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Developmental Origins of Fusion-Negative Rhabdomyosarcomas

Ken Kikuchi^{*}, Brian P. Rubin^{†,‡}, and Charles Keller^{*}

^{*}Department of Pediatrics, Pape' Family Pediatric Research Institute, Oregon Health and Science University, Portland, Oregon, USA

[†]Pediatric Cancer Biology Program, Department of Anatomic Pathology, Taussig Cancer Center and Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA

[‡]Department of Molecular Genetics, Taussig Cancer Center and Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA

Abstract

Rhabdomyosarcomas (RMS) are very heterogeneous tumors that can be divided into three major groups: alveolar rhabdomyosarcoma, embryonal rhabdomyosarcoma, and pleomorphic rhabdomyosarcoma. Concerted efforts over the past a decade have led to an understanding of the genetic underpinnings of many human tumors through genetically engineered models; however, left largely behind in this effort have been rare tumors with poorly understood chromosomal abnormalities including the vast majority of RMS lacking a pathognomonic translocation, i.e. fusion-negative RMS. In this chapter, we review the characteristic genetic abnormalities associated with human RMS and the genetically engineered animal models for these fusion-negative RMS. We explore not only how specific combinations of mutations and cell of origin give rise to different histologically and biologically distinguishable pediatric and adult RMS subtypes, but we also examine how tumor cell phenotype (and tumor “stem” cell phenotype) can vary markedly from the cell of origin.

1. Introduction

Soft-tissue sarcomas are malignant tumors believed to be of the mesodermal lineage, and therefore derived from nonepithelial, nonhematopoietic tissues. While extremely rare in adults, rhabdomyosarcomas (RMS) are one of the more common neoplasms in children and adolescents (Parham and Ellison, 2006). RMS, as the name suggests, are presumed to be associated with the skeletal muscle lineage because of myogenic marker expression. The names of many soft-tissue sarcomas imply a particular line of differentiation (i.e., the tissue that the tumor most closely resembles), without any information about the cell of origin.

Histologically, RMS are very heterogeneous tumors that can be divided into three major groups: alveolar RMS (ARMS), embryonal RMS (ERMS), and pleomorphic RMS (Parham and Ellison, 2006). Pleomorphic RMS affect mainly adults, while ARMS and ERMS affect children and adolescents. Clinically, ARMS is more common in older children, involves the trunk and extremities, and has a worse prognosis, while ERMS presents at an earlier age, mainly in the orbit, head, and neck and retroperitoneum, and is associated with a better

prognosis (Parham and Ellison, 2006); nevertheless, metastatic ERMS portends only 40% overall survival (Breneman *et al.*, 2003).

Beside clinicopathological differences, these tumors also differ at the molecular level. In approximately one-third of soft-tissue sarcomas, a specific translocation drives sarcomagenesis (Borden *et al.*, 2003). This subset of RMS, which accounts for 80–90% of ARMS, is characterized by specific chromosomal translocations having either a $t(2; 13)$ or a $t(1; 13)$ translocation, involving *PAX3:FKHR* or *PAX7:FKHR* fusion genes, respectively. Further, the fusion type correlates strongly with outcome, since *PAX3: FKHR* is associated clinically with more aggressive tumors than *PAX7: FKHR* (Sorensen *et al.*, 2002). The remaining two-thirds of RMS (including ERMS and pleomorphic RMS) do not have a pathogenetic translocation. Instead, these tumors are often characterized by complex karyotypes with inactivation of the p53 tumor suppressor pathway (Borden *et al.*, 2003).

A hypothesis that is currently in vogue in the field of carcinogenesis is that a small, but identifiable population of cells within a tumor provides the “self-renewal” phenotype of cancer. Many names have been used to identify this population but the term cancer stem cell has received broad acceptance. Cancer stem cells have been defined as “a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of tumor cells that comprise the tumor” (Clark *et al.*, 2005). These two definitive biological properties are what make the cancer stem cell the prime candidate for initiation of relapse, thereby becoming a crucial target for the development of novel therapies. An extension of this hypothesis is that these cancer stem cells most closely resemble the cell of origin for a given tumor (Reya *et al.*, 2001). Based on this hypothesis, the originating cell of a tumor is increasingly believed to be a primitive cell with the ability to undergo division with high-phenocopy fidelity and retention of multi-potency. Meanwhile, there is an ongoing debate over whether cancer stem cells represent a mature tissue stem cell which has undergone malignant change or whether more differentiated cells reinitiate a “stemness” programme as part of, or following, malignant transformation. Until we have this information, it is important to consider independently the concepts of cell of origin and cancer stem cell, as defined purely by self-renewal and capacity to differentiate.

We previously reported a conditional mouse model of ARMS in which *Pax3:Fkhr* was activated and *Tp53* was inactivated in maturing myoblasts (Keller *et al.*, 2004). In these mice, cells expressing high levels of *Pax3:Fkhr* were most capable of repopulating tumors at metastatic sites (Nishijo *et al.*, 2009). These results suggested that *Pax3:Fkhr* fusion-positive ARMS tumors display heterogeneity in *Pax3:Fkhr* expression, and that *Pax3:Fkhr* overexpressing cells might be tumor repopulating cells, that is, cancer stem cells. Meanwhile, cancer stem cells of fusion-negative RMS, including ERMS and pleomorphic RMS, have not yet either described in great detail either. In this review, we will consider the genetically engineered tumor models of fusion-negative RMS using transgenic and germline mice with oncogenic mutations, and conditional mice that express oncogenes. Based on these models and corresponding human RMS biology, we will make an effort to understand the cell of origin, tumor phenotype, and cancer stem cell of fusion-negative RMS.

