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Review Article

Challenges in modeling EWS-FLI1-driven transgenic mouse model for Ewing sarcoma

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Abstract: EWS-FLI1 is a master regulator of Ewing sarcoma (ES) oncogenesis. Although EWS-FLI1 represents a clear therapeutic target, targeted therapeutic inhibitors are lacking. Scientific literature has indicated accumulating information pertaining to EWS-FLI1 translocation, pathogenesis, function, oncogenic partnerships, and potential clinical relevance. However, attempts to develop EWS-FLI1-driven human-like ES mouse models or in *vivo* systems ended up with limited success. Establishing such models as preclinical screening tools may accelerate the development of EWS-FLI1 targeted therapeutic inhibitors. This review summarizes the current scenario, which focuses on the limitations, challenges, and possible reasons for past failures in model development and also plausible *interim* alternatives.

Keywords: EWS-FLI1, Ewing sarcoma, mouse models, fusion protein, tumor cell of origin, progenitor cells

Introduction

Ewing sarcoma (ES) is a malignancy associated with bone and soft tissue, which generally affects children and young adults [1]. ES is the second most common primary bone malignancy in pediatric patients [2]. This disease is typically aggressive with frequent cases of micrometastasis at presentation, which in turn is associated with an increased risk of systemic relapse [3-5].

Over the past thirty years, patient response with localized disease has improved dramatically from 10% with surgery and radiotherapy alone to 70% with chemotherapy and multimodal approaches. Despite rapid improvement in treatment regimens, survival rates have remained unacceptably low even in patients with localized disease [6]. Metastases and local recurrence still develop in 30-40% of cases [7-9]. Patients with metastasis, who relapse or have a poor histological response to initial therapy, were found to show an overall poor prognosis [10, 11]. Contemporary chemotherapeutic treatment regimens are harsh and aggressive, and survivors are at an elevated risk of morbidity and mortality due to secondary treatment-associated malignancies [12, 13]. It is estimated that most of the childhood to aged cancer survivors exhibit severe, disabling, or life-threatening conditions as a result of therapy or mortality due to long-term complications [14-16]. Therefore, poor outcome of ES patients with the existing treatment modality presents a clear and compelling need for targeted therapies.

Around 85-90% of ES exhibits a karyotypic abnormality, t(11;22)(q24;q12), due to the fusion of a potent EWS transcriptional activation domain encoded on chromosome 22 with the FLI1 DNA binding domain encoded on chromosome 11 [17-19]. This translocation results in the formation and expression of EWS-FLI1 chimeric fusion protein, which functions as an oncogenic transcription factor. Although alternative chromosomal translocations can be found in a small fraction of Ewing family of tumors, EWS-FLI1 is the most common translocation among the EWS-ETS family translocations. EWS-FLI1 primarily functions as an oncogenic transcription factor, regulating the expression of several genes involved in cancer pro-

gression [18, 20-23], and its successful inhibition has led to tumor regression [24, 25].

Significant scientific literature exists with regard to the molecular biology of EWS-FLI1 in the development of ES. Nevertheless, there is a lack of a robust in *vivo* system to study the role of EWS-FLI1 in driving ES oncogenesis. In *vivo* systems serve as an important screening tool to rapidly facilitate the development of novel compounds to effectively antagonize EWS-FLI1 and to identify novel alternative mechanisms to inhibit ES. This review summarizes the current challenges in developing an EWS-FLI1-driven ES mouse model and suggests possible alternatives.

Challenges in modeling EWS-FLI1

EWS-FLI1 is an attractive therapeutic target due to its absence in normal cells. However, there are many challenges in targeting EWS-FLI1 directly. First, EWS-FLI1 structure was predicted to be highly disordered, hampering structural analysis of the protein. Second, EWS-FLI1 protein exhibits poor solubility characteristics, due to its overall size (68 kDa). Hence, EWS-FLI1 has been a difficult macromolecule to directly analyze under in *vitro* conditions [24, 26]. Consequently, such features hamper the development of rational drug design against EWS-FLI1. To hasten drug discovery process, computer-assisted drug designing programs were widely applied. Indeed, computation-based tools have predicted the structure of EWS-FLI1 [24], associated genes, and pathways [27]. However, a target-based virtual drug design approach has faced drawbacks, since most of the drugs developed by structure-guided approaches have been associated with serious toxic side effects [28]. By definition of a whole living system, a mouse model representing the disease would be a better choice, which can act as a surrogate preclinical screening tool.

