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Molecular Pathogenesis of Ewing Sarcoma: New Therapeutic and Transcriptional Targets

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Abstract

Approximately one-third of sarcomas contain specific translocations. Ewing sarcoma is the prototypical member of this group of sarcomas; it was the first to be recognized pathologically as a singular entity and to have its signature translocation defined cytogenetically, which led to the identification of its key driver alteration, the *EWS-FLI1* gene fusion that encodes this aberrant, chimeric transcription factor. Here, we review recent progress in selected areas of Ewing sarcoma research, including the application of genome-wide chromatin immunoprecipitation analyses, to provide a comprehensive view of the *EWS-FLI1* target gene repertoire, the identification of *EWS-FLI1* target genes that may also point to therapeutically targetable pathways, and data from model systems as they relate to the elusive cell of origin of Ewing sarcoma and its possible similarities to mesenchymal stem cells.

Keywords

EWS-FLI1; Ewing sarcoma; oncogenesis; transcription; chromatin immunoprecipitation

INTRODUCTION

Ewing sarcoma is the prototypical translocation sarcoma; it is one of the first members of this group of cancers to have been recognized pathologically as a singular entity (25), to be defined cytogenetically by a signature translocation (5), and to have its translocation breakpoints defined at the molecular level (19). It is also the most common cancer within this diverse subgroup of primitive sarcomas. Notably, although originally defined as a primary bone sarcoma, Ewing sarcoma is also commonly observed in extraskeletal sites, including organ primaries (e.g., kidney and pancreas). Recently, our understanding of the molecular pathogenesis of Ewing sarcoma has greatly progressed, and there have been some promising therapeutic advances. These are the topics of the present review; other aspects of Ewing sarcoma have been covered by several excellent recent reviews (7, 45, 65, 74).

Ewing sarcoma is defined by chromosomal translocations that fuse *EWS* (*EWSR1*), located at 22q12, and a gene of the ETS family of transcription factors. In 90% of cases, the fusion

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gene is *EWS-FLI1*, which encodes the N-terminal portion of EWS and the C-terminal portion of FLI1, including the ETS DNA-binding domain. In approximately 10% of cases, the fusion gene is *EWS-ERG*, in which the *ERG* gene from 21q22 substitutes for *FLI1*, which is located at 11q24 (51, 82, 93, 101). Rare cases of Ewing sarcoma show fusions of *EWS* to other *ETS*-family genes [such as *ETV1*, *E1AF* (*ETV4*), and *FEV*], or similar fusions of the EWS-related gene *FUS* (*FUS-ERG* or *FUS-FEV*) (11, 62, 80, 94). On the basis of the general rule for sarcomas with chimeric transcription factors that all translocation variants associated with a specific sarcoma involve genes from the same transcription factor family, sarcomas with these rare EWS-ETS fusions are generally subsumed into the broad Ewing sarcoma--like category. However, this elegant rule may not hold because Ewing sarcoma--like tumors with non-ETS fusions have recently been reported, however, there are too few of them to draw any firm conclusions. (84, 94)

EWS-ETS fusions, as the presumed initiating oncogenic event in Ewing sarcoma, are required for proliferation and tumorigenesis (4, 49). The EWS amino-terminal domain provides a strong transactivation domain, and its promoter is ubiquitously activated, which leads to relatively unrestricted high-level expression of the resulting fusion genes (1, 69), in contrast to the highly regulated and lineage-restricted expression of native FLI1 (91). Three aspects of recent work on the biology of Ewing sarcoma are of special interest: (*a*) the use of genome-wide chromatin immunoprecipitation analyses to provide a comprehensive view of the *EWS-FLI1* target gene repertoire, (*b*) the identification of *EWS-FLI1* target genes that may also represent therapeutic targets, and (*c*) the emerging evidence for Ewing sarcoma as a cancer of aberrant reprogramming of mesenchymal stem cells (MSCs).

EWING SARCOMA MODEL SYSTEMS AND THE ELUSIVE CELL OF ORIGIN

EWS-FLI1 functions primarily as a transcription factor to regulate gene expression. Therefore, by deregulating the expression of specific repertoires of target genes, EWS-FLI1 may orchestrate multiple oncogenic hits. Indeed, because the fusion protein regulates critical genes involved in Ewing sarcoma tumorigenesis, it is important to identify those genes and to define their function in tumor development. Some progress has been made in this regard since the original identification of EWS-FLI1, but the recent development of new model systems and the application of modern pangenomic technologies have allowed for much more rapid progress over the past few years.

