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PAX3-FOXO1 Fusion Gene in Rhabdomyosarcoma

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Abstract

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood and adolescence. The predominant histologic variants of this disease are termed embryonal (eRMS) and alveolar (aRMS), based on their appearance under light microscopy. Of the two, aRMS is associated with an more aggressive disease pattern and a higher mortality, mandating a better understanding of this cancer at the molecular level. The *PAX3-FOXO1* fusion gene, resulting from the stable reciprocal translocation of chromosomes 2 and 13, is a signature genetic change found only in aRMS, and thought to be responsible at least in part for its malignant phenotype. This review will discuss the clinical significance of the *PAX3-FOXO1* fusion gene, the pertinent historical and current models used to study its oncogenic contributions, the transcriptional targets that are thought to mediate these contributions, and the cellular mechanisms impacted by *PAX3-FOXO1* that ultimately lead to aRMS.

1. Introduction

Nonrandom stable reciprocal chromosomal translocations represent some of the most enigmatic mutational events found in human cancer cells. In these chromosomal translocations, previously unrelated genomic sequences are rearranged so that they are apposed, in some cases fusing the 5' coding portion of one gene with the 3' coding portion of another. Many of these translocation events are in-frame, generating de novo fusion genes that produce functional transcripts and, after translation, functional fusion proteins. Because these chromosomal translocations and their resulting fusion genes are found reproducibly in specific cancers, it is thought that each fusion gene is not only characteristic of a specific cancer, but also part of its genesis.

The *PAX3-FOXO1* fusion gene is a signature genetic change of the pediatric cancer alveolar rhabdomyosarcoma (aRMS). *PAX3-FOXO1* results from the stable reciprocal translocation of chromosomes 2 and 13, which fuses in-frame the DNA binding domain of *PAX3* with the transactivation domain of *FOXO1*. Since both *PAX3* and *FOXO1* are transcription factors, this fusion results in the generation of a novel transcription factor with altered transcriptional power, potentially altered transcriptional targets, and certainly altered post-translational regulation. Although not the focus of discussion here, it should be noted that a small proportion of aRMS tumors alternatively express the *PAX7-FOXO1* fusion gene, which results from a stable translocation between chromosomes 1 and 13. As prior reviews have focused on the details of the translocation locus([1;2]), and the aberrant subcellular localization that occurs in response to altered phosphorylation on the *FOXO1* moiety of the *PAX3-FOXO1* fusion protein

([3;4]), this review will focus on the recent investigations delving into the functional contributions of PAX3-FOXO1 to aRMS tumorigenesis.

2. Clinical significance

PAX3-FOXO1 (formerly known as *PAX3-FKHR*) was cloned in 1993, after identification and further characterization of the t(2;13)(q35;q14) translocation, and also proven to generate the predicted fusion protein[5;6]. As with other fusion genes, *PAX3-FOXO1* was found to be specific to aRMS, and not found in any other cancer. It was not immediately clear whether the fusion gene was a bystander of the tumorigenic process of aRMS, or whether it played a role in its development. As molecular biology methods improved, it became possible to assay RMS tumors that were deemed alveolar, based on light microscopy analysis of their morphology, for expression of *PAX3-FOXO1*. In some cases, small round blue cell tumors that were highly undifferentiated and difficult to place in a single diagnostic category by light microscopic or flow cytometric examination could be identified as aRMS by the expression of the *PAX3-FOXO1* fusion gene[7]. And while the testing for *PAX3-FOXO1* is not yet a routine part of the pathological assessment of all pediatric sarcomas, the technology to search for this fusion gene, whether by fluorescence in situ hybridization or PCR, has become more widely available.

