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New insights into signalling-pathway alterations in rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children and young adults. Several recent studies have shed new light on the alterations in signalling pathways and the downstream effects of these pathway alterations in RMS. Many of these effects converge on the fibroblast growth factor and insulin-like growth-factor pathways. These new findings improve the current understanding of RMS, thus offering novel potential therapeutic targets and strategies that may improve the outcome for patients with RMS.

RHABDOMYOSARCOMA

Rhabdomyosarcoma (RMS) is a highly malignant cancer that is relatively rare, but is the most common form of soft-tissue tumours in children and young adults. The annual incidence is ~350 cases in the USA. Rhabdomyosarcoma can arise as a consequence of myogenic precursors failing to differentiate into normal muscle, but it is also possible that the tumour cell of origin may be a non-myogenic cell (reviewed in Keller and Guttridge (2013)). Rhabdomyosarcoma is characterised by two major subtypes, embryonal RMS (ERMS) and alveolar RMS (ARMS). Embryonal RMS is the most common form of the disease and has a more favourable prognosis than ARMS. A wide range of genetic aberrations have been described in ERMS including a loss of heterozygosity at the 11p15 locus and chromosome gains including chr. 2, 8, 12, and 13 (reviewed in Wang (2012)). Alveolar RMS is the most aggressive form of RMS with a poorer prognosis and a higher rate of metastasis. Alveolar RMS is characterised by t(2;13)(q35;q14) or t(1;13)(q36;q14) translocations, which fuse the 5′ portion of the paired box proteins 3 or 7 (PAX3 or PAX7), including an intact DNA-binding domain, to the transactivation domain of a forkhead transcription factor (FOXO1), creating novel PAX3-FOXO1 (t(2;13)(q35;q14)) or PAX7-FOXO1 (t(1;13)(q36;q14)) fusion proteins (reviewed in Wang (2012)). The presence of the fusion protein in ARMS has led to the designation of fusion-positive *vs* fusion-negative RMS. Many studies have highlighted the importance of the PAX-FOXO1 fusion in ARMS biology (reviewed in Marshall and Grosveld (2012)). This is further supported by a recent study which showed that PAX3-FOXO1 is dynamically expressed throughout the cell cycle and that the higher expression of PAX3-FOXO1 during G2 is permissive for G2/M checkpoint adaptation, which allows a cell to divide and survive following a sustained checkpoint arrest despite the presence of unrepairable DNA breaks such as would be induced following radiation or DNA break inducing chemotherapy, thus suggesting that PAX3-FOXO1 may enhance the survival of tumour cells in response to chemotherapy and may allow disease progression and relapse in ARMS (Kikuchi *et al*, 2014).

In addition to the PAX-FOXO1 fusions found in ARMS, both ERMS and ARMS cells express PAX3/7 together with the myogenic regulatory factors (MRFs) that drive myogenesis. PAX3 and PAX7 are normally expressed in muscle progenitor cells. During normal myogenesis, the PAX genes are downregulated concomitant with an upregulation of the MRFs, a group of four highly related bHLH transcription factors composed of Myf5, MyoD, Myf6, and myogenin that are required for myogenesis (reviewed in Kablar and Rudnicki (2000)). MyoD and myogenin are expressed in almost all RMS tumours including both major histological subtypes and are thus used as diagnostic markers for RMS. However, the MRFs are unable to promote differentiation and the multiple mechanisms responsible for the impaired differentiation of RMS cells have been recently reviewed (Keller and Guttridge, 2013). We have also recently identified a downregulation of MEF2D, a member of the myocyte enhancer factor family, which function synergistically with the MRFs in skeletal muscle differentiation, in RMS (Zhang *et al*, 2013).