Because the cell of origin of Ewing sarcoma is unknown, many studies have relied on heterologous cell types to study the fusion protein. The earliest studies used NIH3T3 immortalized murine fibroblasts as a model system. NIH3T3 cells are readily transformed by the expression of EWS-FLI1 and were considered a useful system in which to study the oncoprotein (58, 59). Indeed, key aspects of EWS-FLI1function were originally defined in this system, and numerous EWS-FLI1-regulated genes were identified (3, 13, 20, 57, 85, 103). Unfortunately, recent data have raised questions as to the utility of EWS-FLI1-transduced NIH3T3 cells as a model system for Ewing sarcoma. For example, the gene-expression profile of NIH3T3 cells expressing EWS-FLI1 (as determined by microarray analysis) does not mimic the global profile of Ewing sarcoma tumor samples from patients (14, 33). Additionally, key genes that are regulated by EWS-FLI1 in patient-derived Ewing sarcomas and that are required for the tumorigenic phenotype, such as *NKX2.2* and *NR0B1*, are not regulated by EWS-FLI1 in the murine system (28, 67). Finally, some genes that are critical for oncogenic transformation in the NIH3T3 system, such as *PDGFC*, do not appear to be critical in Ewing sarcoma (92, 103). One explanation for these discrepancies is that EWS-FLI1 may trigger a generic ETS-mediated transformation process in NIH3T3 cells rather than a Ewing sarcoma-specific process.

Many groups have developed model systems in which EWS-FLI1 is expressed in various human cell types, rather than mouse cells. These models include human telomerase reverse transcriptase (hTERT)-immortalized primary human fibroblasts, human MSCs, human rhabdomyosarcoma cells, and human neuroblastoma cells (36, 53, 75, 77). In each case, the gene-expression changes associated with EWS-FLI1 expression were significantly similar to those of Ewing sarcoma tumor specimens (33, 36, 53, 75, 77). This finding raises the interesting possibility that the effects of EWS-FLI1 expression depend more on the organism used (e.g., mouse versus human) than on the specific cell type used (e.g., normal versus cancerous). Indeed, Ewing sarcoma appears to be exclusively a disease of humans---with the possible exception of a single case report of a camel developing a Ewing sarcoma-like tumor (95)! The reasons underlying this species specificity are unknown but could be due to differences in microsatellites across organisms (see "EWS-FLI1 Transcriptional Target Genes as Therapeutic Targets" section below).

Each human cell model has provided an opportunity to study different aspects of Ewing sarcoma development. As an example, expression of EWS-FLI1 in hTERT-immortalized human fibroblasts results in a growth arrest that is p53 dependent (53). This result suggests that EWS-FLI1 is toxic when expressed in the improper cellular context. Mutation in p53, or other components of the p53 pathway, may then allow for stable expression of EWS-FLI1 and for the growth and survival of those cells. Although mutations in p53 itself are present in only 10% to 15% of Ewing sarcoma cases, other alterations in the p53 pathway (including loss of $p14^{ARF}$/$p16^{CDKN2A}$, or amplification of HDM2) may be present in additional cases (37, 46, 50). In addition to genetic alterations in the p53 pathway, p53 activity in Ewing sarcoma cells may be modulated by EWS/FLI-mediated inhibition of Notch pathway signaling (8).

Another important lesson learned from EWS-FLI1 studies in heterologous cells comes from work performed in rhabdomyosarcoma and neuroblastoma cells. In these two settings, expression of the fusion protein caused changes in the differentiation characteristics of the target cells. Thus, rhabdomyosarcoma cells expressing EWS-FLI1 adopted a morphology that was similar to the small round cell appearance of Ewing sarcoma, and these cells upregulated genes that are typically expressed in Ewing sarcoma (such as the neuron-specific microtubule gene *MAPT*, the parasympathetic marker cholecystokinin, and the epithelial marker keratin 18) while inhibiting markers of muscle differentiation (36). In the case of neuroblastoma cells, there was an inhibition of neuroblastoma-specific markers and an upregulation of Ewing sarcoma-specific markers (77). These observations suggest that EWS-FLI1 itself induces the neural crest phenotype of Ewing sarcoma; this phenotype may not therefore reflect a neural crest cell of origin.