Despite the enigmatic nature of PAX3-FOXO1, and the lack of understanding of its contribution to tumorigenesis, it appears that the expression of PAX3-FOXO1 in aRMS tumor samples portends a worse outcome for the patient. Early studies examining the association of expression of aRMS gene fusions with clinical phenotype suggested that those patients with the *PAX7-FOXO1* fusion had a better survival than those expressing *PAX3-FOXO1*[8;9]. A larger study demonstrated a statistically significant correlation between gene fusion status and prognosis, with the *PAX3-FOXO1* subgroup identifying a high-risk subgroup[10], although the convenience sample approach used in this analysis might not adequately represent all-comers[11]. An improved understanding of the molecular mechanism of PAX3-FOXO1-mediated tumorigenesis should provide insight into potential new therapeutic options for patients with aRMS.

3. Model systems to study *PAX3-FOXO1*

Following its cloning, the earliest studies of the oncogenic potential of *PAX3-FOXO1* were performed in avian and murine embryo fibroblasts, which showed that expression of PAX3-FOXO1 could cause transformation as assessed by anchorage-independent growth in soft agar [12;13]. However, when expressed as a single genetic change, it could not cause tumorigenesis in vivo, a finding that has been validated in subsequent studies, suggesting that although it may contribute to oncogenesis, alone PAX3-FOXO1 cannot cause tumorigenesis, and requires additional genetic lesions.

Later studies took advantage of improved DNA transduction technologies, and examined the impact of *PAX3-FOXO1* when it was ectopically expressed in cell lines derived from human embryonal rhabdomyosarcoma (eRMS) tumors, which do not naturally express this fusion gene. These gain-of-function studies suggested that PAX3-FOXO1 contributed to tumorigenesis not by a single mechanism, but through multiple paths, including stimulation of proliferation, support of cellular survival, and/or inhibition of differentiation (discussed below). Loss-of-function studies for PAX3-FOXO1, in the era prior to small interfering RNAs (siRNA), relied on the use of the transcriptional repressor PAX3-KRAB, in which the highly conserved eukaryotic transcriptional repressor KRAB (*K*rüppel-associated *b*ox) was fused to PAX3, thus designed to compete with PAX3-FOXO1 binding and therefore silence PAX3 target genes. As studied in human aRMS Rh30 cells, which naturally express PAX3-FOXO1, this showed for the first time through in vitro and in vivo experiments, that by interfering with PAX3 or PAX3-FOXO1 function, the malignant phenotype of aRMS cells expressing PAX3-

FOXO1 could be repressed[14]. Others used anti-sense technology against PAX3, in a similar fashion attempting to inhibit the function of PAX3-FOXO1[15]. In both these approaches, however, the inhibiting reagent not only influenced PAX3-FOXO1, but also the endogenous PAX3, which complicated interpretation of the experiments. Despite the success of siRNA technology for other mammalian target sequences, knock down of PAX3-FOXO1 by either siRNA oligonucleotides or short hairpin RNA sequences has been difficult. Indeed, only recently has there been described a 19mer siRNA oligonucleotide that knocks down PAX3-FOXO1 expression at the protein level[16] in vitro in aRMS Rh30 cells.

While patient-derived RMS cell lines are relatively plentiful, easy to propagate, and manipulable in culture, they pose some natural obstacles towards understanding the oncogenic contributions of a single genetic mutation such as *PAX3-FOXO1*. This is because aRMS cell lines, like all human cell lines, are derived from individuals with diverse genetic backgrounds, have been cultured in many different environments, may have acquired ex vivo mutations no longer reflecting the original disease, and represent the culmination of genetic events leading to aRMS, therefore prohibiting the study of early events in aRMS tumorigenesis. Thus, some groups have turned to mouse models as an alternative approach to dissect the contribution of PAX3-FOXO1 to aRMS. In support of the prior observations that PAX3-FOXO1 cannot by itself cause aRMS, neither transgenic overexpression of *PAX3-FOXO1* under a *Pax3* promoter/enhancer, nor knock-in into the *Pax3* locus was sufficient to cause tumors in mice; rather, both genetic manipulations resulted in severe anatomic defects presumably because of interference with the normal developmental functions of Pax3[17;18]. However, using a conditional knock-in murine allele of *Pax3-Foxo1* that was activated late in embryogenesis and postnatally in terminally differentiating skeletal muscle, aRMS did occur at a low rate. aRMS tumor incidence was increased in this mouse by crossing with conditional *Tp53* or *Cdkn2a* loss of function, suggesting, again, that PAX3-FOXO1 requires cooperating mutations to form aRMS[19].

