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Twenty Years On – What Do We Really Know About Ewing Sarcoma And What Is The Path Forward?

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Abstract

Ewing sarcoma (ES) is a highly aggressive bone and soft tissue tumor with peak incidence in adolescents and young adults. Despite advances in local control and systemic chemotherapy, metastatic relapse after an initial clinical remission remains a significant clinical problem. In addition, metastasis at the time of presentation or at relapse continues to be the leading cause of death for patients diagnosed with ES. Since the discovery over 20 years ago of the pathognomonic *EWS-FLII* fusion gene, much has been learned about the molecular and cellular biology of ES pathogenesis. In addition, more recent exploitation of advances in stem cell and developmental biology has provided key insights into the cellular origins of ES and the role of epigenetic deregulation in tumor initiation and maintenance. Nevertheless, the mechanisms that drive tumor relapse and metastasis remain largely unknown. These gaps in our knowledge continue to hamper the development of novel therapeutic strategies that will improve outcomes for patients with relapsed and metastatic disease. In this chapter we will review the current status of ES biology research, highlighting areas of investigation that we propose have the greatest potential to yield findings that will translate into clinically significant advances.

Keywords

Ewing sarcoma; metastasis; genetics; epigenetics

I. INTRODUCTION

Ewing sarcoma (ES) is a highly aggressive bone and soft tissue tumor with peak incidence in adolescents and young adults. In the pediatric population it represents the second most common malignant bone tumor, and in adult patients it more commonly presents in soft tissues.^{1,2} Intensification of systemic chemotherapy and local control measures have improved survival rates for patients with localized tumors to greater than 70%.³ However, event free and overall survival rates for patients with metastatic disease remain dismal at

around 20%.⁴ In addition, for patients who relapse following an initial clinical remission there is little to offer in the way of salvage therapy.^{5,6} Finally, the acute and long term toxicities associated with current therapeutic regimens are considerable and ES survivors are left with significant health morbidities and shortened life expectancies.^{7,8} Thus, future improvements in survival and quality of life for patients with ES demand that we better understand the biologic processes that drive tumor relapse and metastasis and that we develop less toxic approaches to ES therapy that will spare normal developing tissues.

In this chapter, we will review the current state of knowledge regarding mechanisms of ES initiation and metastatic progression. We will specifically focus on areas of recent discovery and on identifying areas of investigation that, in our opinion, have the greatest potential to lead to clinically relevant therapeutic advances.

II. TUMOR INITIATION

ES is widely believed to arise from malignant transformation of stem and/or progenitor cells of mesoderm and neural crest lineages.^{9,10} Specific cells that have been identified as candidate cells of origin include bone marrow-derived mesenchymal stem cells,^{11,12} neural crest-derived stem cells,¹³ bone progenitors,¹⁴ and most recently, osteochondrogenic progenitors.¹⁵ ES tumors express characteristic fusion oncogenes that likely act as the tumor-initiating mutations when expressed in the appropriate cellular and developmental context (see next section and Table 1).¹⁶ Given the near universal and ES-specific nature of their expression, detection of EWS-ETS fusions is now routinely used for tumor diagnosis. Studies of the direct and indirect biologic effects of EWS-ETS fusions have provided critical insights into ES pathogenesis and these are discussed below and summarized in Figure 1.

A. Genetics of ES

In ~85-90% of ES cases, a somatic reciprocal t(11;22)(q24;q12) chromosomal translocation is observed that fuses *EWSR1* to the *FLI1* ETS family gene to generate *EWSR1-FLI1* fusion transcripts.^{17,18} The EWS-FLI1 chimeric oncoprotein is an aberrant transcription factor that deregulates the expression of key genes involved in ES oncogenesis.^{19,20} Variant *EWS-ETS* gene fusions are also described, including the t(21;22)(q22;q12) associated EWS-ERG fusion that occurs in 10-15% of cases,²¹ as well as rarer EWS-ETV1, EWS-ETV4, and EWS-FEV gene fusions.²²⁻²⁵ Rare cases involving FUS, which like EWS, is a member of the FET (FUS, EWS, and TAF15) family of RNA-binding proteins²⁶ have also been described, including FUS-ERG or FUSFEV.^{27,28} In addition to such FET-ETS gene fusions, cases of so-called “ES-like” tumors are described in which EWS is fused to non-ETS proteins, and others that contain translocations without similarity to EWS-based fusions, such the t(4;19)(q35;q13) generated CIC-DUX4 fusion.^{16,29-34} How ES-like tumors relate to ES remains contentious.^{16,35} The current list of ES- and ES-like tumor associated fusions is summarized in Table 1.

