

# Fusions Involving PAX and FOX Genes in the Molecular Pathogenesis of Alveolar Rhabdomyosarcoma: Recent Advances

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**Abstract:** Rhabdomyosarcoma is the most frequent soft tissue sarcoma in the pediatric population. Two main histopathologic variants have been described, embryonal (ERMS) and alveolar (ARMS), which demonstrate clinical and genetic differences. In particular, most ARMS but not ERMS tumors are characterized by the presence of recurrent chromosomal translocations, which have been cytogenetically defined as t(2;13)(q35;q14) and t(1;13)(p36;q14). These translocations form PAX3-FKHR and PAX7-FKHR gene fusions, which encode chimeric transcription factors. These chimeric proteins are hypothesized to generate a novel transcriptional program in the target cell, thereby contributing to multiple aspects of ARMS tumorigenesis. This review highlights recent advances in numerous areas of biomedical investigation that are providing new insights into the biology, molecular pathology, and translational science of ARMS: the identification of downstream targets of PAX3-FKHR and collaborating events in the process of tumorigenesis and metastasis; generation of animal models based on the gene fusion and collaborating events; development of new assays for diagnosis, prognosis, and detection of minimal disseminated disease; and exploration of immune recognition of this tumor and the fusion protein. These findings highlight the continued importance of the fusion proteins in understanding the biology of this tumor and developing improved diagnostics for this tumor, and have led to the initiation of efforts to explore therapeutic strategies based on the increasing understanding of the biology of these fusion proteins.

Rhabdomyosarcoma (RMS) is the most frequent malignant soft tissue tumor in children and young adults with an annual age-adjusted incidence of five new cases per million [1]. Two main morphologic subtypes have been described, alveolar (ARMS) and embryonal (ERMS), which have distinct clinical features [2]. ARMS is often diagnosed in adolescents or young adults, with primary tumors localized in the extremities and trunk. Most importantly, the ARMS subtype has an aggressive clinical behavior with early dissemination, poor response to treatment, and frequent relapses after treatment. In contrast, ERMS generally is diagnosed in young children with primary tumors in sites such as the head and neck, genitourinary tract, and retroperitoneum, and is usually associated with a good long-term outcome.

Genetic studies revealed marked differences between ARMS and ERMS tumors [3]. Cytogenetic studies demonstrated recurrent 2;13 or 1;13 chromosomal translocations in ARMS tumors. These chromosomal translocations have been suggested to be key determinants of the biologic behavior of ARMS. While ERMS tumors do not have recurrent translocations, molecular genetic analyses revealed consistent loss of heterozygosity on the short arm of chromosome 11, localized to chromosomal region

11p15.5, consistent with the hypothesis that a tumor suppressor gene relevant to ERMS tumorigenesis is located in this region [4].

Recurrent chromosomal translocations are a common feature of a large subset of sarcomas, which includes ARMS [5]. The elucidation of the genes involved in these chromosomal translocations, and the subsequent analysis of the molecular and phenotypic consequences of these tumor-specific genetic alterations, has contributed to the understanding of the process of tumorigenesis in these sarcoma categories. Furthermore, translational studies of these tumor-specific fusion products have provided reagents for improved sarcoma diagnosis, with potential new markers for prognosis, and have led to new directions for design of novel therapies.

In a previous review article, we presented a comprehensive overview of the genetic and biology studies related to the recurrent chromosomal translocations in ARMS up to 2001 [3]. Though the findings of pertinent earlier studies will be discussed, this review will focus on studies after 2001 that have advanced the field investigating these recurrent translocations and the associated genetics and biology of the ARMS subtype.

## CHROMOSOMAL TRANSLOCATIONS IN ARMS

Two specific recurrent chromosomal translocations have been identified in ARMS tumors, t(2;13)(q35;q14) and t(1;13)(p36;q14) [3]. The genes

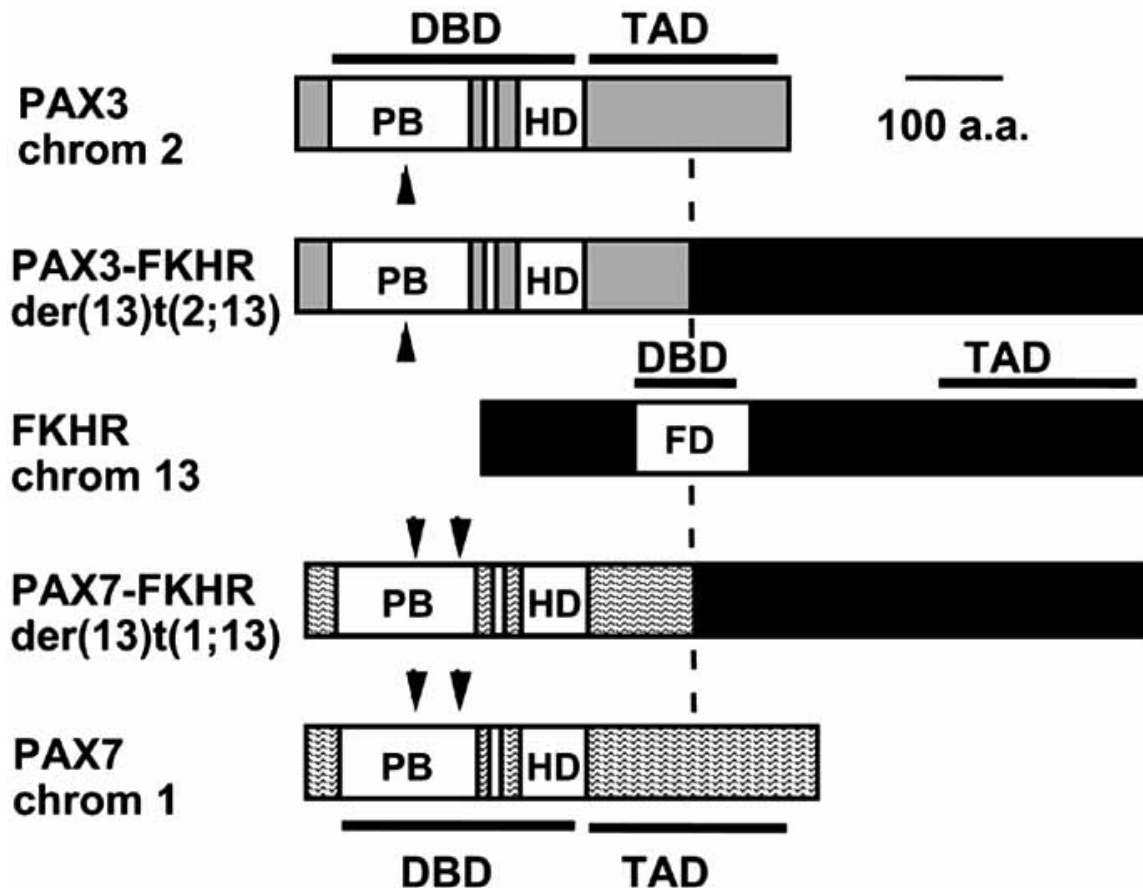
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identified at the translocation breakpoints were *PAX3* and *PAX7*, on chromosome 2 and chromosome 1, respectively, and *FKHR* (*FOXO1A*) on chromosome 13 [6-8]. *PAX3* or *PAX7* and *FKHR* encode transcription factors that are derived from two distinct families, the paired box or PAX family and the forkhead or FOX family, respectively. Furthermore, these wild-type transcription factors are normally involved in very distinct pathways.