To our knowledge, most of the EWS-FLI1-driven transgenic ES mouse models failed and a robust model was not successfully generated [29, 30]. In an effort to identify other potential therapeutic targets alternative to EWS-FLI1 and to develop more effective ways to treat this disease, it is also equally important to understand the role of EWS-FLI1 cofactors and target genes [20, 24]. Indeed, there are studies which have demonstrated the interference of EWS-FLI1 with other interacting downstream partners [25, 31, 32]. Yet, therapeutic applications directed towards eliminating or inactivating EWS-FLI1 have not reached the clinic.

Importance of EWS-FLI1-driven ES mouse model

The current status of research requires appropriate disease models resembling human ES, to suitably understand ES etiology and functional drug targeting [29, 30]. While mouse models have been successfully developed for other cancers involving tumor-specific translocations [33-39], a mouse model for EWS-FLI1-driven ES appears to be very challenging due to the fact that mere expression of EWS-FLI1 in different body tissues *per se* is lethal to animal survival [29]. EWS-FLI1-driven mouse model should serve the purpose as a preclinical screening tool to screen and identify novel compounds that could functionally target EWS-FLI1. Another potential application of this model would be that it should allow the flexibility of studying the etiopathology and biology of ES, such as therapeutic drug response, role of EWS-FLI1 target genes, associated pathways, mutations, involvement of growth factor receptors, cell surface receptors, and their signaling pathways, which can potentially favor the overall growth of the disease.

Difficulties in modeling ES mouse models

An animal model should allow the flexibility to study the function of EWS-FLI1 and its role in tumorigenesis, mimicking actual disease condition in *vivo*. Genetically engineered models (GEMs) are widely used as disease models. In GEMs, the genetic profile should be favorably altered to allow overexpression of the mouse counter part of the gene of interest. There are several problems associated with such models. For instance, the mice must carry the same or similar oncoprotein of human tumors. Second, the oncoprotein should be engineered within the endogenous locus and the expression should be targeted to the correct cell type during embryogenesis and early postnatal development. Next, the oncoprotein should be expressed in specific target tissues. Optimal host/tumor microenvironment is one of the most important criteria that cannot be achieved in GEMs. GEMs cannot fully reproduce the

genetic complexity of human tumors and hold a poor predictive value. Moreover, GEMs as disease models take time to develop, and there may be no correlation between the predictive therapeutic response of a particular drug generated in mice and its outcome in the clinic. To our knowledge, viable whole animal EWS-FLI1-driven GEMs have not been successfully generated for ES until now.

A possible solution to the development of reliable models is the inclusion of the human counter part of EWS-FLI1 into a mouse model to allow the disease to phenotypically and histologically resemble human Ewing tumor. A mouse model was developed with Cre-inducible expression of EWS-FLI1 from the ubiquitous Rosa26 locus, which demonstrated rapid in *vivo* oncogenic activity. In this model, Mx1-Cre was used to drive EWS-FLI1 resulting in strong expression in the bone marrow, liver, spleen, and other hematopoietic tissues after polyinosinic. poly(C) administration [40]. Unfortunately, this model developed myeloid/erythroid leukemia and a series of abnormalities leading to death. A conditional transgenic mouse expressing EWS-FLI1 under the control of Prx1 promoter exhibited severe developmental deformities of limbs [41]. In a study, Cre/loxP-mediated recombination was employed to induce specific translocation between Ewsr1 and Fli1 loci in systemic organs of both adult mice and embryos. These mice did not develop any tumors, suffered from cardiomyopathy, and died due to chronic cardiac failure. EWS-FLI1 expression was found to be decreased in bone and liver, while bone being a common site of ES occurrence. Moreover, EWS-FLI1 expression is not directed to a particular mesenchymal tissue type. This could explain the reason for non-development of tumors and also indicates that generalized expression of EWS-FLI1 is toxic to cardiac tissue [42]. Mx1-Cre mice expressing inducible EWS-FLI1 developed leukemia [43]. Mosaic expression of EWS-FLI1 in a transgenic zebra fish model led to tumor formation at low incidence with similar histological architecture typical to human ES. However, in this model, EWS-FLI1 expression under the control of heat-shock promoter exhibited embryonic lethality, developmental defects, and pericardial edema [44]. A mega study [29] presented data from six independent laboratories seeking an alternative approach to express EWS-FLI1 in different murine tissues. These studies used Runx2, Col1a2.3, Col1a3.6, Prx1, CAG, Nse, NEFL, Dermo1, P0, Sox9, and Osterix promoters to target EWS-FLI1 or Cre expression. Additional approaches included the induction of an endogenous chromosomal translocation, in *utero* knock-in, and injection of Cre-expressing adenovirus to induce EWS-FLI1 expression locally in multiple lineages. Some of the models developed embryonic lethality or severe developmental defects. Further, few models resulted in no phenotype at all, while others led to the formation of other tumor entities like leukemia or fibrosarcoma [29]. EWS-FLI1-induced apoptosis, promoter leakiness, lack of potential cofactors, and difficulty of expressing EWS-FLI1 in specific sites were considered as primary reasons for failed attempts. Despite all these efforts, an EWS-FLI1-driven transgenic ES mouse model has not been successfully developed (**Table 1**). As a result, the need for ES mouse models to faithfully recapitulate human ES and the requirement of a community platform for such models have been strongly felt [30].