Gene fusion detection has emerged as an extremely powerful tool for ES diagnosis. RT-PCR for EWS-FLI1 and EWS-ERG fusions³⁶ theoretically should account for >99% of cases. Fluorescence *in situ* hybridization (FISH) using an EWS “break-apart” probe or dual color EWS and FLI1 probes is also utilized, but must take into account other EWS-rearranged

tumors, such as desmoplastic round cell tumor, myxoid chondrosarcomas, myxoid liposarcoma, or clear cell sarcoma³⁷ Given the above variant fusions, the absence of molecular confirmation of EWS-FLI1 and EWS-ERG fusions may not rule out the diagnosis of ES. Recent next-generation sequencing based approaches may increase the capacity to screen for multiple fusions simultaneously.^{38,39}

Additional chromosomal abnormalities in ES include gains of chromosome 8 in up to 50% of cases,^{28,40,41} chromosome 12 and 1q gains in 25%,^{41,42,43} gains in chromosome 20 in 10-20%,⁴⁴ and 1p36 losses.⁴¹ In ~20% of cases, a t(1;16) chromosomal translocation with variable breakpoints occurs and is associated with 1q gains and 16q losses.⁴⁵⁻⁴⁸ Finally, mutations in TP53 and CDKN2A are detected in 10-20% of cases and may be associated with aggressive disease.⁴⁹⁻⁵¹

B. Biology of EWS-ETS Fusion Proteins

Numerous studies have endeavored to dissect the role of EWS-ETS fusion proteins in ES oncogenesis.⁵² EWS-ETS chimeric proteins are oncogenic in NIH3T3 fibroblasts¹⁸ and function as aberrant transcription factors binding to ETS consensus sequences of target genes.^{18,53-55} Among early reported targets of EWS-ETS mediated transcriptional activation are stromelysin 1, cytochrome P-450 F1, cytokeratin 15, manic fringe, E2-C, Id2, PIM3, uridine phosphorylase, and p21WAF1/CIP1.^{52,56} More recent targets include NKX2.2,¹⁹ NROB1,⁵⁷ GSTM4,⁵⁸ and STEAP1.⁵⁹ EWS-FLI1 and other EWS-ETS proteins down-regulate the *TGF beta type II receptor (TGFB2)*, a putative tumor suppressor gene,^{60,61} pointing to important roles for EWS-ETS repression of gene subsets. Loss of TGFB2 expression may provide ES cells with a mechanism to elude programmed cell death. IGFBP-3, an inhibitor of insulin-like growth factor 1 (IGF-1) mediated proliferation and survival signaling, is also a repressed target of EWS-ETS proteins.²⁰ Another mechanism of IGF pathway deregulation in ES appears to be through EWS-FLI1 repression of microRNAs that otherwise negatively regulate the expression of IGF1 and the IGF1 receptor (IGF1R).⁶² In human mesenchymal stem cells, a potential cell of origin of ES, EWS-FLI1 induces the expression of *OCT4*, *SOX2*, and *NANOG* embryonic stem cell genes¹² as well as the polycomb repressor *EZH2*.⁶³ The significance of these observations in the context of ES biology is under intense investigation and although these discoveries have yielded important insights into disease pathogenesis and new molecular tools for diagnosis, they have not yet significantly impacted ES treatment and outcome.