Such findings have also been observed in other systems. Expression of EWS-FLI1 in murine pluripotent bone marrow progenitors blocked these progenitors' ability to differentiate into adipogenic or osteogenic lineages (88), perhaps due to the recently described binding of EWS-FLI1 to RUNX2 (a protein required for osteogenic differentiation) (54). Similarly, expression of the fusion in murine C2C12 myoblasts blocked their myogenic differentiation capability (23). Even with the caveats noted above about the use of murine cells, these studies are consistent with the emerging concept that EWS-FLI1 may inhibit normal differentiation and may induce the so-called Ewing sarcoma phenotype.

These studies suggested that Ewing sarcoma may arise from a pluripotent precursor cell and that the tumor phenotype may be induced by EWS-FLI1, whereas the normal differentiation pathways of that pluripotent cell may be inhibited by the fusion protein. Although the cell of origin of Ewing sarcoma remains uncertain, a recent hypothesis is that the tumor may arise from MSCs. MSCs can differentiate into numerous mesodermal cell types, including

chondrocytes, osteoblasts, and adipocytes (Reference 26 and references therein). They appear to be particularly prevalent in the bone marrow but are also present in other tissues. Thus, their distribution mimics that of Ewing sarcoma tumors. Consistent with this hypothesis, inhibition of EWS-FLI1 expression in patient-derived Ewing sarcoma cell lines, through the use of RNA interference (RNAi), induces a gene-expression pattern that is similar to that observed in MSCs (40, 86). Furthermore, although control Ewing sarcoma cell lines cannot differentiate into osteoblasts or adipocytes in tissue culture, RNAi-mediated inhibition of EWS-FLI1 in those cells allows such differentiation to occur. In another series of studies, introduction of EWS-FLI1 into murine MSCs induced oncogenic transformation, whereas introduction of the fusion into human MSCs did not (73). Again, the issues concerning the use of murine cells render these data somewhat difficult to interpret, but when taken together with the other data described above, MSCs remain a likely candidate for the long-sought-after Ewing sarcoma cell of origin. The inability to transform human MSCs by EWS-FLI1 expression may reflect simply a requirement for additional mutations in, or activation of, cooperating pathways for expression of the full oncogenic phenotype. The definitive identification of the cell of origin would ultimately allow for a bottom-up approach to modeling Ewing sarcoma, in which EWS-FLI1 and additional alterations could be introduced into those precursor cells and the contributions of each alteration to tumorigenesis could be studied in detail.

Recent studies of the generation of nonembryonic stem cells by inducing pluripotency through nuclear or lineage reprogramming with a minimal set of transcription factors (typically OCT4, also known as POU5F1; SOX2; KLF4; and MYC) (30, 98) may also improve our understanding of primitive sarcomas of uncertain lineage, such as Ewing sarcoma. For example, in human MSCs, EWS-FLI1 induces expression of the embryonic stem cell genes *OCT4*, *SOX2*, and *NANOG* (76) and upregulates the polycomb repressor *EZH2* (72). This finding suggests that EWS-FLI1 itself may contribute to a pluripotent, undifferentiated state in Ewing sarcoma by regulating these stem cell-related genes. Perhaps significantly, *EWS* also, rarely, fuses with *OCT4* itself in undifferentiated bone sarcoma (27, 97), in myoepithelial tumors of soft tissue (2), and in certain salivary gland tumors (60).

The advent of RNAi technology has allowed for the development of a complementary top-down approach to studying the development of Ewing sarcoma (47, 67). In this approach, investigators begin with patient-derived Ewing sarcoma cells, knock down EWS-FLI1 expression with RNAi, and study the changes that occur following this manipulation (and others). Studies using this approach have demonstrated that diminished EWS-FLI1 expression causes decreased cell survival, proliferation, and oncogenic transformation (16, 42, 70, 81). Interestingly, on the basis of the combination of the specific RNAi approach (e.g., transfected transient RNAi versus stable knockdown using retrovirus-mediated RNAi) and/or the specific Ewing sarcoma cell line used, there appear to be some differences in the phenotypes observed in these studies. The reasons for such differences are not known.