Lack of precise cell of origin

While the oncogenic activity of EWS-FLI1 is well established, the cell of origin (COO) has been perplexing due to cytotoxicity of EWS-FLI1 when expressed in many of the primary cell types and tumorigenicity in completely unrelated cell lines such as fibroblasts [45-48]. Unlike other cancers [49, 50], a precise COO has not yet been reported and ES was considered as both soft tissue and bone cancer. Previous studies have identified certain primary cell types as presumed COO, permissive for EWS-FLI1 expression such as mesenchymal, neural crest, hematopoietic, muscle, and osteo-chondrogenic progenitor cells (OCPCs) [51-54]. ES was once thought to be arising from a primitive neuroectodermal origin [55, 56]. Later, it was found that neuroectodermal characteristics of ES cells are due to EWS-FLI1 expression and not based on the COO [55, 57]. ES tumors that arise in soft tissue are morphologically and molecularly indistinguishable from those that arise in bone. Therefore, COO for ES is likely to be a primitive, multipotent cell that can give rise to bone or cell types found in soft tissues. Hence, mesenchymal cells are widely accepted as COO [56, 58-60]. Tirode and colleagues

Table 1. Abnormalities associated with the various attempts to establish EWS-FLI1-driven transgenic ES mouse models

S. No	Promoter	Expressing gene	Outcome	Target Tissue	Abnormality Noted	Reference
1	Prx1-Cre	EWS-FLI1	No tumor induced	Primitive mesenchymal cells of the embryonic limb bud	Developmental defects of limbs including shortening of the limbs, muscle atrophy, cartilage dysplasia, and immature bone	[41]
2	CAG-Cre	EWS-FLI1	No tumor induced	Ubiquitous	Dilated cardiomyopathy and chronic cardiac failure	[42]
3	Mx1-Cre	EWS-FLI1	No tumor induced	Targeted bone marrow, spleen, and liver	Myeloid/erythroid Leukemia With dense hepatic and splenic infiltrations	[40, 43]
4	Dox-inducible Rosa-M2rtTA/Col1a1 or Rosa-tetO-EWS-FLI1-IRES-mCherry	EWS-FLI1	No tumor induced	EWS-FLI1 expressed in a wide variety of organs and tissues including bone marrow and cortex of the bone	Some mice immediately died after EWS-FLI1 induction and accompanied by dysplastic changes of intestinal cells due to impaired differentiation	[104]
5	Pcp2-cre	EWS-FLI1	No tumor induced	Expressed in Pcp2-lineage cells	Displayed ataxia and exopthalmia	[107]
6	Tamoxifen inducible EF; CreER+	EWS-FLI1	No tumor induced	Ubiquitous	Dose-dependent EWS-FLI1 expression triggered early onset of apoptosis in kidneys and acute lethality	[48]
7	Runx2-cre	EWS-FLI1	No tumor induced	Osteoblast precursor	Embryonic lethality	[29]
8	Tetracycline off-Osterix 1-cre	EWS-FLI1	Induced leukemia and osteosarcoma	Osteoblast precursor	Embryonic lethality on p53 and Rb-/- background and facial bone deformities on p53 and Rb+/+ background	[29]
9	Col1a2.3-Cre, Col1a3.6-Cre	EWS-FLI1	No tumor induced	Osteoblast	Embryonic lethality	[29]
10	EWS	EWS-FLI1	No tumor induced	Ubiquitous	Embryonic lethality	[29]
11	Pgk-1	EWS-FLI1	No tumor induced	Ubiquitous	Embryonic lethality	[29]
11	Nse	EWS-FLI1	No tumor induced	Neuronal tissue	Embryonic lethality	[29]
12	NEFL	EWS-FLI1	No tumor induced	Neuronal tissue	-	[29]
13	Zinc chloride inducible metallothionein	EWS-FLI1	No tumor induced	Ubiquitous	-	[29]
14	Tetracycline-off-PLAP	EWS-FLI1	No tumor induced	Ubiquitous	Embryonic lethality	[29]
15	Tetracycline inducible TRE	EWS-FLI1	No tumor induced	Ubiquitous	EWS-FLI1 toxicity during spermatogenesis	[29]
16	Prx1-Cre	EWS-FLI1	No tumor induced	Limb bud Mesenchyme	Embryonic lethality	[29]
17	Dermo1, Prx1, P0, Col1a2 or Sox9-Cre	EWS-FLI1	No tumor induced	Mesenchymal and neural Crest tissue	-	[29]
18	Retroviral LTR	EWS-FLI1	Fibrosarcoma	MSC	-	[29]
19	Piggybac-cmv	EWS-FLI1	Fibrosarcoma	MSC	-	[29]