*PAX3* and *PAX7* encode a distinct subfamily of transcription factors within the larger paired box family [3]. This subfamily is characterized by an N-terminal DNA binding domain consisting of a paired box, a short conserved intervening octapeptide motif, and a complete homeobox, as shown in Fig. (1). These two proteins also have a proline-, serine- and threonine-rich C-terminal transactivation domain. From a developmental standpoint, both Pax3 and Pax7 are involved in the development of the nervous system and skeletal muscle lineages, though these two proteins have distinct expression patterns and timing. For example, in somite compartments, Pax3 expression is activated before Pax7 expression, and Pax7 expression persists longer [9, 10]. In the adult mouse, Pax7 is commonly expressed by most myogenic satellite cells, while Pax3 is expressed in

satellite cells in only a subset of muscles [11, 12]. The significance of Pax3 in development is evidenced by genetic defects in the human Waardenburg syndrome and the mouse *spotch* phenotype, both caused by mutations affecting functional domains of *PAX3/Pax3* and thereby inhibiting transcriptional activity [3]. In mice homozygous for this mutation, in addition to abnormalities of the neural tube and neural crest derived structures, the limb musculature demonstrates a failure to develop whereas the axial musculature shows varying degrees of abnormality [13]. *Pax7* mutations were generated in the mouse by gene targeting and, in addition to abnormalities in neural crest-derived structures, these mutations caused abnormalities in the skeletal musculature due to satellite cell deficiency [11].

*FKHR* (*FOXO1A*) encodes a member of a subfamily of widely expressed forkhead transcription factors that regulates metabolic, cell proliferation, and apoptosis pathways [14]. Notably, the subcellular localization of these transcription factors is regulated by phosphorylation of conserved sites by the AKT/phosphatidylinositol 3-kinase pathway and other signaling pathways. Furthermore, the three members of this subfamily are all involved in



**Fig. (1).** Comparison of wild-type and fusion products associated with the 2;13 and 1;13 translocations. The paired box, octapeptide, homeobox and fork head domain are indicated as open boxes, and transcriptional domains (DNA binding domain - DBD, transcriptional activation domain - TAD) are shown as solid bars. The alternative splice in the paired box is shown by an arrowhead. The vertical dash line indicates the translocation fusion point.

cancer-associated chromosomal translocations [3]. In particular, in addition to the fusion of *FKHR* to *PAX3* or *PAX7* in ARMS, *MLL7* (AFX, FOXO4) and *FOXO3A* (FKHRL1) are fused to the *MLL* gene in small subsets of acute leukemia.

In the 2;13 and 1;13 translocations, the structure of the resulting *PAX3-FKHR* and *PAX7-FKHR* gene fusions are very similar, as shown in Fig. (1) [3]. The translocation breakpoints consistently disrupt the seventh intron of the nine exon-containing *PAX3* or *PAX7* gene and the first intron of the three exon-containing *FKHR* gene. Though there are two reciprocal gene products, the product fusing the 5' end of *PAX3* or *PAX7* to the 3' end of *FKHR* is the important product of these translocations. This juxtaposition of these two regions has important consequences at several biological levels. First, the *PAX3-FKHR* and *PAX7-FKHR* chimeric transcripts encode fusion proteins containing the intact *PAX3* or *PAX7* DNA binding domain and the *FKHR* transcriptional activation domain. These fusion proteins activate transcription from *PAX*-binding sites but are more potent as transcriptional activators than the wild-type *PAX3* or *PAX7* proteins due to the insensitivity of the *FKHR* activation domain to inhibitory effects of N-terminal *PAX3* or *PAX7* domains [15]. In addition, the fusion products are also expressed in ARMS tumors at higher levels than the corresponding wild-type products due to two distinct mechanisms [16]. *PAX3-FKHR* overexpression results from copy number-independent increased fusion gene transcription whereas *PAX7-FKHR* overexpression is associated with fusion gene amplification. Finally, though the subcellular localization of the wild-type *FKHR* protein is regulated by an AKT-dependent signaling pathway, the fusion proteins are resistant to this regulatory pathway and show exclusively nuclear localization [17]. These findings indicate that the chromosomal changes in these tumors result in high levels of nuclear chimeric transcription factors that inappropriately activate transcription of genes with *PAX3* and *PAX7* DNA binding sites to induce tumorigenic behavior.

In conclusion, *PAX3*, *PAX7* and *FKHR* are transcription factors performing cellular functions that once dysregulated result in aberrant transcriptional pathways leading to tumor development. The fusion genes encode potent highly expressed chimeric transcription factors that are localized constitutively in the nucleus. All these features suggest that "gain of function" events involving these transcription factors are integral steps in ARMS tumorigenesis.

## DOWNSTREAM TARGETS OF THE FUSION TRANSCRIPTION FACTOR

Based on studies finding the truncated portion of the *FKHR* DNA binding domain to be inactive and able to be deleted from the fusion protein without apparent loss of function [3], the DNA binding specificity of the *PAX3-FKHR* or *PAX7-FKHR*

transcription factors appears to be provided mainly by N-terminal *PAX3* or *PAX7* domains. However, there is also evidence that the binding function of these N-terminal domains is modified by C-terminal *FKHR* domains [18]. In addition, there are frequent alternative splicing events within the region encoding the paired box that result in coexpression of two *PAX3-FKHR* isoforms (differing by one glutamine residue) and four *PAX7-FKHR* isoforms (differing by a glutamine residue +/- a glycine-leucine dipeptide) [19]. Though findings indicate that these small structural changes in the fusion protein DNA binding domains may modify binding affinity and transcriptional activity, it is unknown whether these changes affect target gene selection. A major long-term goal has been to identify transcriptional targets of the wild-type and fusion proteins relevant to the function of these proteins in normal development and in tumor development. Though earlier studies used a variety of standard approaches to elucidate downstream transcriptional targets of *PAX3*, *PAX7*, *PAX3-FKHR* and *PAX7-FKHR*, only a few genes (such as *MET*) have consistently proven to be targets with multiple approaches in these earlier studies [3].

Improvements in technology have provided faster and higher throughput approaches to identify downstream targets and pathways of these chimeric transcription factors. Earlier studies identified binding sites for the *PAX3* paired box or entire N-terminal DNA binding domain from a library of oligonucleotides by an iterative procedure of DNA binding, isolation of bound fragments, and PCR amplification [20-22]. As an extension of this procedure to identify genomic DNA binding targets of *PAX3* or *PAX3-FKHR*, a direct binding approach was used to screen libraries of mouse or human genomic DNA fragments with *in vitro* translated protein [23]. Fragments containing putative cis-regulatory elements were bound by either *PAX3* or *PAX3-FKHR*, and then subjected to the cyclic binding, isolation, and amplification procedure. High throughput sequencing of these genomic clones permitted determination of the identify and origin of these genomic clones. Detailed analysis of specific clones resulted in detection of the *PAX3* binding sites, and elucidation of participation in *PAX3*-dependent regulation of gene expression. An example of a potential target revealed by this analysis was *FLT1*, which encodes the receptor for vascular endothelial growth factor.

Some recent studies utilized a genome-wide approach, in which many potential targets are investigated at the same time. Several studies, in particular, used microarrays and expression profiling to determine downstream consequences of the wild-type *PAX3* and *PAX3-FKHR* fusion proteins. In a cell culture-based approach to identify targets, the wild-type gene or fusion gene is transferred and ectopically expressed in a cell line. One such study focused on the SaOS-2 osteosarcoma cell line [24]. Following expression of either the *Pax3-FKHR* or wild

type Pax3 cDNA, a set of target genes were identified which were regulated only by Pax3-FKHR (such as PLN) in addition to target genes similarly regulated by both the wild-type and fusion proteins (such as cannabinoid receptor-1 [CNR1]). In addition, a group of genes were identified (such as PTHLH) which were repressed by Pax3 and induced by Pax3-FKHR. The results from chromatin immunoprecipitation analyses confirmed that some of these downstream genes are direct targets of Pax3-FKHR, including CNR1, EPHA4, and EPHA2. In these ectopic expression studies, an important finding is that the specific downstream target genes were dependent on the cell line in which the fusion gene was ectopically expressed. An example of this cell type specificity is the downregulation of BMP4 by Pax3-FKHR in SaOS-2 cells and the upregulation of BMP by Pax3-FKHR in RD ERMS cells. Therefore, the issue must be critically assessed as to the degree to which any given ectopic cell culture system provides a model of the actual ARMS tumor environment.