C. Oncogenic Hubs Downstream of EWS-ETS Fusions

With the advent of genome-wide chromatin immunoprecipitation (ChIP-seq) and RNA interference technologies it has now been established that EWS-FLI1 binds DNA at diverse regions across the genome, including both gene promoter as well as inter- and intra-genic regions,^{64,65} and that it represses as many genes as it induces.⁶⁶⁻⁶⁸ Thus, EWS-ETS proteins have the potential to modulate gene and protein expression through non-transcriptional mechanisms and, as discussed below, these alternative mechanisms are now recognized to function as critical oncogenic hubs in ES tumorigenesis.

1. Epigenetic Deregulation—Coordinated regulation of gene expression *via* epigenetic mechanisms is essential for normal development and these regulatory elements are frequently disturbed in both adult and pediatric tumors.^{69,70} In particular, the actions of polycomb group, histone deacetylase (HDAC) and nucleosome remodeling complexes all contribute to normal regulation of gene expression by altering chromatin structure^{71,72} and EWS-ETS fusions have been shown to impact on all of these epigenetic hubs.

As mentioned above, the polycomb group protein EZH2 is a direct transcriptional target of EWS-FLI1.^{63,73} EZH2 represses transcription by methylating histone 3 at lysine residue 27 (H3K27me3) and alterations in H3K27me3 promote oncogenesis.⁷⁴ In addition, BMI-1, another polycomb group protein, is over-expressed by ES and can be induced by EWS-FLI1, although the mechanism of induction is indirect.^{13,75,76} BMI-1, in complex with its polycomb partner protein RING1B, ubiquitinates histone 2A, resulting in chromatin compaction and gene silencing.⁷⁴ The net effect of these polycombedependent modifications is repression of differentiation and maintenance of stemness, two driving forces in the process of malignant transformation.^{13,63,77}

EWS-ETS proteins also alter HDAC-dependent gene regulation. The direct EWS-FLI1 target gene NKX2.2 represses transcription by recruiting transcriptional corepressors and HDACs to gene promoters and many EWS-FLI1-repressed genes are repressed as a consequence of NKX2.2-HDAC-dependent repression.⁶⁶ Preclinical studies with HDAC inhibitors show anti-tumor effects and therapeutic clinical trials are in development.^{66,78} In addition, cellular biochemical studies have revealed a key role for HDAC deregulation in the context of the NuRD (nucleosome remodeling and histone deacetylase) complex.⁷⁹ Repression of the tumor suppressor genes *LOX* and *TGFBR2*, among others, is mediated by EWS-FLI1-dependent recruitment of the NuRD complex to gene promoters. Transcriptional repression by NuRD is in part dependent on the histone demethylase, lysine-specific demethylase 1 (LSD1, *KDM1A*).^{79,80} LSD1 is over-expressed by ES⁸¹ and inhibition of LSD1 in ES by genetic or pharmacologic means results in de-repression of EWS-ETS-suppressed genes and inhibition of tumorigenicity.⁸⁰ Interestingly, LSD1 inhibition also leads to down-regulation of EWS-FLI1-induced genes demonstrating the complex interplay between chromatin activating and chromatin repressive marks and revealing the central role that EWS-ETS proteins play in disrupting this balance.⁸⁰ Given its role as a downstream hub of EWS-ETS fusions, and the ongoing efforts by several small and large pharmaceutical companies to develop LSD1 inhibitors, LSD1 is highly promising as a novel target for therapeutic intervention in ES.

Finally, accessibility of transcription factors to DNA is also determined by nucleosome position and structure.⁷² Studies of chromatin structure in ES cells as well as EWS-FLI1 transduced endothelial cells revealed that the fusion can alter nucleosome occupancy, especially in regions adjacent to its own binding sites.⁶⁵ This ability of EWS-FLI1 to confer an open chromatin structure indicates that, in addition to altering post-translational modifications of histones, the fusion protein can alter epigenetic regulation of gene expression by interfering with nucleosome remodeling.