2. Mutations Seen in Fusion-Negative RMS

2.1. Congenital syndromes associated with fusion-negative RMS and mutations detected in sporadic fusion-negative RMS

Although no consistent chromosomal rearrangement have been identified in ERMS and the majority of ERMS occur as sporadic cases, these tumors have been reported in hereditary conditions with tumor predisposition such as Li-Fraumeni syndrome (LFS), Beckwith-Wiedemann syndrome (BWS), Costello syndrome, Neurofibromatosis type-1 (NF1), Noonan Syndrome, and Gorlin syndrome (Tables 2.1 and 2.2).

TP53, a transcription factor, is a tumor suppressor gene product involved in apoptosis, cell cycle withdrawal, differentiation, and cellular senescence. p53 is activated by a number of cellular stresses such as DNA damage. Germline mutations of *TP53* are the underlying etiology of LFS, which is associated with high risk of a diverse spectrum of childhood and adult-onset malignancies. One study, based on five families with LFS, estimated age-specific cancer risks as 42% at ages 0–16 years, 38% at ages 17–45 years, and 63% after age 45 years; overall lifetime cancer risk was calculated at 85% (Le Bihan *et al.*, 1995; Schneider and Garber, 2010). Individuals with LFS are at increased risk of developing soft-tissue (i.e., muscle and connective tissue) sarcomas (e.g., RMS, liposarcoma) and sarcomas of bone (e.g., osteosarcoma, chondrosarcoma). Almost all types of sarcomas have been noted in families with *TP53* mutations with the exception of Ewing sarcoma, which is not associated with LFS. The median age of soft-tissue sarcomas in individuals with LFS is 14 years by comparison with sporadic sarcomas, which is 61.3 years (Olivier *et al.*, 2003). Somatic mutations of *TP53* (Diller *et al.*, 1995; Felix *et al.*, 1992; Taylor *et al.*, 2000) and dysregulation of its associated regulatory proteins have also been implicated in the development of ERMS. Loss of p53 tumor suppressor function in sporadic RMS may result from overexpression of *MDM2*, since *MDM2* encodes a protein capable of binding and inactivating p53. *MDM2*, along with other genes at the 12q13–15 locus, have been found to be amplified in ERMS (Taylor *et al.*, 2000). Several studies have also found that *MDM2* is overexpressed at both mRNA and protein levels (Keleti *et al.*, 1996; Miyachi *et al.*, 2009), again suggesting a role for *MDM2* in ERMS. Another member of the p53 pathway is *ARF*, which blocks *MDM2* function and stabilizes the p53 protein. Although mutations of *ARF* have not been reported in RMS, homozygous deletions of the 9p21 region, which contains the *CDKN2A* common locus for both *P16^{INK4A}* and *ARF*, were found in 25–33% of ERMS cases (Gil-Benso *et al.*, 2003; Iolascon *et al.*, 1996).

BWS is a disorder of growth characterized by macrosomia, macroglossia, visceromegaly, omphalocele, neonatal hypoglycemia, ear creases/pits, adrenocortical cytomegaly, renal abnormalities, and embryonal tumors. Children with BWS have an increased risk of mortality associated with neoplasia, particularly Wilms' tumor and hepatoblastoma, but also neuroblastoma, adrenocortical carcinoma, and RMS (ERMS and fusion-negative ARMS; Smith *et al.*, 2001). Also seen are a wide variety of other tumors. The estimated risk for tumor development in children with BWS is 7.5%. The increased risk for neoplasia seems to be concentrated in the first 8 years of life. Tumor development is uncommon in affected individuals older than 8 years of age (Shuman *et al.*, 2010). The diagnosis of BWS relies

primarily on clinical findings. Clinically available molecular genetic testing can identify several different types of 11p15 abnormalities in individuals with BWS: (1) loss of methylation at *KCNQ1OT1* (*DMR2*) is observed in 50% of individuals; (2) gain of methylation at *H19/IGF2* (*DMR1*) is observed in 2–7%; (3) paternal uniparental disomy for chromosome 11p15 is observed in 10–20%. Testing reveals mutations in the *CDKN1C* gene in 40% of familial cases and 5–10% of sporadic cases. Molecular analyses of polymorphic loci in sporadic cases of ERMS revealed frequent allelic loss of 11p15 (Davicioni *et al.*, 2009; Koufos *et al.*, 1985). Comparison of the allelic loss pattern in ERMS tumors to the allelic status of the patients' parents revealed that ERMS tumors preferentially maintain the paternally inherited allele and lose the maternal allele (Scrabble *et al.*, 1989). *H19* and *CDKN1C* are preferentially expressed from the maternally inherited alleles and *IGF2* is imprinted in the opposite direction so that the paternally inherited alleles are preferentially expressed. Some alterations lead to overexpression of the *IGF2* fetal growth factor, while others serve to mutate or inactivate expression of growth suppressive genes such as *H19* and *CDKN1C*. However, definitive genetic alteration in this region has not been identified in ERMS, and whether BWS and ERMS share common genetic mutations is yet to be proven.

