed in triplicate are shown.

Effects of Oncogenic RAS Genes on Cell Numbers and Clonogenic Growth of RMS13 Cells

Next, we investigated the effects of mutant *RAS* genes on cell numbers. Ectopic expression of *NRAS12V*, *KRAS12V*, and *HRAS12V* all caused a significant increase in cell numbers compared to cells expressing empty control vector (**Figure 2A**). In addition, overexpression of mutant *RAS* genes significantly increased cell viability as determined by MTT assay (**Figure 2B**). Besides MTT assay, which relies on mitochondrial activity and may not reliably assess cell viability under oxidative stress, we also used crystal violet assay as another assay to determine cell viability, which yielded similar results (**Figure 2C**). In addition to these short-term assays, we also assessed long-term effects using colony assays to determine clonogenic survival. Of note, ectopic expression of

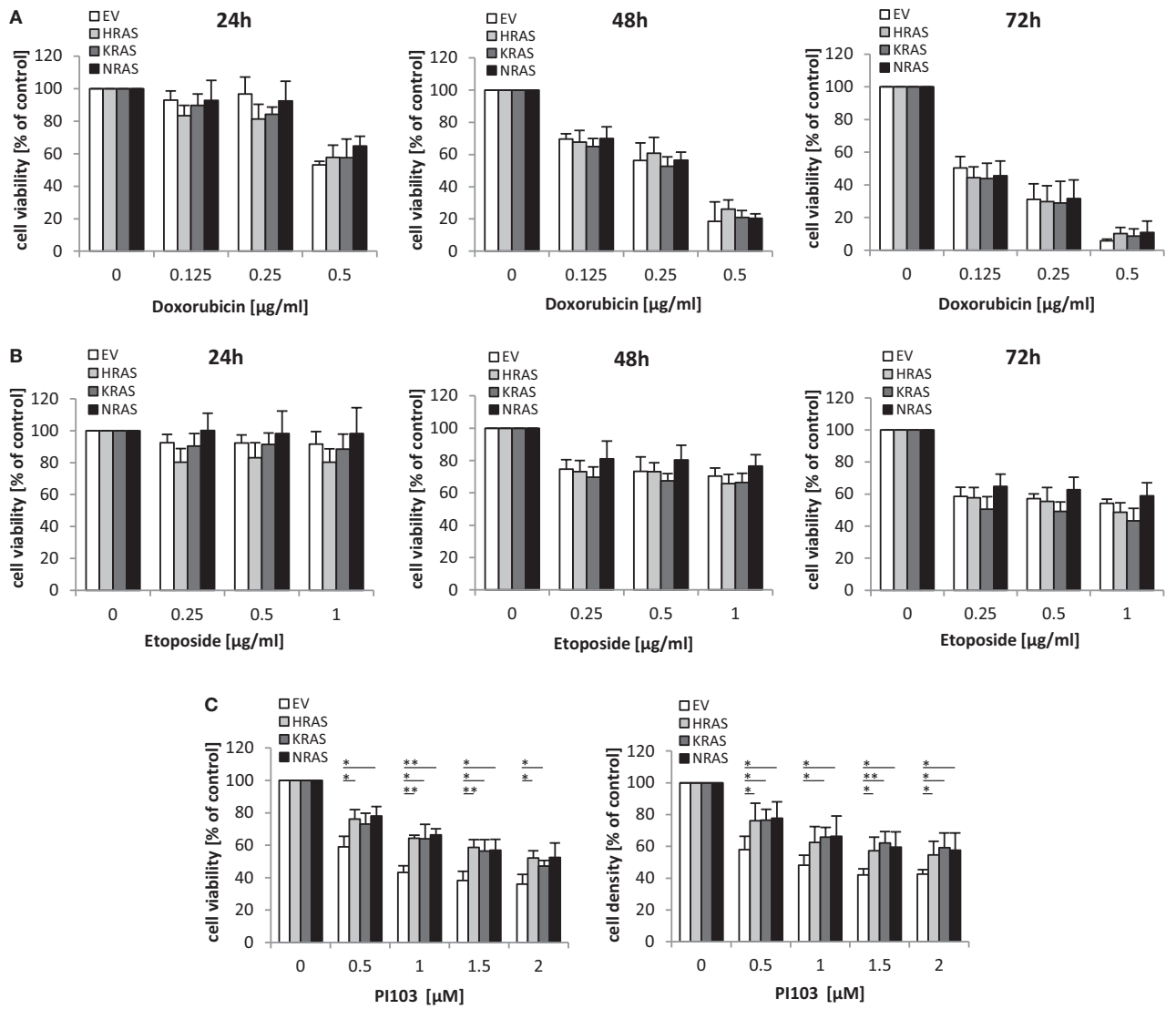

FIGURE 4 | Oncogenic *RAS* genes rescue dual PI3K/mTOR inhibitor-mediated cytotoxicity. RMS13 cells expressing EV, HRAS12V, KRAS12V, or NRAS12V were treated for 24–72 h **(A,B)** or 48 h **(C)** with indicated concentrations of Doxorubicin **(A)**, Etoposide **(B)**, or PI103 **(C)**. Cell viability was determined by MTT assay and cell density by crystal violet assay; results are expressed as percentage of untreated cells. Mean + SD of three independent experiments performed in triplicate are shown; $^{*}p < 0.05$; $^{**}p < 0.01$.

NRAS12V, KRAS12V, and HRAS12V resulted in a significant increase in colony numbers compared to cells transduced with empty control vector (**Figure 2D**). This set of experiments shows that overexpression of mutant RAS genes increases cell numbers and clonogenic survival of RMS13 cells.

Effects of Oncogenic RAS Genes on Spontaneous Cell Death of RMS13 Cells

Since RAS has been implicated in the regulation of cell death in addition to cell growth, we also determined spontaneous cell death of untreated RMS13 cells in the absence of any cytotoxic stimulus. Analysis of DNA fragmentation, used as a characteristic marker of apoptotic cell death, showed no significant changes in DNA fragmentation upon overexpression of NRAS12V, KRAS12V, or HRAS12V compared to cells expressing empty control vector (**Figure 3A**). Similarly, overexpression of mutant RAS genes did not result in enhanced plasma membrane permeability as assessed by PI staining that was used as a marker of non-apoptotic cell death (**Figure 3B**). Based on these results, we conclude that ectopic expression of NRAS12V, KRAS12V, and HRAS12V does not increase spontaneous cell death of RMS13 cells.

Oncogenic RAS Genes Rescue Dual PI3K/mTOR Inhibitor-Mediated Cytotoxicity

Next, we investigated the question whether oncogenic RAS mutants affect the sensitivity of RMS13 cells toward anticancer agents. To this end, we tested the cytotoxicity of Doxorubicin or Etoposide, two chemotherapeutic drugs that are commonly used in clinical protocols for the treatment of RMS.

Dose response and kinetic analysis showed that Doxorubicin and Etoposide reduced cell viability of RMS13 cells in a concentration- and time-dependent manner irrespective of whether or not NRAS12V, KRAS12V, or HRAS12V were ectopically expressed (**Figures 4A,B**). By contrast, overexpression of NRAS12V, KRAS12V, or HRAS12V significantly rescued loss of cell viability or cell density upon treatment with the dual PI3K/mTOR inhibitor PI103 (**Figure 4C**).

Oncogenic *RAS* Genes Protect Against Oxidative Stress Stimuli

Since oncogenic RAS has been implicated in regulating the sensitivity of cancer cells to oxidative stress (10), we extended our study to several agents that interfere with antioxidative defense mechanisms and thereby increase reactive oxygen species (ROS) levels. Interestingly, we found that overexpression of NRAS12V, KRAS12V, or HRAS12V significantly protected RMS13 cells against loss of cell viability and reduction of cell density upon treatment with Auranofin (**Figure 5A**), an inhibitor of thioredoxin reductase (13). Also, RMS13 cells engineered to overexpress NRAS12V, KRAS12V, or HRAS12V were significantly more resistant to RSL3 (**Figure 5B**), a pharmacological inhibitor of glutathione (GSH) peroxidase 4 (GPX4) (14). GPX4 is the only GPX that specifically reduces hydroperoxides within membranes (15). In addition, RMS13 cells exhibiting oncogenic RAS mutants were significantly less susceptible against Erastin (**Figure 5C**). Erastin is an inhibitor of system x_c^-, a cysteine/glutamate amino acid transporter at the plasma membrane (10), and inhibits

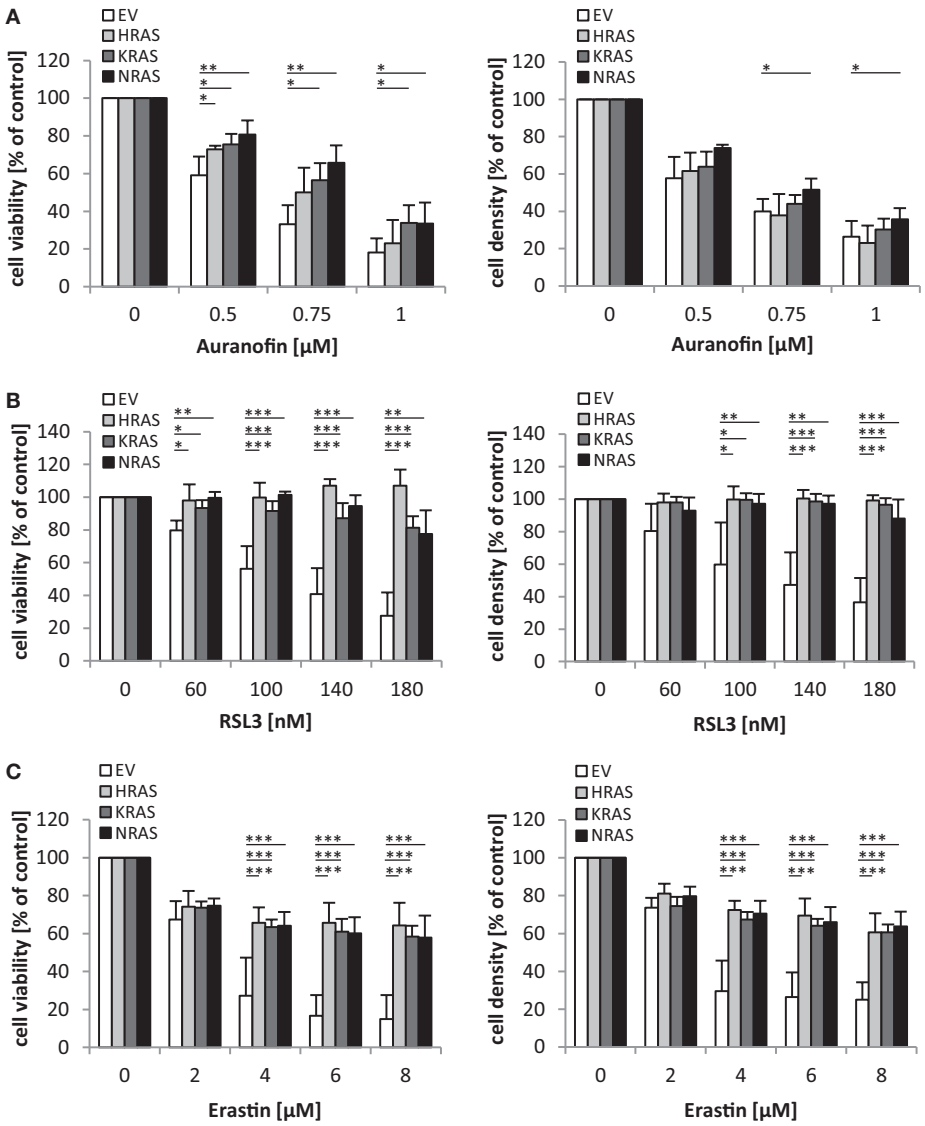

FIGURE 5 | Oncogenic *RAS* genes protect against oxidative stress stimuli. RMS13 cells expressing EV, HRAS12V, KRAS12V, or NRAS12V were treated for 48 h with indicated concentrations of Auranofin **(A)**, RSL3 **(B)**, or Erastin **(C)**. Cell viability was determined by MTT assay and cell density by crystal violet assay; results are expressed as percentage of untreated cells. Mean + SD of three independent experiments performed in triplicate are shown; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

GSH synthesis by blocking cysteine uptake. Together, this set of experiments demonstrates that oncogenic RAS mutants protect RMS13 cells against several oxidative stress stimuli.

Oncogenic *RAS* Genes Protect Against Ferroptotic Cell Death

We noted that oncogenic RAS mutants conferred protection against both RSL3 and Erastin, which either directly (i.e., RSL3) or indirectly through GSH depletion (i.e., Erastin) inhibit GPX4 (14). Since GPX4 has recently been identified as an essential regulator of ferroptosis (14), an iron-dependent non-apoptotic mode of cell death (16), we asked whether RSL3 and Erastin trigger ferroptotic cell death in RMS13 cells. To address this question, we used Ferrostatin-1, which has been described to block ferroptosis (10). Indeed, addition of Ferrostatin-1 significantly reduced RSL3- or Erastin-induced loss of cell viability (**Figures 6A,B**). To further test whether RSL3 and Erastin engage a non-apoptotic form of cell death, we assessed in parallel plasma membrane permeability using PI-staining and DNA fragmentation as markers of non-apoptotic and apoptotic cell death, respectively. Notably, treatment with RSL3 or Erastin caused a significant increase in plasma membrane permeability as reflected by increased PI positivity (**Figures 6C,D**), whereas only a minor increase in the rate of DNA fragmentation was observed (**Figures 6E,F**), consistent with a non-apoptotic mode of cell death. Together, this set of experiments indicates that RSL3 and Erastin trigger ferroptotic cell death in RMS13 cells.

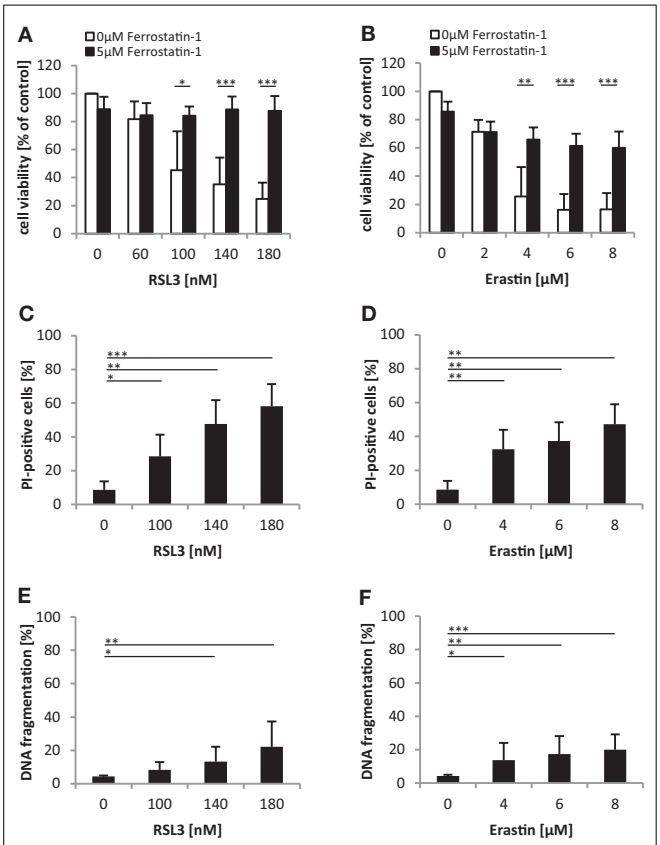

FIGURE 6 | Oncogenic *RAS* genes protect against ferroptotic cell death. **(A,B)** RMS13 cells expressing EV were treated for 48 h with indicated concentrations of RSL3 **(A)** or Erastin **(B)** in the presence or absence of 5 μM Ferrostatin-1. Cell viability was determined by MTT assay; results are expressed as percentage of untreated cells. Mean + SD of three independent experiments performed in triplicate are shown; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. **(C–F)** RMS13 cells expressing EV were treated for 48 h with indicated concentrations of RSL3 or Erastin. Apoptosis was determined by analysis of DNA fragmentation of PI-stained nuclei **(C,D)** and cell death was determined by PI staining **(E,F)** using flow cytometry. Mean + SD of three independent experiments performed in triplicate are shown; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Discussion

In the present study, we investigated the role of oncogenic RAS genes in the regulation of cell death of RMS13 cells. A key finding of our study is the increased resistance to oxidative stress that is conferred by ectopic expression of oncogenic RAS mutants. RMS13 cells engineered to express NRAS12V, KRAS12V, or HRAS12V proved to be significantly less vulnerable to several redox-targeting agents that inhibit antioxidative defense systems responsible for ROS detoxification. This increased resistance to oxidative stress occurs upon inhibition of distinct antioxidative defense pathways, including the GSH system (that is inhibited by Erastin and RSL3) as well as the thioredoxin system (that is inhibited by Auranofin), emphasizing the general relevance of this finding. Interestingly, this form of oxidative stress-induced cell death turned out to be ferroptosis, a recently defined iron-dependent form of programed cell death involving ROS production (16). Our rescue experiments showing that Ferrostatin-1 confers protection against Erastin- or RSL3-induced cytotoxicity underscores that these compounds trigger ferroptotic cell death in RMS13 cells that is attenuated by oncogenic RAS mutants.

Of note, our key finding showing that the RAS mutation status imparts resistance toward treatment with ferroptosis-inducing compounds such as Erastin and RSL3 is in line with recent evidence documenting that the RAS mutation status does not predict sensitivity to Erastin (14). A large analysis of more than a hundred of different cancer cell lines recently documented no selective lethality of Erastin in RAS-mutated cancer cell lines over RAS wild-type counterparts (14). This comprehensive study indicates that oncogenic RAS does not confer sensitivity to Erastin across cancers. By comparison, Erastin has been reported to exhibit greater lethality in human tumor cells harboring mutations in the oncogenes *HRAS*, *KRAS*, or *BRAF* (17) as well as in an individual genetic context using isogenic cell lines with and without oncogenic *RAS* genes (10). Thus, there are likely to be found other more dominant determinants of sensitivity toward Erastin than RAS mutations when analyzing sensitivity across diverse contexts.

In addition to redox-targeting agents, oncogenic RAS mutants also conferred resistance to the dual PI3K/mTOR inhibitor PI103, while they did not alter the response to DNA-damaging chemotherapeutics such as Etoposide and Doxorubicin. This suggests that oncogenic RAS selectively modulates cell death pathways in response to cytotoxic stimuli in RMS13 cells.

Oncogenic forms of RAS have previously been implicated in the control of both proliferation and cell death of cancer cells (9). Consistent with the well-documented role of oncogenic RAS

to drive cell cycle progression and clonogenic growth, RMS13 cells harboring NRAS12V, KRAS12V, or HRAS12V exhibited a significant increase in proliferation and colony formation as compared to cells with wild-type RAS. While oncogenic RAS has been described to also promote cell death under certain circumstances (9), we found no evidence of increased spontaneous cell death in the absence of lethal insults in RMS13 cells, neither apoptotic nor non-apoptotic cell death.

Several genomic studies of RMS samples have revealed a high rate of recurrent mutations in the RAS pathway, which is associated with intermediate and high-risk disease (3). This underscores that RAS signaling is a clinically relevant oncogenic pathway in RMS. Our present study contributes to a better understanding of the biology of oncogenic RAS in RMS. While RAS remains one of the most elusive genes to target directly, RAS mutant cells have been shown to depend on a number of oncogenic signaling pathways that arise as a means of adaptation to RAS-driven intracellular stresses and represent unique vulnerabilities of mutant RAS cancers (18). In RMS, concomitant inhibition of the RAS/MEK/ERK and PI3K/AKT/mTOR pathways has recently been demonstrated in two independent studies to synergistically trigger apoptosis and to inhibit tumor growth *in vivo* (19, 20). Thus, therapeutic targeting of RAS effector pathways and the search for synthetic lethal interactors of mutant RAS may offer exiting opportunities for new therapeutic directions.

Author Contributions

CS performed experiments, analyzed and interpreted data; NC performed experiments; UG analyzed and interpreted data; HH designed research, analyzed and interpreted data; SF designed research, analyzed and interpreted data, and wrote the manuscript; all authors approved the final version of the paper.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fonc.2015.00131

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Hedgehog inhibitors in rhabdomyosarcoma: a comparison of four compounds and responsiveness of four cell lines

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and is divided into two major histological subgroups, i.e., embryonal (ERMS) and alveolar RMS (ARMS). RMS can show HEDGEHOG/SMOOTHENED (HH/SMO) signaling activity and several clinical trials using HH inhibitors for therapy of RMS have been launched. We here compared the antitumoral effects of the SMO inhibitors GDC-0449, LDE225, HhA, and cyclopamine in two ERMS (RD, RUCH-2) and two ARMS (RMS-13, Rh41) cell lines. Our data show that the antitumoral effects of these SMO inhibitors are highly divers and do not necessarily correlate with inhibition of HH signaling. In addition, the responsiveness of the RMS cell lines to the drugs is highly heterogeneous. Whereas some SMO inhibitors (i.e., LDE225 and HhA) induce strong proapoptotic and antiproliferative effects in some RMS cell lines, others paradoxically induce cellular proliferation at certain concentrations (e.g., 10 μM GDC-0449 or 5 μM cyclopamine in RUCH-2 and Rh41 cells) or can increase HH signaling activity as judged by *GLI1* expression (i.e., LDE225, HhA, and cyclopamine). Similarly, some drugs (e.g., HhA) inhibit PI3K/AKT signaling or induce autophagy (e.g., LDE225) in some cell lines, whereas others cannot (e.g., GDC-0449). In addition, the effects of SMO inhibitors are concentration-dependent (e.g., 1 and 10 μM GDC-0449 decrease *GLI1* expression in RD cells whereas 30 μM GDC-0449 does not). Together these data show that some SMO inhibitors can induce strong antitumoral effects in some, but not all, RMS cell lines. Due to the highly heterogeneous response, we propose to conduct thorough pretesting of SMO inhibitors in patient-derived short-term RMS cultures or patient-derived xenograft mouse models before applying these drugs to RMS patients.

Keywords: rhabdomyosarcoma, GDC-0449, LDE225, HhA, cyclopamine

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Introduction

Of the major rhabdomyosarcoma (RMS) subtypes in children, embryonal RMS (ERMS) accounts for approximately two-third of cases. It frequently shows loss of heterozygosity of 11p15.5 and overexpression of IGF2. The other subtype is alveolar RMS (ARMS). Seventy-five percent of ARMS harbor reciprocal chromosomal translocations resulting in fusion genes of *PAX3* or *PAX7* and of

the forkhead transcription factor, *FOXO1*. Twenty-five percent of ARMS are fusion-negative as are all ERMS. ARMS has a very poor prognosis especially when metastatic (<20% long-term survival) (1). Although the majority of ERMS patients experience more favorable outcomes, treatment failure and toxicity remain substantial. Furthermore, long-term survival of patients with metastatic ERMS is still merely 40% (1). The improvement of treatment schemes is therefore important not only in the combat against ARMS but also against ERMS. This can be only assured if we increase our current knowledge of the basic biology of RMS.

We and others recently showed that predominantly ERMS and fusion-negative ARMS are characterized by high HEDGEHOG (HH) signaling activity (2, 3). Thus, the major marker genes of active HH signaling *GLI1* and *PATCHED1* (*PTCH*) are expressed at significantly higher levels in both subgroups compared to fusion-positive ARMS that however also show activation of the pathway. These results implicate that RMS might be sensitive toward a targeted therapy using small molecule inhibitors directed against components of the HH signaling pathway.

The HH signaling pathway plays a critical role in development, cell fate decisions, and tissue growth. Components of the canonical HH signaling cascade are the HH ligand, the transmembrane protein PTCH that acts as a receptor for HH proteins, the seven-pass transmembrane protein and signaling partner of PTCH, SMOOTHENED (SMO), and the family of GLI transcription factors. In the absence of HH, PTCH inhibits SMO. Binding of HH to PTCH suspends this inhibition. Through a series of poorly understood events that involves SMO-trafficking to the primary cilia, SMO activates the transcription factors GLI2 and GLI3 and the expression of downstream target genes [reviewed in Ref. (4)]. The HH signal also induces the expression of GLI1, which thus amplifies the HH signal. Another downstream target of the pathway is PTCH, which in contrast regulates HH signaling in a negative feedback loop. Together *GLI1* and *PTCH* mRNAs are considered as reliable markers for the pathway's activity [reviewed in Ref. (5)]. Finally, also *IGF2* expression can be regulated by HH signaling (6, 7). Gene expression data revealed that IGF2 is frequently overexpressed in ERMS and ARMS and plays also a key role in the formation, proliferation, growth, and metastasis of RMS [reviewed in Ref. (8)].

Several drugs targeting the HH pathway exist that already have entered clinical phase I/II trials. The first inhibitor of the HH pathway discovered was cyclopamine. Cyclopamine is a natural product that can be isolated from corn lilies and that binds and inhibits SMO (9). However, because of its limited potency and poor oral solubility, it is not suitable for clinical development (10). Recently, more potent derivatives of cyclopamine and small molecule antagonists targeting SMO have been identified. One of them, i.e., GDC-0449 (vismodegib) has already been approved for advanced basal cell carcinoma (11) and is also used in clinical trials for adult RMS patients. Another compound is LDE225 (sonidegib), which is well-tolerated by the patients and which is currently evaluated in phase II clinical trials for medulloblastoma and RMS. LDE225 also just met primary endpoint in a trial for patients with advanced basal cell carcinoma. In addition, several other compounds, such as BMS-833923 or PF04449913, are being investigated in a range of advanced cancers (10). Table S1 in Supplementary Material is providing a short overview of the four SMO inhibitors used in this study.

SMOOTHENED inhibitors can vary in their capacity to block canonical HH signaling as estimated by *GLI1* expression. They also can have variable potency in blocking the activity of SMO mutational activating variants. Examples are GDC-0449 and HhAntag (HhA). Whereas GDC-0449 has a robust potency against the SMO-E518K variant, but is weakly active against SMOD473H, HhA is essentially equipotent against all SMO alleles (12). Interestingly, besides inhibiting canonical HH signaling, several SMO inhibitors including cyclopamine and GDC-0449 activate a non-canonical SMO/$Ca2^{+}$/AMPK-dependent signaling cascade that may induce a Warburg-like effect, whereas other SMO modulators such as LDE225 do not (13). Together, these data show that SMO inhibitors differ substantially in their mode of action.

Here, we compared the effects of GDC-0449, LDE225, HhA, and cyclopamine with respect to HH pathway inhibition and their potential to inhibit proliferation, to induce apoptosis and to modulate the differentiation status of four different RMS cell lines. We also compared their impact on the activity of other signaling molecules including AKT and AMPK. Our data reveal that the compounds differ extraordinarily in modulation of the above-mentioned parameters and that the responsiveness of the RMS cell lines is highly heterogeneous.