2. Aberrant Regulation of RNA Splicing and miRNAs—Early studies of EWS-FLI1 suggested that it may play a role in disrupting RNA splicing.^{82,83} This initial observation has been supported by new evidence that links both wild-type EWS and EWS-FLI1 to the RNA splicing machinery.^{84,85} Specifically, wild-type EWS is a key mediator of RNA splicing in response to DNA damage and EWS-FLI1 has been shown to impact expression of splice variants of FAS, Death receptor 4, and VEGF.^{84,86,87} These studies implicate isoform switching as an indirect mechanism of EWS-ETS-dependent tumorigenesis. Abnormal expression and regulation of numerous microRNAs have also been implicated in disrupted gene expression and ES pathogenesis.^{62,88-94} In particular, recent studies have revealed a link between EWS-FLI1-dependent disruptions to miRNA processing and maintenance of a cancer stem cell phenotype.⁹⁵ Specifically, candidate ES cancer stem cells as well as EWS-FLI1-transduced pediatric mesenchymal stem cells display reduced expression of the Dicer complex protein TARBP2, which results in inhibition of miRNA maturation. This fundamental defect in miRNA processing contributes to ES tumorigenicity and provides a potential therapeutic target.⁹⁵

III. TUMOR METASTASIS

There has been little change in overall outcome in ES for almost twenty years, mainly because we do not yet understand the molecular basis of metastasis in these patients. The presence of metastatic disease is the single-most powerful predictor of outcome in ES and other childhood sarcomas, and the prognosis for patients with metastatic disease remains dismal.⁹⁶ *EWS-ETS* gene fusions are present in both localized and widespread disease, and thus they alone cannot account for metastatic behavior.⁹⁷ There is a compelling need to develop curative treatments for ES patients with metastatic cancer. In particular, micrometastatic disease, representing residual tumor cells in the circulation or various organ sites following removal and treatment of the primary tumor, is a very poorly understood stage in cancer progression. Adjuvant chemotherapy, aimed at preventing such recurrent disease, is conceptually designed to eliminate micrometastases. However, the biology of micrometastatic cells, the purported origin of metastatic disease, remains almost completely unknown. Identifying factors that contribute to micrometastasis and metastatic spread, and that can be targeted therapeutically, have tremendous potential to improve outcome. The multistep process of metastasis has been excellently reviewed elsewhere,⁹⁸ and this review will instead discuss new concepts in the metastatic cascade that the ES research community needs to be aware of in order to work towards strategies for potentially preventing or targeting the metastatic process in ES (Figure 2).

A. Genomic Studies of Metastatic ES

The advent of next generation sequencing (NGS) methods can potentially shed light into the metastatic cascade in ES through deep sequencing of patient-matched primary and metastatic lesions to identify genetic drivers of metastasis, and this approach has provided novel insights into metastatic disease in adult malignancies.^{99,100} However, as in other pediatric cancers such as retinoblastoma and neuroblastoma,¹⁰¹⁻¹⁰³ the ES genome is proving to be relatively quiet compared to adult malignancies,^{104,105} and therefore genetic approaches may not delineate the molecular basis of metastatic disease in ES. As will be

described in the next section, epigenetic or other mechanisms may instead underlie this process. Moreover, the issue of tumor heterogeneity suggests that analyzing single primary tumor biopsies by NGS may miss metastatic driver mutations,¹⁰⁰ i.e. relevant clones may not be present in the tumor section subjected to sequencing in a given NGS experiment. Superimposed on this issue, NGS of pediatric medulloblastoma has revealed that primary and patient-matched metastatic disease represents different genetic compartments; i.e. that advanced medulloblastoma is a bicompartamental disease.¹⁰⁶ These studies indicate that metastatic lesions are highly similar to each other across patients, but differ markedly from patient-matched primary tumors, and that only rare clones in the primary tumors are present in the metastases. In fact, so-called post-dispersion events (e.g. mutations or structural variations) may occur after the metastatic clones have migrated from the primary tumor.¹⁰⁶ This has major implications for precision medicine, as actionable mutations identified in primary tumors may have no relevance to metastasis, the major cause of death in that patient population. These studies argue for new approaches to better understand metastatic disease in ES. First, clinical practice must strive to include ES biopsies of metastatic lesions for NGS. Second, as a community we must focus more diligently on biology studies that specifically address metastatic processes in ES. Several recent studies in this regard are now discussed.