As techniques for the genetic manipulation of mice have advanced, providing insight into the impact of genetic mutations on the whole organism level, technology has also improved in the manipulation of primary cells in vitro. Thus, although primary murine cells of skeletal muscle origin have been available for many years, only recently have human primary cells of skeletal muscle origin been easily available for study. This improved availability has translated into a greater understanding of the significant differences between human and murine cancer[20]. The first use of primary human skeletal muscle cells for investigation of rhabdomyosarcoma resulted in the generation of RMS of embryonal histology[21]. Studies are currently underway to use these cells as a starting cell type for the alveolar subtype. However, despite the availability of improved technologies for both murine and primary human cells, the cell of origin of RMS is still not known, and it is not clear whether it is different in eRMS compared to aRMS. Newer technologies enabling fractionation of cell populations through phenotypic assessment by flow cytometric analysis of cell surface proteins may help in this endeavor, as it has in other cancers[22].

4. Transcriptional targets of PAX3-FOXO1

Regardless of the modeling strategy used to study the role of PAX3-FOXO1 in the development of aRMS, it is generally agreed that PAX3-FOXO1 exerts its tumorigenic effect at least in part through altered transcription of target genes. Early studies of its transcriptional ability, as assessed by reporter assays, showed that the fusion of the FOXO1 transactivation domain to PAX3 increased its transcriptional power[3], possibly through constitutive nuclear localization [4], loss of a cis-acting transcriptional repression domain[23], and/or through overexpression of the entire fusion gene through locus-specific mechanisms[24].

Given that PAX3-FOXO1 is thought to alter transcription of PAX3-related targets, much effort has gone in to identifying genes that are upregulated by PAX3-FOXO1. The majority of these studies have been through gene expression profiling studies through cDNA or oligonucleotide microarrays, or other genome wide screens including cyclic amplification and selection of targets (CASTing) or serial analysis of gene expression (SAGE) methods (Table I). Some studies ectopically express PAX3-FOXO1 (in parallel with PAX3) in human or murine cell lines in order to identify PAX3-FOXO1-specific gene changes[25–27], while others have studied actual human-derived RMS tissues[28–31].

Although there are too many potential targets to be listed individually, they cluster as genes that take part in myogenic differentiation, myogenic signaling, muscle contraction, neural-related genes, and transcription factors involved in mesodermal development[32]. SAGE analysis showed that genes expressed in RMS are more parallel to fetal muscle than normal muscle[29], again suggesting that in RMS, embryonic transcriptional programs are illegitimately reactivated. Several groups have also tried to use gene expression profiling as a way to define an aRMS gene expression signature to diagnose those aRMS that do not express *PAX3-FOXO1*[28;30;31;33], and perhaps provide some prognostic information.

Although these genome-wide screens are powerful, they pose several limitations, including that while new potential targets of PAX3-FOXO1 are identified, some of the screens are confounded by the inclusion of aRMS tumors that are *PAX7-FOXO1* positive, or fusion status unknown. In addition, once identified, each target also needs to be validated at minimum by RT-PCR, and in the majority of targets, its upregulation is detected in only a single study. Also, some bone fide PAX3-FOXO1 transcriptional targets that are known to exert profound effects on RMS tumorigenesis, such as the MET proto-oncogene receptor tyrosine kinase, do not emerge from these screens as internal controls. Having said that, there are some PAX3-FOXO1 targets that seem to have emerged through several independent studies regardless of the microarray platform[33]. Thus, the candidates emerging from these genome-wide screens of PAX3-FOXO1-mediated transcriptional upregulation each need to be individually studied for their biological role in aRMS and their suitability as therapeutic targets.