Materials and Methods

Reagents

HhA was from Genentech (San Francisco, CA, USA), GDC-0449 (Vismodegib) from Selleckchem (Munich, Germany) and LDE225 (NVP-LDE225) from Active Biochem (Bonn, Germany). For *in vitro* assays, the drugs were dissolved in DMSO. Cyclopamine was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in Ethanol. All compounds were easy to solubilize in the respective solvents. For proliferation assays, Annexin V/PI staining and Western blot the final DMSO/ethanol concentrations were uniform in all samples, i.e., the medium contained 0.03% (*v*/*v*) DMSO and 0.1% (*v*/*v*) ethanol, whereas for gene expression analysis and WST-1 assay the solvent was DMSO for GDC-0449, LDE225, and HhA and ethanol for cyclopamine. The final drug concentrations used for *in vitro* analysis are indicated in the respective experiments.

Cell Culture

The human ERMS cell lines RD and RUCH-2 and the ARMS cell lines RMS-13 (also known as Rh30) and Rh41 (also called Rh4) were obtained from ATCC [for cell lines see Ref. (14)]. The ERMS and ARMS cell lines were cultured in DMEM and in RPMI, respectively, 10% FCS (20% FCS for Rh41), and 1% penicillin/streptomycin.

For determination of apoptosis, 10^5 cells/well (ERMS) or 15×10^4 cells/well (ARMS) were seeded in six-well-plates. After treatment for 48 h with medium supplemented with drugs or

solvent as indicated in the respective experiments, apoptosis was determined by flow cytometry on a FACSCalibur (BD Biosciences, Heidelberg, Germany) after staining of the cells with Annexin V-FITC (BD Biosciences, Heidelberg, Germany) and Propidium Iodide (PI, Miltenyi Biotec, Bergisch Gladbach, Germany).

For BrdU incorporation and WST assay, 4000 cells/well were seeded in 96-well-plates. For the BrdU assay, cells were incubated for 24 h with the respective drugs in the presence of BrdU. BrdU incorporation was measured using a Cell Proliferation BrdU ELISA (Roche Diagnostics GmbH, Mannheim, Germany). The data are presented as the percentage of the incorporation measured in time-matched solvent-treated controls taken as 100%. For WST-1 assay, cells were incubated for 24 h with the respective drugs. Four hours before the end of incubation, WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added as recommended by the manufacturer. The amount of produced formazan dye (quantitated by spectrophotometer at a wavelength of 450 nm) correlates to metabolic active cells.

Data shown summarize three independent experiments performed as duplicates (apoptosis assay) or triplicates (BrdU incorporation assay) or two independent experiments performed as triplicates (WST-1 assay).

Real-Time Quantitative RT–PCR-Analyses

For gene expression analysis, 10^5 cells/well (ERMS) or 15×10^4 cells/well (ARMS) were seeded in six-well-plates. After incubation of the cells for 24 h, total RNA was isolated using TRIzol Reagent (Invitrogen GmbH, Karlsruhe, Germany) and cDNA was synthesized using Superscript II and random hexamers (Invitrogen, Karlsruhe, Germany). Quantitative RT–PCR of target cDNAs was performed using SYBR-green based assays on an ABI Prism HT 7900 Detection System instrument and software (Applied Biosystems, Darmstadt, Germany). The primers for amplification of target transcripts are shown in the Table S2 in Supplementary Material. All primers used in study were intron-flanking, except of the primers for *18S* and *hMYOD*. Expression levels of *18S rRNA* served to normalize the transcript levels. Each sample was measured in triplicates. Expression of major components of the HH signaling pathway was analyzed once. All other data shown are the summary of two independent experiments performed in duplicates. Graphs represent the mean value of all measurements plus SEM.

Western Blot Analysis

Preparation of cell lysates and determination of protein concentrations were done as described previously (15). Primary antibodies used to detect the individual target proteins and corresponding secondary antibodies are shown in Table S3 in Supplementary Material. All Western blots shown are representative for at least two independent experiments.

Statistical Analysis

When comparing two samples, statistical differences were analyzed using Student's *t*-test. Data were considered significant when $p < 0.05$.

Results

Effects of GDC-0449, LDE225, HhA, and Cyclopamine on HH Signaling Activity and on the Expression of *IGF2* and of Muscle Differentiation Markers

We first examined whether the two ERMS cell lines, RD and RUCH-2, and the two ARMS cell lines, RMS-13 and Rh41, used in the study express the major components of the HH signaling cascade *SHH*, *SMO*, *PTCH*, and *GLI1-3*. Of these genes, *GLI1* and *PTCH* are regulated by the HH signal and thus are HH pathway target genes. As shown in Figure S1 in Supplementary Material, *SHH*, *SMO,* and *PTCH* and the transcriptional effectors *GLI1*, *GLI2*, and *GLI3* were expressed in all cell lines. Expression of *GLI1*, *GLI3*, *PTCH*, and *SMO* was highest in RMS-13 cells that show an amplification of *GLI1* (16). Furthermore, we detected *SHH* expression that was highest in RUCH-2 cells. In summary, all RMS cell lines express the major components of the HH signaling pathway. This is similar to data previously published by Graab et al. (17).

Because *GLI1* and *PTCH* expression were suggestive for canonical HH signaling activity (i.e., via the HH/PTCH/SMO/GLI axis) in the different cell lines, we examined whether it is possible to inhibit HH signaling using the SMO inhibitors GDC-0449, LDE225, HhA, and cyclopamine. For this purpose, we first determined the optimal conditions under which each inhibitor may block HH signaling. In order to measure HH signaling activity, the transcription of the HH pathway indicator *GLI1* was analyzed by qRT-PCR.

In the cell line RD, significant inhibition of *GLI1* expression was revealed for 1, 10, and 50 μM GDC-0449 and LDE225, and for 1–50 μM HhA (**Figure 1**). Interestingly, we found that 30 μM of GDC-0449 or LDE225 did not further decrease HH signaling activity but restored the activity to basal level or above (**Figure 1**). Similarly, cyclopamine increased *GLI1* expression and the increase was significant at 1 μM (**Figure 1**).

In RUCH-2 cells, the dose-response analyses revealed that GDC-0449 did not significantly modulate *GLI1* expression at any concentration, whereas LDE225 and HhA inhibited *GLI1* expression at 30 and 50 μM, and 10 and 50 μM, respectively. In addition, and similar to RD cells, cyclopamine increased *GLI1* expression at concentrations of 0.1, 1, and 5 μM (**Figure 1**).

In RMS-13 cells, which show *GLI1* amplification, the drugs did not decrease *GLI1* expression levels at any concentrations used in the experiments. Rather *GLI1* expression was increased, which was particularly obvious for LDE225 and cyclopamine (**Figure 1**).

In Rh41, we found that GDC-0449 moderately inhibited *GLI1* expression at any concentration whereas LDE225 inhibited *GLI1* expression only at 10 and 30 μM, and increased it at 50 μM. HhA inhibited *GLI1* expression at a concentration of 0.1 and 1 μM, whereas higher concentrations also resulted in restoration or increase in *GLI1* expression. Cyclopamine never inhibited *GLI1* expression but induced it for 1–10 μM (**Figure 1**).

Together, these data show that ERMS and ARMS cell lines show tumor-intrinsic HH signaling activity as estimated by modulation of *GLI1* expression after treatment with SMO – antagonists.

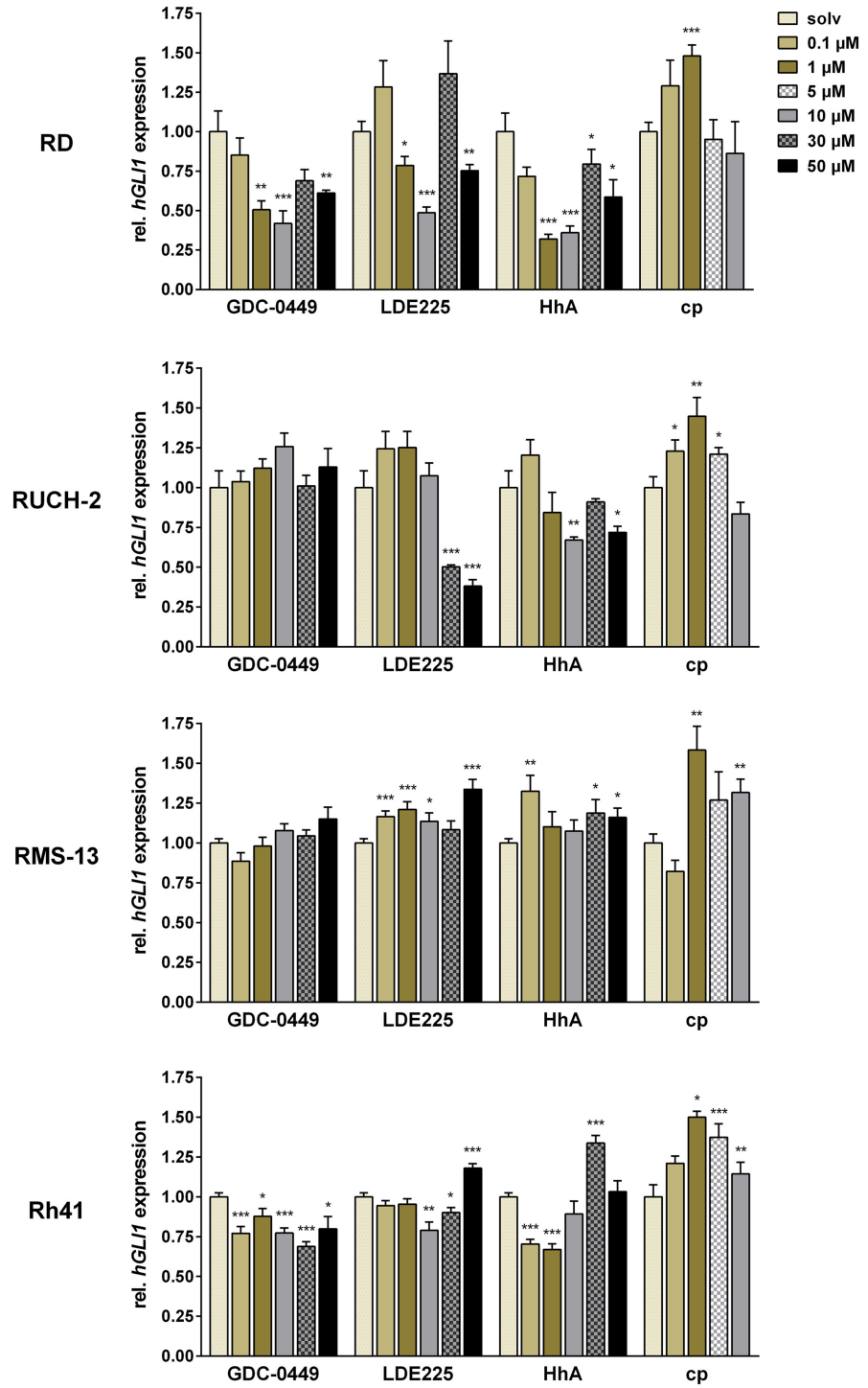

FIGURE 1 | Relative quantification of *GLI1* expression in RD, RUCH-2, RMS-13, and Rh41 after treatment with GDC-0449, LDE225, HhA, and cyclopamine (cp) at the concentration indicated. Gene expression levels were normalized to *18S* rRNA expression levels. The respective solvent-controls (solv) for each experiment were set to 1. All data represent at least two independent experiments performed in duplicates and measured in triplicates. The data are represented as mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Furthermore, our data demonstrate that SMO inhibitors – dependent on the concentration – may paradoxically activate *GLI1* transcription in some RMS cell lines.

We next measured the expression of *IGF2* that plays an important role in RMS pathology (8) and is regulated by HH signaling in specific cellular settings (6, 7). Whereas RUCH-2 cells did not

express *IGF2*, the levels of *IGF2* were regulated by all drugs in the remaining three cell lines, however to a variable extent (Figure S2 in Supplementary Material). In RD cells, *IGF2* transcription was inhibited by 0.1, 30, and 50 μM GDC-0449, by 50 μM LDE225 and by 10, 30, and 50 μM HhA. Cyclopamine had no effect and 0.1 μM HhA increased the *IGF2* levels. In RMS-13, *IGF2* expression was significantly inhibited upon treatment with 50 μM HhA or LDE225 and with 0.1 and 1 μM GDC-0449. By contrast, 0.1 and 1 μM HhA as well as 1–10 μM cyclopamine resulted in a significant up-regulation of the expression of this gene. This was different in Rh41 cells. In this cell line, the *IGF2* mRNA level was significantly decreased only with 10 μM cyclopamine, whereas all other drugs rather increased it. This was specifically obvious after GDC-0449-treatment (Figure S2 in Supplementary Material).

Finally, we also examined the expression of the early and late muscle differentiation markers *MYOD* and *MYH1*, respectively (Figure S3 and S4 in Supplementary Material).

RUCH-2 cells neither expressed *MYOD* nor *MYH1*.

In RD cells, *MYOD* transcription was inhibited by 0.1 and 50 μM GDC-0449, whereas 1 μM GDC-0449 induced it. *MYOD* expression was also inhibited by 0.1, 10, and 50 μM LDE225, by 10, 30, and 50 μM HhA and 0.1 and 1 μM cyclopamine. In RMS-13, *MYOD* expression was significantly inhibited upon treatment with 1 and 30 μM GDC-0449, 30 and 50 μM LDE225 or HhA and with 0.1 and 1 μM cyclopamine. In Rh41 cells, GDC-0449 and LDE225 induced *MYOD* expression at any concentration. *MYOD* was also induced by 10–50 μM HhA, whereas cyclopamine had no effect.

MYH1 transcription in RD cells was inhibited by 30 and 50 μM GDC-0449, by 10–50 μM LDE225, by 1–50 μM HhA, whereas it was induced by cyclopamine at any concentration. In RMS-13, *MYH1* expression was induced by GDC-0449 (significant for 0.1 and 30 μM) and by LDE225 (significant for 1–30 μM). HhA induced the expression at a concentration of 0.1 and 1 μM, whereas 30 and 50 μM inhibited it. Inhibition of *MYH1* expression was also seen with 5 μM cyclopamine. In Rh41, *MYH1* expression was induced by GDC-0449 (significant for 0.1, 10, and 30 μM) and by 1 and 30 μM LDE225, whereas 50 μM inhibited it. HhA induced the expression at a concentration of 0.1 and 1 μM, whereas 30 and 50 μM inhibited it. Inhibition of *MYH1* expression was also seen with 1–10 μM cyclopamine.

Together, SMO inhibitors may – dependent on the concentration – activate or inhibit transcription of *IGF2* and that of muscle differentiation markers in some RMS cell lines. Furthermore, the responses of the cell lines are highly variable and differ enormously from each other.

Effects of GDC-0449, LDE225, HhA, and Cyclopamine on Cellular Proliferation and Apoptosis

Next, we assessed the antiproliferative and proapoptotic effects of SMO inhibitors. For this purpose, we used concentrations that either have decreased or did not impact (10 and 30 μM, respectively) on *GLI1* expression in most experimental settings. Cyclopamine was used only at a concentration of 5 μM.

As shown by BrdU assays, the antiproliferative effects of SMO inhibitors dramatically varied between the cell lines: in RD cells, 30 μM LDE225 and 10 μM and 30 μM HhA reduced the proliferation rate of RD cells approximately by 55% and 30 and 50%, respectively (**Figure 2**). Thirty micromoles of GDC-0449, 10 μM LDE225 and cyclopamine did not show any antiproliferative effects, whereas 10 μM GDC-0449 significantly increased the proliferation rate (**Figure 2**). This was similar in RUCH-2 cells, in which the proliferation was significantly reduced by 30 μM LDE225 and by 10 and 30 μM HhA (**Figure 2**). Ten micromoles of LDE225 and 30 μM GDC-0449 had no effects, whereas 10 μM GDC-0449 and 5 μM cyclopamine significantly increased it (by approximately 50 and 100%, respectively) (**Figure 2**). In RMS-13, proliferation was reduced by 30 μM GDC-0449, by 10 and 30 μM LDE225 and by 30 μM HhA (**Figure 2**). In Rh41, none of the drugs inhibited proliferation (**Figure 2**). However, proliferation-inducing effects were seen for 10 and 30 μM GDC-0449, for 10 μM LDE225 and for cyclopamine (**Figure 2**; for RD and Rh41 the results were confirmed by simple cell counting; see Figure S5 in Supplementary Material).

As far as apoptosis is concerned, 30 μM LDE225 significantly increased the apoptosis rate (as estimated by increase of Annexin V positive cells) of RD cells as did 30 μM HhA (**Figure 3**). Ten micromoles of LDE225 or HhA as well as GDC-0449 and cyclopamine did not trigger apoptosis (**Figure 3**). The substances had similar effects in RMS-13 and Rh41 cells (**Figure 3**). Assessment of apoptosis in RUCH-2 cells was not possible due to unstable results obtained from three measurements.

Together, these data demonstrate that the responsiveness of the cell lines to four distinct SMO inhibitors is very heterogeneous. In general, the results show that cyclopamine at the used concentration neither induces apoptosis nor reduces proliferation of any of the cell lines. It rather seems to increase the proliferation rate in RUCH-2 and Rh41 cells. In addition, GDC-0449 is less effective compared to LDE225 or HhA that have the capacity to induce apoptosis and antiproliferative effects dependent on the cell line and the applied drug concentration.

Effects of GDC-0449, LDE225, HhA, and Cyclopamine on PI3K/AKT/mTOR Signaling Activity and Activation of AMPK and LC3

HEDGEHOG signaling can influence the activation status of PI3K/AKT signaling (18, 19, 20) and can inhibit autophagy (21). In addition, HH inhibitors can impact on a non-canonical SMO/Ca^{2+}/AMPK-dependent signaling cascade (13). Therefore, we examined the phosphorylation status of AKT and AMPK. We also measured the phosphorylation status of S6 that can be taken as surrogate readout for mTOR activity, and the activation status of caspase 3. Autophagy was assessed by the expression levels of the cytosolic 16 kDa large LC3-I and the 14 kDa large LC3-II, the latter being the lipidated form of LC3-I and specifically associates with autophagosome membranes and thus reflect autophagosome numbers (22). In the following, the most important and clear-cut findings from at least two independent Western blot analyses are summarized.

In RD cells, GDC-0449, LDE225, and cyclopamine did not influence the phosphorylation status of AKT or S6 in comparison to the solvent (**Figure 4**). In contrast, treatment with HhA reduced AKT/pAKT levels in RD cells (**Figure 4**). An induction of AMPK phosphorylation was seen with 30 μM GDC-0449 and with 10

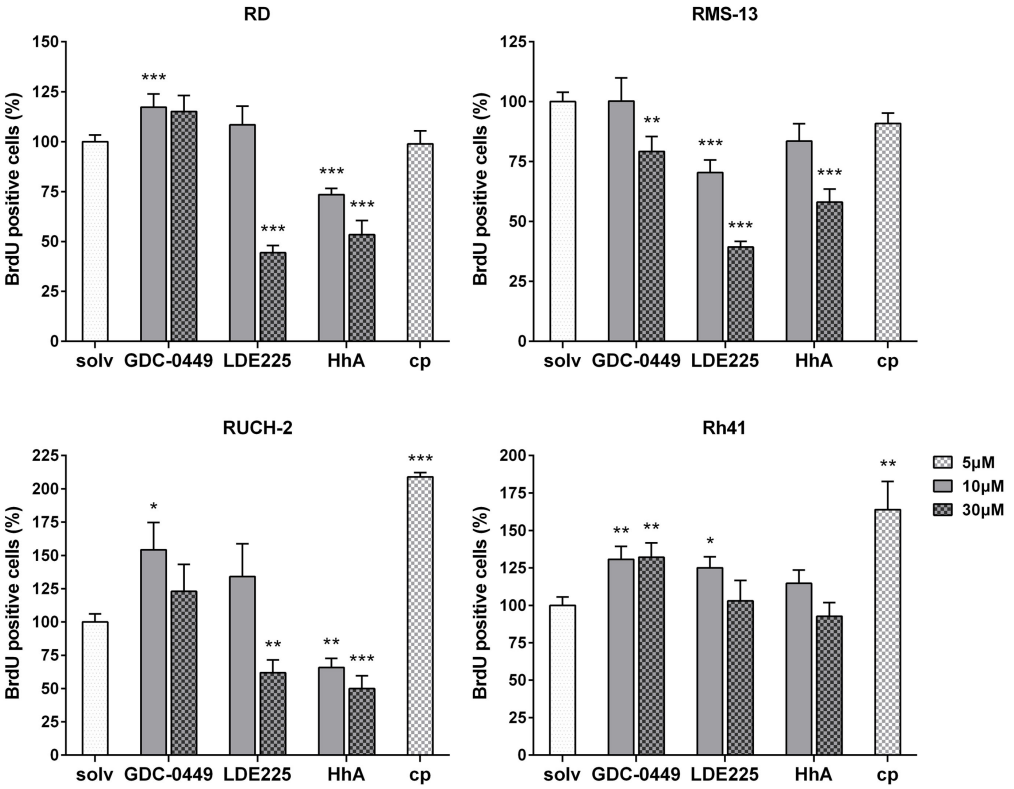

FIGURE 2 | BrdU incorporation in RD, RUCH-2, RMS-13, and Rh41 cells after incubation with GDC-0449, LDE225, HhA, and cyclopamine (cp) at the concentration indicated. BrdU-incorporation is shown as percentage of respective solvent-controls (solv) that were set to 100%. The data are represented as mean ± SEM; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

and 30 μM LDE225 (**Figure 4**). Caspase 3 activity was induced by 30 μM LDE225 as was autophagy (**Figure 4**). Increased LC3-II levels were also seen after treatment with HhA and cyclopamine at any concentration (**Figure 4**).

In RUCH-2 cells and similar to RD cells, HhA reduced AKT/pAKT levels and 30 μM LDE225 induced caspase 3 activity and autophagy (**Figure 4**). Furthermore, 30 μM LDE225 reduced pAMPK phosphorylation (**Figure 4**). An increase in LC3-II expression was also detected with HhA and cyclopamine (**Figure 4**).

In RMS-13 cells, we found a clear-cut effect of 30 μM LDE225 on caspase 3 activation and on LC3-II levels (**Figure 4**). LC3-II was also increased by 30 μM HhA and by cyclopamine at any concentration (**Figure 4**).

In Rh41 cells, GDC-0449 and HhA did not exert any obvious effect (**Figure 4**). LDE225 at a concentration of 30 μM strongly induced caspase 3 activity and increased LC3-II levels (**Figure 4**). Increase in LC3-II was also detected after treatment of the cells with cyclopamine (**Figure 4**).

In summary, these data show that the drugs exert highly heterogeneous effects on the cell lines and that the responsiveness of the cell lines is very heterogeneous as well.

Discussion

The HH pathway controls cell proliferation, differentiation, and tissue patterning. Thus, it is not surprising that a pathological activation of the pathway results in tumor formation. The cancers include those of the skin, brain, prostate, lungs, breast, and also subgroups of RMS. Therefore, the HH pathway is considered a good target for therapy. Several compounds exist and their efficacies are currently tested in clinical studies. One of these drugs is GDC-0449 that has already been approved for advanced and metastasizing basal cell carcinoma (10).

Despite these promising trends, HH inhibitors can rapidly lead to resistance. For example, GDC-0449 resistance has occurred in medulloblastoma through a D473H mutation in SMO, which prevented GDC-0449-SMO binding while maintaining aberrant HH signaling (23, 24). It also has been reported that resistance under LDE225 treatment include amplification of *GLI2*, aberrant up-regulation of PI3K signaling, and *SMO* mutations (25). This indicates that it is important to select those inhibitors for therapy that instantly and very potently inhibit proliferation and/or induce apoptosis of the tumor cells to circumvent the occurrence of these resistance mechanisms.

Our data on RMS show that LDE225 and HhA meet these criteria in RUCH-2, RD, and RMS-13 cell lines at concentrations between 10 and 30 μM, whereas cyclopamine and also GDC-0449 do not. Instead the latter drugs rather *increased* BrdU incorporation and exerted no proapoptotic effects. The *increase* in BrdU incorporation upon cyclopamine and GDC-0449 treatment are hard to explain, especially because it was neither correlated with induction of HH signaling nor IGF2 overexpression. *Vice versa*, the antiproliferative and proapoptotic effects of LDE225 and HhA

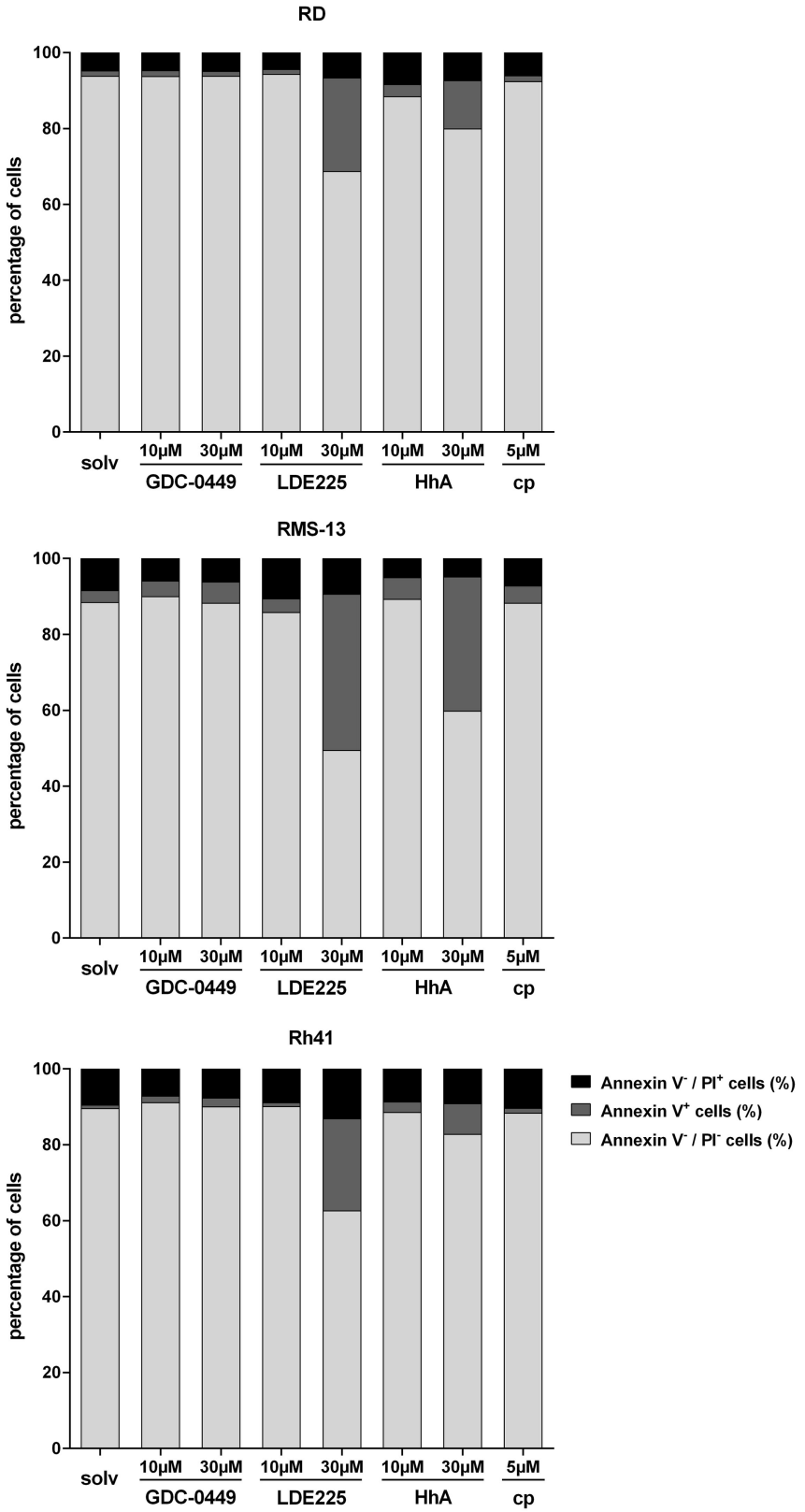

FIGURE 3 | Analysis of Annexin V/PI positivity of RD, RMS-13 and Rh41 cells after treatment with GDC-0449, LDE225, HhA, and cyclopamine (cp) at the concentration indicated.