To identify downstream targets relevant for ARMS, a reasonable strategy is to express the fusion gene in a cell line related to the skeletal muscle lineage. In particular, one readily available set of cell culture resources are ERMS cell lines that are myogenic tumor-derived but lack the 2;13 or 1;13 translocation. Using the RD ERMS cell line, a 4-hydroxytamoxifen inducible cell culture system was developed by constitutively expressing in the cells a protein consisting of PAX3-FKHR joined to the modified estrogen receptor ligand binding domain [25]. Using this cell line in conjunction with a panel of stable PAX3-FKHR expressing clones, systems were available to examine the consequences of transient short-term and long-term stable PAX3-FKHR expression. In one study, these systems were used to demonstrate that PAX3-FKHR upregulates expression of the wild-type *PAX3* gene and chemokine receptor-encoding *CXCR4* gene and downregulates expression of the wild-type *PAX7* gene. In particular, this latter finding provides an explanation for the low *PAX7* expression level in ARMS tumors in comparison to ERMS tumors, and thus should permit reconsideration of a proposal for the cellular origin of ERMS based on the relative wild-type *PAX7* and *PAX3* levels [26].

In a second study of target genes using transfer of PAX3-FKHR into ERMS cell lines, a novel mechanism was revealed by which PAX3-FKHR modulates the downstream program of another transcription factor. Following transfection of PAX3-FKHR into 76-9 ERMS cells, expression profiling experiments demonstrated 31 upregulated and 69 repressed genes [27]. Approximately 40% of these genes were known targets of STAT1 and/or STAT3 that were regulated by cytokine-induced JAK/STAT signaling pathways. These findings were subsequently explained by the finding of a protein-protein interaction between PAX3-FKHR and STAT3 that apparently modulates STAT3 function.

In a study using NIH3T3 fibroblasts, introduction of PAX3-FKHR suppressed expression of CDKN1B (p27Kip1) at the protein level without a corresponding change in mRNA expression [28]. Subsequent experiments demonstrated that protein half-life was substantially decreased secondary to enhanced degradation by the 26 S proteasome system. One of the components of the E3 ubiquitin ligase complex that is involved in the polyubiquitination and degradation of p27Kip1 is Skp2. In PAX3-FKHR-expressing cells, there is increased expression of Skp2 at both the RNA and protein levels. Furthermore, increased SKP2 RNA and protein expression (as well as decreased p27Kip1 protein expression) are also evident in ARMS tumors when compared to skeletal muscle. Therefore, it appears that PAX3-FKHR either directly or indirectly regulates expression of Skp2, which then contributes to decreased expression of p27Kip1.

As important correlates to the ectopic cell line expression studies, phenotypic endpoints resulting from the expression of the fusion protein in various cell lines have also been assessed. In earlier studies, the PAX3-FKHR fusion gene was shown to inhibit the myogenic differentiation of C2C12 murine myoblasts and to induce oncogenic transformation of NIH3T3 murine fibroblasts [29, 30]. Transforming activity was also more recently demonstrated for the four PAX7-FKHR isoforms and the glutamine-lacking isoform of PAX3-FKHR [19]. All isoforms demonstrated comparable transforming activity, except for the glutamine- and glycine-leucine dipeptide lacking isoform of PAX7-FKHR. This latter isoform exerted 2- to 3-fold less transforming activity than the other isoforms, which inversely correlates with the highest level of transcriptional activity in this isoform. As another test of growth stimulating activity, PAX3-FKHR was stably transfected into ERMS cell lines. In cell culture analyses, the PAX3-FKHR stable transfectants demonstrated an improved ability to grow in serum-free conditions and an increased proliferative rate in regular culture conditions compared to vector controls [31]. In xenograft experiments, the PAX3-FKHR-transfected ERMS cells formed tumors that grew faster and were more locally invasive than tumors formed by control-transfected cells. In contrast to growth stimulating activity, PAX3-FKHR also causes growth suppression and/or cell death when expressed in a cell population (without long-term selection) at levels comparable to the levels found in ARMS cells [32]. In assays of NIH3T3 cells with an inducible system or with subpopulations of flow sorted cells, the PAX3-FKHR fusion protein primarily stimulates oncogenic transformation at lower activity levels, and then the phenotype switches to growth suppression as the PAX3-FKHR activity level increases. This association of phenotype with ectopic PAX3-FKHR expression thus should be an important consideration when planning future screens for downstream targets of PAX3-FKHR. Of note, mutagenesis studies

demonstrated that the transforming activity is dependent on an intact homeobox whereas the growth suppression activity is at least partly dependent on an intact paired box. These findings suggest that there may be two separate sets of target genes mediating these two phenotypes as a result of the complex function of the PAX3-FKHR DNA binding domain.

## GENOME-WIDE STUDIES OF RMS GENE EXPRESSION

Gene expression profiling of tumor samples has facilitated the identification of significant genes and pathways deregulated in RMS, and permitted important questions to be addressed regarding the role of the fusion proteins in these expression patterns and resulting pathways. In an oligonucleotide microarray study of 15 ERMS, 10 fusion-positive ARMS (8 PAX3-FKHR and 2 PAX7-FKHR), and 4 fusion-negative ARMS, a strong expression signature was detected by an unsupervised analysis that distinguishes fusion-positive and fusion-negative tumors [33]. Similar results were obtained in a second expression profiling study in which five PAX3-FKHR-positive and five fusion-negative ARMS cases were compared using a cDNA platform with probes mostly derived from muscle tissues; this signature was then used to correctly classify four additional ARMS cases [34]. In the former study, the expression profile appeared to be independent of the PAX gene involved in the fusion; however, the small number of PAX7-FKHR samples included in the study limit the power of such comparisons. This expression information was used to classify a fusion-negative case with a fusion-positive signature in which a *PAX3-NCOA1* variant fusion gene was subsequently identified.

In the oligonucleotide microarray study, subsequent supervised pairwise comparison of the fusion-negative and fusion-positive groups produced a refined signature consisting of 299 genes with  $P < 0.001$  [33]. Of note, *CNR1*, which was previously determined to be a direct target of PAX3-FKHR [24], was identified as the top gene on this list of differentially expressed genes. This list is notable for differential expression of additional tyrosine kinases (such as *FGFR2*, *FGFR4*, and *EGFR*) as well as transcription factors (*TFAP2B* and *FOXF1*). However, few additional downstream target genes of PAX3-FKHR identified in ectopic cell line expression studies were found in this list. This finding indicates that PAX3-FKHR alone may not be sufficient to induce expression of the genes involved in this signature. Either other genetic alterations, unique aspects of the ARMS target cell environment, or aspects of the *in vivo* tumor environment may be important contributors to this gene expression signature.

Another issue considered by various investigators has been the gene expression differences between ARMS, or RMS in general, and a tissue representative of the skeletal muscle lineage. The

goal is to identify expression evidence of fundamental oncogenic events that distinguish these myogenic tumor cells from normal myogenic cells. These studies utilized a wide range of technologies including sequenced cDNA libraries [35], SAGE (serial analysis of gene expression) libraries [36], expression profiling with microarrays [34], and comparative expressed sequence hybridization [37]. The latter technology is a new technique that provides a genome-wide view of relative expression patterns within two tissue samples according to chromosomal location. In each study, a number of genes were found to be either under- or overexpressed in the tumors relative to the control myogenic tissue. The most readily available source of tissue from the myogenic lineage is adult skeletal muscle, which consists mainly of nondividing well-differentiated multinucleated myocytes. However, a substantial subset of the resulting differentially expressed genes simply appear to correspond to abundantly expressed skeletal muscle genes [37]. Furthermore, in the study in which SAGE was employed to compare libraries prepared from four RMS tumors (3 ERMS, 1 ARMS), two adult skeletal muscle specimens, and a fetal muscle specimen, unsupervised analysis by hierarchical clustering revealed that the RMS samples were more similar to the fetal muscle than the adult muscle [36]. This finding supports the view that a more appropriate tissue type for comparing RMS to a "normal" tissue is probably fetal muscle in which there is a higher fraction of mononuclear precursor cells. Similar findings were also reported in the cDNA microarray study of fusion-positive and negative ARMS cases in which a comparison of adult and fetal skeletal muscle demonstrated the expression pattern in an ARMS specimen to be more similar to the fetal muscle specimen [34]. This study then proceeded to compare the expression profiles of all ten ARMS cases (both fusion-negative and positive) to fetal skeletal muscle, and identified 171 differentially expressed genes. Of note, approximately two thirds of these genes, including genes involved in the contractile apparatus, are still expressed at lower levels in the RMS samples compared to fetal muscle. The question arises whether these differences are due to obvious distinctions in functional organization, and thus perhaps systems such as sorted mononuclear cells from fetal muscle may provide a superior assessment of these important comparisons. Finally, a notable finding in the comparative expressed sequence hybridization study was that one region consistently overexpressed in ARMS tumors relative to skeletal muscle was chromosomal region 2p24 [37]. Subsequent RT-PCR analysis showed that the *MYCN* proto-oncogene, which is situated in the 2p24 chromosomal region, is consistently overexpressed at the RNA level in ARMS tumors.