The RAS-mitogen-activated protein kinase (MAPK) pathway is a signal transduction cascade that has been studied extensively during the last decades due to its role in human oncogenesis. Mutations in *RAS* genes have been found in numerous malignancies, including sporadic ERMS (Table 2.2; Chen *et al.*, 2006; Martinelli *et al.*, 2009; Stratton *et al.*, 1989; Wilke *et al.*, 1993). Recently, germline mutations in genes coding for different components of the RAS signaling cascade have been recognized as the cause of several phenotypically overlapping disorders, referred to as the neuro-cardiofaciocutaneous syndromes, Costello (*HRAS* 80–90%), NF1, Noonan (*PTPN11* 50%, *RAF1* 3–17%, *SOS1* 10–13%, and *KRAS* <5%), LEOPARD (*PTPN11* 90% and *RAF1* 3%), and cardiofaciocutaneous (*BRAF* 75–80%, *MAP2K1*, 2 10–15%, and *KRAS* <5%) syndromes all present with variable degrees of psychomotor delay, congenital heart defects, facial dysmorphism, short stature, skin abnormalities, and a predisposition for malignancy including ERMS (Ferrari *et al.*, 2007; Gripp, 2005; Gripp and Lin, 2009; Moschovi *et al.*, 2007; Xia *et al.*, 2002). Costello syndrome is a rare congenital anomaly syndrome and characterized by severe postnatal failure to thrive and short stature. Individuals with Costello syndrome have an approximately 15% lifetime risk for developing a malignant tumor. The most common tumor in Costello syndrome is ERMS, followed by neuroblastoma and bladder carcinoma (Gripp, 2005; Gripp and Lin, 2009).

Gorlin syndrome, an autosomal dominant disorder associated with a predisposition for multiple basal cell carcinomas and other neoplasms such as medulloblastoma and ovarian fibrosarcoma results from a germline mutation in *PTCH1*, a tumor suppressor gene and a negative regulator of the sonic hedgehog signaling (Shh) pathway, which plays an integral role in nervous system development and anterior–posterior limb patterning (Gorlin, 2004). Although rare cases of ERMS have been found in association with Gorlin syndrome, a disease-associated locus has been mapped to 9q22, a region of relatively frequent genomic loss in some series (Bridge *et al.*, 2000; Tostar *et al.*, 2006), and additional supporting evidence for Shh playing a role in a subset of ERMS is provided by observational studies

using human tumors wherein expression of the Shh signaling cascade are altered (Oue *et al.*, 2010; Tostar *et al.*, 2006).

Survivors of hereditary retinoblastoma have substantially increased risks of developing subsequent primary malignancies including osteosarcoma, melanoma, and soft-tissue sarcomas. The predisposition to malignancies in retinoblastoma survivors has been attributed to a germline mutation in the *RB1* gene, which encodes the cell cycle regulatory retinoblastoma protein (pRb; Lohmann and Gallie, 2010). Early studies suggested that pRb abnormalities rarely occur in ERMS or ARMS (De Chiara *et al.*, 1993), but in a contemporary series, 6 of 36 ERMS lacked pRb staining on an immunohistochemical study (Takahashi *et al.*, 2004). A recent study reported that *Rb1* allelic imbalance occurred in 13 of 27 ERMS tested (but much less frequently than in ARMS; Kohashi *et al.*, 2008). However, among patients with hereditary *RB1* mutations, RMS reported to have occurred were more often categorized as RMS “Not Otherwise Specified (NOS)” than ERMS (note that NOS may imply inadequate tissue rather than diagnostic uncertainty), and associated with in the field of radiation (Kleinerman *et al.*, 2007). Indeed, Rubin *et al.* (2011) indicate that while *Rb1* loss of function alone does not lead to tumor initiation, *Rb1* loss of function in combination with other oncogenic factors such as *Trp53* nullizygous with/without *PTCH1* haploinsufficiency is strongly associated with an undifferentiated phenotype, wherein myogenic marker expression is reduced or absent. Tumor cell proliferation (Ki67 positivity) and myodifferentiation capacity under low serum conditions were also severely altered. Therefore, it might be suggested that *Rb1* is best characterized as a “modifier” of phenotype in ERMS.

2.2. Results of RMS subtype by mutation type using transgenic mice

Genetic analyses of sporadically occurring RMS have pinpointed several common alterations (Table 2.3). The role of *TP53* in heritable and sporadic RMS has been confirmed by results of transgenic mouse experiments. *Trp53* nullizygous mice have increased tumor susceptibility in adulthood, and the majority of tumors are lymphomas, whereas RMS is very rare in these mice (Harvey *et al.*, 1993a,b). The low incidence of RMS in individuals carrying *Trp53* alterations indicates that additional genetic lesions and/ or appropriate temporal and tissue specific alterations are required to cause this tumor. Interestingly, however, the frequencies of RMS decrease in mice with dominant negative (R175H) *Trp53* mutation (Lang *et al.*, 2004).

The HER family of genes also appear to play a role in RMS tumor initiation and progression. *HER-1/EGF-R* sustains RMS cell growth whereas *HER-3* induces myogenic differentiation *in vitro*, and both *HER-1* and *HER-3* heterodimerize with *HER-2*. Activation of *HER-2* can lead to transformation *in vitro* and *in vivo* in many cell types, is required for myoblast cell survival and is expressed in approximately one-half of human RMS (Andrechek *et al.*, 2002; Ricci *et al.*, 2000). Nanni *et al.* (2003) described remarkable results in which the combination of *Trp53* inactivation and *HER-2* activation resulted in the induction of ERMS in all male mice. These tumors which arose between 11 and 21 weeks exclusively in the genitourinary tract expressed not only desmin and myosin but also insulin-like growth factor-II.

