

# Animal models of soft-tissue sarcoma

Rebecca D. Dodd<sup>1</sup>, Jeffery K. Mito<sup>1</sup> and David G. Kirsch<sup>1,\*</sup>

Soft-tissue sarcomas (STSs) are rare mesenchymal tumors that arise from muscle, fat and connective tissue. Currently, over 75 subtypes of STS are recognized. The rarity and heterogeneity of patient samples complicate clinical investigations into sarcoma biology. Model organisms might provide traction to our understanding and treatment of the disease. Over the past 10 years, many successful animal models of STS have been developed, primarily genetically engineered mice and zebrafish. These models are useful for studying the relevant oncogenes, signaling pathways and other cell changes involved in generating STSs. Recently, these model systems have become preclinical platforms in which to evaluate new drugs and treatment regimens. Thus, animal models are useful surrogates for understanding STS disease susceptibility and pathogenesis as well as for testing potential therapeutic strategies.

## Introduction

Soft tissue sarcomas (STSs) are a relatively rare, heterogeneous group of tumors that account for <1% of adult human cancer and 15% of pediatric malignancies (Borden et al., 2003). Largely of mesenchymal origin, STSs originate from a variety of tissue types, including muscle, cartilage, adipose tissue, fibrous tissue and blood vessels. This class of tumors is characterized by marked heterogeneity, with more than 75 histopathological subtypes currently recognized (Borden et al., 2003; Helman and Meltzer, 2003). Over 30% of adult patients with STS develop fatal lung metastases, with a median survival of 15 months (Helman and Meltzer, 2003). Treatment for STSs has lagged behind more common epithelial cancers, and survival from the most common STS subtypes has remained unchanged for several decades. The mechanisms associated with sarcoma development remain largely unclear because of the rarity of the disease, its large number of histological subtypes and its varied clinical behavior. As such, preclinical models to dissect mechanisms underlying sarcoma development, progression and treatment are greatly needed.

Sarcomas are traditionally classified by the site of tumor formation. However, recent advances support the view that molecular features are more relevant to tumor biology and treatment regimens (Helman and Meltzer, 2003). Cytogenetic studies have identified two broad groups of STSs. The first group, which makes up approximately one-third of all STSs, is characterized by relatively simple diploid karyotypes with few chromosomal rearrangements. The etiology of these tumors can usually be traced to a chromosomal translocation resulting in an oncogenic fusion gene, such as *Pax3-Fkhr* (*Fkhr* is also known as *Foxo1a*) in alveolar rhabdomyosarcoma (ARMS). By contrast, the second group of sarcomas displays complex karyotypes reflecting global genomic instability. Most human sarcomas, including leiomyosarcoma, belong to this karyotypically complex subgroup

and contain mutations in the p53 tumor suppressor gene pathway. This review will discuss the generation and application of successful animal models for both groups of STSs.

## Lessons from the earliest models of STS

Historically, sarcoma research relied on human sarcoma cell lines and immunocompromised mice. Many human cell lines derived from different subtypes of sarcoma are available, including synovial sarcoma (SW982), rhabdomyosarcoma (RD and RH4), fibrosarcoma (HT1080), liposarcoma (SW872), Ewing's sarcoma (RD-ES) and leiomyosarcoma (HTB88). In vitro studies with these cell lines have been a mainstay of sarcoma research and drug screening efforts (Hoffmann et al., 1999; Morioka et al., 2001; Tirado et al., 2005; Ying et al., 2006), leading to insights into both tumor progression (Toft et al., 2001) and metastasis (Zhang and Hill, 2007; Guo et al., 2008; Taylor et al., 2009). These cells have also been used as subcutaneous and orthotopic xenografts in immunocompromised mice (Sausville and Burger, 2006).

Despite the advances made with xenograft models, these tumor systems have several limitations. First, the patient-derived cell lines come from individuals with diverse genetic backgrounds, and serial propagation in culture can introduce mutations. Second, interactions between tumor cells and the host microenvironment are difficult to model in xenograft systems. For instance, stromal cells might impact tumor growth and development (List et al., 2005), metastasis (Karnoub et al., 2007), and response to therapy (Meads et al., 2009). Injection of a large number of human tumor cells into the non-native microenvironment of the mouse precludes the study of early events in tumor formation. Third, the potential incompatibility between mouse stroma and human tumor cells could also affect the growth of xenografts and their response to treatment (Tzukerman et al., 2006). Finally, development of xenograft tumors in immunocompromised hosts also ignores crucial interactions between the tumor and host immune system that can lead to alterations in tumor progression and development (de Visser et al., 2006). Thus, despite many advances, the use of in vitro and xenograft systems to model human sarcomas has led to

<sup>1</sup>450 Research Drive, Box 91996, LSRC Building, Duke University, NC 27708, USA  
\*Author for correspondence (david.kirsh@duke.edu)

relatively few new drug treatments, in spite of the number of compounds that have shown promise in these models (Gura, 1997). Therefore, there is great interest in studying sarcoma biology in animal models of primary tumors.

One of the earliest primary-tumor mouse models that developed STSs were the Trp53 (p53)-knockout mice (Donehower et al., 1992; Jacks et al., 1994). Like humans that carry mutations in the tumor suppressor p53 and are susceptible to sarcoma development as part of the Li-Fraumeni syndrome, *p53*<sup>-/-</sup> mice are tumor-prone with approximately one-third of tumors identified as sarcomas, including angiosarcomas, undifferentiated sarcomas and osteosarcomas. The p53 mutant mice were crossed with mice carrying a deletion for the neurofibromin (*Nf1*) tumor suppressor gene, which is mutated in patients with neurofibromatosis type 1 and is a negative regulator of the Ras pathway. These *p53*<sup>-/+</sup>; *Nf1*<sup>-/+</sup> mice develop primary malignant peripheral nerve sheath tumors (MPNSTs) (Cichowski et al., 1999). Since these reports, it has become clear that the function of both the tumor suppressor p53 and the Ras pathway play important roles in sarcoma biology.