This overview of the genomic studies of gene expression in RMS should underscore that these studies are still at a relatively early stage in their

development. It is important to note that the concordance in findings between these different gene expression studies is not high. One of the reasons for this low concordance is probably the limited sample size in many of the studies and possibly the use of different statistical analyses. In cases where there is reasonable statistical power, the biological relevance of the genes identified for RMS oncogenesis or progression needs to be analyzed in functional studies. In addition, a fraction of the genes in these signatures are still uncharacterized and thus basic biological annotation of these genes is necessary to start to understand how they may be participating in these processes.

## COLLABORATING EVENTS IN ARMS TUMORIGENESIS

The expression of the PAX3-FKHR or PAX7-FKHR fusion oncoprotein is considered a necessary step for ARMS tumorigenesis. Support for this premise is derived from cell culture studies demonstrating that these fusion proteins induce transformation when introduced into cells in culture [30, 38]. In addition, as demonstrated by cell culture studies with antisense oligonucleotides, PAX3-FKHR also maintains ARMS cell viability by inhibiting apoptosis [39]. However, as described earlier, when expressed in immortalized non-transformed cells at levels comparable to the levels expressed in ARMS tumor cells, PAX3-FKHR exerts growth suppression and cell death in most of the cells in the population [32]. A similar phenomenon was reported for other fusion oncoproteins, such as AML1-ETO in a subset of acute myeloid leukemia, and EWS-FLI1 in Ewing sarcoma [40, 41]. This finding that the level of fusion protein expressed in the tumor cells is toxic in other cells either indicates that the progenitor cells provide a highly and uniquely tolerant environment or that collaborating genetic events must be present in the cells to generate tolerance to the toxic aspects of the oncoprotein. Of note, a study using murine embryo fibroblast lines derived from p53, p16, and p19 (ARF)-mutant mice demonstrates that the growth suppressive and apoptotic effects of EWS-FLI1 are attenuated in the absence of these tumor suppressors [41]. Further studies are needed to determine whether specific frequently occurring genetic alterations in ARMS can be shown to attenuate the toxic effects of the PAX3-FKHR and PAX7-FKHR fusion proteins.

One consequence of the 2;13 and 1;13 chromosomal translocations is the loss of one copy of the *FKHR* gene. Even though one of the two copies of *FKHR* is intact, western blot studies of ARMS tumors and cell lines indicate that the FKHR protein is not detectably expressed [42]. A series of experiments indicated that this loss of protein expression of the second *FKHR* gene copy can be partly attributed to altered transcription or half-life of the FKHR mRNA and partly to posttranscriptional changes resulting in increased proteasomal

degradation. Gene transfer studies demonstrated that transduction of FKHR resulted in morphology changes, cell cycle arrest, and apoptosis in ARMS cells, but not in ERMS cells. The mechanism for induction of apoptosis involves direct transcriptional activation of the gene encoding the effector protease caspase-3. This phenotypic data is consistent with the premise that FKHR is a tumor suppressor specific for ARMS cells.

Comparative genomic hybridization (CGH) is a molecular cytogenetic method for screening a tumor for DNA gains and losses independent of the need for prior knowledge of a gene or sequence from the affected region [43]. This methodology can be used to identify additional genetic alterations that may collaborate with the fusion gene during ARMS tumorigenesis. In CGH methodology, tumor and normal reference DNA preparations are differentially labeled with fluorescent dyes and co-hybridized to either normal metaphase chromosomes or DNA arrays. Assessment of the ratio of tumor to normal DNA hybridization signals along each chromosome or at each element of the array permits identification of regions in the tumor genome with copy number gains or losses. The results from several studies have shown that distinct patterns of whole chromosome gains and whole or partial chromosomal losses are associated with ERMS [44-47]. While chromosome gains and losses were also found in ARMS, this subtype was notable for the very frequent occurrence of genomic amplification events [44, 47, 48]. In particular, frequent amplicons have been noted of chromosomal regions 12q13-15, 2p24, and 13q31 in ARMS tumors, with frequencies of 32%, 28%, and 19%, respectively. Of note, amplicons may occur at a comparable frequency in the anaplastic variant of ERMS [47]. Initial CGH comparisons of small numbers of PAX3-FKHR, PAX7-FKHR, and fusion-negative ARMS cases did not find any significant differences in distribution of amplicons or other genomic imbalances.

Of the amplified regions highlighted by CGH, studies have explored the frequency and significance of amplification of the MYCN gene (situated in 2p24) in ARMS tumors. The MYCN oncogene encodes a phosphoprotein that functions as a transcription factor involved in the regulation of cell proliferation and differentiation in normal as well as in cancer cells. Earlier studies of small numbers of RMS cases, using Southern blot or fluorescent *in situ* hybridization assays, found amplification in 43-67% of ARMS cases, and not in ERMS cases [49-51]. In a recent study evaluating MYCN copy number in RMS cases by real-time PCR, MYCN amplification (>4-fold increase) was detected in 12 of 48 (25%) ARMS cases and 9 of 58 (16%) ERMS cases, with significantly higher genomic copy number changes in the ARMS cases [52]. In addition, a high frequency of low copy number gains (1.5-4-fold) was found in both ARMS and ERMS cases. In cases with MYCN expression measurements, a correlation was noted between expression and copy number in ARMS but

not ERMS cases. Finally, an adverse outcome was significantly associated with either MYCN overexpression or gain of genomic copies of MYCN in the ARMS but not ERMS tumors.

In a final set of potential collaborating events, genetic loci are silenced by epigenetic events involving hypermethylation of CpG islands [53]. These CpG islands are stretches of DNA measuring approximately 1 kb, containing a high frequency of the dinucleotide CpG, and usually located near the 5' end of genes. The cytosine residues in these dinucleotides are usually unmethylated in normal cells, but can become methylated during tumorigenesis, resulting in alteration in the surrounding chromatin structure and transcriptional silencing of the associated genes. In cancers, this CpG island hypermethylation provides an alternative non-mutagenic mechanism for inactivating tumor suppressor genes. Various genes commonly affected by such hypermethylation events have been investigated in a small number of ARMS cases [54-57]. Though commonly hypermethylated genes such as *CDKN2A* were not found to be affected in a significant subset of ARMS cases, recent studies detected a reasonable frequency of hypermethylation of *RASSF1A* (4 of 6 cases), *HIC1* (8 of 8), and *CASP8* (4 of 6). In addition, though the RMS subtypes were not distinguished, the *HIN1* gene was also hypermethylated in a high frequency of RMS cases (11 of 18). Each of these genes encodes a known or putative tumor suppressor, such as *CASP8* encoding caspase 8, which is involved in the apoptotic cascade, and thus these hypermethylation events inactivate expression of these genes and alter the associated suppressive pathways.