(*Continued*)

FIGURE 3 | Continued
Apoptosis was analyzed by FACS after staining of the cells with Annexin V and Propidium Iodide (PI). Vital cells are Annexin V^-/PI^-, early-apoptotic (Annexin V^+/PI^-) and late-apoptotic cells (Annexin V^+/PI^+) were summarized as Annexin V^+ cells and Annexin V^-/PI^+ are necrotic cells, respectively. All data represent at least three independent experiments measured in duplicates. The data are represented as mean ± SEM; solv, solvent.

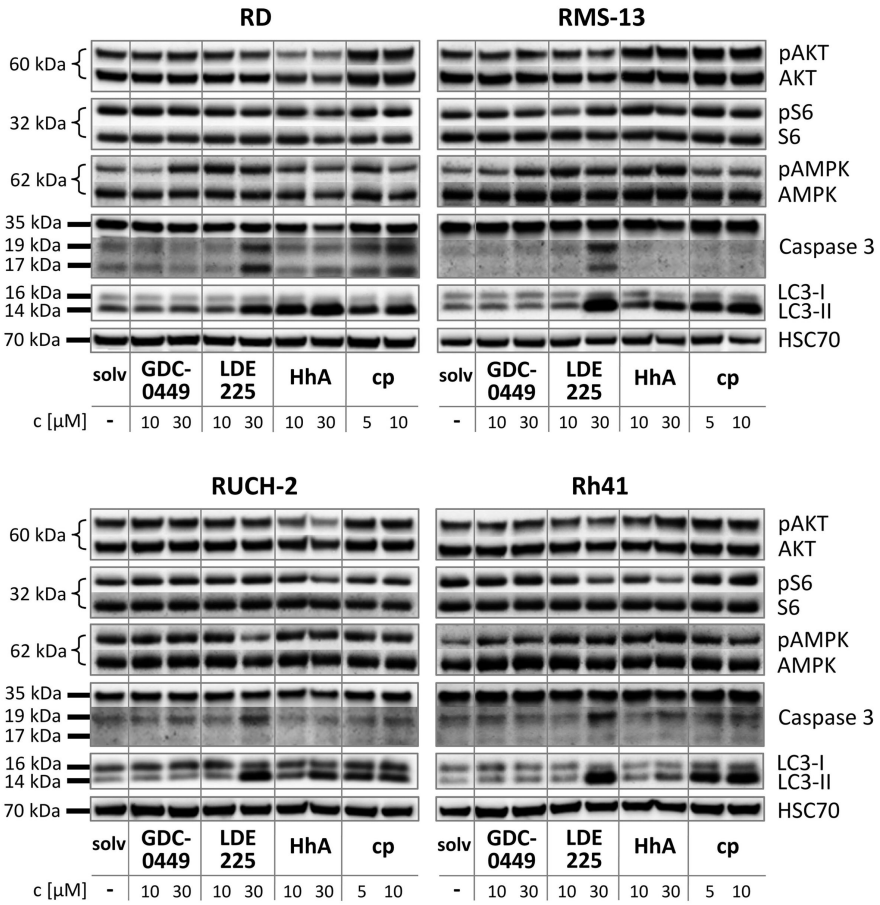

FIGURE 4 | Western blot analysis of RD, RUCH-2, RMS-13, and Rh41 after treatment with GDC-0449, LDE225, HhA, and cyclopamine (cp) at the concentration indicated. solv, solvent.

not necessarily correlated with inhibition of HH signaling. By contrast, we observed that these compounds (and also the other used in the study) had the potency to *increase GLI1* expression levels. This was dependent on the cell line, on the applied drug, and the respective drug concentration. For example, treatment of RMS-13 cells with 30 μM HhA or LDE225 resulted in a strong antiproliferative and proapoptotic effect but in an *up-regulation* of *GLI1*. Curiously, the proapoptotic effects (as measured by Annexin V labeling) were associated with caspase 3 activity in LDE225-treated cells, but not in HhA-treated ones, indicating that HhA may trigger caspase 3-independent apoptosis.

We are not aware of any study showing that SMO inhibitors can *induce GLI1* expression. Although it is possible that this effect is specific for RMS cell lines, it is more likely that the inhibitors influence other signaling molecules that in turn activate *GLI1* expression in a dose-dependent manner. This scenario has been demonstrated for the SMO agonist SAG (26). Thus, Chen and colleagues showed that SAG induced HH signaling activity with an EC50 of 3 nM, but inhibited the activity at concentrations above 1 μM. The authors propose a model, in which SAG may interact not only with SMO, but also with another cellular effector of SMO activation. According to their model, optimal SAG concentrations induce HH pathway activation by facilitating the association of SMO with the respective protein, whereas higher SAG concentrations begin to inhibit this process, as the agonist would independently bind both SMO and the effector (26). Similar models may apply to SMO inhibitors.

It is also possible that the observed concentration-dependent effects of the drugs on *GLI1* expression are off-target effects of the SMO inhibitors. Toxic effects can be rather excluded. A good example are RD cells that show a triphasic *GLI1* expression profile, i.e., a decrease at 10 μM, an increase at 30 μM, and a decrease at 50 μM upon GDC-0449, LDE225, or HhA treatment, but no triphasic changes in metabolic activity that continuously

decreases with increasing drug concentration (compare **Figure 1** and Figure S6 in Supplementary Material). Thus, an increase of *GLI1* expression (at 30 μM) is not correlated with increased cell viability (Figure S6 in Supplementary Material).

Furthermore, Dijkgraaf and colleagues (12) discussed a two-step mechanism for SMO activation that not only requires the transport to the cilia, but also an unidentified second activation step that allows SMO to trigger downstream HH signaling. The authors suggest that SMO antagonists can be subdivided in two classes. Whereas one class of inhibitors influences trafficking of SMO to cilia, the other class affects the activation step. They also discuss the possibility that SMO antagonists can induce slightly different SMO conformations that favor a particular subcellular localization over another (12). This also could explain some of the different effects of the inhibitors used in our study.

HEDGEHOG signaling also regulates the expression of muscle differentiation markers. MYOD expression has been shown to be inhibited by GLIs (27). In addition, HH signaling can inhibit terminal muscle differentiation (28). We therefore hypothesized that inhibition of HH signaling using SMO antagonists should result in up-regulation of *MYOD* and *MYH1* and thus in a more differentiated RMS phenotype. However, our data show that this only applied to some inhibitors at specific concentrations in specific cells lines (e.g., GDC-0449 at any concentration in Rh41 cells), whereas the genes were rather down-regulated in other cell lines (e.g., by LDE225 in RD cells). This again indicates that the drugs have heterogeneous effects.

It also has been demonstrated that SMO inhibitors (besides inhibiting the canonical SMO-GLI axis) can activate a non-canonical SMO/Ca^{2+}/AMPK axis that triggers a rapid Warburg-like catabolic reprograming. Induction of a Warburg-like effect has been shown for cyclopamine and GDC-0449, but not for LDE225, in mature 3T3-L1 adipocytes and the myoblast cell line C2C12 (13). It has been suggested that the physiological effects of these non-canonical endpoints, i.e., Ca^{2+} influx and AMPK-mediated catabolism, are consistent with the two major reported side effects of SMO inhibitors such as muscle cramping and weight loss. Although we did not measure Ca^{2+} influx our data shows that the AMPK phosphorylation was indeed induced in RD cells with 30 μM GDC-0449 and with 10 and 30 μM LDE225. This indicates that also LDE225 can induce Warburg-like effect, which apparently depends on the drug concentration and the cellular context.

Recently, it has been demonstrated that HH signaling can activate PI3K/AKT signaling and that, *vice versa*, AKT can stabilize and thus activate GLI transcription factors (18, 19, 20). In addition, mTOR, which is a downstream target of PI3K/AKT signaling can also trigger GLI activation (29). Therefore, we analyzed whether SMO inhibitors modulate activation of AKT or mTOR in RMS cell lines. Indeed, HhA, but none of the other SMO antagonists, blocked phosphorylation of AKT in both ERMS cell lines. Effects on S6 phosphorylation were not seen. Although this suggests that there is a crosstalk between HH and AKT signaling in at least some RMS, it is possible that HhA induces HH-independent processes that trigger AKT inhibition.

Because HH signaling can inhibit autophagosome synthesis, both in basal and in autophagy-induced conditions (21), we finally investigated whether the applied drugs induced autophagy. Indeed, our data show that HhA, LDE225, and cyclopamine can strongly increase the levels of LC3-II. Induction of autophagy by SMO inhibitors has also been shown in a variety of other cancer cell lines. Examples are hepatocellular and pancreatic carcinoma, in which the GLI inhibitor GANT61 induced autophagy that was accompanied with reduced cell viability and increased apoptosis both *in vivo* and *in vitro* (30, 31). The authors proposed that HH signaling by regulating autophagy plays an important role in determining the cellular response to HH-targeted therapy in pancreatic cancer. However, our data shows that induction of autophagy by HH inhibitors is not necessarily accompanied by antiproliferative or proapoptotic effects. For example, this is demonstrated in Rh41 cells that upon cyclopamine treatment showed increased LC3-II levels, but also increased BrdU incorporation and no alteration in the number of Annexin V positive cells.

Together, our study reveals that the mode of action of the applied HH inhibitors differs substantially from each other. Although HhA and LDE225 are probably the most universal HH inhibitors and exert antitumoral effects in almost all RMS cell lines used in our study, we propose to conduct thorough pretesting of HH inhibitors before applying these drugs to RMS patients. This could be done either in patient-derived short-term RMS cultures or in patient-derived xenograft mouse models.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fonc.2015.00130/abstract

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REVIEW
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Epithelioid sarcoma: opportunities for biology-driven targeted therapy

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Epithelioid sarcoma (ES) is a soft tissue sarcoma of children and young adults for which the preferred treatment for localized disease is wide surgical resection. Medical management is to a great extent undefined, and therefore for patients with regional and distal metastases, the development of targeted therapies is greatly desired. In this review, we will summarize clinically relevant biomarkers (e.g., *SMARCB1*, CA125, dysadherin, and others) with respect to targeted therapeutic opportunities. We will also examine the role of EGFR, mTOR, and polykinase inhibitors (e.g., sunitinib) in the management of local and disseminated disease. Toward building a consortium of pharmaceutical, academic, and non-profit collaborators, we will discuss the state of resources for investigating ES with respect to cell line resources, tissue banks, and registries so that a roadmap can be developed toward effective biology-driven therapies.

Keywords: epithelioid sarcoma, SMARCB1, INI1, BAF47, SWI/SNF complex

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Introduction

Epithelioid sarcoma (ES), first described by Enzinger over half a century ago (1), is a rare neoplasm accounting for <1% of adult soft tissue sarcomas and between 4 and 8% of pediatric non-rhabdomyosarcomatous sarcomas (2, 3). ES is presumed to be a mesenchymal malignancy, but ES characteristically exhibits both mesenchymal and epithelial markers. The cell of origin and molecular drivers are still a matter of debate. ES is divided into two recognizable clinicopathological entities, classic ES (also called distal-type ES), and proximal-type ES (**Figures 1A–E**). These two subtypes are thought be a continuum of disease rather than distinct entities (4). Distal-type ES is histologically identifiable by tumor nodules with central necrosis surrounded by large polygonal cells and spindle cells merging in the periphery (5) (**Figures 1A,B**). Described variants include angiomatoid variant, fibroma-like variant, and myxoid variant. Proximal-type ES is characterized by a multinodular pattern and sheet-like growth of large polygonal cells, often accompanied by a focal or predominant rhabdoid morphology (6) (**Figures 1C,D**). A specific marker has not yet been identified in ES. On immunohistochemistry (IHC), virtually all cases are positive for cytokeratin (CK) and epithelial membrane antigen (EMA) and most cases co-express vimentin. The marker CD34 is expressed in 60–70% of cases. IHC studies are typically negative for S-100, neurofilament protein, carcinoembryonic antigen, factor VIII-related antigen and CD-31, and INI-1 (*SMARCB1*) whose expression is lost in tumor nuclei (7). Establishing a diagnosis of ES can be difficult as tumors can present with a wide range of appearances and immunophenotypes. The differential diagnosis include fibrous histiocytoma, nodular fasciitis, other reactive proliferations, fibromatosis, giant cell tumor of tendon sheath, sclerosing epithelioid fibrosarcoma, and even some carcinomas and melanomas (7). IHC is helpful in differentiating these entities. Epithelioid

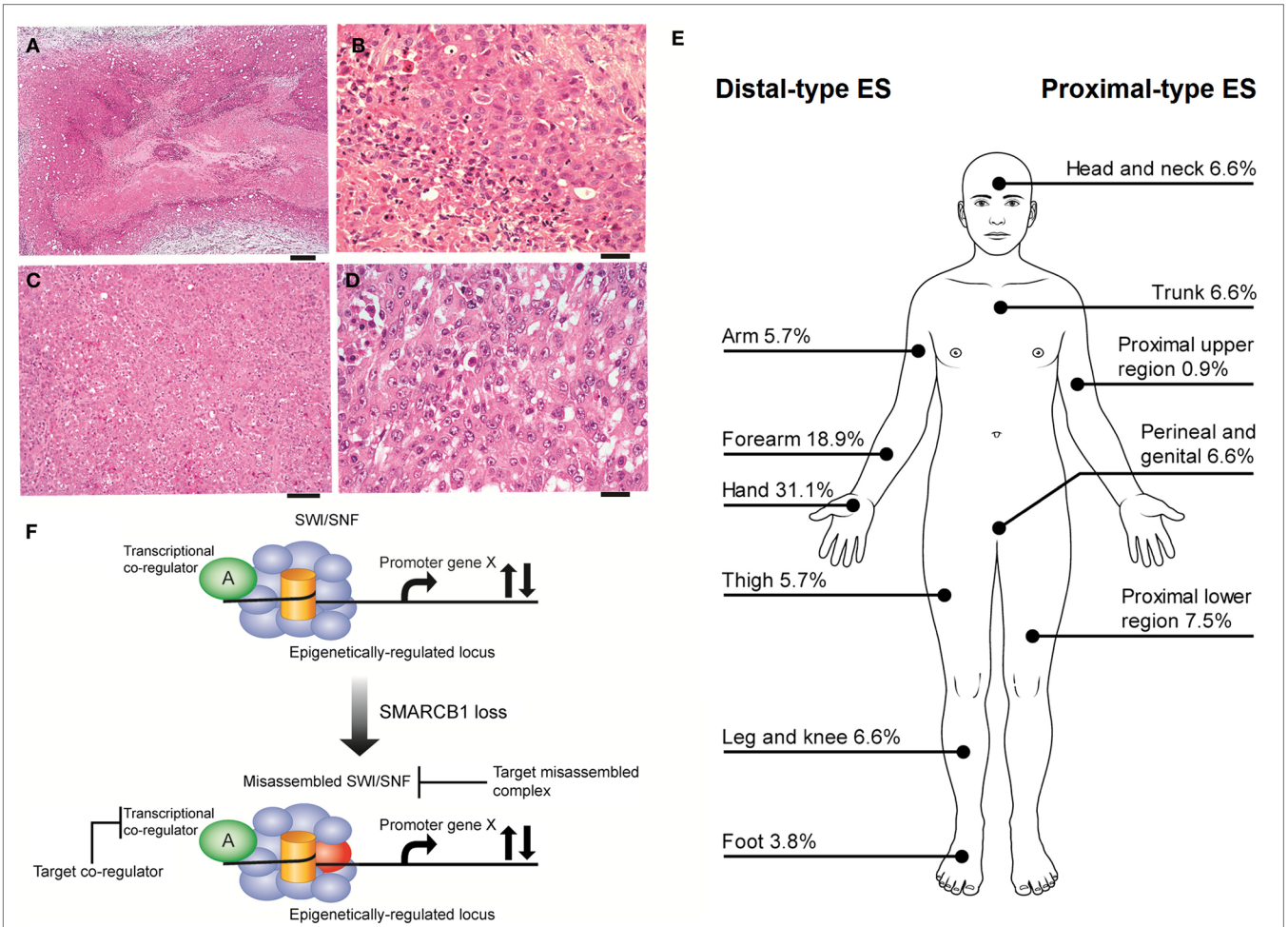

FIGURE 1 | (A,B) Distal-type ES. **(A)** Low power histology shows a nodule of tumor present in the dermis and subcutis, comprising a large area of central geographic necrosis, surrounded by sheets of relatively uniform polygonal neoplastic cells (hematoxylin and eosin, ×40). Scale bar, 500 μM. **(B)** At higher power, these are medium-sized, rounded cells, with ovoid vesicular nuclei with even chromatin, and small nucleoli. This example is cellular, but more sparsely cellular neoplasms can appear subtle, and the neoplastic cells may be confused with inflammatory cells. The characteristic necrosis is seen abutting the tumor cells (bottom left of field) (hematoxylin and eosin, ×200). Scale bar, 50 μM. **(C,D)** Proximal-type ES. **(C)** At low power, proximal-type ES comprises sheets or lobules of medium-sized to large round cells, and is seen to lack the more defined architecture and geographic central necrosis of the distal-type variant (hematoxylin and eosin, ×40). Scale bar, 20 μM. **(D)** At higher power, this is characterized by a sheet-like growth of large polygonal cells, often with focal rhabdoid morphology, and which have ovoid vesicular nuclei, prominent large nucleoli, and relatively abundant eosinophilic cytoplasm. The cells are often more pleomorphic than those of the distal-type variant. On morphology alone, these cells are difficult to distinguish from other malignant epithelioid cells, such as those of carcinoma, melanoma, rhabdomyosarcoma, or epithelioid angiosarcoma, and therefore immunohistochemistry is crucial for establishing a correct diagnosis (hematoxylin and eosin, ×200). Scale bar, 50 μM. **(E)** Distributions of ES subytpes, adapted from the largest series reported by the French Sarcoma Group (9). **(F)** Vulnerabilities in the misassembled SWI\SNF complex when *SMARCB1* is absent. Using epithelioid sarcoma as well as rhabdoid tumor as a basis for this model of *SMARCB1* null tumors, the misassembled SWI/SNF complex has the potential to dysregulate target loci that may be co-regulated by other transcription factors (36, 38–40, 43) and thereby present indirect ways to drug target the misassembled complex.

vascular tumors can resemble ES and efforts must be made to exclude a diagnosis of epithelioid hemangioendothelioma. In epithelioid hemangioendothelioma, the unique translocation t(1;3)(p36;q25), resulting in the fusion of *WWTR1* with *CAMTA1I* establishes a firm diagnosis (8).

The reported overall peak incidence of ES is around 35 years of age (9, 10). Distal-type ES is more frequently diagnosed and tends to affect a younger (20–40 years of age) and more predominantly male population compared to proximal-type ES, which is usually found in an older population (20–65 years of age) (9, 11). Distal-type ES can present itself as superficial, slow growing painless firm nodules leading to chronic non-healing ulcers affecting mostly the hands and arms. Distal-type ES can also arise as deep-seated slow growing tumors in the extremities or in the tenosynovial tissues. Proximal-type ES is more often diagnosed as deep infiltrating soft tissue masses affecting axial proximal regions and is thought to be associated with a more aggressive course (6). **Figure 1E** illustrates the sites of involvement of disease. In the largest reported cohort, a majority of ES patients (47%) had localized disease at presentation (2). ES is one of the rare sarcomas that regularly spread to lymph nodes (2, 12, 13). The course of disease is characterized by multiple local recurrences and eventual metastatic spread in 30–50%

of cases with the lungs being the primary site of involvement (11). It might be said that local recurrence is the gateway to metastasis.

Management and Prognosis

Optimal management of this rare sarcoma remains to be defined. The cornerstone of treatment of localized disease is wide surgical resection (14). Neo-adjuvant or adjuvant radiation therapy is often administered in an attempt to reduce local relapses (15, 16). The role of adjuvant chemotherapy is unclear (13, 14, 17, 18). Despite multimodal management, the relapse rate remains high and recurrences tend to occur many years later following initial therapy. Reported local relapse rates are ~35% (11, 18, 19). Patients with localized disease fare better compared to regional disease [5-year overall survival (OS) of 75 vs. 49%]. Pediatric patients seem to have a favorable prognosis [5-year OS of 92.4%] as they are more likely to be diagnosed with localized distal-type ES and are less likely to have nodal or metastatic involvement at presentation (3).

Even though reasonable control of localized disease is possible, metastatic spread is seen in approximately half of patients (2). The available literature on palliative chemotherapy in ES is limited to case reports and small retrospectives studies. The most commonly administered chemotherapy regimens are single-agent anthracycline therapy or the combination an anthracycline with ifosfamide (20). A single group reported activity of a regimen combining gemcitabine with docetaxel, but the experience is limited to a small number of patients (21). The activity of navelbine was raised in a case report and may warrant further investigation (22). Partial responses are rare. Most patients achieve stable disease at best with palliative chemotherapy. In one study, tumor regression was only seen in distal-type disease (20). However, another group reported high-response rates in proximal-type ES using doxorubicin-ifosfamide combination (23). With the medical evidence being limited to small retrospectives studies, it is difficult to draw definitive conclusions regarding the chemosensitivity of this histological subtype.

Despite the administration of palliative chemotherapy, patients with metastasis have a poor prognosis. The reported median survival is ~52 weeks and the 1- and 5-year survival rates are 46 and 0%, respectively (2, 20). Therefore, a substantial unmet need exists to improve the medical management of ES patients by establishing novel systemic regimens and exploring novel targeted therapy. In this review, we will summarize our current understanding of the underlying biology of this rare disease by highlighting implicated signaling pathways and potential actionable biomarkers (**Table 1**). In order to establish a roadmap that can be developed toward effective biology-driven therapies, we will highlight therapeutics opportunities and drugs with promising activity.

Cytogenetic Analyses

Cytogenetic analyses were first attempted to better understand the biology of ES (24–29). The karyotype analysis on clinical

TABLE 1 | Potential actionable biomarkers in clinical epithelioid sarcoma samples.

Biomarker	Clinical relevance and incidence of biomarker	Available/potential diagnostic	Reference
p53	84% moderate-high nuclear expression by IHC	IHC via TMA	(73)
Cyclin D1	96% expression by IHC 0% amplification by FISH	IHC, FISH	(89)
EGFR	77%-93% expression by IHC; absence of amplification via FISH; absence of mutation by PCR	IHC, FISH, PCR	(59, 66)
VEGF-A	73% by IHC	IHC via TMA	(73)
VEGF-C	96% by IHC	IHC via TMA	(73)
mTOR (via p4EBP1 and pSRP)	100% expression by IHC via TMA	IHC via TMA	(59)
PTEN	Loss of expression in 40% by IHC via TMA	IHC via TMA	(59)
β-Catenin	31% nuclear expression by IHC; 81% cytoplasmic expression by IHC	IHC via TMA	(73)
Interleukin2-Rβ	86% expression by IHC	IHC	(90)
SMARCB1 (INI1)	Lost expression in 85–93% by IHC; 21% mutation by PCR	IHC, FISH, PCR	(9, 49, 50, 91, 92)
SALL4	Expression in 24% of proximal-type by IHC	IHC	(93)
ERG	Expression in 38–68% by IHC; no found rearrangement by FISH	IHC, FISH	(93–95)
FLI1	95% expression by IHC	IHC	(94)
PBRM1	Lost expression in 83% by IHC	IHC	(96)
GLUT1	Expression in 50% by IHC	IHC	(91)
NRAS	Mutated in one case report by sequence assay	Sequence assay	(97)
Dysadherin	54% expression by IHC	IHC	(70)
E-cadherin	Absent expression	IHC via TMA	(70, 73)
SYT-SSX1	Low expression by RT-PCR in one patient; negative by FISH	RT-PCR	(98)

TABLE 2 | Epithelioid sarcoma potential targets and corresponding experimental model systems.

Biomarker	Human cell line(s)	Reference
ALK	YCUS-5	(31)
AKT	VAESBJ, Epi544	(58, 59)
c-MET	ASRA-EPS, VAESBJ	(58)
Dysadherin	HS-ES-1M, YCUS-5, ES-OMC-MN, SFT-8606	(70)
EGFR	VAESBJ, Epi544, GRU-1	(59, 99)
HGFR/MET	VAESBJ	(44)
IL-6 and IL-6R	ES-OMC-MN	(100)
LRP	ES-OMC-MN, SFT-8606	(85)
Metal free protoporphyrin IX	Va-es-bj	(101)
MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-4	GRU-1	(59, 102)
MUC gene	FU-EPS-1, SFT-8606	(80)
mTOR	VAESBJ, Epi544	(59)
PDGF	GRU-1	(99)
RAR-α, RAR-β, and RAR-γ	GRU-1	(103)
TGF-α	GRU-1	(99)
TGF-β/Smad signaling and CD 109	ESX	(99, 104)
TNF receptors	GRU-1	(103)
Tyrosine hydroxylase gene (TH)	YCUS-5	(31)

samples or cells lines varied greatly and was mostly done in adult cases. A minority of samples were diploid, some polypoid, while a great majority had complex patterns consisting of multiple numerical and structural rearrangements (see **Table 3**). Pediatric cytogenetic analyses seem to indicate less complex genetic alterations compared to adults and may therefore offer an explanation of their more favorable prognosis (30, 31). Translocations t(8;22)(q22;q11) in distal-type ES and t(10;22) in proximal-type ES were found (24, 32). However, compared to other translocation-driven sarcomas, there is no unique identifiable reoccurring cytogenetic pattern in ES. The only identified recurrent breakpoints have been structural rearrangements involving 18q11 and 22q11. The observation that a substantial number of ES had either rearrangements or deletions of 22q led to the hypothesis that this region may contain a tumor suppressor gene (32, 33). Further studies identified *SMARCB1* as being involved in the tumorigenesis of ES (34).