B. Metastatic Pathways in ES

Surprisingly, few studies have focused on metastatic disease in ES at the mechanistic level, although recent studies have begun to identify candidate proteins and pathways that promote a metastatic ES phenotype. The zinc finger E-box binding homeobox 2 (ZEB2) transcription factor was recently shown to promote metastasis of ES cell lines *in vivo* and to be more highly expressed by patients with metastatic disease.¹⁰⁷ Dickkopf 2 (DKK2) has also been implicated in ES metastasis *in vivo*, either through activation of its effector matrix metalloproteinase 1 (MMP1), or through upregulation of genes known to be involved in invasion and metastasis such as *CXCR4*, *PTHrP*, *RUNX2*, and *TGF β 1*.¹⁰⁸ *CXCR4*, a chemokine receptor that is known to bind the CXCL12 chemokine, was also shown to promote migration and invasion of ES cell lines *in vitro* in a Cdc42 and Rac1 dependent manner.¹⁰⁹ *CXCR4* expression has previously been associated with ES metastatic disease in patient samples,¹¹⁰ and with ES angiogenesis.¹¹¹ However, links to metastasis have been disputed by others.¹¹²

Analysis of micrometastasis in ES has led to several insights into potential drivers of metastasis. *ERBB4* [(HER4) receptor tyrosine kinase, a member of the epidermal growth factor receptor (EGFR) family], activation suppresses *anoikis*, a hallmark of transformation, in ES cell lines.¹¹³ *Anoikis*, or programmed cell death after detachment from the extracellular matrix,¹¹⁴ is thought to be critical for cancer cells to survive under anchorage independent conditions such as in the circulation or the lymphatics, prior to establishment of overt metastases.¹¹⁵ ES cell lines suppress *anoikis* and survive as multicellular spheroids in suspension cultures¹¹⁶ in an E-cadherin dependent manner that correlates with increased activation of the PI3 kinase (PI3K)-Akt pathway, upregulation of *ERBB4*, and enhanced chemoresistance.¹¹³ Significantly, *ERBB4* is transcriptionally upregulated in cell lines derived from recurrent or metastatic ES, and increased *ERBB4* protein expression was

identified in metastatic compared to primary patient-matched biopsies and was associated with reduced disease-free survival in clinical cases.¹¹⁷ In addition, ERBB4 overexpression enhanced migration and invasion of ES cells *in vitro* and increased lung metastases *in vivo*.¹¹⁷ Upregulation of ERBB4 in ES is dependent on hyperactivation of the PI3K-Akt cascade and on focal adhesion kinase (FAK). Critical for ERBB4-dependent metastasis is the Rac1 GTPase, which is activated through PI3K-Akt and FAK dependent pathways in ES cells *in vivo*.¹¹⁷ Therefore both DKK2-SDF1 α -CXCR4-Rac1 and ERBB4-PI3K-Akt-FAK-Rac1 pathways are potential drivers of metastasis in ES. It will be important to determine whether blocking these pathways pharmacologically, or inhibiting common downstream effectors such as Rac1, can reduce the burden of metastatic disease in ES.

C. Cell Plasticity

Tumor cell heterogeneity that contributes to metastasis can be a result of genetic changes, as discussed above, or non-mutational heterogeneity.¹¹⁸ Heterogeneity that is not driven by differences in DNA sequence is evidenced by inter-cellular differences in gene and/or protein expression and can be mediated by factors ranging from altered epigenetic regulation to altered protein processing.^{118,119} CXCR4 is heterogeneously expressed in ES and expression is induced under conditions of stress.¹⁰⁹ In addition, ERBB4 is preferentially activated under conditions of serum deprivation and anchorage independence.^{113,117} Thus, these studies together suggest that induction of CXCR4 and ERBB4 may be part of an ES stress response that contributes to tumor metastasis. In breast cancer and other epithelial malignancies, phenotypic transitions between non-invasive and invasive states are mediated in part by epithelial to mesenchymal (EMT) and mesenchymal to epithelial (MET) switches. EMT is induced in response to extracellular signals such as TGF- β and is dependent on epigenetic regulation.¹²⁰ Given the critical role that epigenetic deregulation plays in ES pathogenesis, it is highly likely that epigenetic switches promote cell state transitions in ES and contribute to ES cell heterogeneity. Determining how metastasis drivers such as CXCR4 and ERBB4 are dynamically induced in ES will support efforts to block these transitions as a novel approach to metastasis inhibition.