Fos is a zinc finger transcription factor consisting of AP-1 and regulates various biological processes by transcriptional activation of a number of target genes downstream of signaling pathways such as protein kinase C. *Fos*^{-/-} mice display osteopetrosis due to lack of osteoclasts. Overexpression of *Fos* in transgenic mice causes them to develop chondrosarcoma and osteosarcoma (Grigoriadis *et al.*, 1993; Wang *et al.*, 1991). Fos is also known to regulate apoptotic-signaling pathways. Fleischmann *et al.* (2003) generated *Trp53/Fos* compound mutant mice (*Trp53*^{-/-} *Fos*^{-/-}) and these mice initially developed osteopetrosis similar to the phenotype seen in *Fos*^{-/-} mice (Grigoriadis *et al.*, 1994). However, *Trp53*^{-/-} *Fos*^{-/-} mice started to develop tumors of the facial and orbital regions at 10 weeks of age and the tumor penetrance was 93% at 25 weeks. These tumors contained cells with polygonal or elongated shapes and expressed cell cycle-associated proteins and a number of skeletal muscle markers such as desmin and MyoD. These characteristics are very similar to human embryonal RMS. Cell lines isolated from these tumors expressed *Pax7*, which was significantly reduced by *Fos* reexpression. In addition, overexpression of *Fos* in the primary myoblasts also downregulated muscle specific gene expression, and *Pax7* gene expression was decreased to below the detection limit. These results suggest an interesting molecular mechanism by which *Fos* is the repressor for *Pax7* gene transcription and upregulation of *Pax7* gene expression may result in increased myoblast proliferation and prevent apoptosis in this experimental context. Recently, Singh *et al.* investigated Wnt signaling for these *Trp53*^{-/-} *Fos*^{-/-} mouse tumors, and demonstrated a critical role for suppression of the canonical Wnt pathway in human ERMS cell lines that was mediated by AP-1 (Singh *et al.*, 2010).

Another RMS-associated mutant mouse strain carries a germline knockout of the *Cdkn2a* locus (Sharp *et al.*, 2002; Sharpless *et al.*, 2001). This locus encodes two unrelated proteins with tumor suppressor function, p16^{Ink4a} and p19^{Arf}. p16 regulates the cell cycle pathway through modulation of CDK4 and CDK6 and p19 regulates p53 checkpoint function. It is not surprising that *Ink4a/Arf* knockout mice are prone to cancer, but they develop mainly hematopoietic tumors, some melanomas and fibrosarcomas and, rarely, RMS (Serrano *et al.*, 1996).

Activation of the *c-MET* signaling pathway occurs in several types of human cancers, and affects cell motility, proliferation and survival in experimental systems. Activation of *c-MET* can occur through overexpression or activating mutations of the *c-MET* receptor itself or by autocrine expression of *HGF/SF*. Transgenic *HGF/SF* mice develop tumors rarely, and these are mostly melanomas, although a few RMS also develop (Takayama *et al.*, 1997). The surprise came from mice that expressed the *HGF/SF* transgene and, in addition, were deficient for *Ink4a/Arf*. The doubly mutant mice developed ERMS with nearly a 100% frequency with a mean age of onset of 3.3 months. Intriguingly, this study demonstrated the presence of preneo-plastic hyperplastic satellite cells in the skeletal muscle of *HGF/SF* *Ink4a/Arf*^{-/-} mice aged 6–10 weeks, which suggested that these myogenic precursors were the source of RMS.

LIG4 syndrome is a rare autosomal recessive disorder arising from mutations in the *LIG4* gene, which plays a critical role in the repair of DNA double-strand breaks by nonhomologous end-joining. It is characterized by chromosomal instability,

immunodeficiency, developmental delay, and an increased risk for lymphoid malignancies. Most commonly, *Lig4* *Trp53* null mice die from pre-B cell lymphomas (Frank *et al.*, 2000). Sharpless *et al.* described results in which *Ink4a/Arf*^{-/-} – *Lig4* haploinsufficient mice developed soft-tissue sarcomas including RMS, which possess clonal amplifications, deletions, and translocations. Because reduced *Lig4* gene dosage is sufficient to facilitate transformation, this data suggests that tumors with *Lig4* mutations would have increased genomic instability even in the setting of a retained and expressed wild-type allele. *LIG4* appears to constitute a new class of tumor suppressor protein that does not conform to the Knudson paradigm. The authors concluded that either germline or somatic loss of NHEJ proteins might underlie chromosomal aberrations known to be responsible for human malignancies.