The role of other tumor suppressor pathways in sarcomagenesis is less clear. Greater than 60% of osteosarcomas display a loss of heterozygosity at the retinoblastoma (*Rb*) tumor suppressor locus (Wadayama et al., 1994). Consistent with a role in osteosarcoma development, mouse models have shown that loss of *Rb* can potentiate the development of osteosarcomas in the context of mutant p53 (Berman et al., 2008; Walkley et al., 2008). However, despite the clear role of *Rb* in osteosarcoma development, attempts to identify a role for *Rb* in STSs have been met with conflicting reports (Cance et al., 1990; Karpeh et al., 1995; Dei Tos et al., 1996; Kohashi et al., 2008). Attempts to generate STSs in transgenic animals have not demonstrated a causative role for *Rb* in their development. Mutation in *Rb* alone in the mouse limb bud was not sufficient to generate STSs without cooperating mutations in p53 (Lin et al., 2009). Therefore, a recurring theme reflected in the animal models of sarcoma discussed below is mutation of p53 and of components of the Ras pathway, both of which have been shown to have definitive roles in the development of STSs.

The primary-tumor models of STS described in this review use a variety of genetic strategies to express oncogenes. For example, an oncogene can be expressed from either a transgene inserted into the genome (i.e. *Efla* inserted in myxoid liposarcoma) or knocked-in to the endogenous promoter (i.e. *Pax3-Fkhr* in ARMS). A gene can be mutated throughout the entire animal or can be controlled by a tissue-specific promoter. Also, a gene can be expressed throughout development or can be activated in the adult animal through a temporally regulated inducible mechanism (i.e. by CreER, as discussed below). In some cases, expression of a single gene is not sufficient to cause STS formation, suggesting a 'second-hit' of additional mutations might be required to initiate tumorigenesis.

### New models of cytogenetically simple STS: renegade transcription factors

The subgroup of STSs that display tumor-specific chromosomal translocations includes ARMS, synovial sarcoma, myxoid liposarcoma and Ewing's sarcoma (Table 1). Most of these chromosomal translocations result in fusion genes that act as renegade transcription factors and are central to the pathogenesis

of these tumors. In most cases, these fusion genes are composed of the DNA-binding domain of a transcription factor with a transactivation regulatory domain.

### ARMS model

ARMS is one of the most common forms of pediatric sarcomas, occurring predominantly in adolescents. It also provides one of the best-known examples of fusion-gene-driven sarcomas. A t(2;13) chromosomal translocation fuses the paired-box transcription factor Pax3 (or, less commonly, Pax7) with the transactivation domain of the forkhead transcription factor FKHR (Barr, 2001). The *Pax3-Fkhr* fusion gene results in aberrant activation of Pax3 gene targets via the Pax3 DNA-binding motif. Both Pax3 and Pax7 are expressed throughout the developing embryo, including in skeletal muscle precursors and the neural crest. In adults, they mark satellite cells, the quiescent stem cells of skeletal muscle. Thus, the oncogenic function of *Pax3-Fkhr* is postulated to involve aberrant activation of an embryonic myogenic developmental pathway.

Early studies in avian fibroblasts demonstrate that expression of the *Pax3-Fkhr* fusion gene can transform cells, leading to anchorage-independent growth. However, cooperating mutations were necessary to drive tumor formation in vivo (Scheidler et al., 1996). This suggests that the *Pax3-Fkhr* fusion gene might be required, but not sufficient, for ARMS tumorigenesis. Indeed, other mutations have also been found in ARMS, including amplification of *MDM2* (Forus et al., 1993) and *MYC* (Driman et al., 1994). Several studies have identified Pax3-FKHR targets to include genes involved in myogenic differentiation (Khan et al., 1999). Other studies have demonstrated that disruption of Pax3 function or its downstream myogenic differentiation program in ARMS cells induced apoptosis and inhibited tumor formation in immunocompromised mice (Bernasconi et al., 1996; Fredericks et al., 2000; Taylor et al., 2009). These data imply that the Pax3-FKHR fusion protein is necessary for tumor maintenance of ARMS.

To study the oncogenic function of the *Pax3-Fkhr* fusion gene in a primary-tumor model, mice were generated by knocking-in the *Fkhr* gene downstream of the *Pax3* locus. This knock-in strategy allows the Pax3-FKHR fusion protein to be expressed from the identical promoter and tissue type as found in human ARMS (Lagutina et al., 2002). This fusion gene was expressed in the neural-crest and muscle precursor cells, faithfully reflecting the endogenous expression pattern of Pax3 during development. However, after 1.5 years, no tumors developed in these animals. These results suggest that additional gene mutations might be necessary to generate ARMS.

An alternative mouse model of ARMS used *Cre-loxP* technology to obtain tissue-specific expression of the fusion gene (Keller et al., 2004b; Keller et al., 2004a). Cre is a site-specific recombinase that recognizes *loxP* sequences, resulting in excision of DNA that is flanked by two *loxP* sites (Fig. 1A). This technique is widely used to model cancer in the mouse by deleting tumor suppressors or activating oncogenes in specific tissue types. Using a conditional knock-in approach, a silenced portion of the *Fkhr* gene was inserted downstream of the full-length *Pax3* gene. Under normal conditions, *Pax3* is faithfully transcribed. However, in the presence of Cre recombinase, the floxed silencing element is removed, resulting in *Pax3-Fkhr* fusion gene expression. To determine a cellular pool involved in ARMS development, the *Pax3-Fkhr* mice were bred to