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SIGNALLING-PATHWAY ALTERATIONS AND CELL-CYCLE CONTROL

A hallmark of cancer cells is a self-sufficiency of growth signals. Rhabdomyosarcoma cells display many defects in cell-cycle checkpoints and growth-factor signalling pathways that lead to accelerated proliferation. Several deregulated signalling pathways enhance cell growth by modulating cell-cycle regulatory factors in RMS. The most frequently affected signalling pathways include the insulin-like growth factor (IGF), fibroblast growth factor (FGF), hepatocyte growth factor, and platelet-derived growth factor (reviewed in Wang, 2012). In ARMS, PAX-FOXO1 activates these pathways by transcriptional activation of receptor genes including *IGFR1*, *FGFR4*, *MET* (c-Met), and *PDGFRA*.

The impact of signalling-pathway alterations in RMS was recently reinforced in a genome-wide study, which characterised the profile of somatic alterations in 147 RMS tumour samples. This study showed that the overall burden of somatic mutation is low, especially in fusion-positive tumours (Shern *et al*, 2014). The authors also found that alteration of the receptor tyrosine kinase/RAS/phosphoinositide 3-kinase (PI3K) axis affected 93% of RMS cases and that alterations in this axis appeared to hinge on the FGF and IGF receptor pathways. These data strongly suggest that the receptor tyrosine kinase/RAS/PI3K axis may represent a novel therapeutic target and that continued biological investigation and pharmacological targeting of this axis may expand available therapeutic options. The study also revealed two additional novel recurrent mutations in F-Box and WD repeat domain containing 7 (*FBXW7*) and BCL6 co-repressor (*BCOR*) genes, in addition to previously identified mutations in the *RAS*, *FGFR4*, *PIK3CA*, and *CTNNB1* genes. *PIK3CA* encodes a catalytic p110 subunit of the PI3K and *CTNNB1* encodes β-catenin.

In another recent large scale study, a chemical screen for novel drugs which suppress cell growth and self-renewal was performed in ERMS cells. Six major classes of drugs were identified that included inhibitors of glycogen synthase kinase 3 (GSK3), Raf/MEK protein kinase, PI3-kinase/AKT protein kinase, Hedgehog pathway, histone deacetylases (HDACs), and also included DNA damaging agents (Chen *et al*, 2014). Glycogen synthase kinase 3 inhibitors were found to function through the activation of the WNT/β-catenin pathway as both treatment with recombinant WNT3A and expression of a constitutively active form of β-catenin induced differentiation of ERMS cells. Intriguingly, GSK3 has also recently been shown to directly phosphorylate myogenin and repress its activity (Dionyssiou *et al*, 2014). In this work, the authors also found that expression of the PAX3-FOXO1 fusion found in ARMS enhances the activity of GSK3. Thus, the presence of enhanced GSK3 activity in ARMS acts to repress the activity of myogenin, which is required for terminal differentiation.

Fibroblast growth factors are highly overexpressed in RMS and function to drive proliferation. Fibroblast growth factors play a fundamental role in embryonic development including a key role in normal skeletal muscle development. Fibroblast growth factors and their receptors (FGFRs) are essential regulators of cell proliferation, survival, migration, and differentiation. Fibroblast growth factor encompasses a large family of 18 ligands that bind to four homologous high-affinity FGFRs (FGFR1–FGFR4). Rhabdomyosarcoma cells express FGFs and the receptor tyrosine kinase FGFR4 is highly expressed in human RMS tumours and correlates with advanced stage, poor differentiation, and reduced survival of cancer patients (reviewed in Wesche *et al* (2011)). *FGFR4* is a transcriptional target of PAX3 and the PAX3-FOXO1 fusion protein found in ARMS. Fibroblast growth factor signalling through their receptors activates multiple key downstream pathways including: RAS–RAF–MAPK, PI3K–AKT, and phospholipase Cγ (PLCγ). Since FGF signalling is known to influence a multitude of cellular functions including proliferation and survival, aberrantly high FGF signalling may mediate the response to RMS therapy. Recent work has shown that FGF signalling can rescue a subset of ARMS cells from apoptosis induced by compounds targeting the IGF1-R–PI3K–mTOR pathway (Wachtel *et al*, 2014). The different behaviours of the ARMS subsets in this study were found to be based on differences both in the pro-apoptotic machinery and FGFR4 activated signalling. Importantly, this work not only revealed the presence of tumour heterogeneity in the response to potential chemotherapeutic approaches due to alterations in signalling pathways and the cellular machinery, but also implicated FGF signalling in the escape from apoptosis induced by therapeutic agents. The work suggests that inhibition of FGF signalling may offer a new approach to enhance the efficiency of RMS treatments.