5. Contribution towards tumorigenesis

Through modulation of its targets, the *PAX3-FOXO1* fusion gene appears to modify key transcriptional programs in a susceptible cell, and turn on cellular pathways that endow the cell with characteristics that can contribute to the oncogenic process. While the original studies in avian and murine embryo fibroblasts showed that PAX3-FOXO1 could be transforming, they did not shed light on mechanism. As originally proposed[34], in order to form cancer, human cells need to acquire discrete characteristics that enable unbridled proliferation, which can be accomplished through self-sufficiency in growth signals, or insensitivity to anti-growth signals (such as through inhibition of differentiation signals). In parallel, these proliferating cells need to be able to evade apoptotic programs. Additionally, cancer cells need to acquire immortality through telomere stabilization, either through reactivation of telomerase or alternate mechanisms. Next, to grow beyond a size that is limited by oxygen diffusion, cells need to acquire the capacity for sustained angiogenesis. And finally, to grow beyond the primary tumor, cells need to acquire an ability to invade local tissue and to metastasize to distant sites. PAX3-FOXO1 seems to promote several of these characteristics and therefore likely contributes to aRMS tumorigenesis in several ways.

5.1. Stimulation of cell proliferation

Self-sufficiency in growth signals is most often acquired by activation of autocrine growth factor-receptor loops that promote proliferation and enable independence from environmental stimuli. In support of this, stable ectopic expression of PAX3-FOXO1 in RD and HX170C

eRMS cells resulted in an increased proliferation rate in vitro, and enabled growth in low-serum conditions[35], while siRNA knockdown of PAX3-FOXO1 in Rh30 aRMS cells decreased proliferation independent of cell survival[16]. Studies of human aRMS tumor specimens expressing PAX3-FOXO1 show immunohistochemical evidence for increased proliferative rate[36]. Of the possible PAX3-FOXO1 targets that could stimulate proliferation, the receptor tyrosine kinase MET is perhaps the most attractive to study. MET is a bona fide transcriptional target of PAX3/PAX3-FOXO1[37–39], and is known in myoblasts to be part of a natural autocrine loop[40] and in other human cancers to play roles in proliferation, cell survival and invasion. In standard soft agar assays of primary murine fibroblasts or C2C12 myoblasts, ectopic expression of PAX3-FOXO1 caused colony formation in the presence of 10%, but not 2%, FBS. However, addition of the MET ligand HGF could rescue growth, suggesting that, at least in part, MET receptor occupancy could drive proliferation. Because an inducible knock-down of MET in these same cells also caused a significant increase in apoptosis[41], it is not clear to what extent MET impacts overall proliferation by enabling self-sufficiency in growth signals versus promoting cell survival (see next section).

Impact on proliferative rate has also been measured by effect on cell cycle, as shown by studies in which ectopic expression of PAX3-FOXO1 in C2C12 murine myoblasts induced cell cycling [42], and in fibroblasts accelerated the transition from G₀/G₁ to S phase by increasing the degradation of the cyclin-dependent kinase inhibitor 1B (CDKN1B, formerly known as P27KIP1)[43]. Other CDK inhibitors may be affected by PAX3-FOXO1 expression, including the cyclin-dependent kinase inhibitor 1C (CDKN1C, formerly known as P57KIP2), which was decreased in primary myoblasts isolated from transgenic mice expressing *PAX3-FOXO1* driven by the *PAX3* promoter. The decrease in murine myoblast *Cdkn1c* was found to be due PAX3-FOXO1 (but not wild type PAX3 or FOXO1)-mediated destabilization and proteasomal degradation of the intermediary transcription factor Egr1, which normally stimulates *Cdkn1c* transcription[44]. Intriguingly, the authors show that this effect of PAX3-FOXO1 on Egr1 is not through transcription, but through protein-protein interactions, and hypothesize that in this case PAX3-FOXO1 may act as a misfolded protein that can lead associated proteins to the proteasome. Finally, although not shown to directly impact its expression, PAX3-FOXO1 was found to cooperate with the loss of the cyclin-dependent kinase inhibitor 2A (CDKN2A, formerly known as p16INK4A)[45], and enable primary human myoblasts to bypass the tissue-culture induced senescence checkpoint. These findings together suggest that PAX3-FOXO1 is able to most effectively stimulate inappropriate proliferation when key inhibitors of the cell cycle are silenced, providing for the organism a proliferating pool of cells that are not yet transformed, but primed for further genetic insults that ultimately lead to aRMS.