**Table 1. Animal models of sarcomas that have simple karyotypes**

Oncogene	Promoter	Genetic strategy	Mode of Cre activation	Second hits	Tumor developed	Details of model	Reference
<b>ARMS</b>							
<i>Pax3-Fkrd</i>	Endogenous locus	Knock-in activated by Cre activity	<i>Myf6-Cre</i>	<i>p53<sup>+/-</sup></i> , <i>p53<sup>-/-</sup></i> or <i>Ink4a/Arf<sup>-/-</sup></i>	ARMS	Low tumor incidence (1/228) with wild-type p53	Keller et al., 2004
<i>Pax3-Fkrd</i>	Endogenous locus	Knock-in activated by endogenous promoter activity	–	NA	No tumors formed	–	Lagutina et al., 2002
<b>Synovial sarcoma</b>							
<i>SYT-SSX2</i>	<i>ROSA26</i>	Knock-in activated by Cre activity	<i>Myf5-Cre</i>	Not required	Synovial sarcoma	100% penetrance of tumors	Haldar et al., 2007
<i>SYT-SSX2</i>	<i>ROSA26</i>	Knock-in activated by Cre activity	CreER + tamoxifen	Not required	Synovial sarcoma	Tumors display longer latency than Myf5-Cre-activated tumors	Haldar et al., 2009
<b>Myxoid liposarcoma</b>							
<i>FUS-CHOP</i>	<i>EF-1a</i>	Constitutively expressed throughout animal	None	Not required	Myxoid liposarcoma	100% penetrance of tumors	Perez-Losada et al., 2000
<b>GIST</b>							
<i>Kit<sup>Y641E</sup></i>	Endogenous locus	Constitutively active Kit expressed from endogenous promoter	None	Not required	GIST	Kit heterozygotes developed GISTs less frequently than homozygotes	Rossi et al., 2006
<i>Kit<sup>Y558A</sup></i>	Endogenous locus	Constitutively active Kit expressed from endogenous promoter	None	Not required	GIST	–	Sommer et al., 2003
<b>Ewing's sarcoma</b>							
<i>EWS-ETS</i>	<i>EWS</i> locus	Knock-in activated by Cre activity	<i>Rag1-Cre</i>	Not required	Leukemia	–	Forster et al., 2005
<i>EWS-FLI1</i>	<i>ROSA26</i>	Knock-in activated by Cre activity	<i>Mx1-Cre</i>	Not required	Leukemia	–	Torchia et al., 2007
<i>EWS-FLI1</i>	<i>ROSA26</i>	Knock-in activated by Cre activity	<i>Dermo1-Cre</i> , <i>Col1a2-Cre</i>	NA	Embryonic lethal	–	Torchia et al., 2007
<i>EWS-FLI1</i>	<i>ROSA26</i>	Knock-in activated by Cre activity	<i>Prx1-Cre</i>	<i>p53</i> flox resulting in <i>p53</i> -null	Poorly differentiated sarcoma	–	Lin et al., 2008

The table reviews models discussed in the article, including the oncogene, promoter and method of Cre activation (if any) used to create the animals. Also noted are second hits (if any) required for tumor development.

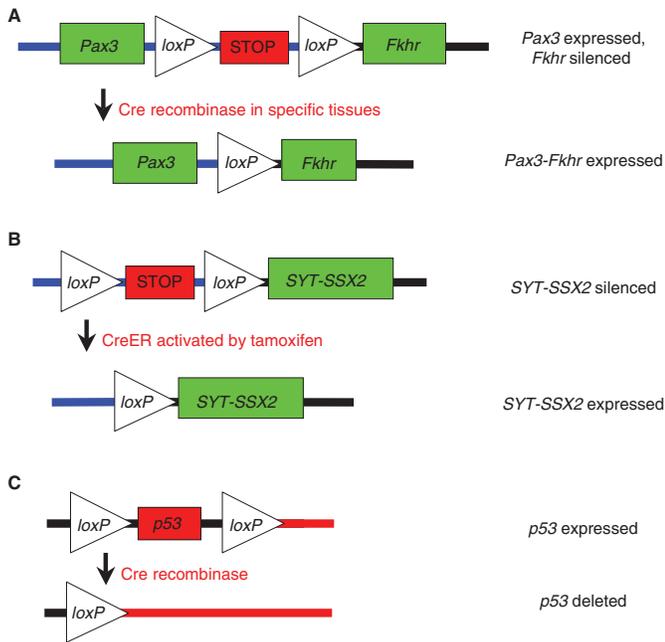
mice expressing Cre under distinct myogenic-lineage-specific promoters. No tumors were observed when crossed with *Pax7-Cre* mice that express Cre in the perinatal muscle satellite cells. However, expression of *Pax3-Fkhr* in mature, differentiating muscle cells (driven by *Myf6-Cre*) resulted in ARMS in a small number of mice (1/228). Tumor incidence was greatly accelerated by pairing of the *Myf6-Cre; Pax3-Fkhr* homozygous mice with knock-out alleles of either *Trp53* (3/17 mice) or *Ink4a/ARF* (*Cdkn2a*; 4/27 mice). This model was later shown to authentically recapitulate the histological features and transcriptional profiles of the human disease (Nishijo et al., 2009). This work supports previous findings (Lagutina et al., 2002) that second hits in tumor suppressor pathways are required to develop *Pax3-Fkhr* ARMS in mice.

### Synovial sarcoma

Synovial sarcomas occur mainly in adolescents and young adults. The majority of synovial sarcomas display a specific t(X;18) chromosomal translocation resulting in fusion of the *SYT* (*SS18*) gene with that encoding an SSX protein, either SSX1, SSX2 or SSX4.

The *SYT* gene is a putative transcriptional coactivator, whereas the SSX family members are believed to be transcriptional co-repressors (Haldar et al., 2008). Consistent with the genetic data from human tumors, expression of the human *SYT-SSX* fusion gene in rat 3Y1 fibroblast cells resulted in the formation of synovial-sarcoma-like tumors in nude mice (Nagai et al., 2001).

Although some synovial sarcomas develop near joints, these tumors bear little resemblance to the surrounding synovial lining. For this reason, the tissue of origin remains unclear, although a cell of the muscle lineage has been proposed as a potential source. To examine this possibility, a mouse knock-in model of synovial sarcoma was generated so that the *SYT-SSX2* fusion gene could be expressed from the ubiquitous *Rosa26* promoter at various stages throughout muscle development (Haldar et al., 2007). In this model, expression of the *SYT-SSX2* fusion gene was initiated by excision of the upstream *loxP*-flanked transcriptional stop cassette via Cre recombinase. Conditional expression of the fusion gene was achieved by tissue- or cell-specific expression of Cre recombinase. In addition, the *SYT-SSX2* gene was followed by an *IRE5-eGFP*