EWS-FLI1 TRANSCRIPTIONAL TARGET GENES AS THERAPEUTIC TARGETS

The top-down approach has allowed for the identification of genes that are comprehensively regulated by EWS-FLI1. Early work (performed in the NIH3T3 cell model) demonstrated that EWS-FLI1 functions as a transcriptional activator to mediate oncogenic transformation (52, 58, 59). Surprisingly, initial gene-expression studies using the top-down approach indicated that EWS-FLI1 downregulates many more genes than it upregulates (70, 81). Nevertheless, these studies provided the first comprehensive views of EWS-FLI1-mediated gene regulation in Ewing sarcoma cells. Importantly, the gene-expression signatures were

very similar to those identified for Ewing sarcoma tumors from patients, which validated the use of Ewing sarcoma cell lines as a model for the disease.

Equally importantly, these models have been used to identify many EWS-FLI1-regulated genes that are involved in the transformed phenotype of Ewing sarcoma. Although space constraints do not allow for a discussion of all of these genes, we illustrate a few here to illustrate some key themes that appear to be relevant for the disease.

As discussed below, there is substantial interest in the role of the insulin-like growth factor (IGF) pathway in Ewing sarcoma. Although this pathway is often considered to be a parallel or complementary pathway to the EWS-FLI1 oncoprotein, EWS-FLI1 itself may regulate the activity of the pathway in Ewing sarcoma. For instance, IGFBP3 (IGF-binding protein 3) is downregulated by the fusion protein (70). The working model for such downregulation posits that such downregulation allows more free biologically active IGF-1 to be available, in an autocrine or paracrine fashion, to stimulate tumor growth and survival. Consistent with this working model, the cell death observed in one model of Ewing sarcoma following EWS-FLI1 knockdown partially depended on IGFBP3 (70). Furthermore, the exogenous administration of IGFBP3 to Ewing sarcoma cell lines blocks both cell growth and oncogenic transformation (10). Work performed in murine MSCs demonstrated that EWS-FLI1 regulates the expression of IGF-1 itself, although the relevance of this finding to human cells is uncertain (18).

Another critical pathway to emerge from the top-down approach is the sonic hedgehog (Shh) pathway via NKX2.2. Early microarray studies demonstrated that *NKX2.2* is upregulated by EWS-FLI1 and that ongoing NKX2.2 protein expression is required for the transformed phenotype of Ewing sarcoma cells (81). NKX2.2 is a transcription factor that harbors a homeodomain-type DNA-binding domain, as well as transcriptional activation and transcriptional repression domains. Structure-function studies demonstrated that NKX2.2 functions solely as a transcriptional repressor in Ewing sarcoma cells to mediate the transformed phenotype (66). As expected, a large fraction of the genes that are repressed by NKX2.2 in Ewing sarcoma are also downregulated by EWS-FLI1. This finding implicates one mechanism for the large number of EWS-FLI1-downregulated genes identified in the aforementioned transcriptional profiling studies: EWS-FLI1 does not directly repress genes but rather causes gene downregulation by upregulating repressors. Note, however, that the NKX2.2 downregulated signature makes up only a small part of the EWS-FLI1 downregulated signature. Thus, other proteins or mechanisms probably play a role in transcriptional downregulation in Ewing sarcoma.

An important consequence of the identification of NKX2.2 as a transcriptional repressor is that this function could be targeted as a therapeutic approach. NKX2.2-mediated gene repression depends on protein-protein interactions with TLE corepressor family members (21). TLE proteins interact with histone deacetylases, which are at least partially responsible for the observed transcriptional repressive function (17). In one study, blockade of histone deacetylase function with the small molecule vorinostat reversed the gene-expression pattern of NKX2.2 and blocked cell growth and oncogenic transformation in vitro (66). Unfortunately, although preclinical testing in a series of pediatric cancer models (including Ewing sarcoma) demonstrated activity in vitro, there was no significant activity against in vivo xenograft models (41). On the basis of the in vitro work, the levels of vorinostat achievable in vivo were probably below the likely IC_{90} (the inhibitory concentration 90%, which is the concentration at which 90% of histone deacetylase activity is inhibited).