## METASTATIC PATHWAYS IN ARMS

As in many cancers, metastatic disease represents one of the main clinical problems in ARMS with these patients having a poor outcome and few options to improve this prognosis. As the final step of tumor progression, the metastatic cascade is presumed to involve complex alterations in the expression of multiple genes. The identification of the genes and biological pathways involved in this process could guide efforts to design possible new therapies. During the last few years, some genes and pathways involved in metastatic pathways of ARMS have been described.

Two established downstream target genes of PAX3-FKHR, *MET* and *CXCR4*, encode cell surface receptors that function in signaling pathways with an impact on metastatic behavior [58, 59]. *CXCR4* is a G-protein coupled chemokine receptor whose ligand is stromal derived factor-1 (SDF1), whereas *MET* is a member of the tyrosine kinase family of receptors whose ligand is hepatocyte growth factor/scatter factor (HGF/SF). Of note, both of these ligands are secreted by the bone marrow, an important site of ARMS metastasis. The *CXCR4*-SDF1 signaling

pathway is involved in the homing of normal cells to hematopoietic sites and the *MET*-HGF/SF signaling pathway is involved in the proliferation and motility of various cell types. For these reasons, both signaling pathways have been proposed to be usurped in metastatic pathways of various cancers. Cell culture studies have explored the influence of *CXCR4*-SDF1 and *MET*-HGF/SF signaling on the metastatic behavior of ARMS cells. SDF1 or HGF treatment of ARMS cell lines was shown to induce cell culture changes in relevant properties including motility, adhesion, chemotaxis, and invasion. In an *in vivo* experiment, more ARMS cells were chemoattracted to and seeded lethally irradiated bone marrow than ERMS cells in association with upregulation of HGF and SDF1 in irradiated bone marrow stroma. Finally, as a model of a pathway-specific therapeutic strategy, a *CXCR4*-specific inhibitor blocked SDF1-directed adhesion and chemotaxis in ARMS cells.

A model of RMS arising in transgenic mice that overexpress HGF/SF and are deficient in *Ink4/Arf* provided another opportunity to identify genes relevant to RMS metastasis [60]. Isolation of tumor cell lines and metastasis testing by tail vein injection or orthotopic transplantation permitted identification of highly and poorly metastatic cell lines [61]. Comparison of gene expression profiles of these two types of cell lines identified a series of 44 differentially expressed genes (28 overexpressed in the highly metastatic cells and 16 underexpressed in these cells). Subsequent analysis focused on two overexpressed genes, *Vil2*, which encodes ezrin, and *Six1*. Ezrin is an adhesion molecule and member of the ERM family. It promotes cytoskeletal reorganization as part of signal transduction pathways and participates in different pathways linked to survival, motility, invasion as well as adherence. In contrast, *Six1* is a homeodomain-containing transcription factor that participates in the development of several lineages. *Six1* is of particular note because it was previously shown to be a downstream target of PAX3-FKHR in NIH3T3 cells and is expressed by ARMS cells [62]. Furthermore, the gene encoding ezrin has subsequently been shown to be a direct transcriptional target of *Six1* [63]. These gene expression relationships are confirmed by studies of tumor samples in which both genes are generally expressed at higher levels in fusion-positive RMS tumors than in fusion-negative RMS tumors. In addition, expression of both genes in RMS tumors is correlated with clinical stage, which is consistent with a role in tumor progression and metastatic behavior [61]. For direct proof of this role, experiments with direct transfer of either gene to low metastatic RMS lines from the HGF transgenic system demonstrated increased metastatic activity. Similarly, inhibition of expression or function of either gene product in highly metastatic lines with shRNA or a dominant-negative inhibitor, respectively, resulted in decreased metastatic activity. Finally, subsequent studies demonstrated that ezrin is necessary for *Six1* to exert its metastatic function thus further confirming

the downstream relationship of ezrin to Six1 [63]. Therefore, these studies suggest a pathway leading from the fusion proteins to Six1 to ezrin to metastatic activity.

One of the main differences between the two morphologic subtypes of RMS is the higher frequency of metastasis at diagnosis in ARMS [64]. In addition to larger expression profiling studies described above, a more directed study compared expression of genes involved in invasion and motility between ARMS and ERMS [65]. In particular, this study focused on matrix metalloproteinases (MMP) and their inhibitors and vascular endothelial growth factor and its receptor in ARMS and ERMS cell lines. Of the genes studied, MMP2 was notable for being generally more highly expressed in the ARMS cell lines than the ERMS lines. In addition, the high expression of MMP2 was correlated with a more invasive behavior in the ARMS cell lines. To determine the relationship of these findings to the fusion protein, the PAX3-FKHR cDNA was transfected into the RD ERMS cell line, and resulted in higher levels of MMP2 and increased invasiveness.

## ANIMAL MODELS

Several studies have utilized a variety of approaches to investigate the consequences of expression of PAX3-FKHR during development of

the mouse. These studies span the technologies from standard transgenic to conditional knock-in, and are summarized in detail in Table 1. Four of these experiments specifically analyzed the phenotypic changes caused by expressing the fusion protein from Pax3 expression elements, and the results show a relatively broad set of phenotypes among the four experimental systems [66-69]. These differences can be partly explained by the differences in the expression characteristics of the fusion product achieved by these experimental approaches, such that the transgenic approach with its exogenous and thus most adaptable expression cassette resulted in the most variable expression pattern [66], whereas the standard knock-in requiring consistent expression in all expressing lineages resulted in probable loss of embryos with high expression and retention of embryos with low expression [67]. The two conditional knock-in studies appear to have comparable expression patterns but are constructed with significant differences in placement of the knock-in, source of the FKHR sequence, and presence of other sequences such that there are multiple possible sources of the differences in embryonic phenotype [68, 69]. In some studies, the phenotypic changes are indicative primarily of involvement of neural and neural crest-derived lineages whereas in other cases, the changes are indicative of the involvement of early myogenic precursors. Furthermore, some of the

**Table 1. Mouse Models Expressing PAX3-FKHR During Embryonic and/or Fetal Development**

Approach	PAX3-FKHR expression	Phenotype	Mechanism	Ref.
Transgenic 14 kb mouse Pax3 promoter/ enhancer	Variable expression level among lines	Variable phenotype Pigmentary disturbances Neurological alterations Embryonic lethality Phenotypic severity increased by <i>spotch</i>	Neural crest phenotype Variable phenotype associated with variable fusion expression	[66]
Gene targeting 3'FKHR cDNA following Pax3 exon 7	Poorly expressed	Perinatal lethality CNS malformations Cardiac defects Diaphragm defects Tongue malformations	Neural crest phenotype	[67]
Conditional knock-in PAX3-FKHR-IRES-LacZ following Pax3 exon 1 PGK-cre (ubiquitous expression)	Normal Pax3 expression pattern	No live born heterozygotes Disorganized somites Ectopic muscle differentiation Rib defects Rescues early <i>spotch</i> phenotype PAX3-FKHR phenotype rescued by mutant met	Overexpression of met leads to constitutive activation of met signaling Alterations in myogenic precursors	[68]
Conditional knock-in 9 kb 3' genomic region of Fkhr locus following Pax3 exon 7 RajCreTg (ubiquitous and early expression)	Normal Pax3 expression pattern	Embryonic lethality Defects of skull, brain, and head, Hypoplastic limb muscle and thinned diaphragm Hypoplastic dorsal root ganglia	Some defects similar to <i>spotch</i> Partial dominant negative action of Pax3-Fkhr on Pax3 by suppressing wild-type Pax3 expression Other effects by activating other targets (e.g. Met)	[69]
Conditional knock-in 9 kb 3' genomic region of Fkhr locus following PAX3 exon 7 Pax7 cre	Pax7 expression domain	Severe postnatal growth retardation Extreme reduction of muscle mass Midface defects – bone hypoplasia Die generally by young adulthood (~3-4 mo)	Smaller satellite cell pool suggests Pax7 deficiency Pax7 expression often increased Pax3-Fkhr may work downstream of Pax7	[69]

phenotypic changes appear to be the result of loss of Pax3-related functions and other changes appear to be the result of gain of Pax3-related functions. Though some of these issues may be specific to each experimental model, others may be indicative of cell type-specific effects. One of the major similarities among all four of these experiments is that the Pax3-FKHR fusion protein exerts toxic effects. The mechanism for this toxicity may differ among experiments and may in fact differ among cell types. A clear problem with these approaches is that expression throughout a lineage causes lineage-wide dysfunction and often severe consequences. Another major related similarity between all four of the experiments is that tumors were not caused by the fusion protein. In the following discussion of a successful tumor model, data will be presented to indicate that even in the successful mouse model of ARMS tumorigenesis, tumors occur at a very low frequency with a very long latency [70]. Therefore, it seems very unlikely that there would be sufficient time for a tumor to develop in these systems in which there was usually premature lethality. Furthermore, it was not even clear that the fusion protein was expressed and functional in the myogenic precursors in some of these model systems. Finally, it is possible that the susceptible lineage may not even arise until later in development or even postnatally, and thus the early lethality would prevent the possibility of expression of the fusion protein in these potential cell types.