Table 2. List of progenitor cells as presumed COO for ES and their role in other cancers

S. No	COO	Reference	Role in other cancers	Reference (for other cancers)
1	Endothelial origin	[108]	Angiosarcoma	[109]
2	Developing myelocytes	[110]		
3	NCC	[53, 57]	Neuroblastoma	[50, 60]
4	MSC	[55]	osteosarcoma	[111]
5	OCPC	[54]		

[61] have shown that EWS-FLI1-silenced ES cells could exhibit features of trilineage differentiation and resemble phenotypes of mesenchymal progenitor cells and can be mobilized from the marrow compartment to populate extra skeletal sites, making these cells good candidates for COO [56].

ES occurs at almost equal frequencies in flat bones and the diaphysis of tubular bones [54]. This suggests that mutations related to the proliferation of bony tissue might not contribute to the genesis of ES. EWS-ETS fusion, a primary genetic event, might occur at an earlier stage of bone development, and ETS families of genes are important for transcriptional regulation in mouse embryonic and perinatal limb skeletogenesis. Dysregulated expression due to abnormal chromosomal translocation of EWS-FLI1 might result in accumulation of defective progenitor cells which exhibit increased proliferative potency. Thus, ES precursors are likely found to be highly enriched and more confined to OCPCs [54]. At the same time, studies have indicated that mesenchymal stem cells (MSCs) [52, 62, 63] and neural crest cells (NCCs) [53, 57, 64] can also be considered as presumed COO (**Table 2**).

Role of progenitor cells

It is apparent that pediatric cancers do not involve lifestyle factors. Predominantly younger age at incidence is a unique feature of several sarcomas including ES, alveolar rhabdomyosarcoma, clear cell sarcoma, synovial sarcoma, and myxoid liposarcoma. Such a scenario raises the likelihood that these pediatric cancers might originate due to abnormal accumulation of inherent deregulated progenitor cells, presumably cancerous, at an early stage of development. These deregulated progenitor cell populations are expected to exhibit an increased, uninhibited proliferative potency, manifesting as cancers at a younger age [54]. ES is an important model for cancers originating from progenitor-type cells or with progenitor-like cell features controlled by chimeric transcription factor oncogenic fusions [65]. These progenitor cells might be expected to harbor the necessary chromatin conditions and its entire cellular machinery essential for tumor-specific translocation and oncogenic activation of EWS-FLI1. Until recently, precise progenitor COO was not known for ES. Nevertheless, many presumed progenitor precursor COOs for ES were known to date [52-54, 57, 62-64]. This presumption is based on the fact that these MSCs, NCCs, and OCPCs are receptive to EWS-FLI1 expression and are able to transform into tumor cells instead of being susceptible to EWS-FLI1 toxicity [54, 66]. A report showed that stable expression of EWS-FLI1 oncogenic fusion protein in mouse mesenchymal progenitor cells was adequate enough to generate ES-like tumors [62]. Comparison of gene expression profiles of EWS-FLI1-silenced *versus* non-silenced ES cell lines indicated that EWS-FLI1-silenced gene expression signatures matched with those of mesenchymal progenitor cells [61, 67]. However, simple expression of functional EWS-FLI1 in progenitor cells, as a key factor, further requires a complicated multiple-step transformation process to achieve features of ES rather than ending up with no tumor development or development of other cancer types [40, 52]. Ideally, cancer stem cells/progenitor cells transformed to cancer cells should retain some of the features indicative of their COO. In fact, reports consistently suggested that ES tumors have originated from primary MSCs and indeed retained the features of primary MSCs [52, 61, 62]. Though the precise ES COO remains enigmatic since, EWS-FLI1-transduced pediatric bone marrow mesenchymal cells [68] and NCCs [53] matched the gene expression profiles of ES tumors.