SMARCB1

The *SMARCB1* gene, located at 22q11, codes for BAF47, a core subunit of the SWI/SNF ATP-dependent chromatin remodeling complex and acts as a tumor suppressor gene (35). Components of the SWI/SNF complex are mutated in 20% of cancers, most notably rhabdoid tumor (36). This complex regulates genes by enabling the nucleosome to reposition itself in relation to the DNA sequence (37). Inactivation of *SMARCB1* leads to neoplastic transformation by transcriptional deregulation of target genes implicated in regulating genomic stability, cell-cycle progression, and other signaling pathways in cooperation with transcriptional co-regulators (e.g., MyoD, Olig2) (36, 38, 39). *SMARCB1* was shown to transcriptionally regulate p16INK4a and/or p21 and repress cyclin D1, thereby suppressing E2F activity and its target genes (40–42). *SMARCB1*-deficient cells have been implicated to have aberrant Hedgehog signaling pathway activation (40, 43). Brenca et al. demonstrated that loss of *SMARCB1* expression in the ES cell line VAESBJ was caused by homozygous deletion of *SMARCB1* through mutations of exon 1. They also identified equally prevalent homozygous deletion of *CDKN2A* and *CDKN2B* loci, responsible for encoding p16, p14, and p15 proteins. Restoration of *SMARCB1* led to a reduction of cell proliferation and cell migration and to an increase in sensitivity to genotoxic stress, thereby providing evidence to support *SMARCB1* inactivation in the tumorigenesis of ES (44). For rhabdoid tumor, SWI/SNF disruption is sufficient to cause neoplastic transformation (45). However, in the context of ES, loss of *SMARCB1* by itself is not sufficient. Interestingly, knockout of *SMARCB1* in primary fibroblast cells causes rapid growth arrest and p53-mediated programed cell death (46). However, when mutations of *TP53* co-exist, tumor proliferation is dramatically increased (47). Brenca et al. demonstrated that the VAESBJ cell line retains wild-type *TP53*, but hypothesized that the homozygous loss of *CDKN2A* which leads to impaired p16/RB and p14/TP53 responses likely contributes to the genomic instability seen in this cell line (44). Hence, other signaling pathways may contribute to tumor progression in ES as witnessed by the complex genetic landscape reported in cytogenetic studies. Whether the *SMARCB1*-deficient SWI/SNF complex exists in a misassembled state as it does in rhabdoid tumor (48), and to what extent the misassembled complex aberrantly deregulates loci that are not normally associated with the SWI/SNF complex remains to be investigated. Most certainly, the milieu of transcriptional co-regulators in ES will be different than in rhabdoid tumor.

Targeting *SMARCB1* is complicated by the different mechanisms of loss of expression. IHC studies demonstrated that the loss of expression of *SMARCB1* ranges from 85 to 93% of cases (9, 34, 49, 50). Allelic homozygous deletions varied from 5 to 71%; however, the true value may be ~10% (51–53). Papp et al. identified different mechanisms to explain the loss of expression of *SMARCB1*: 13% of cases had biallelic deletions, 33% showed single-allelic deletion, and 4% had point mutations (52). In 59% of cases, both alleles were intact and no cases had promoter hypermethylation nor post-translational modification. The authors went on to show that loss of *SMARCB1* protein expression in those cases is due to epigenetic gene silencing by oncomiRs. Three of the overexpressed miRNAs (miR-206, miR-381, and miR-671-5p) could silence the *SMARCB1* mRNA expression in cell cultures (54). The role of oncomiRs was also validated by another group where miR193a-5p could equally inhibit the mRNA expression of *SMARCB1* (55). Beyond targeting the misassembled SWI/SNF complex, transcriptional co-regulators are also theoretical targets (**Figure 1F**). In summary, loss of *SMARCB1* has a crucial role in the pathogenesis of ES (along with other signaling pathways) and therefore is an interesting target to pursue for the development of new therapies. Acknowledging that the restoration of *SMARCB1* function is likely the primary therapeutic opportunity in ES, in the paragraphs to follow we discuss other therapeutic opportunities

TABLE 3 | Demographic and biological features of human epithelioid sarcoma cell lines.

Cell line name	Histology	Age (years)	Sex	Primary site	Metastatic	Cell line source	Select chromosomal marker(s)	Mutation(s)	Primary reference (PMID or other)	Related references (PMID or other)	Originating investigator (and institution) or commercial source(s)	Reference
RM-HS1		37	M	Left foot					2432306		Reeves	(105)
HX 165 c		28	M	Penile		Local recurrence			3179184		Kelland (Institute of Cancer Research, UK)	(106)
GRU-I		32	F	Left buttock	Yes	Para-iliac lymph-node			1688830	7525493	Gerharz (University of Mainz)	(107)
SARCCR 2		33	F	Knee		Local recurrence	Chromosomes 13, 14, 16, 18, and 22 were deleted in all cells		8099901		Roché (Centre Claudius Regaud)	(83)
HS-ES-1M	Proximal-type	60	M	Right perineum nodule	Yes	Local recurrence	All exhibited the identical abnormal karyotype of 46, XY, 1i(8)(q10),221, del(22)(q12)		9216728		Sonobe (Kochi Medical School)	(28)
ES020488		26	M		Yes	Cutaneous metastasis	39–83 chromosomes, with various abnormalities but no specific pattern		7685133		Sonobe (Kochi Medical School, Japan)	(108)
Va-es-bj		41	M	Epidural tumor	Yes	Bone marrow aspirate	Chromosomal triploidy with marker chromosomes		21552805	8572585	Helson (St Agnes Hospital)	(109)
ES-OMC-MN	Distal-type	44	F	Right leg nodule	Yes	Chest wall	Modal chromosome number was 45, X, in 74% of metaphases. Other chromosome numbers were 47, XXX, in 14% of metaphases, and 46, XX, in 12% of metaphases Except for a number of sex chromosomes, the chromosomes had no chromosomal anomaly		9143739		Kusakabe (Osaka Medical College)	(100)
YCUS-5	Proximal-type	3	F	Neck mass	no	Neck mass	48, XX, t(2;7)(p23;q32 ~ 34), ?del(6)(q2?5), +7, +8	expression tyrosine hydroxylase gene (TH) expression of ALK	10398195		Goto (Yokohama City University School of Medicine)	(31)
SFT-8606	Distal-type	75	M	Left elbow	yes	Primary tumor	Complex numerical and structural aberrations, including add(8)(p23), add(9)(p13), der(12)t(12;14)(p13;q22), +i(21)(q10), der(22)t(18;22)(q11;p11.2)		8908166		Iwasaki (Fukuoka University School of Medicine)	(26)
Stenman cell line		64	M	Left forearm	yes	Axillary lymph node	No <14 different marker chromosomes were found, of which all but four resulted from terminal deletions Most frequent del(1) (p21-22), found in about 25% of the cells karyotyped	Elevated p21 expression was probably due to an overexpression of the N-ras gene	2196989		Stenman (Gothenburg University)	(110)

(Continued)

TABLE 3 | Continued

Cell line name	Histology	Age (years)	Sex	Primary site	Metastatic	Cell line source	Select chromosomal marker(s)	Mutation(s)	Primary reference (PMID or other)	Related references (PMID or other)	Originating investigator (and institution) or commercial source(s)	Reference
FU-EPS-1		21	M	Right upper arm	Yes	Axillary node	Hyperdiploid karyotype with the following chromosomal abnormalities: +i(5)(p10), −8, +13, der(13)t(8;13)(q?;p11), +der(19)t(9;19)(?;?), and del(22)(q13). Gains of 5p, 9q, 19q, and 22q and a loss of 8p		16010416		Nishio (Fukuoka University Faculty of medicine)	(111)
NEPS	Classical	32	M	Forearm		Primary tumor			19756736		Hoshino (Niigata University Graduate School of Medical and Dental Sciences)	(80)
Epi-544				Foot			Modal chromosomal number of 45 (range, 42–45), monosomy of chromosomes 2, 8, 13, and X, trisomy of chromosome 5, and the following structural abnormalities: del 7q, del 9q, del 12p, 16q, t(9q;14q), and t(2q;?)		21357725		Sakharpe (University of Texas MD Anderson Cancer Center)	(73)
ESX	Proximal-type	73	F	Left thigh	Yes	Primary tumor	65 ~ 68, X, −X, or -Y, add (X)(q22), +1, add(1) (p32), add(1) (q21), add(1)(q42), add(1)(q42), der(4;10)(q10;q10), add(8)(p11.2), −9, add(9)(p22), der(11)t(11;14)(p13;q13), −13, add(13)(q22), −14, −15, add(16)(p13.1), −17, −18, add(18)(q21), +21, add(22)(q13), +4 ~ 6mar	CD109 mRNA expression	24376795		Emori (Sapporo Medical University School of Medicine)	(104)
Asra-EPS	Angiomatoid ES	67	M	Right elbow mass	No	Primary tumor	Karyotype showed near-tetraploidy with some chromosomal translocations and fragments No recurrent chromosomal translocation was detected. 90, XXYY, −4, +5, +8, +9, −10, −13, t(13;15), +14, −15, −15, −20, −22, −22, +1mar		23915498		Imura (Osaka University Graduate School of Medicine)	(112)

related to consistent alterations in other signaling pathways that may also contribute to the pathogenesis of ES.

PI3K–AKT–mTOR Signaling Pathway

The phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway has been studied extensively and is activated in a myriad of cancers. This signaling pathway's signaling regulates cell proliferation, differentiation, cellular metabolism, and cytoskeletal reorganization leading to apoptosis and cancer cell survival (56). A previous study done on *SMARCB1*-deficient tumor cells revealed persistent AKT activation (57). This finding led Imura et al. to further investigate the importance of this signaling pathway in ES (58). By studying two *SMARCB1*-deficient cell lines (VAESBJ and Asra-EPS), this group has shown that AKT/mTOR pathway is constitutively hyperactivated. Results demonstrated that silencing mTOR by transfecting cell lines with anti-mTOR-specific siRNAs suppressed cell proliferation. However, inhibition of mTOR with everolimus caused tumor growth delay without shrinkage. Blocking the mTOR signaling pathway with everolimus caused an increase in AKT and ERK activity, which was subsequently shown to be dependent of c-MET activation. Blocking c-MET activation had a variable effect on growth inhibition on studied cell lines. This variability could be partially explained by the degree of loss of PTEN, which is thought to contribute to resistance to c-MET inhibitors through sustained AKT activation upon mTOR blockade. Combining agents to block both AKT and c-MET were more effective in inducing tumor arrest compared to using either one alone. The importance of AKT and c-MET/HGF pathways was also highlighted through immunohistochemical analysis of random clinical samples. The variability of AKT activation and loss of PTEN expression in different cell lines were also confirmed by another group and thought to correlate with sensitivity of rapamycin (59). This heterogeneity could highlight the complex genetics of the disease as well as the variable importance of PI3K/AKT/mTOR signaling pathway in the tumorigenesis of ES. *In vitro* sensitivity to mTOR inhibitors is likely an imperfect surrogate for clinical activity. Nonetheless, these preclinical data are interesting and may warrant additional studies before pursuing clinical trials. Resistance to single-agent mTOR inhibitors can not only be a potential issue but can also possibly be overcome by simultaneously targeting other pathways. These findings are consistent with the shortcomings of targeting mTOR signaling pathway in general and highlight the importance of patient selection and identification of putative biomarkers (60).

EGF Pathway

The human epidermal growth factor signaling pathway regroups four distinct receptor tyrosine kinases, HER1 (ErbB-1, EGFR), HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4) and is implicated in cell proliferation, apoptosis, and angiogenesis (61). The role of EGFR in malignant transformation of carcinomas has been extensively studied. Recently, EGFR expression was revealed to be present in soft tissue and bone sarcomas (62, 63). However, subsets of disease demonstrating tyrosine kinase domain mutations were rare (64, 65). These findings sparked an interest in studying EGFR in ES. Cascio et al. showed that 93% of clinical samples (including distal and proximal-type ES) expressed EGFR by IHC (66). This high level of expression of EGFR was also corroborated by Xie et al. (59). Furthermore, Cascio et al. went on to show an absence of EGFR amplification via FISH studies. Sequencing of the *EFGR* gene tyrosine kinase domain revealed no point mutations, insertions, or deletions. Xie et al. further investigated the role of EGFR pathway in the tumorigenesis of ES. EGF-induction contributes to cell-cycle progression partly through upregulation of cyclin D1. EGFR activation also causes an increase in migration and invasion of ES cells where high levels of expression of MMP2 and MMP9 were found. Next, this group tested whether EGFR inhibition with erlotinib would be a viable therapy. Exposure to erlotinib caused tumor growth delay without causing tumor arrest. An explanation for this incomplete response is given by the cooperation of HGFR/MET pathway with EGFR in sustaining AKT and ERK phosphorylation. Dual inhibition of both those pathways had a synergistic effect in decreasing cell proliferation (44). Combining inhibition of EGFR pathway with erlotinib and mTOR pathway with rapamycin also proved to be synergistic causing cell-cycle arrest as well as an increase in apoptosis (59). Targeting solely the EGFR pathway may not translate to a possible clinical benefit. However, combined inhibition of EFGR with either mTOR or HGFR\MET may worth investigating further through preclinical animal studies.

Other Possible Actionable Pathways and Targets

Dysadherin is a cancer-associated cell membrane glycoprotein shown to downregulate E-cadherin cell-mediated adhesion and to promote metastasis (67). Dysadherin contributes to metastatic progression through autocrine activation of CCL2 expression in part through activation of the nuclear factor-kappaB pathway (68). Dysadherin also has the ability to attribute stem-cell like properties to cancer cells (68, 69). Higher mRNA expression levels of dysadherin were documented in cell lines derived from proximal-type ES compared to distal-type ES (70). This difference in expression in levels of dysadherin may offer a possible explanation to the poor prognosis associated with proximal-type ES. Interestingly, in breast cancer cell lines, dysadherin overexpression was shown to possibly enhance AKT activation. Subsequently, inhibiting AKT reduced dysadherin's ability to promote cell mobility and tumor cell invasion (71). Targeting dysadherin could be potentially exploited to treat ES, but further work is needed. Agonists of the CCL2 receptor, CCR2, such as PF-04634817, may be one area to begin investigation.

The role of Wnt/β-catenin signaling pathway in cancer is well documented. APC deficiency or β-*catenin* mutations preventing its degradation lead to constitutive activation of β-catenin signaling, which in turn contribute to stem-cell renewal and proliferation (72). In ES, IHC studies revealed low expressions of nuclear β-catenin (73). Furthermore, no β-*catenin* gene mutations were found (74). Therefore, the proliferative abilities of ES cells are

probably related to other mechanisms than Wnt/β-catenin signaling pathway. This finding is in contradistinction to the β-catenin activation seen in rhabdoid tumor (39). Interestingly, IHC studies identified a complete loss of E-cadherin (70, 73). E-cadherin is a calcium-dependent glycoprotein responsible for cell–cell adhesion (75). E-cadherin/β-catenin protein complexes have an active role in epithelial-to-mesenchymal transition (EMT), an important mechanism for the subsequent development of metastasis (76, 77). Further studies are needed to elucidate the importance of loss of adhesion molecules in tumor progression in ES.

CA125 was first identified and used as a serum marker for epithelial ovarian carcinoma (78). IHC studies revealed high positivity and specificity of CA125 in ES compared to other sarcomas (79). High expressions of the *MUC16* gene were identified by RT-PCR in ES cell lines. Serum levels of CA125 also seem to correlate with disease progression (80). Measuring CA125 serum levels is well-established and routinely available and could potentially be useful in monitoring disease status and evaluating response to therapy. Targeted immune strategies toward CA125 and MUC16 are active areas of research in ovarian cancer and any potential breakthroughs could possibly be applicable in treating ES (81, 82).

Tyrosine Kinase Inhibitors

As stated previously, chemotherapy has a limited role in the management of ES. Early studies explored the reasons underlying chemotherapy resistance. A study of the SARCCR2 cell line showed overexpression of P-glycoprotein, an ATP-binding cassette (ABC) chemotherapy export pump. Using verapamil and cyclosporine A to reverse multidrug resistance, the authors showed increased sensitivity to doxorubicin and vincristine (83). For the GRU cell line, expression of P-glycoprotein and MRP could also be observed (84). However, one study identified an absence of expression of P-glycoprotein and MRP in the ES-OMC-MN and SFT-8606 cell lines (85). In contradistinction, these studies demonstrated the presence of lung resistant protein (LRP), which mediates multidrug resistance (MDR). Results showed that reversing MDR with cyclosporin A increased sensitivity to actinomycin D, vincristine, and adriamycin. The use of tyrosine kinase inhibitors, the newest ABC inhibitors, to reverse multidrug resistance remains unexplored and may potentially enhance the efficacy of chemotherapy in ES.

The medical evidence for the utility of tyrosine kinase inhibitors impacting ES is scarce. To our knowledge, only one case was reported in the medical literature. Sunitinib showed reasonable disease stabilization in a patient with metastatic ES (86). The underlying reasons why sunitinib was active in this patient are unknown and cannot be explained with what is currently known about the biology of this disease. Pazopanib, a recent oral tyrosine kinase inhibitor approved for the treatment of soft tissue sarcoma (87), is worth investigating prospectively as its activity is similar to sunitinib. Work is still needed in mapping out active signaling pathways and identifying actionable tyrosine kinase domain mutations. Polykinase inhibitors remain therefore greatly unexplored in the management of ES and may one day improve outcome.

Future Perspectives

Researching and developing new treatment strategies in rare cancers is a challenge, but possible with technology and resources available today and regulatory agency incentives (88). ES is a perfect model to envision what personalized medicine promises for the future. The intent of this review was to draw a roadmap to develop efficient biology-driven therapy. Achieving this will start with the selection of representative cell lines and mouse models of ES (Available cell lines and potential actionable targets are summarized in **Tables 2** and **3**). Many of the potential targets highlighted in this article were based on IHC-expression or reverse-transcriptase PCR studies. DNA deep-sequencing projects may demonstrate underlying genomic amplification and mutations that can be targeted. Partnership with pharmaceutical companies would allow screening of thousands of compounds on selected cell lines presenting mutations or other actionable targets. Active drugs may then undergo preclinical testing. Those most promising can be prioritized for clinical trials. Drugs being developed in other cancers that share common signaling pathways aberrations with ES may also prove to be useful. It is possible to perform basket trials in rare cancers, and this could be a way of evaluating novel agents in this extremely rare disease. On the way of developing new therapies, possible pitfalls can be expected. As demonstrated on ES cell line models, targeting a single signaling pathway may be insufficient. The complexity of the genetic landscape and the crosstalk between multiple signaling pathways contribute to resistance. This can be overcome by targeting multiple signaling pathways simultaneously. Only through international collaboration between pediatric and adult units, we can remain hopeful that targeted and immune therapy will have a major impact in the management of ES in the near future.

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REVIEW
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Bone Tumor Environment as a Potential Therapeutic Target in Ewing Sarcoma

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Ewing sarcoma is the second most common pediatric bone tumor, with three cases per million worldwide. In clinical terms, Ewing sarcoma is an aggressive, rapidly fatal malignancy that mainly develops not only in osseous sites (85%) but also in extra-skeletal soft tissue. It spreads naturally to the lungs, bones, and bone marrow with poor prognosis in the two latter cases. Bone lesions from primary or secondary (metastases) tumors are characterized by extensive bone remodeling, more often due to osteolysis. Osteoclast activation and subsequent bone resorption are responsible for the clinical features of bone tumors, including pain, vertebral collapse, and spinal cord compression. Based on the "vicious cycle" concept of tumor cells and bone resorbing cells, drugs, which target osteoclasts, may be promising agents as adjuvant setting for treating bone tumors, including Ewing sarcoma. There is also increasing evidence that cellular and molecular protagonists present in the bone microenvironment play a part in establishing a favorable "niche" for tumor initiation and progression. The purpose of this review is to discuss the potential therapeutic value of drugs targeting the bone tumor microenvironment in Ewing sarcoma. The first part of the review will focus on targeting the bone resorbing function of osteoclasts by means of bisphosphonates or drugs blocking the pro-resorbing cytokine receptor activator of NF-kappa B ligand. Second, the role of this peculiar hypoxic microenvironment will be discussed in the context of resistance to chemotherapy, escape from the immune system, or neo-angiogenesis. Therapeutic interventions based on these specificities could be then proposed in the context of Ewing sarcoma.

Keywords: Ewing sarcoma, bone remodeling, bisphosphonate, RANKL, microenvironment, tumor bone niche, 3D models

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INTRODUCTION

Ewing Sarcoma: A Clinical Presentation

Ewing sarcoma was first described by James Ewing in 1921. It is a high-grade neoplasm, and it is the second most common primary bone malignancy in both children and adolescents (1). With peak incidence at 15 years, this disease accounts for 2% of childhood cancers (2). Ewing sarcoma is defined as a bone tumor, which may occur at any site within the skeleton but preferentially affects the trunk and the diaphysis of long bones (2). However, it may occur in extra-skeletal soft tissue in 15% of cases. It is characterized by rapid tumor growth and extensive bone destruction (**Figure 1**) that can

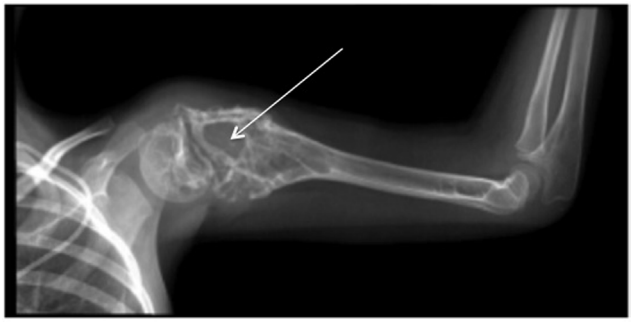

FIGURE 1 | X-ray of typical severe osteolytic lesions in a Ewing sarcoma patient (arrows: severe osteolytic lesions).

result in bone pain and pathological fracture (3). At the histological level, Ewing sarcoma appears as small, poorly differentiated, round tumor cells positive for the transmembrane glycoprotein CD99 staining (4).

The molecular event that initiates the Ewing's family of tumors is a typical chromosomal translocation that occurs in cells of mesenchymal origin and that fuses the *EWS* gene on chromosome 22q12 to a member of the erythroblast transformation sequence (ETS) transcription gene family, most commonly *FLI-1*, on 11q24 in 85% of cases (5–7). This translocation leads to the production of the oncogenic fusion gene *EWS-FLI1*, an aberrant transcription factor that promotes tumorigenicity (8, 9). The presence of this fusion gene, which represents the Ewing sarcoma signature, is used as a specific diagnostic marker of the Ewing's family of tumors thanks to fluorescence *in situ* hybridization and RT-qPCR (10). Numerous biological pathways, such as those involving insulin-like growth factor receptor (IGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), Sonic HedgeHog (SHH) pathway activation, Wnt, and transforming growth factor (TGF)-β receptor II pathway inhibition, are modulated by EWS-FLI1 activity, leading to proliferation, angiogenesis, immune system escape, metastatic potential, and treatment resistance that contribute to the Ewing sarcoma malignant phenotype (11).

Therapeutic Limits

The on-going treatments for Ewing sarcoma patients are effective in more than 70% of patients with localized disease. They elicit clinical responses in patients with metastatic disease but are not curative due to acquired resistance. Before the 1970s, amputation was the main therapeutic option, with 5-year survival of <20%. The introduction of first radiation and then chemotherapy in the 70s has modified the prognostic significantly, with the 5-year event-free survival rate for localized tumors at around 65%, and the overall survival rate close to 75%. However, the survival rates decrease to 15–25% when metastases are detected at diagnosis, or in patients presenting resistance to treatment or with relapsed disease. In the past three decades, conventional therapies seem to have attained a survival plateau for these metastatic patients (12).

Improved poly-chemotherapy has made it possible to limit surgery and salvage limb, but in about 20% of cases, bone sarcomas have already disseminated at the time of diagnosis. In most cases, the distant metastases are located in the lungs, followed by the skeleton. Although Ewing sarcoma patients with lung metastases have overall survival of 45% at 5 years, those with bone or bone marrow metastases have very poor prognosis, with <25% overall survival at 5 years. In the past, when therapy was limited to local control (surgery), nearly all patients who initially appeared to have a localized tumor developed distant metastases (13). Ewing sarcoma thus needs to be considered as a systemic disease, requiring systemic treatment, i.e., combination chemotherapy, as a rule. However, systemic therapy can never replace definitive local control with surgery and/or radiotherapy. The therapy used for Ewing sarcoma therefore requires a combination of surgery or radiotherapy for localized control and high-intensity chemotherapy for localized and disseminated disease. The most recent protocol for Ewing tumors was the European Ewing tumor Working Initiative of National Groups 99 protocol (EuroEWING99, clinicaltrials.gov no. NCT00020566), which tested the benefits of a different chemotherapy combination involving vincristine, ifosfamide, doxorubicin, and etoposide (VIDE). The protocol was composed of six sequences of VIDE treatment followed by surgery when possible. The histological response to chemotherapy was then evaluated and patients were divided into three arms depending on the localization of the tumor at diagnosis, the volume for unresected tumors, and the percentage of residual cells after treatment. The R1 arm included patients with localized disease and a good response to chemotherapy (<10% of residual cells) or with a volume of <200 ml. The R2 arm included patients with lung metastases and patients with localized tumors and a poor response to chemotherapy, or with a volume of more than 200 ml. Finally, the R3 arm included patients with bone, bone marrow, or multifocal metastases. The current survival rate for EuroEWING patients has attained 80% for localized disease of small volume (R1). Unfortunately, the 5-year survival rate for patients with metastases detected at diagnosis remains around 25% and even around 10% when relapse occurs within the first 2 years following treatment.

The current protocol for Ewing sarcoma patients is the EuroEWING2012 (clinicaltrials.gov no. NCT00987636), which started in December 2014 in Great Britain, with two randomizations: the first compares two chemotherapy protocols (with surgery and/or radiotherapy) and the second randomizes patients with or without bisphosphonate zoledronate (zometa®).

Given that survival rates had not evolved in more than three decades, especially for metastatic patients with a very poor initial prognosis, there was an urgent need to define new therapeutic targets for Ewing sarcoma patients. In addition to the tumor cells themselves, targeting the bone tumor microenvironment appears to be promising.

The Bone Microenvironment Is a Favorable "Niche" for Tumor Progression in Bone

Recently, there has been a dramatic increase in the importance given to the theory that the bone microenvironment participates

in determining the "bone niche" in the progression of bone tumors, and in establishing resistance processes to conventional therapies. The concept of "bone niche" is well-recognized in the context of hematological malignancies, such as leukemia (14) or multiple myeloma (15). The "niche" is a functional microenvironment able to both promote the emergence of cancer stem cells and provide all factors required for their development. However, the bone niche is composed of numerous cell types (pre-osteoclasts, pre-osteoblasts, endothelial cells, macrophages, etc.) that are located in the bone matrix, and their functional coordination is a pre-requisite for maintaining the bone and bone niche microarchitecture.

Much research has been published on the role played by the bone microenvironment in establishing metastases in these organs, especially from breast or prostate carcinomas. The concept of bone niche is also currently under discussion in the case of solid tumors, and strengthens the "seed and soil" theory proposed by Paget in 1887, in which tumor cells ("seeds") colonize receptive foci ("soil") (16). These data are supported by the fact that specific molecules (such as cadherin and osteopontin) play a part in stabilizing cancer cells in bone niches, mimicking the cell interactions that take place during hemopoiesis, as identified in the pre-metastatic niche in breast carcinoma (17, 18). In addition, carcinoma cells grow well in bone, which stores a variety of cytokines and growth factors, and thus provides an extremely fertile environment for growing tumor cells (19, 20).

The "seed and soil" theory can be also envisaged for primary bone tumors, as tumor growth and metastasis often require constant interactions between tumor cells and their surrounding microenvironment (21–25). This hypothesis has been largely documented in the case of osteosarcoma (26, 27) and chondrosarcoma (28), but very little information is currently available for Ewing sarcoma.