A mouse model of ARMS was successfully developed using a conditional knock-in approach that targeted expression mainly in differentiated skeletal muscle [70]. This approach used a knock-in allele consisting of a 9 kb fragment of the 3' genomic region of the *Fkhr* gene (flanked by *Lox* recombination sites) inserted after *Pax3* exon 7. The *Pax3* gene was normally transcribed until the Cre recombinase converted *Pax3* to *Pax3-Fkhr*. The Cre recombinase was introduced by mating with a mouse that expressed Cre from *Myf6* expression elements. *Myf6* is normally expressed predominantly in terminally differentiated skeletal muscle and thus these expression elements result in generation and expression of the *Pax3-Fkhr* fusion gene in this nondividing myogenic tissue. The viability and fertility of these mice indicate that *Pax3-Fkhr* is not toxic in this setting. The tumorigenicity of the fusion gene in these mice is evidenced by the finding of one tumor in one of 228 mice occurring at one year of age. The tumor appeared radiologically to arise from muscle and microscopically demonstrated a small blue round cell appearance consistent with the solid variant of ARMS. Furthermore, immunohistochemistry revealed staining with antibodies specific for several myogenic markers, including myogenin, thus confirming the diagnosis of RMS. To test the role of *Fkhr* as a tumor suppressor, a cross was prepared with mice carrying an *Fkhr* conditional knock-in. In these mice with *Pax3-Fkhr* and *Fkhr* haploinsufficiency, there were no tumors detected. To evaluate other collaborating

events, the *Pax3-Fkhr* mice were crossed with mice that had conditional knockouts for *Cdkn2a* (*Ink4a/Arf*) (which modulates both Rb and p53 pathways) or *Trp53*. Based on the finding of highly increased tumor frequency in *Pax3-Fkhr* mice that were homozygous for either *Cdkn2a* or *Trp53*, there appears to be an important role for at least p53 pathway disruption in collaborating with Pax3-FKHR in multistep ARMS tumorigenesis. Of note, homozygosity of *Pax3-Fkhr* was necessary to achieve these enhanced effects suggesting either the need for increased *Pax3-Fkhr* dosage or an inhibitory role for wild-type Pax3 in this system. This animal model will have great utility in further investigating the genetic and biological changes involved in multistep ARMS tumorigenesis as well as providing a tool for testing therapeutic and diagnostic reagents. However, as for the question of cell of origin, the model suggests the possibility that tumorigenesis in these mice may initiate within the terminally differentiated myocyte, but does not rule out that there may be less abundant dividing *Myf6*-expressing populations in which the initiating event could also occur.

## RMS DIAGNOSIS AND PROGNOSIS

The accurate diagnosis of the different subtypes of RMS is essential to select the best treatment available and in this manner to achieve an optimal response with the available therapies. Since the original identification of the PAX3-FKHR and PAX7-FKHR fusion genes, multiple studies have found that detection of these fusions is both a sensitive and specific test for the diagnosis of the ARMS subtype [3]. In a recently published study of RMS cases from the IRS-IV protocol, these gene fusions were not found in any of the 93 cases of RMS (or undifferentiated sarcoma) with a diagnosis other than ARMS, and were detected in 77% of the 78 ARMS cases in the study [71]. In these ARMS cases, the fusion status was 55% PAX3-FKHR, 22% PAX7-FKHR and 23% fusion-negative.

The RT-PCR assays for detection of these gene fusions can be improved by recent technological innovations in PCR methodology [72]. Several technologies have been developed which permit a PCR reaction to be monitored during the course of the reaction with actual quantitative assessment of the accumulation of the PCR product. The advantage of such real-time PCR assays is that gel electrophoresis or hybridization performed as part of the post-PCR testing phase is no longer required and thus there is a reduction of time and cost. Initial real-time RT-PCR protocols have been developed for the detection of the gene fusions in ARMS and other fusion-positive sarcomas [73, 74], and will likely be improved as more laboratories gain experience with this technology.

Immunohistochemistry (IHC) is another technology that may aid in not only the diagnosis of RMS but also in the identification of RMS subtype. A panel of

myogenic markers specific for the skeletal muscle lineage and suitable for IHC has accumulated and improved over time to facilitate the specific and sensitive diagnosis of tumors in the RMS family. Of specific utility for RMS diagnosis are antibodies that detect the myogenic transcription factors MyoD1 and myogenin, which show positive nuclear staining in virtually all cases of RMS and essentially no other tumor [75]. Although all cases of RMS stain with antibodies to these two myogenic transcription factors, several studies noted differences in the myogenin staining patterns between ARMS and ERMS such that most tumor cells in ARMS tumors stained positive with myogenin whereas fewer tumor cells in ERMS tumors stained positive [76-78]. However, the question arises whether this distinction is tight enough to separate ARMS and ERMS in a consistent manner. A recent study compared myogenin IHC and gene fusion RT-PCR assays in 109 RMS cases (45 ARMS and 64 ERMS) [74]. Less than 50% myogenin-positive cells were found in 35 ERMS cases, all of which were fusion-negative, and were not found in any ARMS cases. In contrast, greater than 50% myogenin-positive cells were found in 41 ARMS cases (of which 39 were fusion-positive) and 20 ERMS cases (of which 5 were fusion-positive). Therefore, this study indicates that myogenin IHC is not sufficient to specifically identify only ARMS cases but can be used to select cases for molecular testing.

Based on recent expression profiling studies, additional new IHC markers for distinguishing fusion-positive ARMS and ERMS have been identified [79]. In particular, antibodies were selected for two proteins, AP2 and P-cadherin, which are preferentially expressed in fusion-positive ARMS and not detectably expressed in ERMS as well as fusion-negative ARMS. In addition, antibodies were selected for two proteins, EGFR (epidermal growth factor receptor) and fibrillin-2, which are preferentially expressed in ERMS, detected at lower levels or not at all in fusion-negative ARMS, and not detectably expressed in fusion-positive ARMS. The characteristics of these IHC assays were analyzed on a tissue array with samples from 252 RMS (193 ERMS, 43 fusion-positive ARMS and 16 fusion-negative ARMS). The maximal specificity for detecting fusion-positive ARMS or fusion-negative ERMS was achieved when the two assays of each set were considered in combination. In this way, the IHC assays for AP2 and P-cadherin in combination detected fusion-positive ARMS with a sensitivity of 64% and a specificity of 98% whereas the IHC assays for EGFR and Fibrillin-2 in combination detected fusion-negative ERMS with a sensitivity of 60% and a specificity of 90%. Finally, when the results of these assays were compared with prognosis, AP2 and P-cadherin double positivity was associated with a poor outcome ( $P=0.0044$ ) and EGFR and Fibrillin-2 double positivity was associated with a good outcome ( $P=0.0254$ ).