EWS-FLI1 has been demonstrated to induce the expression of Oct4, Sox2, and Nanog in pediatric human MSCs [68]. EWS-FLI1 also inhibited classical tumor suppressors pRB [69] and p53 [70] and deregulated Wnt/β-catenin

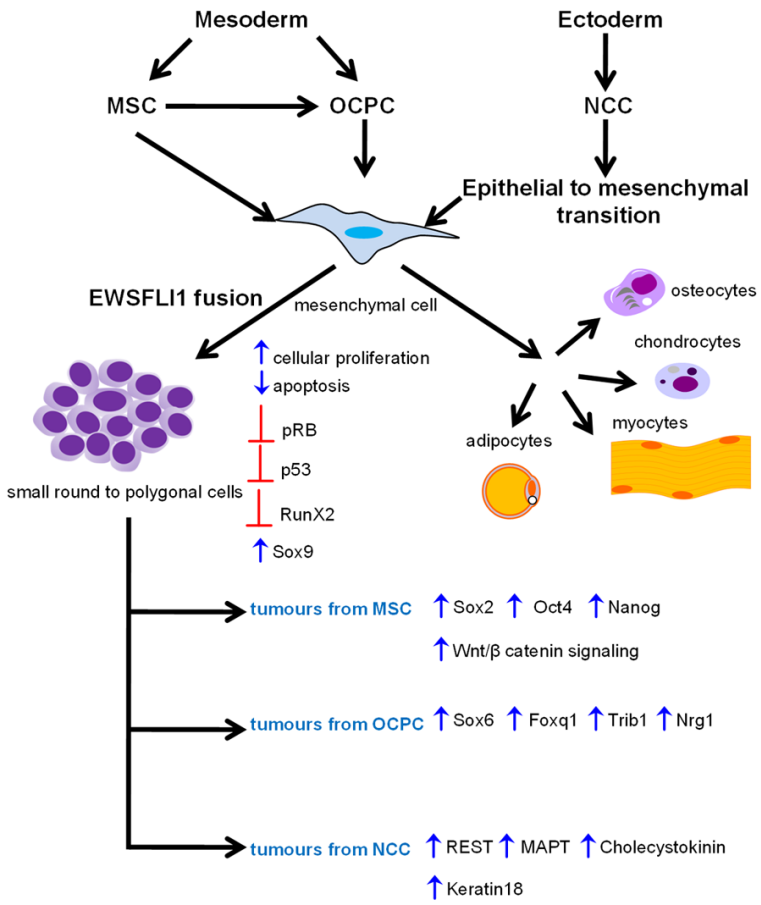

Figure 1. Scheme of EWS-FLI1-deregulated downstream transcription factors and signaling pathways in progenitor cells. Ectopic expression of EWS-FLI1 oncogenic fusion protein deregulated several downstream transcription factors and signaling pathways leading to ES oncogenesis in MSC, OCPC, and NCC progenitor cells. Such oncogenic changes resulted in a characteristic morphological transformation of mesenchymal spindle shape cells to small round-to-polygonal tumor cells.