The Concept of the Vicious Cycle in Ewing Sarcoma

Ewing sarcoma is characterized by extensive bone destruction, mainly due to osteolysis (**Figure 1**). Because Ewing sarcoma cells cannot directly degrade bone, osteoclast activation and subsequent bone resorption may be responsible for the clinical features of bone destruction in this pathology (3). Bone degradation is controlled by osteoclasts, whose differentiation and activation are mainly mediated by receptor activator of NF-kappa B ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily (TNFSF11) after it binds to its receptor RANK expressed at the surface of mature osteoclasts and osteoclast precursors (29) (**Figure 2**). Osteoprotegerin (OPG) acts as a decoy receptor inhibiting osteoclast formation, function, and survival by preventing the binding of RANKL to its receptor RANK (26).

Interaction between tumor cells, tumor-derived humoral factors, and the bone marrow in the bone niche has been shown to be essential for bone tumor initiation and promotion (30, 31). Targeting the bone microenvironment, and particularly osteoclast activation, may therefore be a promising adjuvant strategy for treating bone tumors, including Ewing sarcoma. The vicious cycle between osteoclasts, bone stromal cells/osteoblasts, and cancer cells has been hypothesized during the progression of primary bone tumors (32) (**Figure 2**). Tumor cells produce osteoclast activating factors, such as interleukin (IL)-6, TNF-α, or ParaThyroid Hormone-related Peptide (PTH-rP), which induce osteoclast differentiation and activation. When osteoclasts resorb bone, they allow the release of growth factors stored in the bone matrix (TGF-β, IGF-1, PDGF, etc.), which in turn activate tumor cell proliferation (32). Accordingly, inhibiting osteoclast activity is a promising approach for breaking the vicious cycle, and thus indirectly limiting local cancer growth.

In addition, new therapeutic options targeting hypoxia, angiogenesis, bone cells, or mediators in the particular bone microenvironment have been studied extensively at the preclinical level, with the more promising now being proposed in clinical trials. This review will describe the most recent developments in such therapeutic options for Ewing sarcoma patients.

TARGETING BONE CELLS IN EWING SARCOMA

Therapeutic agents that target the bone environment and modulate bone metabolism have been studied in preclinical models of primary bone sarcomas, demonstrating a certain degree of efficacy in both osteosarcoma and Ewing sarcoma. Two main strategies are currently being developed: (i) the first directly targets osteoclasts (differentiation, activation, and functions), mainly using bisphosphonates (BPs), and (ii) the second targets the cytokine RANKL, the pivotal cytokine for regulating osteoclast activation.

Bisphosphonates

Bisphosphonates are the synthetic analogs of endogenous pyrophosphate, with a high resistance to protease degradation, and the ability to strongly inhibit bone resorption (33). They are composed of two phosphonate groups. The central oxygen atom in pyrophosphate is replaced by a carbon atom, which allows the substitution of two side groups, one of which is often an hydroxyl group, and the other defines the BP generation (**Figure 3**). Two main families can therefore be distinguished: nitrogen- and non-nitrogen-containing BPs, which act on osteoclasts by means of different molecular mechanisms. In both cases, the final result – common to both – is the induction of osteoclast apoptosis. BPs act either by inhibiting the recruitment, proliferation, and differentiation of pre-osteoclasts or by impeding the resorptive activity of mature osteoclasts (34–37). Zoledronic acid (ZOL) belongs to the third generation of BPs, which is the most efficient for preventing bone lesions (38–40). As for other nitrogen-containing BPs, ZOL inhibits the farnesyl diphosphate and geranylgeranyl diphosphate synthases, two enzymes involved in the mevalonate pathway necessary for the prenylation of small intracellular GTPases, such as Ras, Rho, or Rac (41). As the prenylation of these GTPases is essential for osteoclast function, their inhibition leads to osteoclast apoptosis as a result of the loss of the survival signal (42–44). Moreover, BPs may also inhibit bone resorption by increasing the production of OPG by human osteoblasts (45). OPG is the decoy receptor of RANKL,

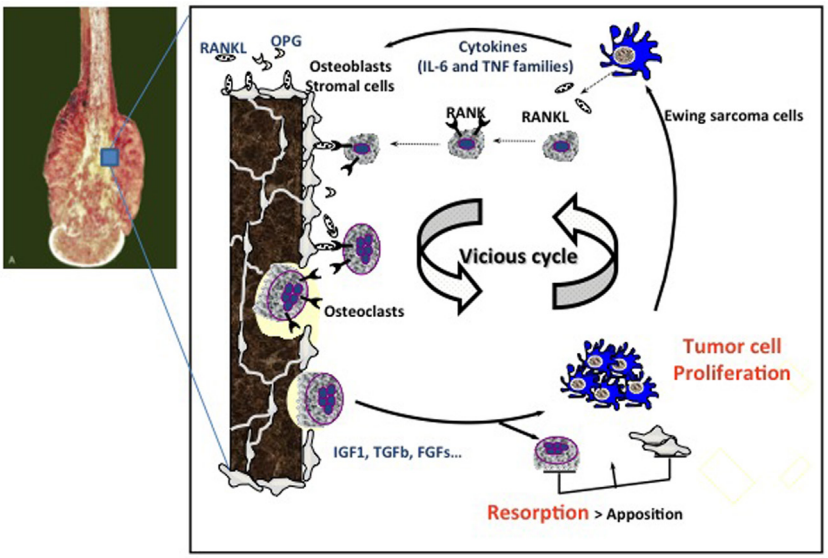

FIGURE 2 | Vicious cycle between Ewing sarcoma cell proliferation and osteoclast activation. Tumor cells produce osteoclast activating factors (IL-6, TNF-α, etc.) that will induce osteoclast differentiation and activation. When they resorb bone, osteoclasts allow the release of growth factors stored in the bone matrix, such as IGF-1, FGFs, and TGF-β, which in turn activate tumor cell proliferation. This is the theory of the so-called "vicious cycle." The molecular OPG/RANKL/RANK triad plays a pivotal role in the regulation of bone resorption. OPG and RANKL are produced by osteoblasts and/or stromal cells, whereas RANK is expressed at the surface of osteoclasts and their precursors. OPG, osteoprotegerin; IL-6, interleukin-6; TNF, tumor necrosis factor; RANK, receptor activator of NF-kB; RANKL, RANK-ligand; IGF1, insulin-like growth factor1; TGF-β, transforming growth factor-β; FGFs, fibroblast growth factors.

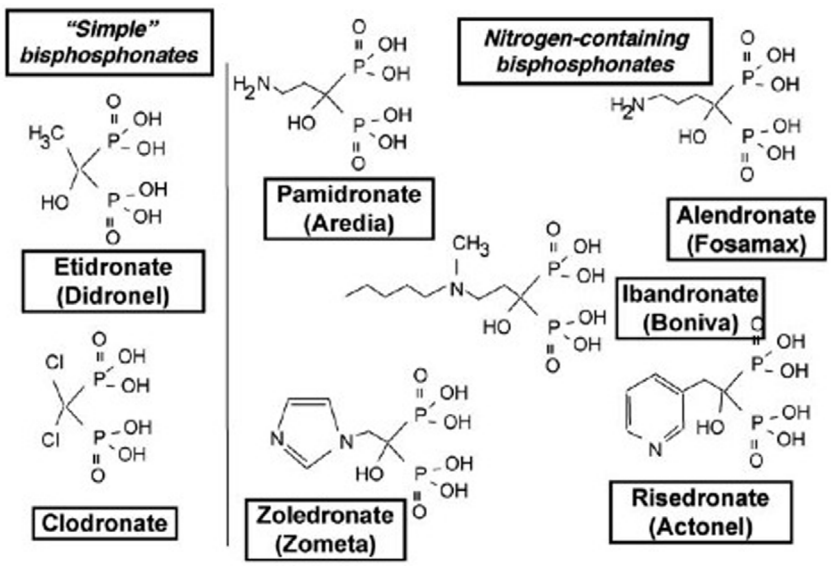

FIGURE 3 | Schematic representation of the different bisphosphonate (BP) families: simple non-N-containing BPs (etidronate and clodronate) and N-containing BPs (pamidronate, alendronate, idandronate, risedronate, and zoledronate).

which inhibits the RANK/RANKL interaction that is essential for osteoclast differentiation and activation. In addition to the antiresorptive effect of ZOL, it has been shown to induce the death of tumor cell lines, such as myeloma, and breast and prostate carcinoma cells in several preclinical studies (39). It also appears to exert an inhibitory effect on cancer cell invasion and angiogenesis (46).

Despite several side effects reported after long-term treatment with BPs, including osteonecrosis of the jaw, BPs are currently under investigation in postmenopausal bone loss and bone

lesions of tumoral origin, such as bone metastases from breast and prostate cancer with variable clinical benefits (47, 48). A significant decrease in bone resorption was observed in these studies but with no unequivocal effects on survival or the occurrence of metastases. The clinical effects of BPs on bone metastases from lung cancer are also discussed, as are their effects on visceral metastases (49–52). The encouraging results reported on bone remodeling, as well as the ability of BPs, and in particular ZOL, to induce tumor cell death *in vitro*, make them good candidates for a therapeutic strategy in primary bone tumors. ZOL may effectively inhibit both bone resorption and tumor proliferation in the vicious cycle, making it more efficient. With regard to primary bone tumors, several studies have already demonstrated the benefits of using ZOL in osteosarcoma (53–56), in particular, promising preclinical results have been reported on survival and tumor growth (56). In this context, ZOL has recently been combined with conventional chemotherapy and surgery for adult and pediatric patients in the French OS2006 phase III randomized clinical trial for osteosarcoma treatment. Following these results, other preclinical and clinical studies demonstrated the beneficial effect of BP treatment in osteosarcoma (57–61).

In Ewing sarcoma, despite improvements to chemotherapy protocols, the survival rate for patients with bone metastases remains very low. In this context, combining ZOL with current conventional chemotherapy may be a promising therapeutic option for both limiting tumor-associated osteolysis and preventing the development of bone metastases, which is currently the main factor for a bad prognosis for this pathology (62). Few fundamental and preclinical studies have demonstrated an antitumoral effect for ZOL on Ewing sarcoma cell lines (63, 64). Of these studies, our team has recently shown that ZOL significantly inhibits tumor cell viability by blocking the cell cycle in S-G_2M phase transition and by promoting caspase-3 activation (65). Using preclinical models of Ewing sarcoma induced in athymic mice by injecting human Ewing sarcoma cells either in bone site or in soft tissue, ZOL alone significantly inhibited tumor development in bone sites, decreasing osteolytic lesions and improving mouse survival (65). On the contrary, the same doses of ZOL had no effect on Ewing sarcoma progression in soft tissue. These results can be explained by the high tropism of BPs for the calcified bone matrix, leading to their elevated concentration in bone tissue and their rapid clearance from blood and soft tissue. These data correlate with other studies on soft tissue tumors or visceral metastases (39). On the other hand, we demonstrated the synergistic effect of a combination of ZOL and ifosfamide, a conventional drug used in Ewing sarcoma clinical protocols, on tumor progression in soft tissue (65). These results correlate with previous studies showing a synergistic effect between BPs and chemotherapeutical agents and demonstrate the great benefit of using ZOL in Ewing sarcoma treatments as a means of reducing the chemotherapy doses and as a consequence, their side effects (58, 66–68).

With regard to invasion and migration, we have already published that treatment with ZOL inhibits Ewing sarcoma cell migration *in vitro* in Boyden chambers and diminishes MMP-2 activity as revealed by zymography (69). In addition, less pulmonary metastases were observed in mice treated with ZOL compared to untreated animals, in a model of spontaneous metastases disseminated from primary Ewing sarcoma induced in bone (69).

For the transfer to clinical practice, one phase II study evaluating the combination of chemotherapy and pamidronate in osteosarcoma patients has demonstrated little impact on patient survival, but has been shown to improve the durability of limb reconstruction (61). In a recently completed phase I study, ZOL combined with conventional multi-drug chemotherapy was safe, but failed to reveal any significant differences in event-free or overall survival in patients with newly diagnosed metastatic osteosarcoma (70). There are three phase II/III trials currently in progress, evaluating the efficacy of ZOL as a single agent or an adjuvant to chemotherapy in localized and metastatic osteosarcoma (NCT00691236 and NCT00470223) and in Ewing sarcoma (NCT00987636).

However, long-term use of BPs may impact bone growth and tooth eruption in young patients. In our laboratory, we have carried out preclinical studies on newborn mice treated or not with ZOL, using a protocol that reproduces the frequency and doses administered in humans. ZOL induces a reversible arrest in bone growth that was also observed in young patients treated with zometa® (71). For tooth eruption, irreversible inhibition was observed (72).

As several side effects have been reported with the clinical use of BPs (49, 50), another approach to decrease bone resorption could therefore be to target RANKL, the main cytokine involved in osteoclast differentiation.

Anti-RANKL Strategies

Bone remodeling is strongly regulated thanks to the molecular triad OPG–RANKL–RANK (26). The binding of RANKL to its receptor RANK, expressed on the surface of osteoclast precursors, induces osteoclast differentiation *in vitro* in addition to macrophage-colony stimulating factor (M-CSF), suggesting that this differentiation plays an important role in bone biology. In the bone microenvironment, RANKL is produced by bone marrow stromal cells and osteoblasts, while in a bone tumor environment, it can be produced by other cell types, such as fibroblasts, epithelial cells, or T-lymphocytes, in which RANKL appears to be the final effector of osteoclast-mediated bone resorption (26). Cells from many tumor types, including multiple myeloma, prostate cancer, or even human neuroblastoma, can also express RANKL themselves (73–75). Moreover, many of the chemokines, cytokines, hormones, and growth factors produced by tumor cells are able to induce an increase in RANKL expression through PTH-rP, and a decrease in OPG production, thus aggravating the vicious cycle in bone metastases. RANK is one of the signaling molecules associated with worse outcomes in osteosarcoma. High expression of RANKL is associated with reduced survival in osteosarcoma, and it has been reported that osteosarcoma cell lines and biopsies show high expression of functional RANK, suggesting a potential autocrine stimulation of this pathway (76, 77). Inhibition of RANKL using the shRNA strategy reduced motility and anoikis resistance in osteosarcoma cell lines, whereas overexpression of RANK increased OS cell motility without affecting cell proliferation (78). One study reported the

preventive effect of siRNA–RANKL on tumor progression when associated with the chemotherapeutic agent ifosfamide in a preclinical model of osteosarcoma (79). For Ewing sarcoma, only a few studies are available, but it seems that these cells express only a low level of RANKL (3). In our case, preliminary preclinical studies evidenced localized but strong expression of RANKL in a paratibial model of Ewing sarcoma induced by an intramuscular injection of human A673 cells in Nude mice (80). The advantages of targeting RANKL have previously been reported in both bone metastases and primary bone tumors and might be a promising target in Ewing sarcoma (73, 74, 81, 82). Several molecules targeting RANKL have already proved their efficiency in other malignant bone pathologies, such as osteosarcoma, and might be a potent therapeutic agent in Ewing sarcoma.

Osteoprotegerin, a member of the TNF receptor super-family, is a ubiquitous secreted homodimeric cytokine able to bind RANKL and then inhibit the RANK/RANKL interaction, as well as any further osteoclast differentiation and activation (83–85). A disruption in the RANKL/OPG ratio in favor of RANKL has been shown to be responsible for severe osteolysis in a tumoral context (86). Accordingly, overexpressing OPG to restore this equilibrium between OPG and RANKL expression appears to be a promising approach for limiting tumor-associated bone lesions. For the first time, our team has shown significant therapeutic benefits of OPG in primary bone tumors. In a preclinical model of osteosarcoma, OPG delivered by non-viral gene transfer effectively inhibited tumor growth and tumor-associated osteolysis, significantly increasing animal survival (81). Several studies have tested OPG overexpression in OS and Ewing sarcoma preclinical models with promising results, especially in osteosarcoma (81). Moreover, despite its clinical efficiency in preventing osteolytic lesions, a major issue for OPG-Fc administration as an adjuvant therapeutic agent in a tumor context is its ability to inhibit the apoptosis induced by TNF-related apoptosis inducing ligand (TRAIL) (87). The dual effect of OPG may inhibit TRAIL-induced apoptosis of tumor cells, a natural mechanism for preventing tumor development (88). In addition, TRAIL's ability to both induce apoptosis in sensitive Ewing Sarcoma cell lines and prevent tumor development has already been demonstrated *in vitro* by Wietzerbin's team and *in vivo* by our team in a preclinical model induced by intratibial injection of Ewing sarcoma cells in nude mice (89, 90). To avoid the potential protumoral effect of OPG, the recombinant protein RANK-Fc, the soluble form of RANK, could be used in Ewing sarcoma to block RANKL activity. RANK-Fc is unable to bind TRAIL, and its efficacy has already been demonstrated in preventing tumor-associated osteolysis and, indirectly, tumor growth in preclinical models of bone metastases, such as prostate, lung, and breast cancer (91–93). Our team also showed how RANK-Fc, when delivered by non-viral gene transfer, is able to prevent osteolytic lesions and tumor development, thus inducing an increase in animal survival in a preclinical rodent model of osteosarcoma (82). The same efficacy can be expected in Ewing sarcoma but remains to be tested. For clinical transfer, denosumab is a monoclonal antibody specific for human RANKL, which was initially developed to treat osteoporosis (94). It was then used for painful bone metastases with effective results (95–98). It was subsequently found to also be effective for giant cell tumor of bone, a benign but destructive neoplasm with severe osteolytic lesions, in which transformed mononuclear cells secrete high levels of RANKL, causing osteoclast hyperactivity (99).

TARGETING OTHER ASPECTS OF THE BONE MICROENVIRONMENT IN EWING SARCOMA

Besides bone cells themselves, the tumor microenvironment of primary bone tumors provides factors that are favorable for tumor initiation, progression, therapy resistance, or metastatic dissemination. Of the different constituents or aspects of this peculiar microenvironment, special attention has been paid to hypoxia, escape from the immune system, angiogenesis, growth factors from the microenvironment, and modification of the microenvironment itself by therapeutic agents that may interfere with tumor progression (**Figure 4**).

Hypoxia is an important condition in the tumor cell microenvironment associated with a more aggressive phenotype and poor prognosis of many cancers in adults. For example, intratumoral hypoxia is a common finding in breast cancer associated with a significantly increased risk of metastasis and patient mortality (100). Hypoxia-inducible factors activate the transcription of a large battery of genes encoding proteins that promote primary tumor vascularization and growth, stromal cell recruitment, extracellular matrix remodeling, pre-metastatic niche formation, cell motility, local tissue invasion, extravasation at sites of metastasis, and maintenance of the cancer stem cell phenotype that is required to generate secondary tumors. It is also known that severe and long-lasting hypoxia results in necrosis, thus being correlated with unfavorable outcome. Concerning Ewing sarcoma, a clinical study previously reported a strong correlation between the presence and the amount of necrotic areas in the tumor with the risk of metastases (101). In addition, Aryee et al. reported that HIF-1α expression was detectable in 18/28 primary tumors from the Ewing sarcoma family and that EWS-FLI1 was up-regulated in a HIF-1α-dependent manner (102). In addition, this study revealed that hypoxia stimulated the invasiveness and soft agar colony formation of Ewing sarcoma cells *in vitro*. Further studies suggest that EWS-FLI1 regulation in an hypoxic environment may occur at the posttranscriptional level, which is supported by the observation that HIF-1α-activated genes, such as VEGF, Aldolase-C, GLUT-1, CA9, and IGFBP3, were increased under hypoxia, whereas EWS-FLI1 RNA expression remained unchanged (103). It is also suggested that hypoxia increases Ewing sarcoma malignancy through enhancing invasive and colony-formation capacities. Furthermore, it could be proposed that hypoxia may contribute to the aggressive metastatic behavior of Ewing sarcoma, as HIF-1α and EWS-FLI1 may function together in both synergistic and antagonistic cross-talk under hypoxia conditions. Therefore, drugs that target hypoxia need to be tested in Ewing sarcoma models.

Crosstalk between the bone niche and the immune system, known as "osteoimmunology," has been suggested as being a potential target for bone tumor treatment. There is a well-recognized link

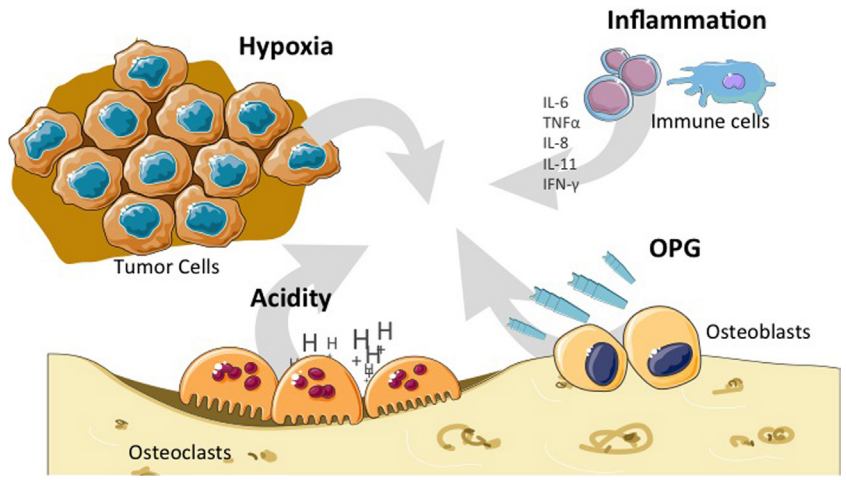

FIGURE 4 | Particular bone microenvironment that may affect tumor initiation, progression, or dissemination. Bone microenvironment is characterized by high hypoxia, high acidity, OPG release, and the influence of the cytokines (IL-6, IL-8, IL-11, and TNF-α) produced by cells from the immune system. IL, interleukin; TNF, tumor necrosis factor; OPG, osteoprotegerin.

between bone constituents and the immune system, leading to recent efforts to elucidate the functions of molecules expressed in both bone and immune cells. A recent review nicely describes the complexity of the interaction between the skeletal and immune systems, suggesting that their interdependency needs to be taken into consideration when designing therapeutic approaches for either of the two systems (31). For example, denosumab, which was originally used to specifically target bone resorption, is now under evaluation for its effect on the long-term immune response. As both the bone and immune systems are often disrupted in cancer, they may be crucial in regulating tumor growth and progression. Certain therapies, such as BPs and RANKL-targeted drugs that aim to reduce pathological osteolysis in cancer, may interact with the immune system, thus providing favorable effects on survival. Another interesting publication reported that dynamic tumor–host immune interactions within the tumor microenvironment may polarize immune responses *in situ*, influencing tumor development and/or progression (104). They studied the nature of tumor–host immune interactions within the Ewing sarcoma microenvironment, analyzing the presence and spatial distribution of infiltrating CD8(+)(/)CD4+ T-lymphocytes in therapy-naive Ewing sarcoma. They observed that tumor-infiltrating T-cells contained significantly higher percentages of CD8(+) T-lymphocytes than stroma-infiltrating cells, suggesting preferential migration of this type of T-cell into tumor areas. Their results indicated that an inflammatory immune microenvironment with high expression of type 1-associated chemokines may be critical for the recruitment of CD8(+) T-lymphocytes expressing the corresponding chemokine receptors. The observed impact of tumor-infiltrating CD8(+) T-lymphocytes is consistent with there being a role for adaptive anti-tumor immunity in preventing Ewing sarcoma from progressing. Recognizing the merits and exploitation/induction of an inflammatory microenvironment may thus improve the efficacy of natural responses against, and (adoptive) immunotherapeutic approaches for, Ewing sarcoma.

With regard to angiogenesis, VEGF-165 expression in the tumor microenvironment has been shown to influence the differentiation of bone marrow-derived pericytes, which play a part in the vasculature of Ewing sarcoma (105). One year later, the same team demonstrated that VEGF-165 contributed to the osteolytic process in Ewing sarcoma by upregulating RANKL (106). They showed that VEGF-165, together with EWS-FLI1, increased RANKL promoter activity. This increase in *RANKL* gene expression in the bone marrow microenvironment during the metastatic process may be involved in tumor-induced bone osteolysis.

Other *growth factors* present in the bone microenvironment, such as basic FGF, may play a part in tumor progression as they enhance cell motility and invasion of the Ewing sarcoma family of tumors by activating the FGFR1–PI3K–Rac1 pathway (107). The authors therefore conclude that the bFGF–FGFR1–PI3K–Rac1 pathway in the bone microenvironment may have a significant role in the invasion and metastasis of the Ewing sarcoma family of tumors.

Conversely, *therapeutic agents*, such as ZOL, are able to modify the bone microenvironment surrounding primary or disseminated tumor cells, as has been reported in breast cancer recurrence in bone (108). Treatment of mice with ZOL induced a rapid increase in trabecular bone volume versus controls, which was reflected by a significant reduction in osteoclast and osteoblast numbers per millimeter in trabecular bone, and reduced bone marker levels in serum. Pre-treatment with ZOL caused an accumulation of extracellular matrix in the growth plate associated with a trend for preferential homing of tumor cells to osteoblast-rich areas of bone, but without affecting the total number of tumor cells. The number of circulating tumor cells was reduced in ZOL-treated animals. Although this study concerns breast cancer, osteoblasts may be key components in the bone metastasis/tumor niche, and therefore a potential therapeutic target, at least in breast cancer. This hypothesis therefore needs to be studied extensively in primary bone tumors, including Ewing sarcoma.

BONE MICROENVIRONMENT MODELING IN EWING SARCOMA

As the microenvironment, and especially the bone tumor microenvironment, can both inhibit and facilitate tumor growth and metastatic dissemination, better modelization of the tumor bone niche is needed to characterize tumor cell–stroma interaction in depth. It has been shown that osteoblasts, osteoclasts, fibroblasts, myeloid cells, and mesenchymal stem cells (MSCs) play essential roles in primary tumor growth and metastasis (109, 110). However, current *in vitro* approaches are far from replicating the native *in vivo* milieu in which tumors develop, a necessary condition for advancing cancer research and translating new therapies into clinical practice. Most preclinical anti-neoplastic drug testing is still carried out on conventional 2D cell culture systems. Although these systems mimic some of the phenotypic traits observed clinically, they are limited in their ability to model the full range of microenvironmental interactions, such as 3D cell–cell and cell–extracellular matrix interactions. Several teams have thus established *ex vivo* 3D bone tumor models that closely mimic the morphology, growth kinetics, and protein expression profile of human tumors, including Ewing sarcoma (111–113). For example, Ewing sarcoma cells cultured in porous 3D electrospun poly(ϵ-caprolactone) scaffolds were not only more resistant to traditional cytotoxic drugs than cells in 2D monolayer cultures but also exhibited remarkable differences in the expression pattern of the IGF-1R/mTOR pathway (111). This 3D model of the bone microenvironment may therefore have broad applicability for mechanical studies of bone sarcomas and shows the potential for increasing preclinical evaluation of anti-neoplastic drug candidates for these malignancies. In the same way, Villasante et al. described a bioengineered model of human Ewing sarcoma that mimics the native bone tumor niche with high biological fidelity (113). In this model, cancer cells that have lost their transcriptional profiles after monolayer culture re-express genes related to focal adhesion and cancer pathways. The bioengineered model recovers the original hypoxic and glycolytic tumor phenotype and makes possible re-expression of angiogenic and vasculogenic mimicry features that favor tumor adaptation. Differentially expressed genes between the monolayer cell culture and native tumor environment may thus be potential therapeutic targets that could be explored using the bioengineered tumor model.