**Fig. 1. Gene activation or deletion by Cre recombinase.** Cre recombinase deletes DNA that is flanked by *loxP* sites. (A) Fusion-gene activation by tissue-specific Cre. Removal of a silenced STOP cassette by Cre allows for expression of the *Pax3-Fkhr* fusion gene (Keller et al., 2004b). (B) Gene activation by CreER activity. Addition of tamoxifen activates CreER, allowing for removal of the silenced *loxP-STOP-loxP* cassette. This results in expression of the oncogene *SYT-SSX2* (Haldar et al., 2007). (C) Gene deletion via Cre recombinase. Cre activity removes the *p53* gene flanked by two *loxP* sites (Jonkers et al., 2001).

element to facilitate cell-lineage tracing via the fluorescent marker. Crossing the *SYT-SSX2* mouse to *Myf5-Cre* animals resulted in a high-fidelity synovial sarcoma model with 100% penetrance. *Myf5* is expressed in myoblasts, a committed progenitor cell for skeletal muscle. Use of Cre drivers genetically upstream of *Myf5* – including *Hprt-Cre* (early-stage embryo), *Pax3-Cre* and *Pax7-Cre* (both satellite cell specific) – resulted in embryonic lethality. Breeding the *SYT-SSX2* mouse to those containing Cre drivers of genes expressed in differentiated skeletal muscle lineage – including *Myf6-Cre* (*Mrf4*), which is expressed in myocytes and fused myofibers – led to myopathy. These genetic data suggest that *SYT-SSX2* can initiate synovial sarcomas from a cell derived from the muscle lineage.

The *SYT-SSX* knock-in model has provided insight into the development of the distinct subtypes of synovial sarcoma tumors. Human synovial sarcomas can be classified as monophasic (composed of spindle cells) or biphasic (containing a mixture of spindle and epithelial cells). Both histologies were present in the tumors from *SYT-SSX2; Myf5-Cre* mice (Haldar et al., 2007; Haldar et al., 2008). As tumor size increased, the tumors gained more biphasic histology, suggesting a progression from a monophasic to a biphasic subtype as tumors progress.

In a more recent publication, an alternative *SYT-SSX2* mouse tumor model used tamoxifen-inducible CreER technology to activate the oncogenic fusion gene (Haldar et al., 2009). In this system, the CreER protein can provide temporal activation of *SYT-SSX2* expression in the adult through exogenous application of

tamoxifen (Fig. 1B). This system contrasts with the previously discussed promoter-driven Cre system (*Myf5-Cre*, etc.), in which Cre activity is constitutively found within all cells of a specific tissue. The distinction between these two model systems could be important for defining the events necessary for sarcoma development. In human tumors, the genetic mutation (i.e. *SYT-SSX*) occurs only in a small group of neoplastic cells within a given tissue. As such, in a model utilizing tissue-specific Cre drivers, a developing neoplasm would be surrounded by an artificial microenvironment comprised of cells expressing identical Cre-induced oncogenes. By contrast, the CreER system allows Cre activation to occur in a small population of cells at a precise time. Thus, this strategy might better mimic the natural pathogenesis of cancer through activation of the oncogene in a subset of cells that are surrounded by a microenvironment of relatively ‘normal’ tissue.

Using a mouse strain expressing CreER from the ubiquitous *Rosa26* promoter, tamoxifen injection of *SYT-SSX2; CreER* mice resulted in formation of soft-tissue tumors that histologically resemble synovial sarcomas. Although tamoxifen activates CreER in multiple tissue types, mice expressing the *SYT-SSX2* gene only developed synovial sarcoma. Interestingly, the incidence of tumor development in *SYT-SSX2; CreER* mice was similar regardless of whether the mice were injected with tamoxifen, suggesting that tumors developed after spontaneous activation of CreER. Despite the distinct modes of Cre activation in the models described above, microarray analysis revealed a high genetic similarity between the *SYT-SSX2; CreER* tumors (Haldar et al., 2009) and the *SYT-SSX2; Myf5-Cre* tumors (Haldar et al., 2007). Nevertheless, the two models did display important differences. The *SYT-SSX2; CreER* mice display longer survival (1 year vs 3.5 months), but developed larger tumors, which caused lethality. CreER-driven tumors were concentrated in the paraspinal region and face, whereas *Myf5-Cre* tumors arose most frequently in the intercostal region. Both strains developed tumors in the limbs with lower frequency than in other parts of the body. Tumors in both models occurred in proximity to skeletal tissue, suggesting a role for this microenvironmental niche in the development of synovial sarcoma. The differences in tumor size, latency and anatomical location suggest that some synovial sarcomas can arise from a non-myogenic cell-of-origin.

### Myxoid liposarcoma

Liposarcomas are a diverse group of STSs that histologically resemble white adipose tissue. Although most subsets of liposarcomas have not been studied in animal models, a mouse model of myxoid liposarcoma has been generated. Myxoid liposarcoma is characterized by a t(12;16)(q13;p11) chromosomal translocation resulting in fusion between the *FUS* and *CHOP* (also known as *DDIT3* and *GADD153*) genes in more than 90% of patients (Perez-Mancera and Sanchez-Garcia, 2005). *FUS* encodes a constitutively expressed RNA-binding protein that might regulate splicing. The *CHOP* gene expresses a stress-regulated basic-leucine-zipper-domain transcription factor with anti-apoptotic properties. The *FUS-CHOP* fusion gene replaces the RNA-binding domain of *FUS* with the activation domain of *CHOP*. Overexpression of the *FUS-CHOP* oncogene in primitive mesenchymal cells – including human fibrosarcoma cells (Engstrom et al., 2006) and murine primary bone-marrow-derived mesenchymal progenitors (Riggi et

al., 2006) – produced myxoid or round cell liposarcomas in xenograft experiments. These results indicate that the *FUS-CHOP* oncogene can initiate myxoid liposarcoma development.

Mice with ubiquitous expression of the *FUS-CHOP* fusion gene from the *Efla* promoter developed large liposarcomas with 100% penetrance (Perez-Losada et al., 2000). The level of the adipocyte regulatory protein PPAR- $\gamma$  was highly elevated in these tumors, suggesting an adipocytic lineage. These tumors were always found within white adipose tissue, and other organs did not show abnormal growths, despite ectopic expression of the oncogene with the *Efla* promoter. By contrast, driving expression of the oncogene with the *aP2* promoter of immature adipocytes did not result in tumors, although mice did accumulate more white adipose tissue (Perez-Mancera et al., 2007). This suggests that the cell of origin in the *Efla*-driven liposarcomas is not an immature adipocyte or their descendant.