Insulin-like growth factor is required for RMS cell growth and IGF2 is expressed in an autocrine manner by the tumour cells (reviewed in Rikhof *et al* (2009)). Insulin-like growth factor is necessary for FGF-induced proliferation in other cells (Arsenijevic *et al*, 2001), suggesting that the signalling pathways may be interconnected in RMS cells as well. In normal skeletal muscle, IGF has both a pro-proliferative and pro-differentiation effect on cells (Mourkioti and Rosenthal, 2005), which suggests that the pro-differentiation function of IGF is blocked in RMS cells. The precise role of IGF in RMS cells is unclear, but IGF clearly promotes the proliferation of RMS cells and blocks to IGF signaling suppress the growth of RMS cells *in vivo*. The IGF2 locus shows a loss of imprinting in both ERMS and ARMS tumours and expression of PAX3-FOXO1 can induce the upregulation of IGF2, thus enhancing the activation of IGF signalling pathway in ARMS (reviewed in Marshall and Grosveld (2012)). The expression of the IGF receptor, IGF-1R, is indicated in the pathogenesis of RMS as well as several other types of sarcoma (reviewed in Maki (2010)). Intriguingly, IGF-1R localises to both the cell surface and nucleus of ARMS cells and cells with high nuclear IGF-1R expression established tumours more efficiently *in vivo* (Aslam *et al*, 2013). Other studies have shown that nuclear IGF-1R localises to the nucleus of humor tumour cells where it associates with the chromatin, suggesting a biological function in transcription regulation (Aleksic *et al*, 2010). Drugs that inhibit IGF signalling are in clinical trials for RMS, but mouse models suggest that drug resistance is easily achieved (Abraham *et al*, 2011). A recent clinical trial with antibodies against IGF-1R in sarcoma patients showed that some RMS patients initially responded to therapy, but the patients usually progressed rapidly despite the therapy (Pappo *et al*, 2014). Clearly, targeting the IGF pathway is an important strategy in treating RMS, but more needs to be understood about the regulation and function of IGF and its receptor, IGF-1R, in RMS cells in order to develop effective therapies.

Many of the cell signalling pathways such as FGF and IGF converge on cell-cycle regulators such as the cell-cycle regulator cyclin-dependent kinase (Cdk) inhibitor $p21^{CIP1/WAF1}$ (*CDKN1A*), hence referred to as p21, and the cell-cycle regulator $p14^{ARF}$ (human) or $p19^{ARF}$ (murine; *CDKN2A*). An understanding of the signalling pathways and factors that regulate p21 and $p14/19^{ARF}$ is essential for understanding RMS progression and for designing potential strategies to inhibit tumour growth. In normal muscle cells, p21 is induced early in myoblast differentiation and functions to block cell-cycle progression (reviewed in Wei and Paterson (2001)). p21 is regulated by MyoD and myogenin in normal muscle cells and the inactivation of these factors in RMS cells contributes to the silencing of p21 in RMS cells (Otten *et al*, 1997). The MEK/ERK signalling pathway contributes to the activation of p21 expression in RMS cells and is correlated with growth arrest and differentiation of RD cells (Ciccarelli *et al*, 2005). $p14/19^{ARF}$ is a well known tumour suppressor and copy number deletions in *CDKN2A* were present in 2% of the RMS tumours characterised in the recent genome-wide study described above (Shern *et al*, 2014).