NKX2.2 appears to be an indirect target gene of EWS-FLI1. In the developing central nervous system, *NKX2.2* expression is regulated by Shh signaling (15). Normally, Shh

signaling is initiated by the binding of the Shh ligand to the Patched receptor. Patched normally inhibits Smoothened, and so Shh-Patched binding releases this inhibition, resulting in Smoothened activation, and stimulates a signaling cascade culminating in increased function of the GLI family of transcription factors (including GLI1, 2, and 3). Suprisingly, analysis of Shh signaling in Ewing sarcoma suggested that the Shh pathway is not activated at the ligand-receptor level (102). Interestingly, microarray analysis identified *GLI1* as an EWS-FLI1 upregulated gene (81). Subsequent studies demonstrated a pathway in which EWS-FLI1 directly binds and upregulates the *GLI1* promoter while GLI1, in turn, upregulates *NKX2.2* (9). Thus, the canonical Shh signaling pathway is effectively short-circuited by EWS-FLI1. This is one example of how EWS-FLI1 may usurp cancer-relevant molecular pathways to mediate oncogenesis. It is unknown whether other downstream targets of GLI1, in addition to NKX2.2, are also involved in the oncogenic development of Ewing sarcoma.

Another recently identified critical EWS-FLI1 target gene is *NR0B1. NR0B1* was previously identified as being mutated in congenital adrenal hypoplasia and as being duplicated in dosage-sensitive sex reversal (100). Analysis of EWS-FLI1-regulated genes across multiple Ewing sarcoma cell lines demonstrated that *NR0B1* was the most consistently regulated gene by the fusion protein (42). Direct analysis of NR0B1 (also known as DAX1) protein function revealed a crucial role in maintenance of the transformed phenotype of Ewing sarcoma cells (29, 42).

NR0B1 is an unusual member of the nuclear hormone receptor superfamily. It contains a putative ligand-binding domain in its C terminus, although a ligand, if one exists, has yet to be identified. Classic nuclear hormone receptors contain a DNA-binding domain in their N termini. However, in NR0B1, the N terminus does not contain an identifiable DNA-binding domain. Instead, it contains three-and-one-half repeats of approximately 65 amino acids each that contain conserved LXXLL motifs (29). LXXLL motifs are commonly found in coregulators of nuclear hormone receptors and are also referred to as NR (nuclear receptor) boxes (78).

The function of NR0B1 in Ewing sarcoma is not well understood, but current data suggest that it may be a coregulator of gene expression. Microarray analysis of Ewing sarcoma cells in which NR0B1 expression was reduced with RNAi approaches demonstrated an equivalent number of genes that were upregulated and downregulated by the protein (43). Further analysis demonstrated a small but significant overlap between genes that were regulated by NR0B1 and those that were regulated by EWS-FLI1. Interestingly, binding-site analysis demonstrated that the patterns of EWS-FLI1 and NR0B1 occupancy at a small number of genes were identical. This finding led to the identification of a protein-protein interaction between NR0B1 and EWS-FLI1 and prompted the suggestion that each protein modulates the other's transcriptional function.

During the process of understanding the regulation of *NR0B1* gene expression by EWS-FLI1, a unique EWS-FLI1 response element was identified: GGAA-containing microsatellites (28). Microsatellites are sequence elements that consist of multiple consecutive repeating units; each unit consists of a small sequence. Microsatellites make up approximately 3% of the human genome and are sometimes considered junk DNA, that is, DNA without a known function. In the case of the microsatellite found in the *NR0B1* promoter, there are 25 repeating GGAA motifs (28). That particular microsatellite harbors two single-base insertions and is thus 102 base pairs in length. It is found approximately 1.5 kb upstream of the *NR0B1* transcriptional start site. A series of focused studies revealed that the GGAA microsatellite is both necessary and sufficient to confer EWS-FLI1-mediated

regulation of the *NR0B1* promoter. Furthermore, EWS-FLI1 binds this element both in vitro and in vivo. Thus, the GGAA microsatellite is a bona fide EWS-FLI1 response element.

The use of GGAA microsatellites as EWS-FLI1 response elements is not limited to the *NR0B1* gene. Genome-wide localization studies using chromatin immunoprecipitation (ChIP) and analyzed with either microarray (chip) or high-throughput sequencing (seq) demonstrated that EWS-FLI1 binds to many GGAA-microsatellites throughout the genome (28, 31). The initial ChIP-chip studies demonstrated that other genes are similarly regulated by EWS-FLI1 through these elements (28). Furthermore, GGAA microsatellites are enriched in the promoters of genes that were upregulated by EWS-FLI1, but these elements are depleted near genes that were downregulated by the fusion protein. Similar results were observed in subsequent ChIP-seq studies (31).