PTCH1, a member of the patched gene family, is the receptor for SHH, a secreted molecule implicated in the formation of embryonic structures and in tumorigenesis, as well as the desert hedgehog and Indian hedgehog proteins. This gene is thought to function as a tumor suppressor. The experimental demonstration that *Ptch1* haploinsufficiency is linked to ERMS tumor initiation has been reported. Second allele inactivation is thought to occur by methylation (Calzada-Wack *et al.*, 2002; Hahn *et al.*, 1998). Lee *et al.* investigated the role of *Ptch2*, which is highly similar to *Ptch1* in tumor suppression by generating *Ptch2*-deficient mice. In striking contrast to *Ptch1*^{-/-} mice, *Ptch2*^{-/-} mice are born alive and show no obvious defects and are not cancer prone. However, loss of *Ptch2* markedly affects tumor formation in combination with *Ptch1* haploinsufficiency. These data suggest that *Ptch2* mutations enhance tumorigenesis, resulting from defects in Shh signaling due to *Ptch1* heterozygosity, suggesting a cooperation of *Ptch2* loss with *Ptch1* mutation (Lee *et al.*, 2006).

In our own studies, we have examined the influence of *Ptch1* haploinsufficiency on *Trp53* nullizygous muscle and found *Ptch1* loss to be both important as a cooperative initiating mutation for soft-tissue sarcomas, but also as a modifier of myogenic differentiation that depends upon the cellular context. *Rb1* loss in the context of *Trp53* nullizygous (with or without *Ptch1* loss) also appears to be a modifier, resulting uniformly in loss of differentiation. The work of others affirms the assertion that *Trp53* phenotype can be modified by cooperating mutations: *Trp53*^{-/-} – *Fos*^{-/-} mice develop ERMS in facial and orbital regions only. In contrast, *Trp53*^{+/-} – *HER-2* transgenic mice develop ERMS in the male genitourinary tract only.

3. Cell of Origin of RMS

3.1. Pleomorphic RMS

Pleomorphic RMS typically arises in the skeletal musculature of adults and is distinctly uncommon in children. As the name implies, pleomorphic RMS is a high-grade pleomorphic sarcoma composed of pleomorphic spindle cells containing irregular, hyperchromatic nuclei and numerous mitoses, admixed with varying numbers of rhabdomyoblasts, which demonstrate muscle filaments by electron microscopy. Immunohistochemistry confirms the myogenic nature of these tumors, which demonstrate positivity for desmin, MyoD1, and myogenin. Cytogenetic studies reveal complex karyotypes, with rearrangements and

evidence of gene amplification, that offer no distinctions from other adult pleomorphic sarcomas (Parham and Ellison, 2006).

The cell of origin for pleomorphic RMS remains to be defined. Doyle *et al.* developed a mouse model of pleomorphic RMS in which *Cytochrome P450* promoter-driven Cre (AhCre)-activated oncogenic *KRasG12V* can cooperate with *Trp53* mutation and/or loss in pleomorphic RMS development. Frequencies of pleomorphic RMS seen in AhCre *KRasG12V Trp53*^{+/-} mice, AhCre *KRasG12V Trp53R172H*^{+/+} mice, and AhCre *KRasG12V Trp53*^{-/-} were 6%, 88%, and 94%, respectively. AhCre, under the control of the *Cyp1A1* promoter, was originally described as showing expression in the small intestine, liver, and colon following induction with β -naphthoflavone (Ireland *et al.*, 2004). However, spontaneous Cre expression has been seen in other organs (Sansom *et al.*, 2005). Mice expressing AhCre were crossed to mice expressing conditional *GFP*, and GFP expression was seen throughout skeletal muscle. This result indicated that cell of origin for pleomorphic RMS might be a muscle resident cell early in the myogenic lineage (Doyle *et al.*, 2010). Tsumura *et al.* (2006) utilized genetically engineered mice with Cre-inducible oncogenic *Kras* and loss of the *Trp53* allele to generate a spatially and temporally restricted mouse model. In this model, a Cre expression vector is electro-porated into the muscle and 10 weeks later pleomorphic RMS develops at the site of electroporation. The electroporation clearly inflicts tissue damage, followed by mononuclear skeletal muscle cell accumulation, and this subsequently disappears in control mice, but numerous mononuclear skeletal muscle cells continue to proliferate in intramuscular tissue of mice harboring conditional *Kras* and *Trp53* genes. It is presumed that activated *KrasG12V* and loss of *Trp53* creates a premalignant population of myogenic precursors incapable of withdrawing from the cell cycle. As Cre recombinase was locally introduced into muscular tissue by electroporation, cells might have been derived from residential stem cells in the muscle, including satellite cells. Loosely interpreted, these results suggest that the cell of origin for pleomorphic RMS might be an adult satellite cell, and that *Trp53* function impairment and *Kras* activation are crucial for tumor initiation (Fig. 2.1).

3.2. ERMS

ERMS are so-named because of their remarkable resemblance to developing embryonic and fetal skeletal muscle. As such, these tumors are characterized by variable zones of condensation that produce alternating foci of hypocellularity and hypercellularity. Like embryonic muscle, the dense zones typically contain areas of more overt myogenesis, whereas the loose areas more closely resemble primitive mesenchyme and lie in a loose gelatinous matrix (Hall and Miyake, 2000).