The ability of cells to undergo phenotypic switching is a marker of cell plasticity and is a feature of stem and progenitor cells.¹¹⁸ In epithelial tumors there is mounting evidence that cell plasticity is a major determinant of the cancer stem cell phenotype.^{119,121} Nearly all ES cells robustly express BMI-1, a marker of both normal and cancer stem cells.^{75,122} Thus, plasticity may be an inherent property of many, if not all, ES cells. As such, ES cells *in vivo* are also likely to be highly dynamic and responsive to stress. Future studies should focus on developing *in vivo* and *ex vivo* models that will permit testing and validation of tumor cell plasticity and its contribution to ES metastasis in real time in contextually relevant microenvironments.

IV. THE WAY FORWARD

Improved outcomes and quality of life for patients remain the ultimate goals of ES research. Unfortunately, the field has reached an impasse largely as a result of a continued inability to improve outcomes for patients with metastatic disease. In this final section we discuss the

areas of research that we feel have the greatest potential to yield transformative advances that will translate into benefits for patients.

A. Novel Treatments

Early stage clinical trials continue to test molecularly targeted agents in ES but, to date, results have been largely disappointing.⁵ Nevertheless, there is mounting preclinical evidence, and some supportive clinical trial data, that targeting the IGF1 pathway and PARP inhibition will be beneficial for at least some patients.^{5,6} It will be imperative that new agents be tested in combination with other drugs in order to optimize response and minimize emergence of resistant clones.¹²³ Failure to appreciate the absolute nature of emergent resistance to single pathway-targeted therapies will result in failed clinical trials and unwarranted dismissal of promising agents.

Immunotherapy approaches, including PD-1 blockade and chimeric antigen receptor-modified T-cell therapy, are showing tremendous promise in hematologic malignancies as well as some solid tumors.¹²⁴⁻¹²⁶ These findings provide compelling rationale to continue to pursue these avenues in ES, either alone or in combination with epigenetic priming to induce immunogenic antigen expression.^{127,128} In addition, approaches to directly target EWS-ETS fusions themselves, or their key downstream hubs, should continue as areas of focus.⁵ With respect to targeting the fusions themselves, small molecule inhibitors of protein-protein interactions, such as YK-4-279^{129,130} and RNA-interference based technologies^{131,132} provide proof of concept data that this approach is feasible. With respect to targeting the aforementioned downstream hubs of EWS-ETS action, early preclinical studies with inhibitors of LSD1,⁸⁰ HDAC,^{66,133} and DNA methyltransferases,¹³⁴ suggest that targeting epigenetic deregulation will provide therapeutic benefit. Likewise, targeting of the miRNA machinery has shown promise in preclinical models.⁹⁵ Continued investigation of hub-targeted therapies should be prioritized given that they are likely to be less susceptible to the development of therapeutic resistance than single pathway-targeted approaches.

B. Novel Models

A critical need is to develop and better utilize models of metastatic ES, such that metastasis inhibition and time to progression studies can be initiated. There is currently a dearth of strategies to assess *in vivo* metastatic capacity of ES cells. New models for this purpose that should be further developed for these efforts include renal subcapsular implantation¹¹⁷ as well as orthotopic injections into bone.^{107,135} These models more faithfully recapitulate the metastatic process than traditional subcutaneous or tail vein injection models. In addition, epigenetic instability and evolution of cell lines in culture can result in considerable biologic drift that can mask or even erase key physiologic features of the primary tumor. Moreover, response to therapy can be altered by culture-induced changes. For this reason novel therapies and studies of tumor biology should ideally be performed using early passage cells or patient derived xenografts (PDX).¹³⁶ Given the rarity of ES and the need for considerable infrastructure to develop PDX models, the ES research community should strive to develop, test, and share these models as a global resource both for studies of tumor biology as well as for testing of novel therapeutic agents and regimens.