5.2. Promotion of cell survival

The activation of apoptosis is a powerful means to eradicate cells that acquire genetic instability and potentially oncogenic characteristics, thereby protecting the whole organism from the threat of cancer. As such, the ability to ignore apoptotic signals and continue proliferating despite DNA damage portends an advantage for malignant cells[46]. As assessed mostly by loss of function studies, there is some evidence that PAX3-FOXO1 contributes to cellular survival in aRMS. In vitro in Rh30 aRMS cells, downregulation of PAX3-FOXO1 by anti-sense oligonucleotides against *PAX3*, or by the PAX3-KRAB transcriptional repressor, caused apoptosis as assessed by sub-G₁ DNA content[15], or TUNEL and Annexin V staining, respectively[47]. In vivo, the PAX3-KRAB repressor caused tumor regression, at least in part due to increased apoptosis[47]. Interestingly, when expressed transiently in Rh30 cells, the recently described siRNA against PAX3-FOXO1 did not cause apoptosis[16], but only an inhibition of cellular proliferation, suggesting that downregulation of PAX3-FOXO1 independent from PAX3, might have discreet consequences. In actual aRMS human tumor specimens, PAX3-FOXO1 expression was surprisingly associated not with decreased, but with

increased apoptosis, as assessed by TUNEL staining[36]. This apparent contradiction may reflect the reality that tumor formation is the end result of a combination of tumorigenic mechanisms.

Regarding mechanism of PAX3-FOXO1-mediated cell survival, at least two, and probably more, downstream targets of PAX3-FOXO1 play roles promoting cell survival. The in vitro work in Rh30 cells suggests a role for the anti-apoptotic protein BCL2L1 (formerly known as BCL-X_L), as expression of the inducible PAX3-KRAB transcriptional repressor caused decreased BCL2L1 expression at the transcript and protein level[47]. Additional studies showed that BCL2L1 is transcriptionally modulated by PAX3 and PAX3-FOXO1, and ectopic expression of BCL2L1 in cells expressing the anti-sense against *PAX3* can rescue them from apoptosis[48]. Conditional silencing of MET in SJ-Rh30 cells results in apoptosis as assessed by Annexin V staining and appearance of caspases 3 (CASP3) and 7 (CASP7), suggesting that aRMS is “addicted” to MET expression[39]. Thus, there is evidence that PAX3-FOXO1 and its downstream targets contribute to aRMS by suppressing apoptosis and promoting survival. However, since virtually all of these studies were performed in a single cell line (Rh30 or its derivatives), and some of the studies used techniques that impacted both PAX3 and PAX3-FOXO1, further work in additional cell systems will be needed to validate this role for PAX3-FOXO1. In addition, the interplay between PAX3-FOXO1-mediated suppression of apoptosis and other tumorigenic mechanisms will need to be comprehensively examined.