Using Zebrafish, Langenau *et al.* (2007) discovered that a transgene which expressed human *KrasG12D* by a *rag2* promoter rapidly induced tumors that appeared to be skeletal muscle in origin, based on the presence of multinucleated striated muscle fibers and a battery of diagnostic markers (i.e., desmin, myod, met, myf5, mcadherin; Merlino and Khanna, 2007). Gene set enrichment analysis (GSEA), successful in classifying human, mouse, and even zebrafish cancer (Lam *et al.*, 2006), was used to determine whether a gene set derived from their zebrafish model (tumor vs. normal muscle) was enriched in human data sets of a

variety of tumors versus their corresponding normal tissues. Notably, gene sets upregulated in the zebra-fish tumors were significantly associated with human ERMS. The *rag2* promoter is expressed in immature T and B cell lineages, olfactory rosettes, and sperm (Jessen *et al.*, 2001). In this chapter, using *rag2-EGFP-bcl2* and *rag2-dsRED2* transgenic animals revealed that transgene-expressing cells were also detected in the mononuclear component of the skeletal musculature, comprising mononuclear satellite cells, differentiating myoblasts, and rare fusing myoblasts, but not multinucleated terminally differentiated muscle fibers.

Linardic *et al.* (2005) established ERMS model mice with xenografts of human skeletal muscle cell precursors and muscle myoblasts infected with retroviruses encoding SV40 large-T and small-t oncoproteins, the hTERT catalytic subunit of telomerase and HRasG12V. Interestingly, introducing genetic changes characteristic of RMS in cultures of human fetal skeletal muscle cell precursors led to a broad spectrum of sarcomas, ranging from undifferentiated small round blue cell tumors (sarcomas, NOS) to tumors exhibiting differentiation markers characteristic of rhabdomyoblasts, but lacking frank histopathologic features of either ERMS or ARMS. On the other hand, transformation of human adolescent skeletal muscle myoblasts generated an ERMS phenotype.

Recently, Rubin *et al.* generate mouse models of ERMS, through lineage-specific homozygous deletion of *Trp53* with or without heterozygous *Ptch1* deletion by interbreeding *Ptch1* or *Trp53* conditional lines with the myogenic Cre mouse line MCre (Brown *et al.*, 2005), Myf5Cre (Haldar *et al.*, 2008), Pax7CreER (Nishijo *et al.*, 2009), or Myf6Cre (Keller *et al.*, 2004). MCre is specific for the prenatal and postnatal hypaxial lineage of Pax3 that includes postnatal satellite cells (Brown *et al.*, 2005; Relaix *et al.*, 2006). Myf5Cre is specific for the prenatal and postnatal lineage of Myf5 that includes quiescent and activated satellite cells and early myoblasts (Beauchamp *et al.*, 2000; Cornelison and Wold, 1997; Kuang *et al.*, 2007). Pax7CreER is specific for the postnatal lineage of Pax7 that includes quiescent and activated satellite cells (Nishijo *et al.*, 2009), and Myf6Cre is specific for the prenatal and postnatal lineage of Myf6 that includes maturing myoblasts (Keller *et al.*, 2004). When *Trp53* was homozygously inactivated with or without heterozygous *Ptch1* deletion, tumors in all mouse lines developed at a penetrance rate of 13–56% within a 600-day follow-up period. They developed several types of tumors including RMS (embryonal, alveolar, and pleomorphic) and non-RMS soft-tissue sarcoma including undifferentiated pleomorphic sarcoma and osteosarcoma. Interestingly, Myf6Cre *Trp53*^{-/-} lineage gave rise to the highest percentage of ERMS (100%; Rubin *et al.*, 2011).

These results suggest that cell of origin for ERMS is most commonly the differentiating late myoblast (Fig. 2.1). The final stage of transformation might be at this differentiating stage. Indeed, the development of a viable germline *Trp53*^{-/-} mouse model demonstrates that the *Trp53* molecule is not critical during development. Furthermore, White *et al.* (2002) indicated that p53 is not required for the regulation of myoblast proliferation, differentiation, or myotube formation during myogenesis of adult skeletal muscle *in vivo*. Thus, in the context of *p53* mutations, complementary mutations are required to generate RMS and Rubin *et al.* indicate that *PTCH1* and *RB1* mutations are not uncommon in human RMS.

A common impression is that RMS arise in skeletal muscle, but in fact many pediatric examples arise in viscera such as prostate, urinary bladder, and gallbladder, which are devoid of striated muscle fibers. However, revealing new reports of the presence of satellite cells in the urethral rhabdosphincter are emerging (Sumino *et al.*, 2007), and the presence of similar cells in other regions of the bladder, the prostate, and biliary tract are intriguing. RMS occasionally present with diffuse bone marrow involvement and no clear primary tumor suggests that RMS can arise from non-muscle cells, such as a mesenchymal stem cell with the capacity to be pushed down the skeletal muscle lineage; however, almost all of the reported cases associated with marrow involvement, including those presenting as possible acute leukemia, have been of the alveolar, not embryonal subtype (Chen *et al.*, 2004; Lisboa *et al.*, 2008; Sandberg *et al.*, 2001).

Are cell of origin and cancer stem cell the same? Certainly the tumor initiating cell may be able to become a cancer stem cell, but the biology of the “tumor when conceived” may be distinct from the final tumor product. Several different cell types may be capable of becoming RMS by processes of differentiation, dedifferentiation (Odelberg *et al.*, 2000) or trans-differentiation (Lagha *et al.*, 2009; Messina *et al.*, 2009; Wiggan *et al.*, 2002). Will tumors retain characteristics of their originating lineage? It may be dangerous to generalize, but at least in the context of certain mutations, Rubin *et al.* (2011) suggest this to be the case. We speculate that cell of origin (tumor initiation cell) may be able to become a cancer stem cell, but by way of a process that may significantly change its character.