Other xenograft models that are worthy of further investigation in ES are the mouse pulmonary metastasis assay (PUMA) and zebrafish metastasis models. In the PUMA assay fluorescently tagged sarcoma cells are injected through the tail vein, and then lungs are cultured *ex vivo* and assessed for tumor formation.¹³⁷ In zebrafish, assessment of migration capacity of fluorescence-labeled tumor cells can be determined in real time by *in vivo* imaging following xenotransplantation of the cells into embryo yolk sacs.¹³⁸⁻¹⁴⁰ Candidate metastasis genes and pathways that are identified in primary tumors or *in vitro* assays should be tested in appropriate *in vivo* models in order to validate their importance to metastatic progression.

Host immune system, cancer associated fibroblasts, blood vessels and other tumor-associated stromal cells contribute to tumorigenicity, tumor progression and drug response.¹⁴¹ Immune deficient animals and other available experimental models of ES largely fail to consider the contribution of these factors and exploration of innovative approaches that utilize so-called humanized mice should be pursued.¹⁴² In addition, models that rely on the use of bioengineered 3-dimensional scaffolds have recently been described and provide innovative *ex vivo* microenvironments in which to study ES tumor cell biology and drug response.^{143,144} Biologically relevant preclinical models of ES such as these that better recapitulate the cellular and matrix milieu of an established ES tumor should continue to be priorities for research and development. Moreover, preclinical drug testing should optimally be tested in at least one contextually relevant model system in order to improve identification of top promising candidates and expedite successful translation to the clinic.

C. Circulating Tumor Cells

Without systemic chemotherapy localized ES will nearly always recur at distant sites, confirming that micrometastatic disease is essentially universal^{145,146} and supporting a need for a better understanding of micrometastatic disease in ES metastasis. A landmark study in pancreatic adenocarcinoma showed that circulating cells with features of EMT can be identified in the circulation in advance of primary tumor detection.¹⁴⁷ This suggests that micrometastatic cells may be liberated very early in tumorigenesis, reiterating the need to go beyond the analysis of primary tumors to understand mechanisms of tumor spread. To that end, new technologies in nanomaterials and microfluidics that can isolate circulating tumor cells (CTC),¹⁴⁸ tumor exosomes¹⁴⁹ and tumor DNA (ctDNA)^{150,151} should be studied in ES, both in the context of patient-derived peripheral blood samples as well as in animal models of metastasis. Real-time insights into tumor metastasis from its earliest stages will be gained from these studies. In addition, CTC, ctDNA and/or tumor exosomes may prove useful as biomarkers of treatment response and relapse.¹⁵²

V. CONCLUSION

The persistent failure to improve outcomes for patients with relapsed and metastatic ES demonstrates that much remains to be learned with regards to the biology of tumor progression. In addition, the considerable short- and long-term toxicities that are associated with current chemotherapeutic regimens demand that newer approaches to therapy are developed in order to minimize off-target side effects. Successful integration of novel agents

will require their use in the context of rational combination therapies that also consider timing, sequence, and dosing. Ideally, these rational combinations will first be tested in relevant and predictive animal models, thus allowing for rapid prioritization of only the most promising agents and regimens. Finally, there is a critical need for new *in vivo* and *ex vivo* models of ES that are more reflective of tumor biology and tumor heterogeneity than current *in vitro* cell line-based models. With focused efforts to address these gaps in our knowledge the ES research community will be better placed to make discoveries that will favorably impact on patient outcomes.