In addition, Ludwig's team has highlighted a number of innovative methods used to fabricate biomimetic Ewing sarcoma, including both the surrounding cellular milieu and the extracellular matrix. These methods suggest potential applications for advancing our understanding of the biology of Ewing sarcoma, preclinical drug testing, and personalized medicine (112).

Finally, it appears that the bone microenvironment should be modelized in order to analyze the response of bone tumor cells to drug screening under optimal conditions. Currently, few preclinical models of bone cancer, and particularly Ewing sarcoma, mirror the site of the disease in patients, as they are mostly subcutaneous or intramuscular xenografts (114).

For metastasis in Ewing sarcoma, intravenous models induced in non-obese diabetic/severe combined immunodeficient (NSG) mice showed a pattern of disease spread similar to that found in patients, but only 23% of the experimental mice developed assessable bone metastases (115). It is therefore preferable to develop orthotopic models that involve direct injection of Ewing sarcoma cells at the clinically relevant site, i.e., intrafemoral. This type of injection in immunocompromized mice provides a technically feasible and reproducible approach, resulting in tumors that are detectable by palpation or *in vivo* imaging, and that closely resemble those observed in patients (116). The importance of such orthotopic models for testing potential new drugs at the preclinical level was emphasized in the study by Odri et al. (65), comparing how tumor progression responds to ZOL in two models of Ewing sarcoma: one induced by tumor cell injection in the medullar cavity of tibia and the other with initial progression in soft tissue (65). ZOL significantly inhibited Ewing sarcoma cell progression only in the intratibial model and showed no effect in the soft tissue. These results strongly suggest the importance of considering the complete bone microenvironment when testing new drugs, especially in the case of bone tumors, such as Ewing sarcoma. Recently, Vormoor et al. also developed an interesting preclinical orthotopic model of Ewing sarcoma in NSG mice, reproducing the biology of the tumor–bone interactions observed in human disease (117). In this model, the Ewing sarcoma cells have been modified allowing *in vivo* monitoring of disease progression (115). The authors therefore demonstrated the utility of small animal bioimaging for tracking disease progression, making this model a useful assay for preclinical drug testing.

CONCLUSION – PERSPECTIVES

Despite improvements in poly-chemotherapy combinations and surgical approaches preserving limbs from amputation, one group of Ewing sarcoma patients still remains at high risk, with poor survival rates. These patients present with metastatic disease at diagnosis or respond poorly to chemotherapy due to acquired resistance. New therapeutic options are thus needed. Given the growing interest in the microenvironment and its recognized involvement in cancer initiation and progression, it is relevant to propose therapeutic strategies that target molecular and/or cellular protagonists of the bone tumor microenvironment in the case of Ewing sarcoma.

Most on-going studies focus on bone cells, especially osteoclasts, either by directly targeting them or inhibiting RANKL, the main cytokine involved in osteoclast activation. These strategies (BPs, anti-RANKL: denosumab) could be proposed not only to target the bone component of the primary tumor but also to target bone/bone marrow metastases, the worst prognosis factor for Ewing sarcoma patients, as confirmed in the R3 arm of the latest EuroEWING99 trial (survival rate of <20% at 5 years). However, as expected, these strategies have no effect in preclinical models of pulmonary metastases, which remains the main cause of mortality in Ewing sarcoma patients (the prognosis for patients with lung-only metastases is 30% survival at 5 years). However, the strategies could be proposed for pulmonary metastatic patients or patients with soft tissue Ewing sarcoma if they are in synergy with current or targeted therapies, as suggested by our preclinical studies combining ZOL with ifosfamide (65).

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Immunostimulation by OX40 ligand transgenic Ewing sarcoma cells

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Interleukin-2 (IL-2) transgenic Ewing sarcoma cells can induce tumor specific T and NK cell responses and reduce tumor growth *in vivo* and *in vitro*. Nevertheless, the efficiency of this stimulation is not high enough to inhibit tumor growth completely. In addition to recognition of the cognate antigen, optimal T-cell stimulation requires signals from so-called co-stimulatory molecules. Several members of the tumor necrosis factor superfamily have been identified as co-stimulatory molecules that can augment antitumor immune responses. OX40 (CD134) and OX40 ligand (OX40L = CD252; also known as tumor necrosis factor ligand family member 4) is one example of such receptor/ligand pair with co-stimulatory function. In the present investigation, we generated OX40L transgenic Ewing sarcoma cells and tested their immunostimulatory activity *in vitro*. OX40L transgenic Ewing sarcoma cells showed preserved expression of Ewing sarcoma-associated (anti)gens including lipase member I, cyclin D1 (CCND1), cytochrome P450 family member 26B1 (CYP26B1), and the Ewing sarcoma breakpoint region 1-friend leukemia virus integration 1 (EWSR1-FLI1) oncogene. OX40L-expressing tumor cells showed a trend for enhanced immune stimulation against Ewing sarcoma cells in combination with IL-2 and stimulation of CD137. Our data suggest that inclusion of the OX40/OX40L pathway of co-stimulation might improve immunotherapy strategies for the treatment of Ewing sarcoma.

Keywords: Ewing sarcoma, immunotherapy, co-stimulation, OX40/OX40L system, tumor necrosis factor (receptor) superfamily

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INTRODUCTION

Ewing sarcomas (or "Ewing family tumors," EFT) represent bone and/or soft-tissue tumors of uncertain histogenetic origin. The majority of the cases are observed in children and young adults. Today, more than half of the patients with localized EFT can be cured. However, the prognosis for patients with disseminated disease or early relapses remains poor with conventional therapy (1). EFTs are characterized by the expression of tumor-specific oncofusion proteins (2). These fusion proteins are highly tumor specific and might be interesting targets for immunological treatment strategies. However, peptides derived from these proteins have only low binding affinity to common human leukocyte antigen (HLA) class I molecules (3). Using high-density DNA microarrays, we identified additional potential tumor antigens expressed in EFT (4–7). The presence of such tumor-specific antigens alone is not sufficient for the induction of efficient immune responses. Additional co-stimulatory signals are required. We demonstrated that interleukin-2 (IL-2) transgenic EFT cells

can induce immune responses against wild-type tumor cells *in vitro* and in a xenotransplantation model (8–10). However, tumor growth was not completely inhibited in this model, suggesting that additional co-stimulatory signals might be required. One group of such co-stimulatory molecules is represented by members of the tumor necrosis factor (TNF) superfamilies (SF) consisting of the superfamily of TNF receptors (TNFRSF) and the corresponding ligands (TNFSF). The eponymic member of the TNFSF has been initially characterized as a factor with antitumor activity in mice (11). TNF is the prototype of a large gene family, which has several immune-regulatory functions and can augment antitumor immune responses (12, 13). Members of the tumor necrosis factor receptor superfamily comprise a group of type I membrane glycoproteins consisting of more than 50 members that have been identified as co-stimulatory molecules that augment antitumor immune responses. Activation of these surface receptors by the natural ligands or by agonistic antibodies leads to different cellular responses ranging from cell differentiation, proliferation, apoptosis, and survival to enhanced production of cytokines and chemokines (13–16). The differential and unique expression of the TNFRSF molecules on cells of the immune system has made these molecules as ideal targets for new immune therapy strategies (13, 15). OX40 (CD134) and CD137 (4-1BB) and their ligands OX40L (CD252) and 4-1BBL are examples of such co-stimulatory molecules. CD137 (4-1BB) is an activation-inducible TNFRSF member expressed on activated T cells (CD8-positive and CD4-positive T cells) and is also expressed on a variety of immune cell lineages including activated natural killer cells, human macrophages, eosinophils, and dendritic cells (17). The natural ligand for CD137 (4-1BBL) is mostly expressed on professional antigen-presenting cells or in inflamed non-hematopoietic tissues (15).

Recently, we analyzed the effects of the CD137/4-1BBL system in our Ewing sarcoma immune-therapy model (10). 4-1BBL transgenic cells or agonistic antibodies against CD137 can induce rejection of varying tumors *in vivo* (18, 19). In our Ewing sarcoma model, we observed modulation of immunosuppressive indoleamine 2,3-dioxygenase 1 (IDO) expression by stimulation of the CD137/4-1BBL system (10). However, engagement of this co-stimulatory system had only limited efficacy for enhancing the immunostimulatory activity of EFT cells (10). The OX40/OX40L system represents another highly interesting co-stimulatory system. OX40 (CD134) was identified as cell surface molecule on activated T cells (20). OX40 is preferentially expressed on CD4-positive T cells (21–23). Optimal antigenic stimulation induces OX40 expression also on CD8-positive T cells (24). The human OX40 molecule has a molecular weight of 50 kDa and is encoded on chromosome 1p36. Murine and human OX40 have only approximately 62% sequence homology in the intracellular domain and <64% in the extracellular domain (25, 26). OX40 is absent from unstimulated peripheral blood mononuclear cells (PBMCs) and most antigen-presenting cells (27). OX40 expression peaks 48 h after stimulation of naive T cells, whereas memory T cells express high levels 4 h after restimulation (28). In contrast to the OX40 receptor, the ligand OX40L (CD252, TNFSF4) is expressed on several professional antigen-presenting cell types, endothelial cells, and activated T cells (29–32). Human OX40L has a molecular weight of 34 kDa and is located on chromosome 1q25 (25, 26). Activation of the OX40 receptor by OX40L or an agonistic antibody leads to increased expression of antiapoptotic molecules and reduced expression of the inhibitory cytotoxic T-lymphocyte antigen 4 (CTLA4) (25, 33, 34). An important aspect of OX40 for antitumor immune responses is the observation that the OX40/OX40L system favors the development of tumor-specific memory T cells and T cells expressing OX40 have been found in tumor-draining lymph node cells and in tumor-infiltrating lymphocytes from patients with various tumors (15, 35). In addition, direct enhancement of cytotoxic T cells by OX40 stimulation has been proposed (36). Therefore, in the present investigation, we established OX40L overexpressing Ewing sarcoma cells for analyzing the effects of OX40 stimulation in our immunotherapy model.

MATERIALS AND METHODS

Gene Expression Analysis and Cloning of OX40L

RNA from cell lines was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) following manufacturer's protocol. Two micrograms of the RNA was transcribed into cDNA and used as template for polymerase chain reaction (PCR). Reverse transcription of RNA was performed by using the following conditions: 4 μL 5× buffer, 1 μL Oligo-dT12-18 primer, 1 μL dNTP mix (10 mM), 1 μL Revert Aid H-M-MuLV reverse transcriptase (Fermentas, St. Leon Rot, Germany); 37°C, 60 min; and 90°C, 5 min. After reverse transcription, 2 μL cDNA was mixed with 2.5 μL 10× buffer, 1.5 μL $MgCl_2$ (25 mM), 0.2 μL Taq-polymerase (Promega, Mannheim, Germany), 0.5 μL dNTP mix (10 mM; Fermentas), 0.25 μL of sequence specific primers (MWG-Biotech AG, Ebersberg, Germany), and 17.8 μL water. The following primer combinations were used: actin beta (ACTB): 5′-GGC ATC GTG ATG GAC TCC G-3′ and 5′-GCT GGA AGG TGG ACA GCG A-3′; cyclin D1 (CCND1): 5′-AAC TAC CTG GAC CGC TTC CT-3′ and 5′-CCA CTT GAG CTT GTT CAC CA-3′; CD99: 5′-TCC TCC GGT AGC TTT TCA GA-3′ and 5′-TCC CCT TGT TCT GCA TTT TC-3′; OX40L (primer combination 1): 5′-aac tcg agT ATC GCA CGT TCC CCT T-3′ (nucleotides in lower case: *Xho*I restriction site) and 5′-aac cgc ggC CAG GAT CTG CTT-3′ (nucleotides in lower case: *Sac*II restriction site); OX40L (primer combination 2): 5′-GTG AAT GGC GGA GAA CTG AT-3′ and 5′-GCC AGG ATC TGC TTC TTG TC-3′; cytochrome P450 26B1 (CYP26B1): 5′-TGA CAG GAT CCC TGT GTT GT-3′ and 5′-CCA ACA TCG AAA GTG CTT CA-3′; enhanced green fluorescent protein (eGFP): 5′-ACG TAA ACG GCC ACA AGT TC-3′ and 5′-AAG TCG TGC TGC TTC ATG TG-3′; Janus kinase 1 (JAK1): 5′-TGT AAG GAG CTG GCT GAC CT-3′ and 5′-CAC CTG CTC CCC TGT ATT GT-3′; lipase H (LIPH): 5′-GAT GGC TGG GGA GAA TTA CA-3′ and 5′-TGG ATT CTG TGG TGT TTC CA-3′; lipase I (LIPI): 5′-TCC GAG AAT AGA GAC CAT TCT GA-3′ and 5′-GCT CTC TGG TGG TTG CAT TT-3′; neomycin resistance cassette (NeoR): 5′-AGA CAA TCG GCT GCT CTG AT-3′ and 5′-AGT GAC AAC GTC GAG CAC AG-3′. The PCR conditions were as follows: 94°C,

30 s; 60°C, 45 s; and 72°C, 45 s (35 cycles). Each PCR program started with a denaturation step (95°C, 5 min) and was finished with 72°C for 5 min followed by cooling down to 4°C. The PCR products were subjected to agarose gel (1.5%) electrophoresis in the presence of ethidium bromide. DNA microarray data were visualized by using Genesis (37). DNA microarray data from EFT cell lines (4, 38), human embryonic kidney 293 (HEK293) cells (39), neuroblastoma (NB) cell lines (4), acute lymphatic leukemia (ALL) cell lines (40, 41), acute myeloid leukemia (AML) cell lines (42), Hodgkin's lymphoma (HL) cell lines (43), Epstein–Barr virus-immortalized lymphoblastoid cell lines (LCL) (44), and normal PBMCs (45) were used. For cloning of OX40L, cDNA was amplified by using the OX40L primer combination 1. PCR products and vector pIRES2-eGFP (Clontech, Mountain View, CA, USA) were digested with *Xho*I and *Sac*II. After agarose gel purification, ligation, and transformation into *Escherichia coli* XL1-Blue, individual clones were sequenced by using primers 5′-CAA GTC TCC ACC CCA TTG AC-3′, 5′-GTG AAG ATG GAA AGG GTC CA-3′, 5′-aac cgc ggC CAG GAT CTG CTT-3′, and 5′-CAG GGC ATG GAT TCT TCA TT-3′. For sequencing, a 10 μL sequencing mix was used that contained 0.5 μL gene-specific sequencing primers (10 μM), 4.0 μL BigDyeTerminator Cycle Sequencing Kit mix (Applied Biosystems, Foster City, CA, USA), and 10–30 ng DNA. Sequence analysis was performed using ABI Prism™ 310 Genetic Analyzer (Applied Biosystems). A clone with complete error-free OX40L open reading frame was used for further analysis. This clone differs from the reference sequence by a T to C transition in the 3′-untranslated region (corresponding to residue 738 in reference sequence NM_003326).

Cells and Cell Culture

A673 cells and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SK-N-MC cells and SH-SY5Y cells were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Brunswick, Germany). 4-1BBL transgenic A673 cells and stimulation of PBMCs with anti-CD137 antibodies (clone 26G6; a kind gift from R. Mittler) was described elsewhere (10). PBMCs were isolated from healthy donors with informed consent and approval by the local ethics committee as described (45). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and penicillin and streptomycin. For selection of transgenic cells, medium was supplemented with 400 μg/mL geneticin sulfate. PBMCs from HLA-A1,A2 positive donors were isolated as described (44, 45). Stimulation of PBMCs and enzyme-linked immunospot (ELISPOT) analysis was performed as described (7) by using an interferon gamma ELISPOT kit (Becton-Dickinson, Heidelberg, Germany). Transfection of cells was performed using jet PEI (Qbiogene, Carlsbad, CA, USA). MACS separation was performed using anti-PE microbeads (Miltenyi, Bergisch Gladbach, Germany). Statistical analysis was performed with Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

Flow Cytometry

Phycoerythrin (PE)-labeled mouse anti-human-OX40L antibodies were purchased from Anzell (Bayport, USA). Mouse IgG1 isotype control was purchased from Becton-Dickinson. Flow cytometry was performed as described (40), and cells were analyzed on a FACScan flow cytometer (Becton-Dickinson) equipped with CellQuest Pro software (Becton-Dickinson).

RESULTS

Cloning of OX40L into a Mammalian Expression Vector

We screened DNA microarray data of varying cell types for samples with high expression of OX40L. Highest expression was observed in lymphoblastoid B cell lines (LCL) (**Figure 1A**). High expression of OX40L in LCL was validated by RT-PCR (**Figure 1B**). All analyzed LCL expressed OX40L. In contrast, no expression of OX40L was detectable in the B-cell leukemia cell line NALM6 (**Figure 1B**). Primer combination 1 amplifies the complete open reading frame of OX40L. The amplificate from LCL with this primer combination was eluted from agarose gels and cloned into vector pIRES2-eGFP. Functionality of the vector was assessed by transient transfection of HEK293 cells (**Figure 1C**). Wild-type HEK293 cells are negative for OX40L (**Figure 1A**). After transfection with the OX40L-containing vector, HEK293 cells clearly expressed OX40L on the surface (**Figure 1C**). HEK293 cells that had been transfected with the empty vector were not stained with antibodies against OX40L. Green fluorescence of eGFP indicated that these cells were successfully transfected with similar efficiency as the OX40L transfected cells (**Figure 1C**).

Generation of OX40L Transgenic Ewing Sarcoma Cells

We transfected cells from the Ewing sarcoma cell lines A673 and SK-N-MC with OX40L in vector pIRES2-eGFP. Transfected cells were selected with geneticin sulfate and further enriched by staining with PE-labeled anti-OX40 antibodies and immunomagnetic beads directed at PE. After magnet-activated cell sorting (MACS), more than 80% of the cells expressed eGFP and OX40L (**Figure 2A**). For immunotherapy with transgenic tumor cells, it is necessary that the tumor cells can be irradiated without functional impairments. Therefore, we tested the stability of OX40L after irradiation. As shown in **Figure 2B**, OX40L remained stable after irradiation for at least 5 days. Another prerequisite of transgenic tumor cells for immunotherapy is the stability of tumor antigen expression in these cells. We tested the presence of Ewing sarcoma-associated transcripts in the transgenic cells. OX40L transgenic Ewing sarcoma cells showed the same gene expression pattern as wild-type cells or mock-transfected cells (**Figure 3**). In contrast to wild-type cells, OX40L transgenic cells and mock transgenic cells expressed transcripts for the vector marker eGFP and the NeoR. Importantly, transgenic Ewing sarcoma cells stably expressed the Ewing sarcoma-specific oncofusion protein EWS-FLI1 (**Figure 3**). Other genes typically expressed in Ewing sarcoma cells that remained stably expressed after transfection included the putative cancer/testis antigen LIPI (4, 5), LIPH (46), the surface glycoprotein CD99 (47), cyclin D1 [CCND1 (4, 48)], janus kinase 1 [JAK1 (4)], and the retinoic acid metabolizing cytochrome P450 member 26B1 [CYP26B1 (4, 49)] (**Figure 3**).

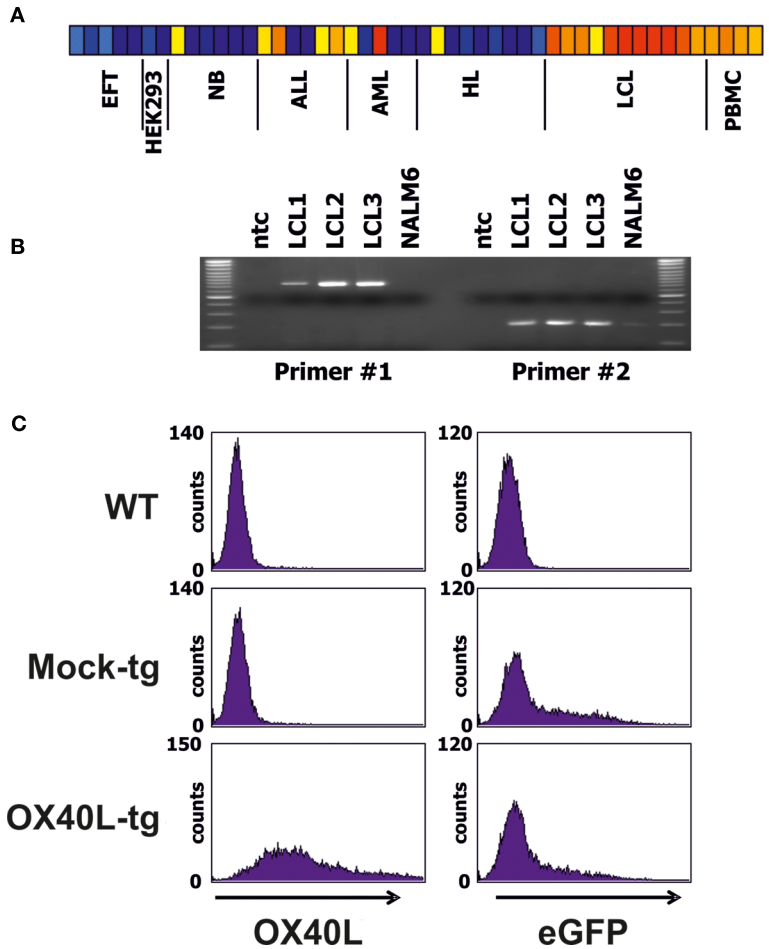

FIGURE 1 | Expression pattern and cloning of OX40L. **(A)** DNA microarray data from a panel of cell lines and normal PBMCs were analyzed for expression of OX40L. Presented is a heat map of OX40L signal intensities form EFT cell lines, HEK293 cells, neuroblastoma (NB) cell lines, acute lymphoid (ALL) and myeloid (AML) leukemia cell lines, Hodgkin's lymphoma (HL) cell lines, lymphoblastoid cell lines (LCL), and normal PBMCs. Red and yellow correspond to high signal intensities and blue corresponds to low signal intensities. From left to right, the following cell lines are shown: A673 (two samples), SK-N-MC, SBSR-AKS (38) (two samples), HEK293 (two samples), CHP-126 (two samples), SiMa, SH-SY5Y (three samples), RPMI, Loucy, Karpas, CALL2, 697, NALM6, U937, Kasumi, KG1, HL60, SKNO, L-428, HD-MyZ, KM-H2, HDLM-2, L-1236 (three samples), L-540 (two samples), 11 independent LCL, and four independent PBMC samples. **(B)** Expression of OX40L was analyzed by RT-PCR in three LCL and NALM6 ALL cells. Two different primer combinations were used. ntc = no template control. **(C)** After amplification of OX40L with primer combination 1 (see Materials and Methods), PCR products from LCL were cloned into vector pIRES2-eGFP. Functionality of the vector was assessed by transfection of HEK293 cell. Empty pIRES2-eGFP without OX40L (Mock) served as control. Transfected cells were stained with anti-OX40L-PE antibodies and analyzed by flow cytometry. eGFP served as marker for transfected cells.

Assessment of the Immunostimulatory Activity of OX40L Transgenic Ewing Sarcoma Cells

For the following experiments, we used A673 Ewing sarcoma cells with the partial HLA type A1,A2 for which HLA-matched PBMCs are easily available (9, 10). HLA-matched (HLA-A1,A2 positive) PBMCs were stimulated with A673 cells in the presence or absence of OX40L transgenic cells, interleukin 2, and antibodies against CD137 or 4-1BBL transgenic A673 cells. Reactivity of the primed cells against wild-type A673 cells was assessed by ELISPOT analysis (**Figure 4**). As expected (9, 10), the presence of interleukin 2 increased the number of cells that reacted with the sarcoma cells. In combination with interleukin 2, OX40L-transfected A673 cells marginally increased the reactivity of the primed cells compared to mock-transfected cells. Addition of antibodies against CD137 enhanced this effect significantly. In the presence of interleukin 2, the combination of anti-CD137 stimulation and OX40L transfected cells showed significantly enhanced immune stimulation in comparison to anti-CD137 antibodies or OX40L-transfected cells alone. The combination of OX40L transgenic cells with 4-1BBL transgenic cells showed a trend for higher stimulatory activity compared to 4-1BBL transgenic cells alone (**Figure 4**). After priming in the presence of OX40L transgenic A673 cells, the cells showed a higher specificity for A673 cells than for SK-N-MC cells or the neuroblastoma cell line SH-SY5Y (**Figure 5**).

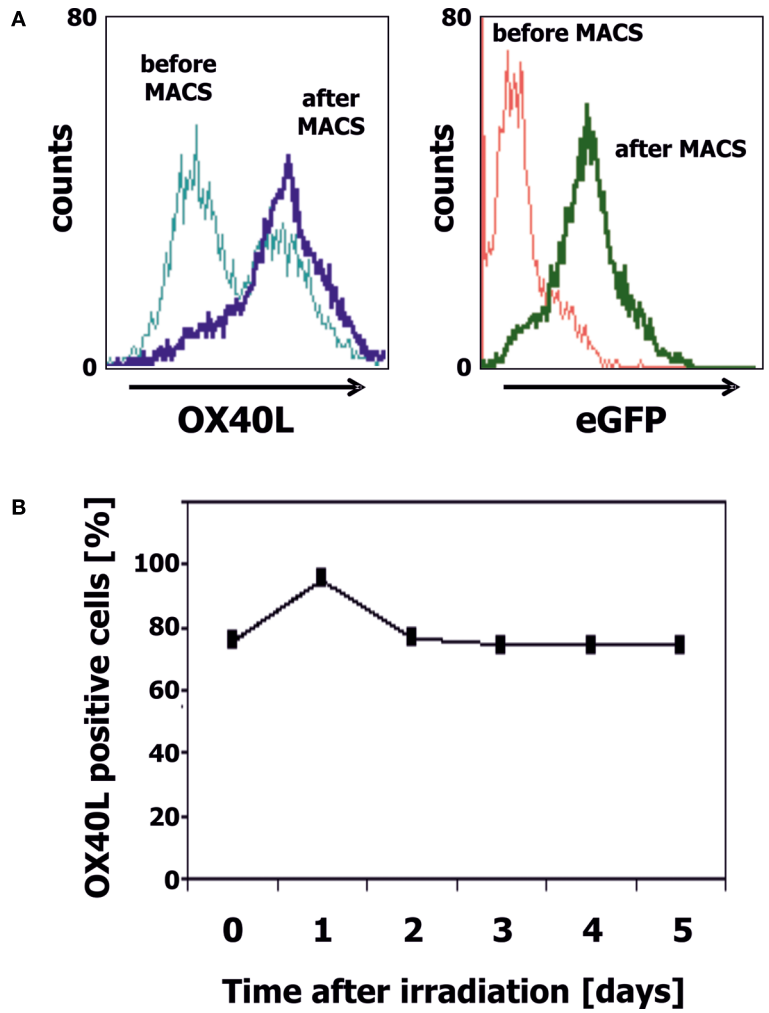

FIGURE 2 | Generation of OX40L transgenic EFT cells. **(A)** A673 EFT cells were transfected with OX40L in vector pIRES2-eGFP. Transfected cells were stained with anti-OX40L-PE antibodies and analyzed by flow cytometry. eGFP served as marker for transfected cells. Transgenic cells were enriched by using anti-PE-microbeads. After enrichment, nearly all cells stained positive for OX40L (dark blue line) and eGFP (green line). **(B)** A673 EFT cells were irradiated with 30 Gy and cultured for 5 days. Stability of OX40L expression after irradiation was assessed by staining with anti-OX40L-PE antibodies and flow cytometry.