4. Tumor Phenotype and Cancer Stem Cells

The hypothesis that malignant tumors grow and progress as a result of rare subsets of tumor repopulating cells that are more tumorigenic than other cancer cells has increasingly gained credence. Presuming that a single cell of origin gives rise to any individual tumor, the basis for this functional heterogeneity has been explained by one of two models, the hierarchy model and the stochastic model (Bomken *et al.*, 2010). The hierarchy model predicts that a malignancy is organized in a manner analogous to the normal tissue hierarchy with tumor/tissue stem cells able to produce identical daughter stem cells with self-renewal capacity, and committed progenitor daughter cells with limited, although potentially still significant, potential to divide. This model, with a rare cancer stem cell at the apex, is essentially synonymous with the cancer stem cell model (Quintana *et al.*, 2008). The stochastic model predicts that a malignancy is composed of a homogeneous population of cells, which generate their heterogeneity in response to particular combinations of endogenous and exogenous factors. Endogenous effects include gene dosage effects and transcriptional and translational control mechanisms, whereas exogenous effects include cytokine concentrations, cell–cell interactions and niche environment. This model has stemness as a functional phenotype, and does not yet predict whether stemness is found truly within each population, or whether cells first undergo a process of dedifferentiation to a more tissue specific stem cell-like phenotype, reacquiring stemness in the process (Gupta *et al.*, 2009). However, the current definitive test for a cancer stem cell is the capacity to propagate tumors as xenografts in immunocompromised mice (Clarke *et al.*, 2006). What factors are responsible for ERMS tumor repopulating cells? Hirotsu *et al.* (2009) reported xenoengraftment of single FGFR3-positive ERMS cells yielded tumor formation. In this

study, cancer stem cells were enriched in RMS subpopulations defined not by side population characteristics (Komuro *et al.*, 2007) or CD133 positive-cells, but FGFR3 alone. It has been reported that bFGF promotes proliferation and inhibits differentiation of muscle satellite cells (Guthridge *et al.*, 1992; Lefaucheur and Seville, 1995), and while FGF ligand and receptor interactions are still being defined, the binding of bFGF to FGFR3 is known to activate FGF signaling (Maric *et al.*, 2007). The mRNA expression of FGFR3 and FGFR4 are higher in quiescent than activated satellite cells (Fukada *et al.*, 2007) suggesting a commonality between tumor repopulating cell and normal muscle stem cell maintenance mechanisms. Further, identification of new FGFR4-activating mutations in 9% human ERMS were identified, and this mutation promoted metastasis and “remote” tumor repopulation in xenotransplanted models (Taylor *et al.*, 2009).

As mentioned previously, Langenau *et al.* (2007) discovered that expression of human *KrasG12D* driven by a *rag2* promoter rapidly induced ERMS in Zebrafish (Merlino and Khanna, 2007). By coinjecting both *rag2-dsRED2* and *rag2-KrasG12D* constructs into α -actin-GFP transgenic zebra-fish embryos, they were able to differentially label RMS cells based on their differentiation status; *rag2* promoter-directed red fluorescence marked mononuclear myogenic precursors (R+), while α -actin promoter-driven green fluorescence labeled more mature muscle cells (G+). R+G+ cells represented cells of intermediate muscle differentiation, while double-negative cells were positive for blood cell markers. Using serial transplantation and limiting-dilution transplantation, functional hallmarks for cancer stem cell behavior, Langenau *et al.* found greater stem-like potential among the less-well-differentiated myogenic population (R+), likely representing the target cells for *Kras* transformation. Based on microarray analysis, the R+ population found in zebrafish ERMS was similar to activated satellite cells and shared pathways involved in normal satellite cell self-renewal. Recently, Rubin *et al.* also found that murine ERMS cells share features of satellite cells. Perhaps the most fascinating aspect of this study is the high concordance of gene expression between murine ERMS and activated rather than quiescent satellite cells (Fukada *et al.*, 2007; Rubin *et al.*, 2011).

Taken together, these data suggest that ERMS cancer stem cells may represent a myogenic precursor state, akin to a satellite cell, and express FGFRs. The notion that the tumor is driven by cancer stem cells has obvious therapeutic implications. The efficacy of tumor response to systemic therapy has traditionally been assessed based on the bulk of tumor cells by monitoring of changes in tumor size. However, if only a small fraction of cancer stem cells are capable of initiating tumor formation, then curative therapy should be designed to target these rare cancer stem cells rather than the bulk of nontumorigenic cells. Analysis of RMS cancer stem cells might thus yield novel and important therapeutic targets.

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Figure 2.1.

Model of skeletal myogenesis and possible cellular origins of fusion-negative RMS. Prenatal muscle development and postnatal muscle maintenance and regeneration are regulated by Pax3, Pax7, and muscle regulatory factors (MyoD, Myf5, Myogenin, and Myf6). Cell of origin for pleomorphic RMS might be an adult satellite cell. Meanwhile, cell of origins for ERMS might be a differentiating late myoblast.