Aside from the prognostic differences between ARMS and ERMS or fusion-positive and fusion-negative RMS, there is also evidence of a difference in outcome between fusion subtypes. In the IRS-IV study, the patients with PAX3-FKHR and PAX7-FKHR-positive ARMS had comparable outcome when these tumors presented as localized disease [71]. In contrast, in patients with metastatic disease, the patients with PAX3-FKHR-positive tumors had a significantly worse outcome than those with PAX7-FKHR-positive tumors (estimated 4-year overall survival rate of 75% for PAX7-FKHR versus 8% for PAX3-FKHR;  $P=0.0015$ ). As a possible source of explanation for this difference between these two groups of metastatic patients, there was a high incidence of bone marrow involvement among the PAX3-FKHR-positive metastatic tumors compared to no bone marrow involvement among the PAX7-FKHR metastatic tumors ( $P=0.044$ ). In addition, another study showed that differentiation markers and cell cycle characteristics are different between PAX3-FKHR-positive and PAX7-FKHR-positive ARMS tumors. In particular, there is evidence of increased cell proliferation and apoptotic activity in PAX3-FKHR-positive tumors compared to PAX7-FKHR-positive tumors [80].

The detection of PAX3-FKHR and PAX7-FKHR in ARMS with highly sensitive molecular techniques showed that ~20% of the cases are fusion-negative. Detailed study of the gene fusions in this group of patients showed that multiple subsets of patients could be distinguished within this "fusion-negative" category: variant fusions of PAX3 or PAX7 with other genes, rare cells in the tumors with typical fusions, atypical presentation of standard fusions, and true fusion-negative cases [81]. Clinical correlative studies are needed to determine if these various subsets of fusion-negative ARMS cases correspond to clinically distinct categories and whether these categories differ clinically from fusion-positive ARMS. As initial evidence of potential clinical differences between fusion-negative and fusion-positive ARMS tumors, two relatively rare and distinct presentations of RMS have been recently suggested to be associated with fusion-negative ARMS tumors. The first presentation is congenital ARMS, a rare and fatal variant in which ARMS presents at birth and often results in cutaneous metastases [82]. Molecular genetic analysis of three cases of congenital ARMS demonstrated all cases to be fusion-negative. A second rare presentation of RMS occurs in association with Beckwith-Wiedemann syndrome, a congenital overgrowth syndrome, which has an increased risk of developing tumors, most notably Wilms' tumor, and hepatoblastoma. The frequency of RMS in this genetic disease is very low. However, three cases of RMS occurring in the context of Beckwith-Wiedemann syndrome were recently reported and all three showed morphological features of ARMS and cytogenetic findings indicating the absence of the characteristic 2;13 and 1;13 translocations [83].

## DETECTION OF MINIMAL DISSEMINATED ARMS AND ITS VALUE AS A PROGNOSTIC FACTOR

Though the presence of disseminated disease at diagnosis is one of the best characterized prognostic factors in ARMS and RMS in general, there is a group of patients that show no evidence of disseminated disease by conventional methods but eventually relapse at distal sites during or after treatment. The suggestion has been raised that these patients have disseminated microscopic or submicroscopic disease at diagnosis that is not detected by conventional methods. Therefore, strategies need to be developed to identify such minimal disseminated disease, particularly early in the patient's course, since intensification of treatment could improve outcome. Bone marrow and peripheral blood are accessible sites for collecting samples for assessment of such disease spread and, for other solid tumors, the presence of micrometastatic disease at such sites at the time of diagnosis has been shown to be an indicator of poor outcome [84-87]. As bone marrow is a frequent site of metastasis for ARMS tumors, this site should be appropriate to assay for minimal disseminated disease in ARMS.

Several markers have been developed in conjunction with several different methodologies to assay minimal disseminated disease in ARMS. Based on the highly specific PAX3-FKHR and PAX7-FKHR gene fusions, initial assays focused on RT-PCR methodology to detect the fusion transcripts. Using cell-mixing studies, these standard RT-PCR assays were reported to detect one tumor cell per  $10^4$ - $10^5$  cells [88]. Though these gene fusion assays are highly useful tools, they cannot be used for monitoring disseminated disease in fusion-negative ARMS nor in ERMS. Thus, there is impetus to develop markers that would detect minimal disseminated disease in all RMS subtypes. One strategy for such markers is to rely on the premise that RMS tumors retain some expression features unique to the myogenic lineage, which are not found in bone marrow or other sites assayed for disease spread [88-93]. The myogenic markers that have been developed for this purpose to date are MYOD1, myogenin (MYOG), and the acetylcholine receptor genes ACHRA and ACHRG. In standard RT-PCR assays, MYOG has a sensitivity of  $1/10^4$  and MYOD1 has an improved sensitivity of  $1/10^5$  [88]. In accord with this higher sensitivity, instances have been reported in which MYOD1 expression is detected without expression of other markers. In addition to standard RT-PCR, real-time RT-PCR assays were developed for MYOD1, ACHRA, ACHRG, and the gene fusions, with a reported improved sensitivity of  $1/10^6$  (for the fusions) to  $1/10^7$  (for the other markers) [93]. However, it should be noted that this increased sensitivity is also associated with detection of low level background expression in sites such as peripheral blood, and the need to set lower detection limits for these assays.

Besides greater sensitivity, an additional advantage of the real-time assays is the ability to monitor quantitative changes in disease load over time and the ability to use the quantitative data to set an appropriate clinically significant threshold rather than the threshold being set by the inherent limits of the assay. Finally, as an alternative to RNA detection, immunocytochemistry assays were developed with MyoD1 and myogenin antibodies on cytopspins of mononuclear cells prepared from Ficoll gradients of marrow aspirates [94]. By processing large numbers of cells and performing multiple counts on multiple cytopspins, a high level of sensitivity was achieved, approximated to be to  $1/10^4$ .

These minimal disseminated disease assays were applied to clinical material in a number of different settings. Several studies have started to address the feasibility of detecting occult disease in the bone marrow, though the studies to date are relatively small in number [88, 91, 93-95]. As shown in Table 2, regardless of the methodology or marker, histologically positive bone marrows were consistently detected by these assays, and thus there does not appear to be a significant false negative rate. For ARMS, which often metastasizes to the bone marrow, the frequency of a positive result in a histologically negative marrow ranged from 15% to 60%. Of note, in ERMS, for which the bone marrow is not seen as a frequent site of metastasis, the rate of positive results in histologically negative marrows was lower but still impressive, ranging from 7% to 33%. In the study involving MyoD1 and myogenin immunocytochemistry of bone marrows from non-metastatic patients, when the RMS patients were grouped together, the follow-up data indicated a clinically significant association between bone marrow involvement and outcome [94]. In particular, occult marrow involvement was associated with a significantly higher risk of recurrence (50% vs. 11%,  $P = 0.011$ ) and poorer overall survival (47% vs. 92%,  $P = 0.01$ ). In addition to these studies, which evaluated bone marrows at the time of diagnosis, one study evaluated additional marrows during and after treatment [88]. These sequential assays demonstrated that 4 of 9 positive marrows were cleared of disease after one treatment cycle and 6 of 8 after were cleared after two cycles. Finally, peripheral blood samples were evaluated with real-time RT-PCR assays in one study and demonstrated positive findings in specific patients at diagnosis, during treatment, and following treatment [93]. Of note, the finding of a positive peripheral blood at the end of treatment was correlated with poor outcome, and the finding of a persistently positive peripheral blood preceded a metastatic relapse.