signaling [71] in MSCs. Sox6, a physiological driver of proliferation of OCPCs, was found to be activated and controlled by EWS-FLI1 in ES tumors and cell lines [72]. EWS-FLI1 upregulated and co-partnered with Foxq1 and collectively deregulated Trib1 and Nrg1 oncogenes in ES48 and ES49 cell lines, derived from human EWS-FLI1-expressing OCPC mouse tumor allografts [73]. RE1-silencing transcription factor (REST) is a neuronal repressor gene that regulates neuronal stem cell differentiation which was found to be elevated in ES tumors and cell lines [74]. EWS-FLI1 upregulated critical genes (microtubule-associated protein Tau (MAPT), cholecystokinin, and keratin 18) in neural crest development and is responsible for neuronal phenotype of ES tumors [57]. EWS-FLI1 was found to block RunX2 hampering osteoblast differentiation in mesenchymal progenitor cells [75]. ES tumors obtained from patients have shown an increased expression of Sox9, which is believed to disrupt the chondrocyte differentiation [76]. EWSFLI1 is known to upregulate several genes (NR-0B1, DKK1, DAB1, CNTNAP2, cMYC, Sox2, CXCR4, NGFR, IGFBPs, and IGF1) essential for maintaining self-renewal, or pluripotency, of embryonic development or function as transcription factors in the development of several tissues [67]. A scheme of transcription factors and signaling pathways of progenitor cells deregulated by EWS-FLI1 is summarized in **Figure 1**. Hence, the change in molecular mechanisms within progenitor cells, dictated by oncogenic transformation of EWS-FLI1, substantiates its role as a driver of ES.

On the other hand, replenishing healthy progenitor cells as therapeutic candidates to ES patients appears to indicate the significance and utility of progenitor cells in ES [77]. In a study, peripheral blood progenitor cell transplantation has been tried out as a therapeutic approach post standard chemotherapy. The study, despite having a low sample size, concluded that replenishment prolonged disease-free survival [78]. Thus, the importance and complexity of progenitor cells as optimum targets for efficient and specific induction of ES are gaining momentum. More information on the progenitor cell transformational behavior through altered physiological signaling pathways, and post harboring the transcription factor oncogenic fusions, should make them likely candidates for targeted therapy.

Importance of molecular differences between mice and humans

A mouse cannot be regarded as a perfect equivalent to a human-like cancer model due to obvious biological, physiological, and anatomical differences. Yet, mouse models are

widely used to mimic human cancers. Though these models work in other cancers [33-39], there exist two major molecular differences between mice and humans as hurdles in the successful generation of a functional ES mouse model. First, GGAA microsatellites are important for gene expression of EWS-FLI1 targets. GGAA microsatellites motifs are not evolutionarily conserved between mice and humans, and this difference may explain the reason behind non-development of tumors, despite EWS-FLI1 expression [79]. Second, CD99 is important for ES pathogenesis, and this gene is only 50% conserved between mice and humans [80, 81]. In addition, differences in specific DNA-binding motifs and enhancers required for sarcomagenesis may also explain the difficulty in modeling ES [82].

These factors can be considered as reasons for failure, when sixteen different approaches of creating transgenic ES mouse models were attempted, with either ubiquitous or localized EWS-FLI1 expression in certain target tissues and through different promoters [29]. On the other hand, knock-in or induced expression of EWS-FLI1 in presumed COO such as OCPCs and MSCs resulted in the transformation of these primary cells into tumor cells and these cells formed tumors in immunocompromised mice. This indicates the possibility that an EWS-FLI1-driven ES mouse model can still be modeled amidst several difficulties. The results indicated [54] appear to be promising with reference to molecular differences which are barriers to model development. Indeed, Tanaka and colleagues observed an increase in NROB1 expression and other downstream target genes in mouse OCPCs transfected with EWS-FLI1, with similar trends as observed in human ES [54, 83]. Human EWS-FLI1 was able to bind enhancer elements in mouse ES48 and ES49 cell lines [73]. EWS-FLI1 binding region distribution patterns enriched with GGAA microsatellites were comparable, but with a lower frequency than human ES [79, 84, 85].

In another study, EWS-FLI1 expression was induced in primary murine MSCs with p53-/- and stag2 knockdown background, which formed sarcomas when these cells were implanted in mice [86]. Similar studies of expressing EWS-FLI1 in murine MSCs generated ES tumors in mice [62, 87]. It is worth noting that in these models [54, 62, 86, 87] there is no threat for the animals survival. These models could be employed as disease model to study ES biology or as a preclinical screening tool. To our knowledge, there is no report on attempts of ectopic EWS-FLI1 expression in murine NCCs. However, ectopic EWS-FLI1 was well tolerated in human NCCs. Upon introduction of EWS-FLI1 in *vitro*, these cells were found to differentially circumvent cellular senescence and undergo transition to ES family of tumors-like state. However, this report failed to clearly demonstrate the formation of ES-like tumors in mice with EWS-FLI1 expression in neural crest stem cells [53].