To determine the protein domain that is sufficient for tumorigenesis, the *FUS* and *CHOP* genes were examined in separate mouse models (Perez-Mancera et al., 2002). White adipose tissue from *Efla-FUS* or *Efla-CHOP* single transgenic mice was normal. Creation of double transgenic *Efla-FUS*  $\times$  *Efla-CHOP* mice resulted in liposarcomas that develop at the same rate as the *FUS-CHOP* fusion gene mice (Perez-Mancera et al., 2002). Tumors were not identified in any other tissues, despite the ubiquitous expression from the *Efla* promoter. These models illustrate the requirement for both *FUS* and *CHOP* function in myxoid liposarcoma tumorigenesis, and demonstrate that these proteins can function in trans to initiate tumor development.

### Ewing's sarcoma

Ewing's sarcoma is a rare, small-round-cell tumor found within bone or soft tissue, and most commonly occurs during childhood and early adulthood. The tumors are defined by a chromosomal fusion between the *EWS* RNA-binding protein and one of several E-twenty six (ETS) transcription factors, with the *EWS-FLI1* fusion gene being the most common (Ordóñez et al., 2009). Direct targets of the *EWS-FLI1* oncogene have been difficult to identify, and the fusion gene is proposed to function through direct upregulation of NKX2.2, a potent transcriptional repressor (Owen et al., 2008). Introduction of the fusion gene into primary human fibroblasts resulted in senescence (Lessnick et al., 2002), whereas *EWS-FLI1* addition to murine bone-marrow-derived mesenchymal progenitors resulted in Ewing's-sarcoma-like tumors (Riggi et al., 2005). Inhibition of *EWS-FLI1* expression by antisense oligodeoxynucleotides in human Ewing's sarcoma cells reduced xenograft tumor growth (Tanaka et al., 1997). These studies imply that *EWS-FLI1* drives sarcomagenesis in a cell-type-specific manner and might also be required for tumor maintenance.

Despite the genetic and xenograft data supporting the role of *EWS-ETS* genes in Ewing's sarcoma, the majority of mouse models expressing these fusion genes develop leukemias. Although the *EWS-FLI1* fusion product has not been identified in human leukemias, a related chromosomal translocation involving the ETS family member *ERG* has been reported (Ichikawa et al., 1994). Mice expressing the *EWS-ETS* fusion gene from the *Rosa26* promoter under the control of the lymphocyte-specific *Rag1-Cre* develop T-cell lymphomas within 5 months (Forster et al., 2005). Additionally, expression of the *EWS-FLI1* gene in the bone marrow by *Mx1-Cre*

induces myeloid or erythroid leukemia in mice (Torchia et al., 2007). The rapid and severe disease progression in these mice might have precluded the development of sarcomas in this model. In an attempt to circumvent this limitation, *EWS-FLI1* mice were crossed to *Dermo1-Cre* or *Col1a2-Cre* mice to express the fusion gene in mesenchymal tissue during embryonic development. However, early expression of the *EWS-FLI1* oncoprotein resulted in embryonic lethality (Torchia et al., 2007).

A mouse model of *EWS-FLI1*-driven sarcoma was developed using a conditional *EWS-FLI1* gene under control of *Prx1-Cre* (Lin et al., 2008). *Prx1-Cre* is expressed in primitive mesenchymal tissues of the embryonic limb bud, and thus avoids the embryonic lethality that was seen with the *Dermo1-Cre* and *Col1a2-Cre* mice described above. The *EWS-FLI1; Prx1-Cre* mouse has truncated limbs, muscle atrophy and an accumulation of immature bone, but does not develop tumors, demonstrating that *EWS-FLI1* expression alone is not sufficient for tumor formation. However, addition of a 'second hit' through deletion of *p53* in triple transgenic mice (*EWS-FLI1; p53<sup>fllox/fllox</sup>; Prx1-Cre*) did result in poorly differentiated STSs (Lin et al., 2008). These mice contain a floxed *p53* allele with exons 2-10 flanked by *loxP* sites (Fig. 1C), resulting in deletion of the *p53* gene in the presence of *Cre* recombinase (Jonkers et al., 2001). This finding suggests that, in some cell types, genetic inactivation of the *p53* tumor suppressor pathway can cooperate with *EWS-FLI1* in sarcomagenesis.

### Gastrointestinal stromal tumors

Gastrointestinal stromal tumors (GISTs) are the most common sarcoma of the gastrointestinal (GI) tract. They are believed to arise from mesenchymal cells within the wall of the GI tract, which are called the interstitial cells of Cajal (ICCs). Although the development of GISTs does not seem to be driven by a fusion gene, these STSs do have relatively simple karyotypes and are classified with the first grouping of STSs. Almost 90% of all GISTs have activating mutations in the receptor tyrosine kinase *Kit* (Helman and Meltzer, 2003). Following the initial identification of *Kit* as an oncogene for feline sarcomas (Besmer et al., 1986), activating mutations in *Kit* have been identified in many human tumors, including in GISTs, leukemias and mastocytosis (Furitsu et al., 1993; Hirota et al., 1998). Additional studies have identified a direct role for *Kit* in the development of hematopoietic stem cells, germ cells and ICCs.

Two independent groups have generated mouse models of GIST through expression of an activated form of the *Kit* gene. A homozygous knock-in of activated *Kit<sup>K641E/K641E</sup>* to the mouse *Kit* locus results in ICC hyperplasia and GISTs, with 100% penetrance (Rubin et al., 2005). *Kit<sup>K641E/+</sup>* heterozygotes did develop small GISTs, although the ICC hyperplasia was much less extensive, illustrating a dose dependency for *Kit* activation. No tumors were identified outside of the GI tract of these mice. An additional study produced a mouse with a *Kit*-activating mutation *Kit<sup>V558Δ/+</sup>*, deleting valine 558 in exon 11 (Sommer et al., 2003). *Kit<sup>V558Δ/+</sup>* mice developed GISTs and had a 50% survival rate of 9 months. These mice had elevated levels of mast cells, although there was no elevation in overall hematocrit or white blood cell levels. This mouse model of GIST has been used to investigate chemotherapeutic treatment of *Kit*-dependent tumors, and will be discussed in later sections (Rossi et al., 2006).