## RMS AND TUMOR IMMUNITY

Based on the lack of evidence for spontaneous regression, sarcomas have been considered to be tumors that do not trigger an effective immune response [96]. Recent studies of the PAX3-FKHR

**Table 2. Detection of RMS Dissemination in Bone Marrow**

Methodology	Markers	Tumor	Detection rate		Reference
			Histologic Positive BM	Histologic Negative BM	
RT-PCR	PAX3-FKHR PAX7-FKHR	ARMS	4/4	2/13	[95]
RT-PCR	Myogenin	RMS, NOS	---	2/9	[91]
Immunocytochemistry	MyoD1 Myogenin	ARMS ERMS	---	5/14 5/23	[94]
Real-time RT-PCR	MYOD1 ACHRA ACHRG PAX3-FKHR PAX7-FKHR	ARMS ERMS	2/2 ---	3/5 3/9	[93]
RT-PCR	MYOD1 Myogenin PAX3-FKHR PAX7-FKHR	ARMS ERMS	6/6 ---	3/15 1/15	[88]

fusion protein provide a possible explanation for tumor-based immunoinhibitory mechanisms in ARMS. As described earlier, PAX3-FKHR interacts with STAT3 and modifies the expression of genes that are normally regulated by the JAK/STAT signaling pathways [27]. This alteration of STAT3 function is reminiscent of the findings in other cancer types in which STAT3 activation contributes to suppression of inflammatory response and immune regulation. In ARMS, PAX3-FKHR modulation of STAT3 activity results in decreased MHC expression and an increase in secreted immunoinhibitory factors that depress local inflammatory and immune phenomena.

Despite these immunoinhibitory mechanisms, evidence for achieving detectable autologous immune response against ARMS, and RMS in general, has been accumulating in recent years. These recent studies have focused on identifying specific tumor-associated antigens for these cancers that could induce an effective immune response and be used for developing successful immunotherapy. Since the immune response, as quantified by different methods, is weak but detectable in sarcomas, the vaccines may require an extra component to boost the immune response. Dendritic cells have been recognized as very attractive additional components because this cell population is the most important means by which an antigen is delivered to the immune system and sustains a primary immune response.

A natural first consideration for a tumor antigen is the PAX3-FKHR or PAX7-FKHR fusion protein, which is present in the far majority of ARMS cases and, unlike the fusions in some other cancers, demonstrates a consistent structure. In one study, a human cytotoxic T-lymphocyte (CTL) line specific for a ten amino acid peptide (RS10) derived from the PAX3-FKHR fusion region was raised from a normal healthy HLA-B7<sup>+</sup> blood donor [97]. This CTL line was raised using autologous dendritic cells pulsed with

the RS10 peptide. The finding that this CTL line kills the HLA-B7<sup>+</sup> RH5 ARMS line in culture provides evidence that the epitope is successfully processed and presented by HLA-B7 in tumor cells expressing the fusion protein. However, it should be noted that another study addressing a similar issue did not find any antigenic determinants in the breakpoint region of the PAX3-FKHR fusion protein presented by HLA-A1, HLA-A2, or HLA-A3 molecules [98]. In a pilot clinical trial of an 18 amino acid peptide from the fusion point region, peripheral blood mononuclear cells were collected from ARMS patients (with recurrent tumors) by apheresis and centrifugal elutriation, and pulsed with the peptide [99]. The washed cells were reinfused along with a continuous intravenous infusion of IL-2. Though toxicity was mild and limited to IL-2-related effects, the tumors did not show any response to this immunotherapy but instead showed progression.

Other studies have explored the utility of additional antigens in other immunotherapy approaches. The expression of the tumor-specific antigens MAGE, GAGE, and BAGE were detected in RMS, and initial efforts showed that RMS cells are able to process MAGE-A proteins and present the resulting peptides to T cells [100]. Based on the high expression level of the fetal type acetylcholine receptor (fAChR) by RMS tumors in contrast to the expression of the adult type by mature skeletal muscle, fAChR has been proposed as another candidate target antigen for immunotherapy [101]. As a strategy to bypass the intrinsic immune resistance of RMS cells, T lymphocytes were engineered to express a chimeric receptor consisting of the antigen-binding domain of a human anti-fAChR antibody combined with the cytoplasmic signaling domain of the human T cell receptor chain. In cell culture studies, RMS cells activated and then were killed by these engineered T cells, thus indicating the potential promise for this approach.

## CONCLUSIONS AND FUTURE DIRECTIONS

ARMS is one of a group of sarcomas in which there are recurrent chromosomal translocations. Among the resulting fusion proteins, PAX3-FKHR and PAX7-FKHR provide perhaps the best model for a swapping of transcriptional domains between two transcriptional factors. The function of the PAX3-FKHR fusion transcription factor has been explored by a combination of studies involving directed analysis of target genes and genome-wide expression analysis of tumors. Expression profiling studies indicate that there is a unique signature that distinguishes fusion-positive ARMS from ERMS and fusion-negative ARMS tumors. In the high throughput microarray systems set up to identify downstream targets of PAX3-FKHR in cell culture systems, the genes whose expression is modulated by the fusion protein in these cell culture systems only account for a small fraction of the fusion-positive ARMS signature. Therefore, more sophisticated approaches are needed to identify relevant PAX3-FKHR target genes and to annotate this fusion-positive ARMS signature with the contribution of other factors, such as cell environment and collaborating events. In addition, within the genes identified as PAX3-FKHR downstream targets, the true transcriptional targets and the genes involved in downstream pathways need to be established to understand the dynamics of these expression events. It is also essential that these expression events be correlated with phenotype in appropriate test systems. One endpoint to which several target genes appear to be contributing is metastasis and thus analysis of test systems that monitor aspects of motility and invasion, as well as actual metastasis, are equally important.

An important realization in the study of the genetics of ARMS is that the gene fusion is not working alone but must collaborate with other genetic alterations. Therefore, an area that must complement these PAX3-FKHR studies is the identification and analysis of other recurrent genetic alterations in ARMS. To date, loss of expression of the second *FKHR* allele, amplification of genomic loci, and hypermethylation of several known or putative tumor suppressor loci have been demonstrated in series of ARMS cases. This new information provides numerous opportunities for investigation of the contribution of these genetic changes to ARMS tumorigenesis. Of great importance, a mouse model of ARMS now exists to study the issue of collaborating events *in vivo*. The incorporation of such collaborating events is one of the essential issues in the development of improved animal models. In turn, the relevance of any particular version of the mouse model can be tested by determining if it naturally develops other secondary events, which were not specifically engineered into the model. In addition, as the model is engineered to more closely resemble the human disease in its genetic features, the model will be a

more reliable tool for use in clinical testing for therapeutic and diagnostic purposes.

From a clinical standpoint, the fusion genes have become well accepted as highly sensitive and specific diagnostic markers of ARMS, but still unclear tools for therapeutic intervention. The RT-PCR assays are becoming more sophisticated, and even "surrogate" immunohistochemical markers that are specific for the fusion-positive ARMS and ERMS categories have been proposed and tested. Beyond the value as a diagnostic marker, there may also be prognostic value in distinguishing PAX3-FKHR from PAX7-FKHR fusions, particularly in patients with metastatic disease. There is also still much to be learned about the category of fusion-negative ARMS cases, which is genetically heterogeneous, but does include a large subset of true fusion-negative cases with true ARMS histology. The fusion genes have also proved useful in the development of assays for minimal disseminated disease in the bone marrow. However, to monitor all RMS patients, minimal disease assays have been developed based on myogenic expression markers, such as MYOD1. Finally, the initial attempts to use fusion peptides to stimulate an immune response have shown mixed results. There may in fact be an active effort by the PAX3-FKHR fusion oncoprotein to suppress an immune response so that additional factors may need to be manipulated before a consistent immune response to the tumor cells is achieved in such protocols. However, in addition to these immune approaches, the biology discussed above involving PAX3-FKHR transcriptional pathways and collaborating events along with powerful test systems such as the mouse model system will provide new directions and opportunities for the development of novel therapeutic approaches for ARMS.

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## ABBREVIATIONS

RMS	=	rhabdomyosarcoma
ARMS	=	alveolar rhabdomyosarcoma
ERMS	=	embryonal rhabdomyosarcoma
SAGE	=	serial analysis of gene expression
CGH	=	comparative genomic hybridization
SDF1	=	stromal derived factor-1
HGF/SF	=	hepatocyte growth factor/scatter factor
MMP	=	matrix metalloproteinases
IHC	=	immunohistochemistry
EGFR	=	epidermal growth factor receptor
MYOG	=	myogenin

CTL = cytotoxic T-lymphocyte  
 AChR = fetal type acetylcholine receptor

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