Unlike ChIP-chip studies using commercial promoter arrays, ChIP-seq is not limited to regions surrounding promoters; this technique finds that the majority of genomic regions bound by EWS-FLI1 are intergenic. Use of the ChIP-seq approach revealed that GGAA microsatellites bound by EWS-FLI1 are not limited to promoter regions close to transcriptional start sites but often are more than 200 kb upstream of the target gene transcription start site. This finding suggests that chromatin looping may bring distant regions together in a transcriptional hub that allows EWS-FLI1 to exert its effects on gene expression.

Microsatellites are known polymorphic sites, so higher numbers of repeats at one or more key target genes may underlie individual or ethnic differences in susceptibility to Ewing sarcoma, such as its rarity in individuals of African descent (28, 96). EWS-FLI1 also binds to more conventional, nonrepetitive ETS motifs, and these sites are associated with either repression or activation of transcription (12). Also, sites for E2F, NRF1, and NFY are overrepresented in a subset of EWS-FLI1 target regions, which suggests that cooperative interactions may occur between EWS-FLI1 and specific cognate transcription factors at these promoters (45). Identifying the direct target genes that are upregulated by EWS-FLI1 can inform focused RNAi-based screens to define the genes that are the most essential for the survival and proliferation of Ewing sarcoma cells. Likewise, an even more global approach may be to perform genome-wide RNAi screens of Ewing sarcoma cell lines and integrate the data with existing ChIP-based, genome-wide EWS-FLI1 target gene data sets to determine which EWS-FLI1 target genes or pathways may constitute therapeutic targets.

As a proof of principle of this concept, recent work used GGAA microsatellites as a hook to identify directly bound EWS-FLI1 target genes. Following a search for GGAA microsatellites within gene promoters, the *GSTM4* gene was identified as a direct EWS-FLI1 target (55). The promoter of this gene contains a GGAA microsatellite that is both necessary, and sufficient, for EWS-FLI1-mediated regulation. The microsatellite is also bound by EWS-FLI1 in vivo.

GSTM4 encodes one member of the glutathione S-transferase family. GST enzymes often mediate the detoxification and excretion of both endogenous and exogenous compounds through conjugation to glutathione. Experimental manipulation of GSTM4 levels demonstrated that reduced levels of the enzyme cause increased sensitivity of Ewing sarcoma cells to some, but not all, chemotherapeutic agents [such as etoposide and fenretinide (55)]. This result suggests that the level of GSTM4 expression in tumors may correlate with patient outcome. Analysis of a limited number of primary tumor specimens from patients supported this association; patients with lower levels of GSTM4 protein expression generally had improved outcomes compared with patients with higher levels of

GSTM4 expression. Whether differences in expression of GSTM4 are associated with GGAA-microsatellite polymorphisms in the *GSTM4* promoter has yet to be determined.

In addition to the significant recent progress on the identification of EWS-FLI1 response elements and DNA binding, there has also been progress in our understanding of the mechanisms of transcriptional regulation by the fusion protein. For example, recent work has demonstrated that EWS-FLI1 undergoes posttranslational modifications by the addition of both phosphates and carbohydrates (O-GlcNAcylation) to the protein (6). These modifications appear to increase the transcriptional activity of the fusion protein.

Protein-protein interactions also appear to be important for the transcriptional function of EWS-FLI1. As discussed above, the EWS-FLI1 upregulated target NR0B1 interacts directly with the fusion protein and modulates its transcriptional activity (43). Interactions between EWS-FLI1 and the coactivator CBP have been described, as have interactions between EWS-FLI1 and one of the RNA polymerase II subunits itself (hsRPB7) (68, 71). Protein-protein and protein-DNA interactions of transcription factors have been historically considered “poorly druggable,” that is, difficult to inhibit with small molecules. However, a recent notable exception, namely the identification of a small molecule that disrupts a critical interaction between the Ewing sarcoma EWS-FLI1 protein and RNA helicase A (RHA), suggests that such approaches may be feasible (24).

Interactions between EWS-FLI1 and RHA have been described (89). Although the exact role of RHA in normal cellular physiology is not well understood, the protein (and its interaction with EWS-FLI1) appears to be important for the transcriptional activation function of the fusion protein. This interaction has particular relevance to oncogenesis in Ewing sarcoma because inhibition of this interaction (via a peptide-based inhibitor or a small-molecule inhibitor) induces apoptosis and inhibits xenograft tumor growth of Ewing sarcoma cell lines (24). This finding suggests that a new therapeutic approach to this disease may be through blockade of the transcriptional function of EWS-FLI1.