## Animal models of sarcomas with complex karyotypes: karyotypic confusion

The second main group of sarcomas is not defined by simple chromosomal translocations or an activating mutation in a single oncogene, but instead display complex karyotypes indicative of global genomic instability. These tumors include embryonal rhabdomyosarcoma (ERMS), undifferentiated pleomorphic sarcoma [UPS; also known as malignant fibrous histiocytoma (MFH)] and leiomyosarcoma (Table 2).

### Embryonal rhabdomyosarcoma

ERMS is the most common subtype of rhabdomyosarcoma, and is histologically and molecularly distinct from the alveolar subtype (ARMS). Common genetic hallmarks of human ERMS include inactivation of the p53 pathway and loss at 11p15.5, which might encode a tumor suppressor gene (Besnard-Guerin et al., 1996). However, no single genetic lesion has been uniformly linked to ERMS. Thus, attempts to genetically model ERMS have been focused on altering pathways known to be mutated in rhabdomyosarcomas, including p53, Rb, MYC and RAS (Merlino and Helman, 1999). Disruption of these pathways in human skeletal muscle myoblasts led to ERMS-like cells that were capable of generating tumors in xenograft models (Linardic et al., 2005). This work is consistent with committed skeletal muscle myoblasts being a cell of origin for ERMS, but it does not rule out other potential cells of origin.

Expression of oncogenic KrasG12D in muscle satellite cells of transgenic zebrafish results in an animal model of ERMS (Langenau et al., 2007). Injection of zebrafish embryos with a constitutively active, oncogenic *Kras* gene (*Kras*<sup>G12D</sup>) under control of the *Rag2* promoter produced tumors in over half of the animals. These masses were highly invasive and were composed of both multinucleated striated muscle fibers and undifferentiated muscle cells characteristic of the ERMS subtype. Microarray analyses identified upregulation of the zebrafish ERMS (zERMS) gene set within human ERMS (hERMS), but not within human ARMS, data sets. Further analysis showed that the zERMS data set is significantly associated with gene sets from other RAS-driven tumors, including human pancreatic adenocarcinoma and murine lung adenocarcinoma. The identification of a *RAS* oncogene signature in the zebrafish model supports a role for this pathway in ERMS formation.

This zebrafish model also provides information about the cell of origin for ERMS. In zebrafish, expression of the *rag2* promoter is found within the mononucleated skeletal muscle cells (satellite cells and differentiating myoblasts), but not in multinucleated terminally differentiated muscle. Although the *rag2* promoter is also found in B- and T-cell progenitors, lymphoid hyperplasia was rarely observed. This model supports a muscle progenitor cell as a potential initiator for the ERMS tumor subtype. No inactivating mutations in the *p53* gene were identified in the zebrafish ERMS tumors, although elevation of p53 suppressors were found,

**Table 2. Animal models of sarcomas that have complex karyotypes**

Genetic mutation	Promoter	Genetic strategy	Mode of Cre activation	Second hits	Tumor developed	Details of model	Reference
<b>ERMS</b>							
<i>Kras</i> <sup>G12D</sup>	<i>Rag2</i>	Constitutively active <i>Kras</i> expressed from <i>Rag2</i> promoter	None	Not required, but <i>p53</i> loss accelerates tumor growth	ERMS	Transgenic zebrafish	Langenau et al., 2007
<i>HGF/SF</i>	Metallothionein	Constitutively expressed via metallothionein promoter	–	<i>Ink4a/Arf</i> <sup>-/-</sup> or <i>Ink4a/Arf</i> <sup>+/-</sup>	ERMS	–	Sharp et al., 2002
<i>Her2 (neu)</i>	MMTV	Constitutively expressed via MMTV promoter	–	<i>p53</i> -null	ERMS	Genitourinary tract of males only	Nanni et al., 2003
<i>Kras</i> <sup>G12V</sup>	<i>Ryr2</i>	Constitutively active <i>Kras</i> expressed from <i>Ryr2</i> promoter	Electroporation of <i>Cre</i> plasmid	<i>p53</i> <sup>-/-</sup> or <i>p53</i> <sup>+/-</sup>	Pleiomorphic rhabdomyosarcoma	–	Tsumura et al., 2006
<b>Leiomyosarcoma</b>							
<i>Pten</i>	Endogenous locus	Deletion of <i>PTEN</i> in smooth muscle	<i>Tagln</i> (smooth muscle actin 22- $\alpha$ )	none	Leiomyosarcoma, no ULMS	–	Hernando et al., 2007
<i>Brca1</i>	Endogenous locus	Deletion of <i>BRCA1</i> in smooth muscle	<i>Amhr-Cre</i>	<i>p53</i> loss required	ULMS	ULMS developed with <i>p53</i> -null alone, but increased with <i>BRCA1</i>	Xing et al., 2009
<i>CR-1</i>	MMTV	Constitutively expressed via MMTV promoter	None	Not required	ULMS	–	Strizzi et al., 2007
<i>LMP2</i>	Endogenous locus	Deletion of <i>LMP2</i> in entire mouse	None	Not required	ULMS	–	Hayashi et al., 2002
<b>UPS</b>							
<i>Kras</i> <sup>G12D</sup>	Endogenous locus	Knock-in activated by Cre activity	Inject adenovirus expressing <i>Cre</i>	Requires <i>p53</i> flox resulting in <i>p53</i> -null	UPS	–	Kirsch et al., 2007

The table reviews models discussed in the article, including the genetic mutation, promoter and method of Cre activation (if any) used to create the animals. Also noted are second hits (if any) required for tumor development.

including Mdm2 and survivin. Tumor formation was accelerated when the *rag2-Kras<sup>G12D</sup>* transgene was injected into p53 loss-of-function mutants. These data support a role for the p53 pathway in acceleration of ERMS tumor growth.