DISCUSSION

The role of cancer immunotherapy that boosts the extraordinary power of our immune system to detect and destroy cancer cells still remains unclear. Acquired adaptive cancer immunotherapy regimens represent the most promising new treatment strategies, which have the ability to detect and kill cancer cells specifically and which have the potential to achieve a long-lasting antitumor response. Although tumor cells express tumor-specific antigens that can be recognized and targeted by T cells, the tumor produces different molecular and cellular mechanisms that reduce the ability of the immune system to recognize or kill tumor cells (50). There are many different mechanisms in the tumor microenvironment that suppress ongoing T-cell functions and enable tumor escape (51, 52). For instance, the tumor microenvironment can reduce activation of T cells, tumor cells can escape immune recognition by downregulation of tumor-associated antigens or antigen-presenting HLA molecules, tumor cells can produce antigen-loss variants, tumor cells can secrete immunosuppressive factors (e.g., indoleamine-2,3-dioxygenase), and co-stimulatory signals can be absent from antigen-presenting cells (53–57). Naive T cells require a strong interaction between the T-cell receptor and antigen-presenting HLA molecules (signal 1) and binding of co-stimulatory molecules expressed on the surface of antigen-presenting cells (signal 2) for optimal activation (58). In the absence of a co-stimulatory signal, T cells typically enter a state of anergy or paralysis (59). Some members of the TNFRSF have been identified as co-stimulatory molecules that augment antitumor immune responses. Activation of these surface receptors by their natural ligands or by agonistic antibodies leads to different cellular responses ranged from cell differentiation, proliferation, apoptosis, and survival to enhanced production of cytokines and chemokines (11, 13, 15, 16). The expression of the TNFRSF molecules on cells of the immune system has made these

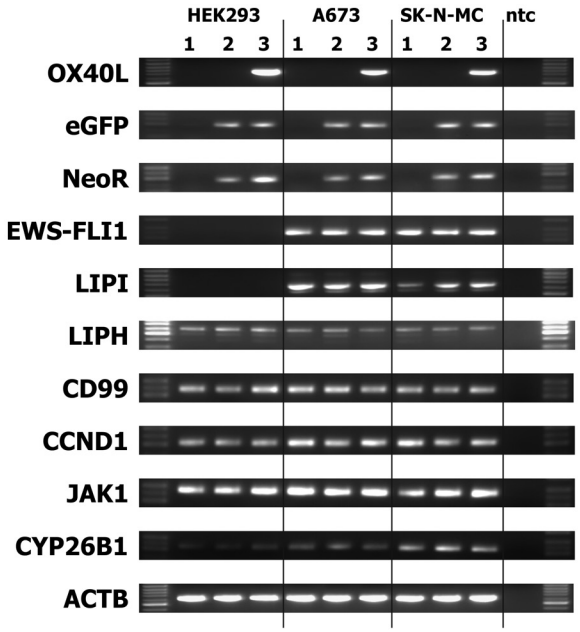

FIGURE 3 | Stability of EFT makers in OX40L transgenic EFT cells. Expression of the indicated markers was assessed by RT-PCR in cells without transfection (1), after transfection with empty pIRES-eGFP vector (2), or after transfection with OX40L in vector pIRES-eGFP (3). Actin beta (ACTB) served as housekeeping control. The neomycin-resistance cassette (NeoR) and enhanced green fluorescent protein (eGFP) served as markers for the presence of the vector in the cells. Transfected and wild-type Ewing sarcoma cells expressed the Ewing sarcoma-specific EWS-FLI1 oncofusion transcripts as well as the Ewing sarcoma-associated factors lipase I (LIPI), lipase H (LIPH), CD99, cyclin D1 (CCND1), janus kinase 1 (JAK1), and cytochrome P450 family member 26B1 (CYP26B1).

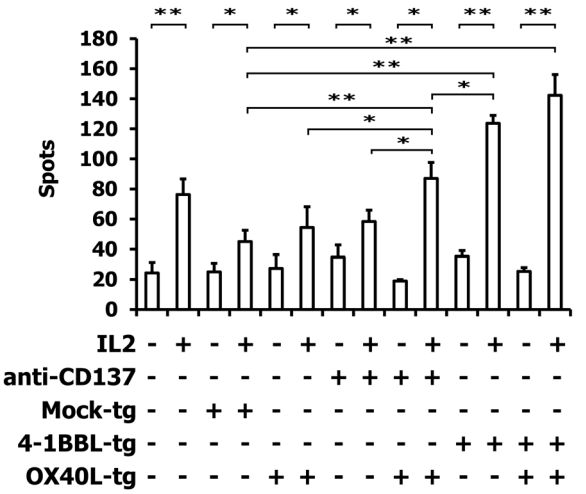

FIGURE 4 | ELISPOT analysis of PBMCs after stimulation with A673 EFT cells. HLA-matched PBMCs were incubated together with A673 EFT cells in combination with the indicated immune stimuli. After 6 days, reactivity against A673 wild-type cells was assessed by interferon gamma ELISPOT analysis. The highest numbers of spots were obtained after stimulation with the combination of OX40L transgenic and 4-1BBL transgenic cells. Presented are means and standard deviations from triplicates from a representative experiment ($N = 3$). Asterisks indicate statistical significance (*$p < 0.05$; **$p < 0.01$; Student's t-test).

molecules as ideal targets for new immune therapy strategies (14, 15). OX40 (CD134) and CD137 (4-1BB) and their ligands OX40L (CD252) and 4-1BBL are examples of such co-stimulatory effective molecules.

We have shown in previous studies that transgenic expression of IL-2 on EFT cells enhances the immunostimulatory activity but could not completely inhibit the growth of the tumor cells (8, 9). Addition of transgenically expressed co-stimulatory molecules on the surface of tumor cells or stimulation with agonistic antibody against the co-stimulatory receptor may enhance the cytotoxic effect of activated T cells (10, 60). In the present study, we present preliminary data that OX40L transgenic EFT cells not only preserve expression of typical Ewing sarcoma-associated antigens but also might enhance the immune response against EFT cells in combination with IL-2 and stimulation of CD137.

The presence of OX40-positive T cells at sites of tumor metastases suggests that engagement of OX40 by OX40L or agonistic antibodies may enhance function of tumor-reactive T cells. In various studies, different tumor cells transfected with OX40L were used as vaccines to induce tumor-specific antitumor immunity [for review, see Ref. (14, 15, 61)]. Andarini et al. treated subcutaneous tumors of melanoma, Lewis lung carcinoma, and adenocarcinoma with intratumoral injection of tumor cells expressing mouse OX40L (62). It was shown that the treatment of tumor-bearing mice with tumor cells expressing OX40L induced significant suppression of tumor growth and enhanced survival of the treated mice (21). Similar results were found with EL4 lymphoma cells or with C26 colon carcinoma cells in combination with stimulation of APC function with granulocyte–monocyte colony-stimulating factor [GM-CSF (15)]. In all these experiments, both CD4-positive and CD8-positive T cells were required for the induction of antitumor immunity and both CD4-positive and CD8-positive tumor-infiltrating T cells (TILs) expressed OX40. Nevertheless, it was unclear whether OX40-mediated signaling in CD8-positive T cells might have been required to induce their cytotoxic effector function (21). It is possible that activation of the OX40 receptor increases the function of tumor-specific CD4-positive T cell and allows more efficient effector function as well as the generation of CD8-positive T-cell memory (63, 64). Furthermore, in several preclinical models, treatment of tumor-bearing hosts with anti-OX40 agonistic antibodies or OX40L-Fc fusion protein resulted in a significant tumor regression [for review, see Ref. (21)]. In these studies, it was suggested that activation of OX40 receptor by agonistic antibody or OX40L transgenic tumor cells pushes regulatory T cells (Treg) in suppressing or depletion depending on the context of simulation and the cytokine milieu (65). This activation of OX40 on different T cells with agonistic antibody or OX40L-expressing tumor cells may lead to decreased inhibitory effects mediated by Treg cells and thereby might promote antitumor responses of CD8-positive T cell which is necessary to maintain long-term antitumor responses. It is possible that several different mechanisms are important for the antitumor

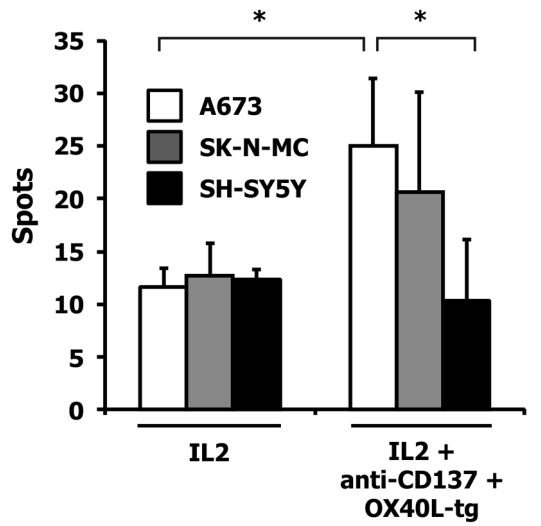

FIGURE 5 | Specificity of the induced immune response. HLA-matched PBMCs were incubated together with A673 EFT cells in combination with the indicated immune stimuli. After 6 days, reactivity against the indicated wild-type cell lines was assessed by interferon gamma ELISPOT analysis. The highest numbers of spots were obtained after restimulation of A673/anti-CD137/OX40L-primed cells with A673 cells. Presented are means and standard deviations from triplicates from a representative experiment ($N = 3$). Asterisks indicate statistical significance ($p < 0.05$; Student's t-test).

effects mediated through the activation of the OX40 receptor on T cells (61).

It is unlikely that the use of only one co-stimulatory molecule will be sufficient for generating immunotherapy strategies to cure patients with different tumor types. However, the use of combinations of several co-stimulatory molecules may be a more effective strategy for producing immunotherapy against cancer. In this context, Cuadros et al. showed a potential benefit of anti-CD137 and anti-OX40 antibodies in enhancing the immune responses in combination with dendritic cell-based vaccines in a Her-2/neu tumor model. The authors showed that joint co-stimulation with anti-CD137 and anti-OX40 agonistic antibodies induce strong effector immune responses depending on both CD4-positive and CD8-positive T cells (66). The combination of activation of two co-stimulatory molecules induces a strong effector immune response by primary CD8-positive T cells that is sufficient to attack established tumors, induce CD4-positive T-cell responses, and generate tumor-specific T-cell memory (66). Furthermore, Pan et al. successfully treated metastatic colon carcinomas with anti-CD137 agonistic antibodies together with IL-12 transfected tumor cells (67). However, the success was limited to small tumors. The addition of anti-OX40 agonistic antibodies to the immunotherapy protocol improved the success also to greater tumors on established colon carcinomas. This triple co-stimulatory combination therapy induced a high CTL activity in the TILs against parenteral tumors, and this effect was partly cell dependent on CD4-positive T cells. These observations suggested that anti-OX40 antibodies enhanced the helper function of CD4-positive T cells that increased the number or activity of CD8-positive T cells against the tumors (67).

We have shown that OX40L-expressing Ewing sarcoma cells preserved the expression of typical Ewing sarcoma-associated antigens and are practicable for immunotherapy protocols with transgenic tumor cells. The stimulated PBMCs exerted some specificity for the tumor cells that were used for stimulation. It remains unclear which antigens are recognized and which cell types are responsible for the effects. In our previous investigations, we observed activation of T and NK cells by IL-2 transgenic Ewing sarcoma cells (9). The high sensitivity of Ewing sarcoma cells for NK cell-mediated lysis (68, 69) might also be responsible for the higher "specificity" of Ewing sarcoma-activated PBMCs for these stimulatory cells. Whether antigenic peptides in combination with major histocompatibility complex (MHC) molecules are recognized by T cells or whether activated NK cells are triggered by receptors like CD226 or CD314 requires further investigations. Our data suggest that OX40L-expressing tumor cells might enhance immune response against Ewing sarcoma cells in combination with IL-2 and activation of the CD137/4-1BBL co-stimulatory pathway. The inclusion of the OX40/OX40L pathway in co-stimulation immunotherapy protocols might improve the immunotherapy strategies against Ewing sarcoma or the development of tumor vaccines.

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REVIEW
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Immunotherapy of childhood Sarcomas

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Pediatric sarcomas are a heterogeneous group of malignant tumors of bone and soft tissue origin. Although more than 100 different histologic subtypes have been described, the majority of pediatric cases belong to the Ewing's family of tumors, rhabdomyosarcoma and osteosarcoma. Most patients that present with localized stage are curable with surgery and/or chemotherapy; however, those with metastatic disease at diagnosis or those who experience a relapse continue to have a very poor prognosis. New therapies for these patients are urgently needed. Immunotherapy is an established treatment modality for both liquid and solid tumors, and in pediatrics, most notably for neuroblastoma and osteosarcoma. In the past, immunomodulatory agents such as interferon, interleukin-2, and liposomal-muramyl tripeptide phosphatidyl-ethanolamine have been tried, with some activity seen in subsets of patients; additionally, various cancer vaccines have been studied with possible benefit. Monoclonal antibody therapies against tumor antigens such as disialoganglioside GD2 or immune checkpoint targets such as CTLA-4 and PD-1 are being actively explored in pediatric sarcomas. Building on the success of adoptive T cell therapy for EBV-related lymphoma, strategies to redirect T cells using chimeric antigen receptors and bispecific antibodies are rapidly evolving with potential for the treatment of sarcomas. This review will focus on recent preclinical and clinical developments in targeted agents for pediatric sarcomas with emphasis on the immunobiology of immune checkpoints, immunoediting, tumor microenvironment, antibody engineering, cell engineering, and tumor vaccines. The future integration of antibody-based and cell-based therapies into an overall treatment strategy of sarcoma will be discussed.

Keywords: pediatric sarcoma, immunotherapy of cancer, antibodies, monoclonal, CAR T cells, tumor vaccines, natural killer cells, osteosarcoma

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Introduction

Sarcomas are a heterogeneous group of malignant tumors arising from bone or soft tissues. More than 100 different subtypes of sarcoma have been described in adults and pediatrics; the majority of cases in children are rhabdomyosarcoma, Ewing's family of tumors, osteosarcoma, and the non-rhabdomyosarcoma soft tissue sarcomas. Although these tumors are rare individually, as a group they account for 10–14% of all childhood cancers (1). While most patients who present with localized disease are highly curable with conventional therapies involving surgery and chemoradiotherapy, those who present with metastatic disease or who relapse post-therapy have an extremely poor prognosis, with little to no improvements in survival seen over the past 20 years. Furthermore, current therapies are highly toxic and associated with significant long-term morbidity in survivors; thus, new and effective therapies are urgently needed for these patients.

History of Immunotherapy for Sarcomas

That the immune system might be involved in cancer control was first observed in sarcoma patients when Wilhelm Busch in Germany reported in 1866 on tumor regressions in sarcoma patients who developed erysipelas infections (2). Immunotherapy for the treatment of sarcomas can be traced back at least as far as 1891, when William Coley, a prominent bone surgeon at Memorial Hospital in New York (now Memorial Sloan Kettering Cancer Center), published his report on the use of what came to be known as "Coley's Toxin" to treat a series of sarcomas of the bone (3, 4). He found that injections with streptococcus organisms (originally live bacteria, later a heat-killed concoction that also included *Serratia marcescens*) could induce remissions in some patients with otherwise inoperable sarcomas. Though use of his toxins was highly controversial and eventually fell out of favor, they are considered by many to be the precursors of today's modern anti-cancer immunotherapy (5). Perhaps the best conceptualization of what has become modern immunotherapy came from Paul Ehrlich in the early 1900s with his description of the "magischen kugeln" – the "Magic bullet" – specific medicines fashioned to attack and kill only the diseased cell while sparing the surrounding normal tissues (6). The increased frequency of lymphoid malignancies in patients with immunodeficiencies also suggests that the immune system plays an important role in carcinogenesis (7). In addition, development of sarcomas has been well described in allograft transplant recipients, with a risk more than double that of non-immunosuppressed patients (8).

Immune System in the Non-Malignant State

Our immune system is a complex organization of immune cells and mediators that interact with each other and with other accessory cells to protect against infections; simultaneously, this system must maintain tolerance toward self. The immune system consists of two layers of defense: the innate and adaptive spheres. The innate immune system includes dendritic cells, mast cells, and macrophages, as well as natural killer (NK) cells, neutrophils, basophils, and eosinophils. Innate immune cells serve as the initial defense against foreign antigens. Once activated, macrophages and mast cells release cytokines that engage additional immune cells and initiate an inflammatory response. Dendritic cells serve as antigen-presenting cells, taking in foreign antigens and subsequently presenting them for recognition by adaptive immune cells, thereby recruiting the second sphere of the immune system. NK cells can also interact with dendritic cells, either activating or eliminating them depending on context, thus they too can influence both the innate and adaptive immune systems.

The adaptive immune system includes B-lymphocytes, CD4+ T helper lymphocytes, and CD8+ cytotoxic T lymphocytes (CTLs). This arm of the immune system requires direct activation through antigen presentation by antigen-presenting cells. Upon antigen presentation and activation, antigen-specific T and B cells are generated. Together, the innate and adaptive pathways eliminate pathogens and remove damaged cells (7, 9). Unlike the innate system, the adaptive immune response requires training, but, once established, is antigen specific, has a memory, and can be recalled to rapid action in the future.

Immune Surveillance and Immunoediting

One of the basic principles of cancer immunosurveillance is that cancer cells possess antigens that distinguish them [or set them apart] from non-transformed cells. These so-called tumor "neo-antigens" can be recognized by the endogenous immune system and targeted for destruction. These tumor antigens are generally products of mutated genes, abnormally expressed normal genes, or genes coding for viral proteins. Unfortunately, transformed cells, under the selective pressure of the normal host response, are sometimes able to evolve evasive or immune-suppressive mechanisms and thus avoid detection and/or eradication. This concept that the immune system, while protecting against cancer, influences tumor immunogenicity and ultimately tumor escape was proposed as the framework for cancer immunoediting (10). This process can be divided into three phases: elimination, equilibrium, and escape. During the elimination phase both the innate and adaptive immune systems work to identify a developing neoplasm and eliminate it, through various mechanisms including activation of innate immune effector cells such as NK cells, and secretion of interferons (IFNs) and subsequent activation of dendritic cells, which in turn promote adaptive anti-tumor immune responses. However, a subset of cancer cells may develop the ability to survive this elimination phase, and thus the developing neoplasm enters the equilibrium phase. Here, the immune system prevents tumor escape, yet fails to eradicate it completely and thus participates in influencing the immunogenicity of these remaining cells. Finally, in the escape phase, those tumor cells that evolved the ability to evade the immune system during the equilibrium phase progressively proliferate and present as clinically apparent tumors. Mechanisms by which this escape may occur include loss of tumor antigens, down regulation of histocompatibility locus antigens (HLA) from the tumor cell surface; altered tumor microenvironment that is immunosuppressive due to the recruitment of regulatory T cells (Tregs); myeloid-derived suppressor cells, tumor-associated M2 macrophages, and others (11–13); upregulation of inhibitory receptors (e.g., PD-1) on T cells; or upregulation of inhibitory ligands (e.g., PD-L1 or B7-H3) on stromal cells or tumor cells.

Immunomodulatory Agents

A variety of immunomodulatory agents have been investigated for the treatment of sarcomas, including cytokines such as interleukin-2 (IL-2) and IFN. The majority of sarcoma studies have been conducted in adult patients with advances fueling interest in the pediatric patient population.

Cytokines

Stimulation of the immune system has been attempted using various cytokines. Cytokines are involved in a wide array of immune functions including modulation of antigen presentation

and T cell activation (14, 15). The list of cytokines continues to expand (15). Although the most widely studied clinically are IFN and IL-2, several other cytokines are also moving into the clinic.

Interleukin 2

Interleukin 2 stimulates T cells proliferation, induces generation of CTLs, and facilitates the maintenance of NK cells (16–18). IL-2 is FDA approved for the treatment of metastatic renal cell carcinoma and melanoma, and responses to IL-2 have been reported in several other cancers including lung and breast cancers (19, 20). In pediatrics, IL-2 has been used most notably for the treatment of high-risk neuroblastoma in combination with an anti-GD2 monoclonal antibody (mAb) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (21). A study of high-dose IL-2 in relapsed pediatric patients included four patients with osteosarcoma and two patients with Ewing sarcoma. Two of the four osteosarcoma patients had complete responses, while the other two and both Ewing sarcoma patients had progressive disease (20). However, use of high-dose infusional IL-2 is greatly hampered by significant toxicity, including capillary leak syndrome; continued use in pediatric sarcoma as a single agent seems unlikely. Several studies are ongoing in pediatrics combining IL-2 given in a variety of different routes and dosages with antibody therapy, vaccines, and adoptive cell therapy.

Interferon

Interferons are a complex family of molecules that bind to IFN receptors; IFNα and IFNβ activate type I receptors, while IFNγ activates type II receptors (15, 22). Both IFNα and IFNβ activate immune cells and increase antigen presentation to T cells. INFα is approved for use in melanoma and has also been studied in sarcomas. Most recently, the large EURAMOS study reported three-year follow-up data on 715 pediatric and adult osteosarcoma patients up to 40 years of age randomized to postoperative chemotherapy ± IFN; there was no survival benefit from IFNα when added to standard three-drug chemotherapy in osteosarcoma patients (74% chemotherapy alone vs. 77% chemotherapy + IFNα; EFS, $p = 0.21$) (23). Further development of IFN as a single agent in pediatric sarcoma seems unlikely; its role in pediatric sarcoma immunotherapy as an adjuvant combined with other immunotherapies such as adoptive cell therapy to increase antigen presentation remains to be defined.

Interleukin 15

Interleukin 15 (IL-15) (24, 25) is a 14–15 kDa glycoprotein that binds to a heterotrimeric receptor that shares the IL-2R/IL-15Rβ (CD122) and the common gamma (γc) chain (CD132) with the IL-2 receptor (26), as well as a unique α subunit (IL-15Rα) that confers receptor specificity. However, unlike IL-2, IL-15 is not required for the maintenance of Tregs (27); it does not induce activation-induced cell death (AICD) of CD8+ effector T cells (28); is required for the differentiation of NK, effector CD8+ and memory phenotype CD8+ T cells; and does not cause capillary leak syndrome (29). IL-15Rα binds to IL-15 with high affinity ($Kd < 10^{-11}$ M) and retains IL-15 on the cell surface. IL-15Rα trans-presents IL-15 to IL-2R/IL-15Rβ-γc on neighboring NK and T cells through immunological synapses (30, 31). IL-15 has diverse immunologic effects (26). It stimulates the proliferation of activated CD4−CD8−, CD4+CD8+) CD4+, CD8+ T cells, induces cytotoxic CTLs, and stimulates the generation, proliferation, and activation of NK cells. Though not essential for the generation of memory CD8+ T cells, IL-15 is required for their homeostatic proliferation over long periods of time (32). IL-15 protects neutrophils from apoptosis, modulates phagocytosis, stimulates mast cell growth, induces B cell proliferation and differentiation partially independent of T cell help, and increases their immunoglobulin secretion, while stimulating secondary cytokine release from macrophages and maturing dendritic cells. When given as the IL15/IL15Rα complex, it is more effective and should be less toxic than the soluble IL15 (33–35). Several preclinical studies have shown that IL-15 may potentiate anti-sarcoma immunotherapy in Ewing and osteosarcoma models (36–38). A clinical trial combining recombinant human IL15 with NK cells for relapsed and refractory pediatric solid tumors, including sarcomas, is currently underway at the U.S. National Cancer Institute (NCT01875601). Although no clinical trial of IL-15 has been conducted specifically for sarcomas, this cytokine will likely play a major role in future immunotherapy strategies.

Liposomal-Muramyl Tripeptide Phosphatidyl-Ethanolamine

The immune modulator liposomal-muramyl tripeptide phosphatidyl-ethanolamine (L-MTP or mifamurtide) has been extensively studied, primarily in osteosarcoma. This compound is a non-specific modulator of innate immunity and is a synthetic analog of muramyl dipeptide derived from bacterial cell walls. It activates monocytes and macrophages leading to an increase of a wide variety of immunomodulatory molecules including: tumor necrosis factor-alpha (TNF-a), interleukin (IL)-1, IL-6, IL-8, IL-12, nitric oxide, prostaglandin E2, lymphocyte function-associated antigen 1 (LFA-1), and intercellular adhesion molecule 1 (ICAM1) (39). Preclinical studies suggested that this inflammatory response triggered by L-MTP could potentially eliminate minimal residual disease. A small study conducted by the EORTC Soft Tissue and Bone Sarcoma Group in the 1990s treated 20 adult patients with soft tissue sarcomas with MTP; there were no responses in that study (40). The largest clinical experience with combination chemotherapy and L-MTP derives from the Intergroup (INT-) 0133 osteosarcoma study. This prospective, double randomization, phase III trial tested first the utility of adding ifosfamide to the standard three-drug chemotherapy regimen (doxorubicin, cisplatin, and high-dose methotrexate); and second the impact on survival with the addition of L-MTP to either assigned chemotherapy arm. No difference in survival was found for patients who received ifosfamide in addition to the standard three-drug chemotherapy. The study did suggest that L-MTP had a beneficial impact on survival, improving the 5-year overall survival rate from 70 to 78% ($p = 0.03$) (41). However, when the 91 patients who had metastatic disease were analyzed separately, the difference in survival between those who did versus those who did not receive L-MTP, though suggesting improvement, did not reach statistical significance. The overall survival at 5 years was 53% for those randomized to receive L-MTP versus 40% for those who did not ($p = 0.27$) (42). Based,

in part, on the updated results of the non-metastatic cohort of INT-0133, the European Medicines Agency granted L-MTP an indication for the treatment of non-metastatic osteosarcoma in 2009; the American Food and Drug Administration (FDA) did not. L-MTP is also approved for use in Turkey, Mexico, and Israel.

Antibody-Based Immunotherapy

Monoclonal Antibodies

Unmodified antibodies specific for tumor-associated surface antigens can engage tumor cells while activating innate immune effector cells, primarily macrophages and NK cells via their Fc receptors (FcγR). Once activated, the effector cell releases cytotoxic granules to kill the target cell, a process known as antibody-dependent cellular cytotoxicity (ADCC). It is important to note that T cells do not possess FcγR and have no affinity for conventional antibodies, and hence cannot be activated by these tumor selective antibodies.

Many mAbs have been developed for various cancer types. While there have been notable successes (for example, anti-CD20 for hematologic malignancies, anti-human epidermal growth factor receptor 2 (HER2) for breast cancer, and anti-GD2 for neuroblastoma), most mAbs have failed to improve outcomes despite their initial promise, especially in pediatric sarcomas.