Blockade of EWS-FLI1 function does seem to be an ideal approach for the treatment of Ewing sarcoma. As discussed above, ongoing EWS-FLI1 expression is required for the transformed phenotype of Ewing sarcoma cells. Approaches to target EWS-FLI1 itself, in vivo, have begun to be investigated. RNAi is an excellent laboratory-based approach, but application in the clinical arena has made only slow progress. An important step in this direction has focused on nonviral delivery of short interfering RNA (siRNA). Nanoparticles have been developed that incorporate cyclodextrin-containing polycations to package the siRNA and protect it from degradation (35). These nanoparticles also incorporate transferrin on their surface, which allows targeting to transferrin receptor-expressing tumor cells (including many cancer types, such as Ewing sarcoma). Administration of nanoparticles prepared with siRNA targeting EWS-FLI1 inhibited the development of metastatic tumor deposits following tail-vein injection of Ewing sarcoma cells, thereby providing a proof-of-principle of this approach.

An alternate approach is to identify agents that disrupt EWS-FLI1 function or expression. As discussed above, blockade of important protein-protein interactions is one such approach. A recent study instead searched for agents that could inhibit the EWS-FLI1-mediated transcriptional signature, without regard for how this process might occur (83). The conceptual benefit of this approach is that molecular-level detail of EWS-FLI1 function is not required, and theoretically, agents that block any activity required for EWS-FLI1 function may be identified. An initial small-molecule screen using this approach identified cytarabine as an agent that inhibits EWS-FLI1 protein expression. The mechanistic basis for this finding is not yet known. However, because cytarabine is a drug that is already

commonly used in the pediatric cancer population (mainly for leukemia treatment), this agent was rapidly tested in a clinical trial setting (22). Unfortunately, the agent proved too toxic for the majority of patients with relapsed or refractory Ewing sarcoma to tolerate, so no efficacy could be shown. Future work in this regard might include devising approaches to administer this agent early in the course of therapy or, perhaps, at lower doses when given in combination with other, potentially synergistic agents. Additional screens could also be conducted to identify other agents.

A still-promising approach to the discovery of therapeutic targets in Ewing sarcoma is the identification of EWS-FLI1 target genes within pathways that encode known drug targets. As more transcriptional targets of the EWS-FLI1 fusion protein of Ewing sarcoma are uncovered, multiple signaling pathways appear to be affected; these pathways include Notch (8), Hedgehog/GLI (9, 39, 102), Wnt/β-catenin (61), transforming growth factor β(32, 38), and IGF-1 receptor (IGF-1R) (34). The last pathway may be deregulated by EWS-FLI1 at several levels, as discussed above. The role of the IGF1R pathway in tumorigenesis has been investigated for years and was initially of interest in Ewing sarcoma due to the overexpression of IGF-1 and IGF-1R in tumor cell lines (79, 99). These findings have provided part of the rationale for trials of several IGF-1R inhibitors (56, 63, 90). Indeed, these trials of anti-IGF-1 receptor agents in Ewing sarcoma patients, either monoclonal antibodies or small molecules (44), have reported remarkable, albeit rarely durable, responses in a subset of patients (48, 64, 87).

CONCLUSIONS

Recently, great progress has been made in our understanding of the molecular pathogenesis of Ewing sarcoma. Much of this progress has occurred because of new and improved technologies, especially in the genomic realm, such as microarray-based gene-expression analysis and deep sequencing approaches. These technologies have allowed for an unprecedented genome-wide view of EWS-FLI1 binding and transcriptional activity and has allowed for new insights into the function of this important oncoprotein.

Although new technologies have allowed for major advances, they have also created unprecedented challenges. The ability to assess the role of specific target genes and EWS-FLI1-bound loci on Ewing sarcoma development is still largely a one-gene/locus-at-a-time endeavor. Again, new technologies and approaches may find utility here. For example, high-throughput RNAi screens could be used to perform rapid initial screens for genes required for various phenotypes associated with tumor development (proliferation, survival, and so on). Similarly, use of the many data generated via high-throughput techniques to identify new therapeutic strategies remains a challenge. Nevertheless, innovative approaches to small-molecule screening have already shown some promise in this regard. Ultimately, we believe that the rapid pace of scientific progress in Ewing sarcoma will allow us to better understand the molecular pathogenesis of this aggressive disease and to use this knowledge to devise better strategies to cure patients.

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