Interestingly, other investigators used the same genes (*Kras* and *p53*) to generate a mouse model of pleomorphic rhabdomyosarcoma, which is a subset of chemoresistant adult rhabdomyosarcoma and often arises in the large muscles of the extremities (Tsumura et al., 2006). This model utilizes a Cre-activatable oncogenic *Kras<sup>G12V</sup>* expressed from the myocardial ryanodine receptor type 2 (*Ryr2*) promoter. Oncogene activation occurs by electroporation of a Cre-expressing plasmid into the muscle of the lower leg. Tumors developed in all *p53<sup>-/-</sup>* mice and in 40% of *p53<sup>+/-</sup>* mice. The tumors stained positive for the rhabdomyosarcoma marker desmin and  $\alpha$ -sarcomeric actin.

Two mouse models of ERMS also report a crucial role for the p53 tumor suppressor pathway. In *Ink4a/Arf<sup>-/-</sup>* mice, expression of HGF/SF, which activates the Met growth factor receptor, resulted in highly penetrant malignant rhabdomyosarcomas (Sharp et al., 2002). These mice exhibit hyperplastic satellite cells at 6-10 weeks of age, suggesting a role for satellite cells in ERMS tumorigenesis. Mice expressing one wild-type allele of *Ink4a/Arf* also developed ERMS, albeit with a longer latency. In an alternative model, activation of the *Her2 (neu)* gene coupled with p53 inactivation produced spontaneous ERMS-like tumors in the genitourinary tract of male BALB/c mice (Nanni et al., 2003). *Her2* is expressed in multiple tumor types, including in ~50% of human rhabdomyosarcomas. Because female mice did not develop sarcomas, this might suggest a gender-specific role for the *Her2* gene in these tumors. Taken together, these animal models support the importance of p53 loss for ERMS development in a mammalian system.

### Undifferentiated pleomorphic sarcoma

UPS (also known as malignant fibrous histiocytoma, or MFH) is one of the most common subtypes of STS in adult patients. The diagnosis of UPS refers to tumors displaying high-grade pleomorphic cells in a storiform growth pattern. Genetically, UPS sarcomas display a complex karyotype reflecting genomic instability. There are no known single oncogenic mutations associated with UPS, although activation of the *Kras* pathway might be involved (Mito et al., 2009). Because the cell of origin for this tumor remains unclear, some have suggested that UPS is not a distinct clinical entity, but instead represents a common undifferentiated state of diverse tumors that are derived from different mesenchymal cells.

A mouse model of high-grade, poorly differentiated primary STS was generated in mice with conditional mutations in both oncogenic *Kras* and mutant *p53* (*Kras<sup>G12D/+</sup>; p53<sup>fllox/fllox</sup>*) (Kirsch et al., 2007). In this model, Cre activates expression of oncogenic *Kras* by deletion of an upstream floxed transcription/translation STOP cassette (termed '*loxP-STOP-loxP*'; or LSL cassette) inserted into the endogenous *Kras* promoter. Cre is expressed by intramuscular injection of an adenovirus (Ad-Cre) instead of a genetic tissue-specific Cre driver. High-grade sarcomas develop at the site of injection 2-3 months after intramuscular Cre delivery. Additionally, up to 50% of these mice develop lung metastases, which are a hallmark of human sarcoma pathogenesis. By spatially and

temporally restricting tumor initiation, sarcomas develop in adult mice at a defined anatomical site. This is similar to advantages described for the CreER technology above (Haldar et al., 2009), but avoids the potential leakiness of genetic CreER systems. Additionally, the site of tumor formation can be tightly controlled by Ad-Cre injection, in contrast to systemic activation of CreER through intraperitoneal tamoxifen delivery. The adenovirus-based mode of Cre delivery is also well-suited to generate tumors with no known cell of origin, precluding the need for tissue-specific Cre drivers.

To determine the subtype of human sarcoma that most closely resembles the Ad-Cre-activated *Kras<sup>G12D/+</sup>; p53<sup>fllox/fllox</sup>* model, gene expression analysis generated a mouse sarcoma signature (Mito et al., 2009). This signature is highly enriched in human UPS compared with other types of STS. Samples of human UPS were also highly enriched for both the mouse sarcoma signature and a genomic signature for Ras pathway activation, compared with other common subtypes of STS. These data suggest that this mouse model is most similar to UPS, and also suggest a link between UPS and Ras pathway activation.

The Ad-Cre-generated *Kras<sup>G12D/+</sup>; p53<sup>fllox/fllox</sup>* model of UPS (Kirsch et al., 2007) discussed above is similar to the Cre-injectable *Kras<sup>G12V/+</sup>; p53<sup>fllox/fllox</sup>* model (Tsumura et al., 2006) of pleomorphic rhabdomyosarcoma. In both models, deletion of *p53* and activation of oncogenic *Kras* resulted in highly aggressive STSs, but apparently of different subtypes (UPS vs pleomorphic rhabdomyosarcoma). Although it is possible that these two models represent different spectrums of the same tumor type, the models differ in several key features. First, different activating *Kras* mutations are used (G12D vs G12V), in addition to different promoters driving *Kras* expression (endogenous promoter vs *Ryr2*). Second, one model activated oncogenic *Kras* expression by Ad-Cre infection, whereas the other model utilized electroporation of a Cre-expressing plasmid. Therefore, it is possible that Cre is expressed in different cells using the adenovirus and the electroporation strategies. Third, a *p53*-null background was required for tumor development in the UPS model, whereas tumors were generated in *p53<sup>+/-</sup>* mice of the pleomorphic rhabdomyosarcoma model. Taken together, the spectrum of tumor types displayed in these models with similar genetic mutations highlights the complexities involved in studying sarcomas in animal models.