It is evident that due to systemic EWS-FLI1 toxicity and inherent molecular differences between mice and humans, a whole animal transgenic ES mouse model has not materialized to date. However, it is still possible if EWS-FLI1 can be targeted to the precise COO in mouse to result in ES formation. Since the exact COO has not yet been elucidated, as a tentative option, a graft of malignantly transformed presumed COO in mice can still be modeled as a viable alternative, with a relatively similar genotypic and phenotypic functionality comparable to human ES.

Interim alternatives

Several research groups failed to develop EWS-FLI1-driven whole animal ES models [29, 30]. Widespread toxicity in mouse models expressing EWS-FLI1 ubiquitously in the entire range of mouse tissues underlines the disadvantage of developing transgenic mice expressing EWS-FLI1 fusion transcript in *vivo* [29, 41, 42, 88]. Moreover, EWS-FLI1 toxicity is evident in *vitro*, when expressed in completely unrelated cells such as cardiac myocytes [42], primary human fibroblasts [47], mouse embryonic fibroblasts [48]. This indicates that whole animal EWS-FLI1 transgenic mice or mere expression of EWS-FLI1 in non-receptive cell lines will not be practically feasible unless the expression of EWS-FLI1 is directed to the appropriate COO. Hence, we propose some *interim* alternatives based on available literature and practical considerations.

As a simple and foremost alternative, orthotopic or heterotopic xenografts can be considered appropriate. Indeed, xenograft models have been widely employed to study the biology and

molecular pathway involved in ES formation [59, 89-91]. These xenografts can be simply generated with commercial American Type Culture Collection (ATCC)-derived ES cell lines or other reliable sources, injected into the flanks of immunocompromised mice. Tumor formation can be identified by simple palpation of the injected area and physically measuring the tumor dimension in a longitudinal period of time [92]. The advantages include reflecting the genetic complexity of human tumor, rapid generation of results, and the flexibility of testing multiple therapeutics. It is obvious that heterotopic xenografts fail to mimic organ-specific microenvironment for which orthotopic xenograft models would be an optimal choice. This can be generated by injecting cell lines into the desired body compartment. However, due to repeated passages, cell lines might have limitations of being already immortalized, adaptation to artificial culture conditions, and undergoing a series of genetic transformations due to continuous passaging. Despite the drawbacks, orthotopic and heterotopic xenografts have been widely used [59, 89-91, 93, 94]. Commercial cell lines can also be engineered to express a reporter protein tag, which enables quantitation of tumor growth [95, 96].

Second, patient-derived xenografts (PDX) can be generated by implanting patient ES tumors either underneath the flank or orthotopically into immunocompromised mice [97-99]. The major advantage of PDX over cell line xenografts is their ability to better predict a patient's response to specific drug. PDX models allow growth and expansion of patient tumors without significant genetic transformation of tumor cells over multiple passages in a murine compartment.

However, expression of EWS-FLI1 and its downstream targets will be heterogeneous in different ES cell lines and may vary between different patient tumors or can fluctuate with time in a fully reversible process between EWS-FLI1 high (triggering active cell proliferation) and low states (where cells have a strong propensity to migrate, invade, and metastasize). Changes in EWS-FLI1 expression and activity due to cellular heterogeneity determine the ES cells to switch between proliferation and migration choices [100].

ES is generally considered to be more responsive to drugs, making chemotherapy the most preferred treatment modality compared to surgery [101]. Hence, the availability of treatment of naïve tumor tissues must be considered before generating PDX. This is due to the fact that EWS-FLI1 patients who have undergone chemotherapy might exhibit unexpected heterogeneity in the behavior and sensitivity to test drugs. If the intention is to develop EWS-FLI1-driven ES mouse model, then there is no opportunity in PDX to track the dynamics of endogenous EWS-FLI1.