5.3. Suppression of terminal differentiation

As opposed to apoptosis, which eradicates genetically unstable cells from the organism, the process of cellular differentiation reprograms damaged cells so that they are still able to take part in organ function, but are removed from the proliferative pool, thus maintaining but protecting the organism. As such, an ability to ignore or suppress differentiation signals in damaged cells can contribute to tumorigenesis[49]. The earliest hints that PAX3-FOXO1 might interfere with differentiation came from studies in C2C12 murine myoblasts, and 10T1/2 fibroblasts transfected with *Myod1*, which showed that PAX3-FOXO1 inhibits low serum-induced myogenic differentiation [42;50]. This property was shared by wild type PAX3, suggesting that the PAX3 moiety is critical for this phenotype[50]. Later studies, using myoblasts isolated from transgenic mice expressing PAX3-FOXO1 under the control of a *PAX3* promoter, were found to be similarly refractory to differentiation in vitro, at least in part due to an indirect loss of function of CDKN1C[44], a cell-cycle regulator known to effect terminal differentiation[51]. However, in this case the ability to inhibit differentiation was not shared by wild type PAX3. In contrast to murine myoblasts, ectopic expression of PAX3-FOXO1 in human myoblasts does not render them refractory to differentiation to myotubes [45], suggesting that, while PAX3-FOXO1 has some influence over the ability to terminally differentiate, the cell type and context in which this is studied must be taken into consideration.

5.4. Other contributions of PAX3-FOXO1

Regarding the contribution of PAX3-FOXO1 to other characteristics of cancer, although there is no data to suggest that it modulates telomere stability, there is some data to suggest that PAX3-FOXO1 can influence angiogenesis. The earliest evidence, based on studies in which the inducible PAX3-KRAB transcriptional repressor was expressed in Rh30 aRMS cells, which were then injected as subcutaneous xenografts in immunocompromised mice, is indirect and qualitatively showed fewer blood vessels in those xenografts expressing the repressor[47]. Regarding mechanism, this is still very early in study, but possibilities include the upregulation of the VEGF-VEGFR axis, as the CASTing strategy mentioned above identified FLT1 (formerly known as VEGFR1) as a potential target of PAX3-FOXO1[26], and several human aRMS cell lines expressing PAX3-FOXO1 (although not all of those studied) showed

upregulation of FLT1[52;53]. Further studies are needed to definitively prove a role for, and provide details into the mechanism of PAX3-FOXO1 in angiogenesis.

Among RMS, the alveolar (aRMS) subtype has the highest propensity to metastasize, with the bone marrow as a common site for distant spread[54]. There is some evidence that PAX3-FOXO1 can promote a metastatic phenotype, since when expressed ectopically in the eRMS cell lines RD or HX170C, the cells showed a qualitative increase in local invasion and/or increased matrix metalloproteinase MMP-2 activity[35;53]. Regarding mechanism, at least one route may be via upregulation of the chemokine receptor CXCR4, which, in concert with its ligand CXCL12 (formerly known as SDF-1), modulates homing to cellular niches such as the bone marrow[55]. CXCR4 is highly expressed in aRMS but not eRMS cell lines, and stable ectopic expression of PAX3-FOXO1 in eRMS RD cells increased CXCR4 levels at both mRNA and protein levels and also increased their responsiveness to CXCL12[56]. Further study of the human aRMS cell lines expressing PAX3-FOXO1, Rh28 and Rh30, showed that they respond to CXCL12-mediated phenotypes, including cellular locomotion, actin cytoskeleton rearrangement, migration through Transwell membranes, adhesion, and matrix metalloproteinase MMP-2 secretion[56]. However, examination of human RMS tumor specimens demonstrated no difference in the level of *CXCR4* mRNA or protein expression in eRMS versus aRMS groups[57], so that the CXCR4-CXCL12 axis cannot be presumed to be the only signaling pathway responsible for the increased metastatic behavior of aRMS. In support of this, inducible knock-down of the MET receptor, well known to participate in invasion and metastasis[58], in SJ-Rh30 and SJ-Rh4 aRMS cell lines (both express PAX3-FOXO1) inhibited migration as assessed by Transwell assays, while treatment of RC2 aRMS cell lines with recombinant HGF stimulated Transwell migration[59]. Since at this time, as a transcription factor PAX3-FOXO1 cannot be pharmacologically inhibited, identifying “druggable” downstream pathways of PAX3-FOXO1-mediated metastasis in aRMS will be important to improve the survival of patients with this disease.