Table 2.1

Congenital syndromes associated with fusion-negative RMS

Cancersyndrome	Locus	Gene	Characteristic malignancies	Frequency	Reference
Li-Fraumeni syndrome	17p13.1	<i>TP53</i>	Sarcomas, breast cancer, brain tumors, adrenocortical carcinoma	1–10% of RMS patients	Le Bihan <i>et al.</i> (1995), Schneider and Garber (2010), Xia <i>et al.</i> (2002)
Beckwith-Wiedemann syndrome	11p15.5	<i>KCNQ1OT1, H19/IGF2, CDKN1C</i>	Wilms tumor, hepatoblastoma, neuroblastoma, adrenocortical carcinoma, rhabdomyosarcoma	7.5% childhood risk for tumor development	Shuman <i>et al.</i> (2010)
Costello syndrome	11p15.5	<i>HRAS</i>	Rhabdomyosarcoma, neuroblastoma, transitional cell carcinoma of the bladder	15% lifetime risk for tumor development	Gripp (2005), Gripp and Lin (2009)
Neurofibromatosis type 1	17q1.2	<i>NF1</i>	Glioma, malignant peripheral nerve sheath tumor, rhabdomyosarcoma	1–6% of RMS patients	Ferrari <i>et al.</i> (2007), Xia <i>et al.</i> (2002)
Noonan Syndrome	12q24, 12p12.1, 3p25, 2p21	<i>PTPN11, SOS1, RAF1, KRAS</i>	JMML, AML, ALL	RMS; three cases	Moschovi <i>et al.</i> (2007)
Gorlin syndrome	9q22.3	<i>PTCH1</i>	Basal cell carcinoma, medulloblastoma, fibroma	RMS; three cases	Gorlin (2004), Xia <i>et al.</i> (2002)
Hereditary retinoblastoma	13q14.2	<i>RB1</i>	Osteosarcoma, soft-tissue sarcomas, melanoma	Standardized incidence ratio of RMS = 279	Kleinerman <i>et al.</i> (2007)

Table 2.2

Mutations detected in sporadic fusion-negative RMS

Locus	Gene	Mutation	Frequency	Percentage (%)	Reference
17p13.1	<i>TP53</i>	Point mutation	5/36	8–50	Diller <i>et al.</i> (1995), Felix <i>et al.</i> (1992), Taylor <i>et al.</i> (2000)
12q14.3-q15	<i>MDM2</i>	Amplification	1/12	8	Taylor <i>et al.</i> (2000)
9p21	<i>CDKN2A/ARF</i>	Deletion	3/10	25–33	Gil-Benso <i>et al.</i> (2003), Iolascon <i>et al.</i> (1996)
9q22.3	<i>PTCH</i>	Deletion	7/20	33–38	Bridge <i>et al.</i> (2000), Tostar <i>et al.</i> (2006)
12p12.1	<i>KRAS</i>	Point mutation	2/65	0–14	Chen <i>et al.</i> (2006), Martinelli <i>et al.</i> (2009), Stratton <i>et al.</i> (1989)
1p13.2	<i>NRAS</i>	Point mutation	10/65	5–21	Chen <i>et al.</i> (2006), Martinelli <i>et al.</i> (2009), Stratton <i>et al.</i> (1989)
11p15.5	<i>HRAS</i>	Point mutation	2/54	0–33	Chen <i>et al.</i> (2006), Martinelli <i>et al.</i> (2009), Wilke <i>et al.</i> (1993)
12q24	<i>PTPN11</i>	Point mutation	2/51	3–5	Chen <i>et al.</i> (2006), Martinelli <i>et al.</i> (2009)
5q35.1-qter	<i>FGFR4</i>	Point mutation	5/58	9	Taylor <i>et al.</i> (2009)
11p15.5	<i>H19/IGF2, CDKN1C</i>	LOH	30/39	77	Davicioni <i>et al.</i> (2009)
13q14.2	<i>RB1</i>	Allelic imbalance	13/27	48	Kohashi <i>et al.</i> (2008)

Table 2.3

Results of rhabdomyosarcoma type by mutation type using transgenic mice

Mutation type	Tumor type	Mouse strain	Onset	Frequency (%)	Reference
<i>Trp53</i> ^{+/−}	RMS	129Sv X C57Bl6	21–78 weeks	3	Harvey <i>et al.</i> (1993b)
<i>Trp53</i> ^{−/−}	RMS	129Sv	17 weeks	6	Harvey <i>et al.</i> (1993a)
<i>Trp53</i> ^{+/−} <i>HER-2</i> <i>tg</i>	Embryonal RMS	BALB/c	11 weeks	100	Nanni <i>et al.</i> (2003)
<i>Trp53</i> ^{−/−}	Embryonal RMS	C57Bl6 X 129Sv	10 weeks	93	Fleischmann <i>et al.</i> (2003)
<i>Fos</i> ^{−/−}					
<i>Ink4a</i> <i>Arf</i> ^{−/−}	Embryonal RMS	C57Bl6 X FVB	3 months	100	Sharp <i>et al.</i> (2002)
<i>HGF/SF</i> <i>tg</i>					
<i>Ink4a</i> <i>Arf</i> ^{−/−}	Sarcomas (including RMS)	129SvJ X C57Bl6	22.9 weeks (average)	47 (sarcomas)	Sharpless <i>et al.</i> (2001)
<i>Lig4</i> ^{+/−}	Embryonal RMS	129Sv X CD-1	6–13 weeks	9	Hahn <i>et al.</i> (1998)
<i>Pch1</i> <i>l</i> ^{+/−}	Embryonal RMS	C57Bl6 X 129Sv	2–14 months	40	Lee <i>et al.</i> (2006)