### Leiomyosarcoma

Leiomyosarcomas are malignant growths of the smooth muscle and can form at multiple sites throughout the body. For instance, uterine leiomyosarcomas (ULMSs) are rare tumors that arise from the smooth muscle of the uterine wall. Deletion of 10q, which contains the tumor suppressor gene *PTEN*, has been found in human leiomyosarcomas (Segal et al., 2003). Indeed, activation of the PI3K-AKT signaling pathway, which is inhibited by PTEN activity, has also been reported in leiomyosarcoma cases (Hernando et al., 2007). To investigate the role of the PI3K-AKT pathway in leiomyosarcomas, a mouse model was generated that genetically inactivates PTEN in the smooth muscle through transgelin [*Tagln*; also known as smooth muscle 22- $\alpha$  (Sm22a)]-Cre activity (Hernando et al., 2007). Deletion of PTEN in the smooth muscle (*Tagln-Cre; Pten<sup>fllox/fllox</sup>*) caused smooth muscle hyperplasia and a rapid onset of leiomyosarcoma in 80% of the mice. Both abdominal

and retroperitoneal leiomyosarcomas were detected, although no uterine sarcomas were observed. No tumors were found in skeletal or cardiac muscle, despite the transient expression of Cre in their precursor cells by the *Tagln* promoter.

A mouse model of ULMS was created using a Cre driver from the promoter of the anti-Mullerian hormone type II receptor (*Amhr2*) gene, which is expressed in tissues that develop into the fallopian tubes, female gonad, uterus and ovary (Xing et al., 2009). Deletion of p53 by using *Amhr2-Cre* resulted in ULMS in over half of the mice within 13 months. Loss of the tumor suppressor gene *Brcal* accelerated formation of these tumors in the triple transgenic *Brcal<sup>fllox/fllox</sup>; p53<sup>fllox/fllox</sup>; Amhr2<sup>Cre/+</sup>* mice. Indeed, loss of BRCA1 protein expression was detected in about one-third of human ULMS samples (Xing et al., 2009).

Another mouse model of ULMS has been reported that utilizes the Wnt-activated growth factor Cripto-1 (CR-1). The CR-1 protein is strongly expressed in ~70% of human leiomyosarcomas (Strizzi et al., 2007). A transgenic mouse driving CR-1 expression from the mouse mammary tumor virus (MMTV) long-terminal-repeat promoter developed ULMSs in ~20% of the females. These MMTV-*CR-1* mice show activation of *CR-1* and of Wnt signaling through elevated levels of phospho-Src, phospho-AKT and phospho-GSK-3 $\beta$  and nuclear accumulation of  $\beta$ -catenin. Interestingly, tumor formation did not require p53 loss, although deletion of p53 might increase the penetrance and tumor development in these mice. In an additional model, spontaneous ULMSs developed in mice deleted for LMP2, an IFN- $\gamma$ -regulated proteosomal subunit. The *LMP2<sup>-/-</sup>* mice also developed hepatocellular carcinomas and lung tumors, albeit at a lower incidence than the ULMSs.

### The promise of new therapies

The animal models of STS discussed above have been instrumental in our understanding of sarcomagenesis, including identification of relevant oncogenes, the cells of origin, and new therapies and treatments. These tools are crucial for the sarcoma field because clinical samples are relatively rare and treatment regimens have not changed in 30 years for many sarcoma subtypes. Several studies that highlight preclinical applications of these models are described below. The discussion presented here is by no means comprehensive, but is intended to emphasize current trends using such models.

In vivo screening of anti-sarcoma drugs is a major preclinical use for animal models. For example, the mTOR pathway is activated in leiomyosarcomas from *Tagln-Cre; Pten<sup>fllox/fllox</sup>* mice, suggesting that these tumors might respond to mTOR inhibitors. Treatment with a rapamycin analog, Everolimus, beginning at 1 month of age significantly delayed tumor growth and extended survival in these mice (Hernando et al., 2007). Post-treatment tumors showed significant downregulation of both phospho-AKT and Ki67 staining. Similar studies have been completed in a model of GIST driven by *Kit<sup>V558 $\Delta$ /+</sup>*. Treatment with either the tyrosine kinase inhibitor imatinib or Everolimus decreased GIST growth (Rossi et al., 2006). The faithful response of these tumors to the Kit inhibitors used in the clinic validates this model as a screening platform for future drug therapies. An additional model was used to study the synergistic effects between radiation therapy and treatment with the tyrosine kinase inhibitor sunitinib (SU) (Yoon et al., 2009). Using

the Ad-*Cre*-driven model of UPS (Kirsch et al., 2007), the efficacy of radiation treatment was enhanced when administered in tandem with SU. This model was well-suited for such a study because changes in tumor volume in response to treatment can be easily measured because the location of the sarcoma was determined by the site of Ad-*Cre* injection.

Further understanding of the heterogeneity of subtypes of STSs will hasten the development of additional animal models. The ability of these models to faithfully recapitulate the spectrum of disease seen within these subtypes will provide vital mechanistic and therapeutic insights that will have a direct impact on sarcoma patients. Specific mouse models of STS might identify novel drug targets that have not been identified by the relatively small number of clinical samples available to investigators, similar to the successful identification of potential therapeutic targets using mouse primary-tumor models of Kras-driven lung cancers (Engelman et al., 2008).

### Conclusion

Animal models are indispensable tools for the study of STSs because a paucity of clinical samples makes large-scale analysis of human samples challenging. Furthermore, the diversity of sarcoma subtypes complicates analyses of human sarcomas. Until recently, studies of sarcoma biology were limited to human cell lines and xenografted tumors. Using the sophisticated tools of mouse genetics, models of many oncogene-driven STSs have been developed. In these models, tumors develop in the native microenvironment of an animal that has an intact immune system. Many advances have been made in determining the signaling pathways activated in sarcomas and in identifying potential cells of origin within these models. The further development of models using advanced genetic techniques will continue to increase our understanding of STS biology. Additionally, preclinical studies of novel drug and radiation therapies will form the foundation for new treatment regimens. Ultimately, continued genomic study of human STS samples might identify novel genetic mutations that can guide the creation of new and even more faithful animal models.

### ACKNOWLEDGEMENTS

We thank Lisa Crose and members of the Kirsch lab for helpful discussions and critical reading of this Perspective. We apologize to authors whose work we could not discuss due to space limitations. This work is supported by a joint ACS-Canary Foundation Postdoctoral Fellowship (R.D.D.), and NIH grants T32-GM-07171 (J.K.M.) and RO1 CA 138265 (D.G.K.). Deposited in PMC for release after 12 months.

### COMPETING INTERESTS

The authors declare no competing interests.

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