6. Conclusions

The *PAX3-FOXO1* fusion gene results from the stable reciprocal translocation of chromosomes 2 and 13, and is found only in the cancer alveolar rhabdomyosarcoma. Expression of this fusion gene, especially in the metastatic setting, portends a very poor outcome for patients with this disease. A variety of model systems have been used to dissect the oncogenic mechanisms of *PAX3-FOXO1*, with the common conclusion that, alone, it is not capable of causing transformation, but must cooperate with other genetic changes to form rhabdomyosarcoma. PAX3-FOXO1 works at least in part through transcriptional activation of targets that turn on aberrant myogenic programs, but many of these targets must still be validated. Regarding contribution to oncogenic characteristics, there is evidence that PAX3-FOXO1 exerts pleiotropic effects, including driving proliferation, promoting cell survival, suppressing terminal differentiation, promoting invasion and perhaps supporting angiogenesis. Further work is needed to precisely define the molecular mechanisms underlying these contributions, and their value as targets of therapeutic intervention.

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Table 1

Gene profiling studies to identify putative transcriptional targets associated with PAX3-FOXO1 in alveolar rhabdomyosarcoma

No.	Platform	Approach	Targets suggested	Reference
1	cDNA microarray	PAX3 and PAX3-FOXO1 ectopically expressed in murine NIH 3T3 cells	<i>Myod1, Myog, Six1, Slug, Igf2, Igfbp5</i>	[25]
2	cDNA microarray with artificial neural networks	Analyzing primary aRMS tumors and cell lines derived from aRMS tissue, including some known to express <i>PAX3-FOXO1</i>	<i>FGFR4, IGF2, MYL4</i>	[28]
3	CASTing strategy	<i>PAX3</i> and <i>PAX3-FOXO1</i> ectopically expressed in RD eRMS cell line	<i>Itm2A, Fath, FLT1, TGFA, BVES, EN2</i>	[26]
4	Oligonucleotide microarray	<i>PAX3</i> and <i>PAX3-FOXO1</i> ectopically expressed in U2OS osteosarcoma and RD eRMS cell lines	<i>CNR1, EPHA2, EPHA4, BMP4</i>	[27]
5	SAGE analysis and cDNA microarray	Comparison of 4 RMS tumors (1 aRMS with <i>PAX3-FOXO1</i>) to normal skeletal muscle	Decrease in genes coding for cell adhesion proteins, increase in genes coding for protein biosynthesis	[29]
6	Oligonucleotide microarray	Analyzing primary RMS tumors and <i>PAX-FOXO1</i> ectopically expressed in RD eRMS cell line	33 “metagene” score for <i>PAX-FOXO1</i> , including genes involved in repressing myogenic differentiation, activating cell survival	[30]
7	cDNA microarray	Analyzing 10 primary aRMS tumors (5 <i>PAX3-FOXO1</i> fusion positive, 5 <i>PAX3-FOXO1</i> fusion negative)	4 discriminating genes that distinguish <i>PAX3-FOXO1</i> negative from <i>PAX3-FOXO1</i> positive tumors (<i>RAC1, CFL1, CCND1, IGFBP2</i>)	[31]
8	Oligonucleotide microarray	Comparison of <i>PAX-FOXO1</i> positive aRMS tumors to <i>PAX-FOXO1</i> negative eRMS tumors	<i>CNR1, PIPOX, DKFZp762M127, DCX, FOXF1</i>	[33]