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ABBREVIATIONS

BMI-1	BMI1 proto-oncogene, polycomb ring finger
CXCR4	chemokine (C-X-C motif) receptor 4
EMT	epithelial to mesenchymal transition
ERBB4	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4
ERG	v-ets avian erythroblastosis virus E26 oncogene homolog
ES	Ewing sarcoma
ETS - E26	transformation-specific family of transcription factors
EWSR1	EWS RNA binding protein 1
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FET	family of RNA-binding proteins that includes FUS, EWS, and TAF15
FLII	Fli-1 proto-oncogene
HDAC	histone deacetylase
LSD1 (KDM1A)	lysine (K)-specific demethylase 1A
MET	mesenchymal to epithelial transition
miRNA	microRNA
NKX2.2	NK2 homeobox 2
NROB1	nuclear receptor subfamily 0, group B, member 1
NURD	Nucleosome remodeling deacetylase
TARBP2	TAR (HIV-1) RNA binding protein 2

TGFB β 2**Transforming growth factor beta receptor II****REFERENCES**

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Figure 1. Model of ES Initiation

An EWS-ETS fusion gene is created by a chromosomal translocation event during cell division. If the event occurs in a cell type that is tolerant of the fusion oncoprotein, such as a mesenchymal (MSC) or neural crest (NCSC) stem or progenitor cell, in a supportive microenvironment, such as developing bone, tumor initiation can begin. Initiation of malignant transformation downstream of the EWS-ETS fusion gene is dependent on both molecular and cellular changes that, in concert, lead to maintenance of an immature cell state, epigenetic deregulation, and unlimited proliferative capacity. Secondary changes evolve over time that support clonal selection and expansion and ultimately lead to full malignant transformation. These secondary changes likely evolve in response to developmental and growth factor stimuli and occur on a cellular background of epigenetic instability. Latency between the original EWS-ETS fusion event and presentation of ES can be either brief or very prolonged depending on the stochastic nature of secondary changes and their relative potency as pro-oncogenic drivers.

Figure 2. Biology of ES Progression

It is likely that all ES cells have the capacity to invade and metastasize as a consequence of the inherent migratory nature of their stem cells of origin. Plasticity in response to cell intrinsic (e.g. metabolic and genotoxic) and cell extrinsic (e.g. hypoxia, nutrient deprivation) stress is a key biologic feature of normal stem cells that is necessary for maintenance of stemness and unlimited proliferative capacity. ES cells, by nature of their cell of origin and epigenetic deregulation, are highly plastic and dynamically respond to stress. Adaptive, reversible responses to stress contribute to metastasis, therapy resistance, and relapse. Epigenetic evolution over time results in the selective outgrowth of clones that have reversibly, or irreversibly, adopted phenotypic changes that are heritably passed on to daughter cells. In this way, metastatic and drug resistant clones emerge in the absence of additional mutation. Finally, irreversible genetic changes that confer a growth advantage, such as loss of tumor suppressor genes or gain of copy number alterations, will be selected for over time and selective pressure for creation and expansion of these clones will be promoted by the DNA damaging effects of chemotherapy.

Table 1

Chromosomal rearrangements found in Ewing sarcoma and Ewing sarcoma-like tumors.

REARRANGEMENT	FUSION GENE
t(11;22)(q24;q12)	<i>EWSR1-FLI1</i>
t(21;22)(q22;q12)	<i>EWSR1-ERG</i>
t(7;22)(p22;q12)	<i>EWSR1-ETV1</i>
t(17;22)(q12;q12)	<i>EWSR1-ETV4</i>
t(2;22)(q35;q12)	<i>EWSR1-FEV</i>
t(16;21)(p11;q22)	<i>FUS-ERG</i>
t(2;16)(q35;p11)	<i>FUS-FEV</i>
t(20;22)(q13;q12) (NB: can occur in ring chromosome and may be amplified)	<i>EWSR1-NFATC2</i>
t(6;22)(p21;q12)	<i>EWSR1-POU5F1</i>
t(4;22)(q31;q12)	<i>EWSR1-SMARCA5</i>
Submicroscopic inv(22) in t(1;22)(p36.1;q12)	<i>EWSR1-ZSG</i>
t(2;22)(q31;q12)	<i>EWSR1-SP3</i>
t(4;19)(q35;q13)	<i>CIC-DUX4</i>