Approximately 50% of osteosarcomas overexpress HER2, and HER2 expression was shown to correlate with a poorer prognosis (43); a phase II study was conducted by the Children's Oncology Group (COG) to evaluate if the addition of trastuzumab (anti-HER2, Herceptin) to standard chemotherapy would improve survival in metastatic osteosarcoma patients. Ninety-six patients were enrolled, and 41 were found to have HER2 overexpression. Unfortunately, no significant difference in survival was seen in patients who received trastuzumab + chemotherapy compared to those who received chemotherapy alone {EFS of 32% in both arms, OS of 50% for chemotherapy alone compared to 59% for chemotherapy + trastuzumab, [$p = 0.54$ for EFS; $p = 0.58$ for OS] (44)}.

Instead of binding directly to tumors, antibodies can neutralize growth factors (e.g., insulin-like growth factor 1 (IGF1) or IGF2) or their receptors (e.g., IGF-1R, -A12). A large body of preclinical and early clinical data suggested that IGF1 and 2 might play an important role in the initiation and progression of a variety of cancers, including pediatric sarcomas (45–47). Several phase I and II studies were conducted evaluating anti-IGF1 mAbs in relapsed and refractory solid tumors including sarcomas, the largest being a phase II study by the COG that enrolled 116 patients, including 20 with rhabdomyosarcoma, 11 with osteosarcoma, and 10 with synovial sarcoma; there were no objective responses in any of the sarcoma patients (48, 49). Finally, a randomized phase II study of standard chemotherapy ± the anti-IGF-1R mAb ganitumab is ongoing within COG for Ewing sarcoma (NCT02306161). However, this agent failed to show improved outcomes in a large randomized phase III trial of adult pancreatic cancer patients (50) and the manufacturer has announced that they will not be pursuing development of this agent. Thus, regardless of the results of the ongoing trial, its future for pediatric sarcoma is unclear.

Although both IGF-1 and IGF-2 activate IGF-1R, the latter shares a similar tetrameric α2β2 structure with insulin receptor (IR). The IR can be expressed in two isoforms (IR-A and IR-B). IR-A binds to IGF-2 with the same affinity as it binds to insulin. In addition, insulin and IGF-1 receptor subunits can form hybrid heterodimeric receptors (51). Antibodies against IGF-1R only partially inhibit IR-A activity by disrupting the IR-A/IGF-1R hybrid, but completely fail to inhibit IR-A homodimers. Failure of IGF-1R inhibition results from two compensatory mechanisms: (1) IGF-2 is increased during treatment with IGF-1R mAb (52) which signals through IR-A, which is known to promote cancer survival (53). (2) Compensatory activation of the epidermal growth factor receptor (EGFR) allowing the cancer to continue to progress despite blockade of the IGF pathway (54). One novel approach to overcome these limitations is to reduce the serum and tissue levels of the IGF ligands, using neutralizing mAbs specific for both IGF-1 and IGF-2. By removing IGF-2, the escape mechanism of IGF-2-mediated IR-A activation can be aborted, suggesting that newer mAbs that target both IGF-1 and IGF-2 may have more success than the first-generation mAbs tested (55, 56).

Several trials of mAbs against the EGFR and the VEGFR (57) alone and in combination with chemotherapy have been conducted in children and young adults with sarcomas. The COG conducted a randomized trial of bevacizumab (anti-VEGFR) combined with vincristine, topotecan and cyclophosphamide in patients with recurrent Ewing sarcoma, as well as a randomized trial of bevacizumab and temsirolimus in combination with vinorelbine and cyclophosphamide in recurrent/refractory rhabdomyosarcoma patients. In the rhabdomyosarcoma trial, the bevacizumab arm was significantly worse than the temsirolimus arm and the study was stopped early (58); results for the Ewing sarcoma trial have not yet been published. Despite preclinical rationale for these targets (59–61), overall, these studies have not shown many significant responses in sarcomas, though some studies are ongoing.

A phase I trial of the anti-tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-2) mAb lexatumumab was conducted by the U.S. National Cancer Institute (NCI) in pediatric solid tumors. This study enrolled 24 patients, including 21 with various sarcomas. No objective responses were seen and this mAb is no longer under clinical development (62).

Given the success of anti-GD2 mAb therapy in neuroblastoma (21, 63) and the expression of GD2 by many sarcomas (64, 65), studies exploring the use of these mAbs in sarcomas, particularly in osteosarcoma, are underway. Current trials include the anti-GD2 mAbs humanized3F8 (NCT01419834 and NCT01662804) and hu14.18K322A (NCT00743496).

Engineered Antibodies Including Bispecific Antibodies

Bispecific antibodies are engineered antibodies linking a tumor antigen recognition domain to a second domain that activates a receptor on immune effector cells, typically T cells (**Figure 1**). The anti-CD19/anti-CD3 bispecific antibody blinatumomab was approved by the FDA for the treatment of precursor B cell acute lymphoblastic leukemia in 2014, making it the first in its class

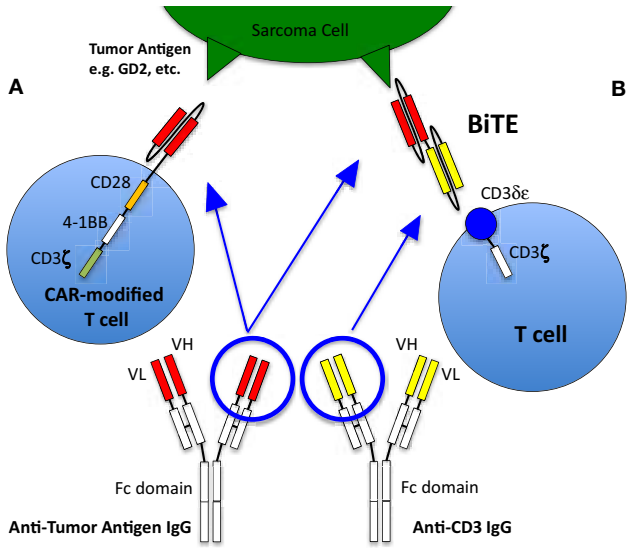

FIGURE 1 | T cell activation and recruitment to tumor cells. Normally, T cells will only target cells via antigenic sequences presented to the T cell receptor via MHC. However, this rarely occurs, especially in pediatric sarcomas, thus native T cells generally are not active in killing tumor cells and other strategies to recruit T cells to the tumor are therefore required. **(A)** T cell with a chimeric antigen receptor to a sarcoma tumor-associated antigen is able to recognize the antigen and activate the T cell, allowing it to kill the targeted cell. The CAR-T cell depicted here is a so-called third generation CAR, because it contains three co-stimulatory domains (CD28, 4-1BB, and CD3ζ). **(B)** Bispecific antibody binding to a sarcoma tumor-associated antigen as well as to the CD3 receptor of a T cell, thus activating the T cell and allowing tumor cell killing. Figure adapted from Suzuki et al. (67).

to be approved in the US. Recently published preclinical data of an anti-GD2 T cell retargeting bispecific antibody showed excellent *in vivo* activity against GD2 expressing neuroblastomas and melanomas (66). Currently, there are limited clinical data on bispecific antibodies in pediatric sarcomas; there is one study that recently began enrolling OS patients (Activated T Cells Armed with GD2 Bispecific Antibody in Children and Young Adults With Neuroblastoma and Osteosarcoma, NCT02173093).

Immunologic Checkpoint Blockade or Inhibitors

Recently, there has been much excitement about the potential of the immune checkpoint inhibitors in solid tumors including pediatric sarcomas following their clinical successes and approvals for treatment of metastatic melanoma and metastatic squamous non-small cell lung cancer.

CTLA-4 Blockade

Ipilimumab is a human IgG4 monoclonal antibody that blocks the anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and was the first of the new generation of checkpoint inhibitors to gain FDA approval (68). CTLA-4 is a member of the immunoglobulin superfamily; after T cell activation, CTLA-4 is expressed on the plasma membrane of cells where it acts to inhibit T cell function through a variety of mechanisms, allowing tumor cells to escape immune surveillance (69, 70). The experience of ipilimumab in pediatric patients is limited; GI toxicity was the major concern. A small phase II study in adults with synovial sarcoma had no clinical or immunological responses (71).

PD-1 Blockade

Antibodies targeting the programed cell death protein 1 pathway (PD-1/PD-L1) (nivolumab, pembrolizumab) function in a similar manner to ipilimumab by removing the brakes on T cells which then can perform active anti-tumor immune surveillance (69, 70). Preclinical studies have demonstrated expression of PD-1L in OS and suggest that high expression levels may correlate with worse clinical outcomes (72); *In vivo* studies using murine sarcoma models with anti-CTLA-4 antibodies have also shown promise for these agents (73). Currently, however, these agents have limited pediatric clinical data available; several trials with these agents for relapsed or refractory pediatric solid tumors are currently ongoing.

Despite the overall successes of checkpoint inhibitors, only subsets of patients with melanoma, lung cancer, ovarian cancer, NHL, and Hodgkin lymphoma have responded. Two important studies have examined the tumors of responders versus non-responders, one in melanoma and one in non-small-cell lung cancer (74–76). In both cases, treatment efficacy was associated with a higher number of mutations in the tumors. In melanoma patients treated with ipilimumab, the investigators carefully examined the tumors of those who responded versus those who did not, and found that the responders had tumors with higher mutation rates and tumor antigens and in particular, those whose tumor neoantigens shared tetrapeptide sequences with viral antigens were most likely to be responders to checkpoint inhibition (75). To improve on the quality of response to immune checkpoint blockade, CTLA-4 and PD-1/PD-L1 antibodies are being tested in combination or when added to other anti-cancer agents such as chemotherapy, targeted therapy, radiotherapy, and other immunotherapy (19, 69, 77). Currently, the COG is conducting a phase I/II study (NCT02304458) of nivolimab alone or in combination with ipilimumab for relapsed and refractory solid tumors including sarcomas.

Although there is much excitement currently surrounding these new agents, caution seems warranted in pediatric sarcomas. In contrast to melanoma and lung cancer, pediatric cancers in general and pediatric sarcomas in particular have an extremely low rate of recurrent mutations (<1 mutation per Mb for pediatric cancers compared to 15 per Mb for melanomas) (78, 79). Furthermore, many sarcomas do not express major histocompatibility complex (MHC) which is required for both the afferent and efferent arms of T cell response (80). Taken together, it seems probable that checkpoint inhibitors may have less efficacy in pediatric sarcomas (especially as single agents) than in melanoma and lung cancer; careful consideration of ideal clinical trial design using these agents will be critical for defining their potential role in the immunotherapy of pediatric sarcomas.

Tumor Vaccines

Vaccines directed against specific tumor antigens were some of the earliest targeted immunotherapies tested. The aim of tumor vaccines is to induce an anti-tumor response through exposure to tumor antigens. Of the high value tumor targets among the 75 candidates derived from the NCI consensus panel, only a few are directly adaptable to sarcoma (81). The most notable are the gangliosides GD2 and GD3, polysialic acid, and translocation breakpoints. In animal models and human clinical trials (9, 82), vaccines have shown efficacy in preventing tumor development or delaying progression, but have generally failed to mediate regressions of established tumors. Single-arm trials have investigated vaccines targeting whole cells, lysates, proteins, and peptides in both adult and pediatric patients with sarcomas. Results from most studies in sarcomas (adult and pediatric) have been disappointing, though some have shown potential benefit with either laboratory evidence of the development of an immune response, or prolonged stable disease or disease-free intervals (83–88). Several additional pediatric sarcoma studies remain ongoing (NCT01241162, NCT01803152, NCT01061840). Promising results of a recent phase I study of a bivalent GD2-GD3 gangliosides vaccine in combination with β-glucan in neuroblastoma patients in second remission (89) suggest that vaccines for sarcomas may be beneficial if given in the setting of minimal residual disease.

Adoptive Cell Therapy

Adoptive cell therapy is the term coined to describe the concept of giving a patient immune cells with cytolytic properties in sufficient numbers to cause an anti-tumor response. There are various strategies to accomplish this, including use of *ex vivo* expanded autologous cells and infusion of donor-derived allogeneic immune effector cells.

Natural Killer Cells

Natural Killer cells are lymphocytes of the innate immune system with both cytotoxic and regulatory functions and are important mediators of immune responses against infections and cancer. Unlike T and B cells, NK cells recognize their targets without prior sensitization and generally do not have the same memory system [with some exceptions (90)] as T or B cells. NK cells are activated through various receptors that recognize proteins that are upregulated by cell stress or are foreign. In turn, NK cells are negatively regulated by inhibitory receptors that primarily bind HLA as a means of preventing self-recognition, thus preventing autoimmunity. NK cell target cytotoxicity is triggered when the overall balance between the various activating and inhibitory signals is weighted toward activation (91). NK cells were initially identified through their ability to kill tumor cells (92); the anti-tumor actions of NK cells have subsequently been documented in many human and animal models. NK cells are neither HLA-restricted nor do they require activation via the adaptive immune system (93). These facts plus their ability to target and kill a wide variety of tumors has led to strong interest in their therapeutic potential (94). The first application of NK-cell-enriched cellular products to treat cancer was performed at the NCI using autologous cells in 1980 (95). Subsequently, clinical trials, primarily in acute myeloid leukemia, confirmed that haploidentical donor-derived NK cells can be expanded *in vivo* and can induce remissions (96). Preclinical data suggest that NK cell strategies may be of benefit in pediatric sarcomas (97). Specifically, various studies have shown that Ewing sarcoma, osteosarcoma, and rhabdomyosarcoma cell lines, including highly chemoresistant lines, are all sensitive to NK cell killing and that cytokine activation greatly enhances this killing ability both *in vitro* and in *in vivo* models (36, 38, 98). Several current NK-cell-based studies are open to pediatric sarcomas and apply various strategies, including post-allogeneic transplant and *ex vivo* expansion and/or cytokine stimulation; however, no results have yet been reported from these trials.

Cytotoxic T Lymphocytes

Cytotoxic T Lymphocytes are highly efficient at targeting and killing specific cells; thus, there has long been much interest in harnessing this ability for cancer immunotherapy. *De novo* T cells are generally of low frequency and incapacitated by the tumor microenvironment. Initial efforts to use T cells for cancer therapy involved *ex vivo* expansion of the so-called tumor infiltrating lymphocytes (TILs) freed from excised tumors. This approach is limited, however, by the fact that they cannot be reliably extracted or be expanded to sufficient numbers from most tumors. To date, there are no studies in pediatric cancer patients (9). Despite their limitations, TILs are an important proof-of-concept of the potential value of T-cell-based immunotherapy as they were the first immunotherapy to induce regressions of bulky tumors (99). To overcome these limitations, polyclonal T cells can be genetically modified to express T cell receptors (TCRs) that recognize tumor peptide antigens in the context of MHC. These transgenic TCRs function like their natural counterparts, but remain restricted by MHC, thus limiting the use of these cells to the patient's specific individual HLA alleles. As approximately 50% of the Caucasian population in the U.S. express HLA A*0201, many studies have focused on associated antigens, particularly the cancer testis antigens. Among these, NY-ESO-1 is one of the most studied with expression found in 70–80% of synovial sarcomas, but only sporadically in other sarcomas (100, 101); in a pilot feasibility study, four of six patients with synovial sarcoma had an objective response (101, 102). Further studies using NY-ESO-1 CTLs in synovial sarcoma are ongoing (NCT01343043).

Chimeric Antigen Receptor-Modified T Cells

Because T cells do not carry Fcγ-receptors, these potent effector cells cannot recognize tumor-bound antibodies, and have therefore traditionally not been recruited by such antibodies to tumor sites. Furthermore, T cells need to recognize tumor peptides in the context of their own MHC antigens to be effective killers. However, many tumors down regulate or lose their HLA, or even tumor peptides, making them transparent to even the primed T cells. To overcome these issues of HLA and to broaden the selection of targets (e.g., to carbohydrates or lipids), chimeric antigen receptors (CARs) can be engineered into T cells. These receptors are not classic TCRs, but derived from conventional antibodies specific for any target. A chimeric molecule consisting of an antibody in the form of single chain Fv (scFv) as the

ectodomain, and T cell signaling machinery as the intracellular domain, forms this artificial receptor through which T cells are activated when they come into contact with the specific antigen, without the necessity of MHC. These CARs are inserted into T cells using viral vectors, DNA transposons, or RNA transfection. In the early versions (so-called First generation) of CAR-modified T cells (CAR T cells), signaling was done through a single activation domain (either the CD3-ζ chain or FcεRIγ). Second- and third-generation CAR T cells contain one or two additional co-stimulatory signaling domains such as CD28, 4-1BB, and OX40 (67) (**Figure 1**). The first-generation CAR T cells did not show significant activity in clinical trials presumably because many tumor cells lack co-stimulatory ligands (103), and because of poor persistence of the T cells, although a phase I study of anti-GD2 CAR T cells in relapsed neuroblastoma patients saw some objective clinical responses including complete remission in three patients (104, 105). Second-generation CAR T cells have shown improvements in T cell proliferation and survival (106) and have shown promising results in hematologic malignancies (107). Several studies with CAR T cells are underway that include pediatric sarcoma patients. Two of the open trials target HER2 expressing sarcomas (NCT00902044, NCT00889954), while two more target GD2 expression (NCT01953900, NCT02107963); it remains to be seen whether similar successes seen in hematologic malignancies can be achieved in solid tumors. The death of a patient receiving third-generation anti-HER2 CAR T cells has raised concerns regarding the safety of highly activatable T cells even when the expression of the antigen in normal tissues was low (NCT00924287) (108).

Challenges

Toxicity

In general, although immunotherapy may have less long-term toxicity than chemotherapy or radiation therapy, which is particular appealing for pediatric cancer, major short-term toxicities can be daunting. These include immediate infusion-related allergic reactions with mAbs, and autoimmune reactions to the checkpoint inhibitors, some of which were life threatening (109). In a recently completed phase I study of ipilimumab (NCT01445379) in pediatric patients with refractory solid tumors including sarcomas, no objective responses were seen but significant autoimmune toxicity was observed, with up to 50% of patients experiencing symptoms (Personal communication, Dr. L. Wexler, 2015); however, no pediatric safety data for these agents are yet published. In adults, enterocolitis, hepatitis, and dermatitis were the most commonly seen toxicities, but autoimmune-related toxicities due to unregulated T cell activity have been reported in nearly every organ system (109). Adoptive cell transfer also carries the real potential for serious adverse events. T cell therapy is highly potent such that even normal tissues with low target antigen expression can become innocent bystanders. These unintended and unexpected toxicities to critical organs can be life threatening (110) and have limited the choice of certain targets for redirected T-cell-based therapy (111). Additionally, T cells have been associated with severe, sometimes fatal, cytokine release. Cytokine release syndrome (CRS) occurs when extremely high levels of immune cells are activated thereby stimulating release of large amounts of inflammatory cytokines, leading to organ dysfunction and death. CRS is particularly seen with second- and third-generation CARs as well as bispecific antibodies (112), but can occur after antibody infusion as well as with other adoptive lymphocyte therapies. Corticosteroids are the mainstay of treatment, while anti-IL6R antibody can also be helpful (113).

Target Selection

Perhaps the most critical first step in designing cancer immunotherapy is identifying appropriate immunologic targets. A good immunotherapy target must be highly expressed on tumor tissues but not on normal tissues. Ideally, a good target will play a role in the underlying oncogenesis of the tumor, though this is not always required. Targets that meet these attributes are rare (81). An alternative approach has been to target markers that are highly expressed on cancers and expressed in the so-called non-vital tissues, such that targeting and loss of these normal cells are tolerated by the patient. Monoclonal antibody targeting of CD20, and CAR T cells and bispecific antibodies targeting CD19 are examples of this approach. Adding further difficulty to target selection is that they by necessity must be present on the surface of the cell for immune recognition, which limits the potential target list. In fact, of the 75 NCI consensus high value targets, two-thirds are internal antigens (81). The only way to target these internal antigens is through their peptides presented on the HLA; hence the description of such antibodies as TCR like. Less than 100 publications have been published on the discovery of such antibodies, but the best characterized are those against the RMFPNAPYL peptide of the Wilm's tumor-1 (WT1) antigen presented on HLA0201 (114). However, this approach is limited by the restriction to specific HLA subtypes. Most pediatric sarcomas lack HLA expression (80), and among those that have it, only individuals with the specific restricted subtype would be sensitive to the immunotherapy. Efforts to mine gene expression databases for potential new antibody targets are promising but still in early stages; validation of these mRNA level exploratory analyses at the protein level will be critical (115, 116). **Tables 1** and **2** list some of the pediatric sarcoma-specific targets, both MHC non-restricted (**Table 1**), and MHC restricted (**Table 2**), currently in preclinical and/or clinical development.

Future Directions

Sarcoma immunotherapy remains in its infancy. To date, while we have not seen the successes seen in other malignancies, there are glimpses of activity which suggest that immunotherapy could be an effective treatment modality. However, to fully realize that potential we believe that the following four areas must be carefully considered:

Target Discovery and Validation

Given the narrow mutation landscape in sarcomas, and especially so among those with translocations, neoantigens derived from gene mutations are predicted to be rare. Translocation fusion sequences have remained difficult to target with T cells, or to be used as vaccines. Without neoantigens, even checkpoint blockades

TABLE 1 | Cell surface targets for MHC non-restricted immunotherapy of pediatric sarcomas.

Target	Tumor Expression	Normal Expression	Comments
GD2	Osteosarcoma (90%) Soft tissue sarcomas (varies)	GD2+ neuronal tissue (peripheral sensory nerves)	Dinatuximab (Ch14.18) FDA approved for NB; trials in OS using hu3F8 and dinatuximab are planned.
HER2	Osteosarcoma DSRT	Low-level lung expression	
FGFR4	Rhabdomyosarcoma	Expressed during muscle development	
Glypican-3, -5	Rhabdomyosarcoma	Rare outside embryonal tissues	
FOLR1	Osteosarcoma, Rhabdomyosarcoma	Luminal cell mem-brane of some epithelial tissues	

Table adapted from Orentas et al. (116).

TABLE 2 | MHC-restricted immunotherapy targets for pediatric sarcomas.

Target	Tumor expression	Comments	Reference
NY-ESO-1	Synovial Sarcoma (70%)	Cancer testis antigen, HLA-A1	(102, 117)
HER2/Neu	Osteosarcoma (60%)		(64)
STEAP (Six-transmembrane epithelial antigen of prostate)	Ewing Sarcoma	% expression data limited	(118, 119)
WT1	Rhabdomyosarcoma (100%) Ewing sarcoma (50%)	HLA-A1, A24, DP5, DR4	(120, 121)
PAX3-FKHR	Alveolar rhabdomyosarcoma (90%)	HLA-B7	(122)
SYT-SSX1, 2	Synovial Sarcoma (100%)	HLA-B7	(123)

Table adapted from Orentas et al. (116).

used at recommended dosage levels might not be effective. By default, differentiation antigens and tissue antigens deserve to be more carefully explored. These include the gangliosides GD2 (124) and GD3 (65), ROR2 (125), HER2 (126, 127), B7-H3 (128), CSPG4 (129, 130), polysialic acid (131), and glypican 3 (132). All of these antigens have established antibodies ready for construction of CAR T cells or bispecific antibodies. Importantly, most of these antibodies have already been tested in humans with acceptable toxicities. Considerations should also be given to novel engineered forms such as bispecific antibodies to retarget T cells (66) or bispecific antibodies for multistep targeting to greatly improve therapeutic index (133). Given the early glimpses of response to IGF1R antibodies and a better understanding as to why tumors escape, the new generation of dual-specific antibodies for IGF1 and IGF2 should be considered (56, 134).

Careful Patient Selection

The majority of clinical trials to date have shown that immunotherapy is generally not effective against large, bulky disease. Thus, it is imperative that the proper patient population is selected for clinical trials moving forward. For example, we are developing a phase II anti-GD2 immunotherapy protocol for osteosarcoma patients in second or greater remission, with the goal of targeting pulmonary minimal residual disease. This is based on our experience in OS patients treated on our phase I protocol where we found that patients with visible metastatic lesions progressed rapidly while those with minimal residual disease have shown increased time to progression compared to historical controls. It would appear that the clinical efficacy of immunotherapy for pediatric sarcoma can best be tested in clinical trials designed to treat patients after their overt disease burden has been reduced as much as possible.

Development of Combined Modality Regimens

To date, the majority of studies using single immunotherapy modalities have not demonstrated significant activity in solid tumors in general and in pediatric sarcomas in particular. However, rational combinations of new immunotherapies are being developed and will need to be carefully explored. Antibodies combined with immunomodulatory agents are the most mature of these combinatorial approaches. Anti-GD2 mAbs combined with GM-CSF or GM-CSF and IL-2 are effective against neuroblastoma (21, 63) with studies planned in osteosarcoma. While checkpoint inhibitors, for reasons described above, are unlikely to be of significant benefit when used alone in pediatric sarcomas, their combination with adoptive cell therapy or bispecific antibodies has the potential to enhance the efficacy of these T cell-based strategies. Additionally, preclinical studies suggest that prior radiotherapy can induce tumor neoantigen expression and increased effectiveness of checkpoint blockade, echoing the abscopal effect in the clinic (135). Studies exploring this strategy in adults are underway and may be warranted in children. T cells could also be combined with NK cells: MHC down regulation by the tumor cells as a means of escape from T cell killing should render these cells more susceptible to NK cell killing, which does not require MHC, but is instead inhibited by high MHC expression (91).

Tolerance of Increased Toxicity

This last point is perhaps the most controversial. However, historical precedent suggests that learning to manage toxicities associated with therapies can allow otherwise effective treatments to be developed. Anti-GD2 immunotherapy is associated with significant infusional toxicities including severe pain; this pain side effect was completely unexpected when these mAbs were first used (136). Fortunately, rather than halting the development of these antibody treatments, ways to overcome the toxicities were developed and as a result, anti-GD2 immunotherapy is now proven effective in neuroblastoma and is in active trials in sarcoma patients. Similarly, it seems likely that newer immunotherapy treatments, especially combination therapies as suggested above, will have both predictable, as well as unexpected, and potentially severe side effects. However, an unwillingness to carefully explore and manage novel toxicities may limit the adoption of some potentially beneficial treatments. With checkpoint blockade, the autoimmune toxicity

seen shows that children do, in fact, have autoreactive T cells that will react with self if the "brakes" are sufficiently released. Since many tumors overexpress normal self antigens, it is plausible that "releasing the brakes" enough (by combining ipilimumab with nivolumab while pushing the dose of both) could allow an autoreactive T cell to target a protein on the tumor that would otherwise be tolerated by the immune system. The currently approved dose of ipilimumab for patients with melanoma, however, achieves the target trough concentration of 20 mcg/mL, the level at which ipilimumab attains maximum CTLA-4 blockade, in only 30% of patients (68), suggesting that increasing the dose could yield improved clinical benefit, if toxicities can be managed. Several clinical trials testing this hypothesis in adults are underway. Similarly, combination therapy with adoptive T cells and checkpoint blockade could have significantly increased toxicity, especially for on-target, off-tumor effects, such that appropriate target selection and clinical trial design to minimize these risks are critical.

Conclusion

Pediatric cancer immunotherapy continues to advance; we believe these advances will improve outcomes in patients who have not benefited from conventional therapy alone. Late toxicities remain a major challenge for those patients who underwent life saving chemotherapy and radiation therapy. Immunotherapy offers an opportunity to consolidate remission while reducing genotoxic therapy. We are cautiously optimistic that immunotherapy will improve not just survival but also the quality of life in children with sarcomas.

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