Next, a model representing the tumor COO could be an option owing to their receptivity to EWS-FLI1 expression. The efficiency of progenitors in constructing a tumor mouse model has been widely justified [54, 62, 102-104]. ES models can be developed by directly injecting EWS-FLI1-expressing progenitor cells into mice [54]. This would be a reliable model since these presumed COOs are generally believed to harbor the transcriptional machinery for tolerating EWS-FLI1. A progenitor cell graft model is safer in many aspects. There is no ubiquitous EWS-FLI1 expression throughout the mouse body districts, and unlikely there will be an organ dysfunction *per se*. Cellular grafts expressing EWS-FLI1 will not be immediately lethal to the animal's survival. A collection of progenitor cell graft models permissive for EWS-FLI1 expression as either ortho or heterotopic grafts can be used to run efficacy studies in parallel to understand the predictive response of any given investigational drug (**Figure 2**). In addition, these progenitor cell-based models can be tagged with bioluminescent or fluorescent reporters which could dynamically signal changes in tumor growth in response to empirical treatment. This would enable the model to be assessed by in *vivo* imaging, which quantifies an increase in photon emission as equivalent to growing tumor. Non-invasive in *vivo* imaging is known to dynamically visualize tumor initiation and progression [105, 106].

Conclusion

Though EWS-FLI1 represents a clear therapeutic target, there is a lack of functional ES models [20, 29, 30]. A mouse model is required for generating pragmatic preclinical evidence for any investigational new drug to inhibit EWS-

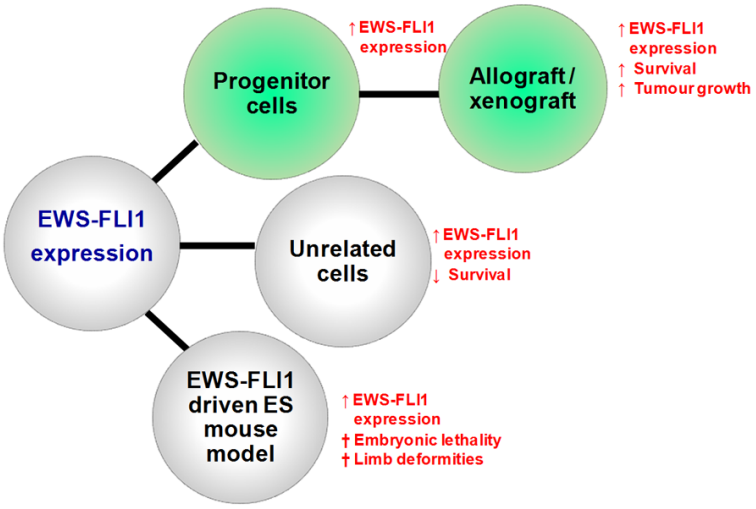

Figure 2. Survival outcomes due to EWS-FLI1 expression in progenitor cells, unrelated cell lines, and EWS-FLI1-driven ES mouse model. Deliberate expression of EWS-FLI1 in mouse models as well as in unrelated cell lines (e.g., cardiac myocytes, human fibroblasts, and mouse embryonic fibroblasts) cannot yield a functional model due to EWS-FLI1 toxicity *per se*. An allograft/xenograft model with progenitors representing presumed COO for ES (e.g., OCPC, NCC, and MSC) would be a viable option owing to their receptivity to EWS-FLI1 expression and better survival without immediate lethality.

FLI1 *per se* by bringing down ES tumor burden and to serve as a tool to facilitate rapid drug screening. The difficulties encountered in generating models have emphasized the fact that it is absolutely impractical to develop an EWS-FLI1-driven whole animal transgenic model on account of well-demonstrated developmental defects and embryonic lethality due to toxicity of ubiquitous or tissue-specific EWS-FLI1 expression [41, 42]. It would be unrealistic to have a mouse model with a silenced EWS-FLI1, appearing physiologically normal during the course of development and expressing a diseased phenotype only in typical ES occurrence sites when desired. On the other hand, cells representing the tumor COO should be capable of tolerating EWS-FLI1. Unfortunately, a precise COO is unknown to date.

Hence, the best alternative at least at this point of time would be the generation of orthotopic or heterotopic models with a collection of cells known to represent the presumed COO (MSCs, NCCs, and OCPCs). Additionally, these models can be designed as reporter models, which can be used as a rapid screening tool with the aid of in *vivo* imaging. In spite of fundamental molecular differences between mice and humans, a graft of EWS-FLI1-transformed COO mouse model could still be the only closest in *vivo* option of being able to mimic a human-like ES, at least owing to its relative molecular similarities. The alternatives suggested have both advantages and limitations. Hence, these models must be carefully chosen after vigilant considerations based on the scientific and technical needs of the study.

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Disclosure of conflict of interest

None.

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