A summary of the known signalling-pathway alterations and effects of the PAX3-FOXO1 fusion present in ARMS is shown in Figure 1.

ONCOGENIC ROLE AND POTENTIAL REGULATION OF TBX2 IN RMS

Understanding the downstream effects of signalling-pathway alterations is central to understanding the pathology of RMS and improving therapeutic strategies for patients. We have recently found that a T-box gene family member, TBX2, is highly overexpressed in both ERMS and ARMS cells (Zhu *et al*, 2014). The regulation of TBX2 is uncharacterised in RMS cells, but is likely to link TBX2 expression to the known deregulation of signalling pathways in RMS. In melanoma cells, TBX2 is regulated by PAX3 (Liu *et al*, 2013) and PI3K signalling is required for PAX3 expression (Bonvin *et al*, 2012), which strongly suggests that the expression of TBX2 may be a downstream effect of PI3K signalling in RMS cells. In embryonic lung fibroblasts, TBX2 has also been shown to be regulated by the PLCγ-activated protein kinase C (PKC; reviewed in Abrahams *et al*, 2010), which represents a large multigene family of serine/threonine kinases. One isoform, PKCι, is upregulated in RMS and contributes to tumour growth (Kikuchi *et al*, 2012). Taken together, the data strongly support further characterisation of the regulation of TBX2 in RMS.

The T-box gene family of transcription factors play a critical role in embryonic development and contains the well known developmental regulator *brachyury* along with 18 different T-box genes with diverse regulatory functions in development and disease. TBX2 and TBX3 function as transcriptional repressors and both have been shown to inhibit myogenesis (Carlson *et al*, 2002; Zhu *et al*, 2014). Abnormal expression of TBX2 has been reported in several cancers including breast, pancreas, and melanoma, where it has been shown to drive proliferation (reviewed in Abrahams *et al* (2010)). As has been previously shown in other cell types, TBX2 was found to induce a downregulation of p14/19^{ARF} and function as a direct repressor of p21 in RMS (Zhu *et al*, 2014). In normal cells, p21 is the effector

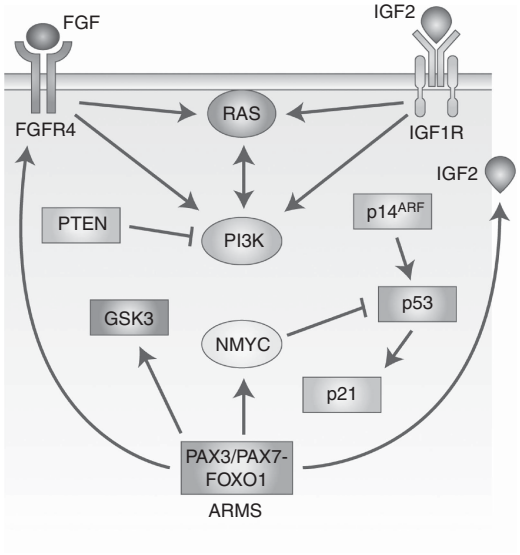

Figure 1. Model of the signalling pathways implicated in fusion-positive ARMS progression. The fusion protein PAX/FOXO1 functions to directly activate IGF and FGF signalling pathways as well as the expression of additional oncogenes such as *NMYC* and *GSK3* to drive tumour cell proliferation and tumourigenesis. Many additional components of each pathway were omitted for clarity.

of p53-mediated growth arrest and expression of p14/19^{ARF} results in the binding of MDM2, stabilisation and activation of p53 and induction of cell-cycle arrest or senescence. Inhibition of p14/19^{ARF} can result in a bypass of senescence and promotion of cell proliferation. Thus, the suppression of both p21 and p14/19^{ARF} by TBX2 may provide a powerful and cooperative anti-senescence signal to cancer cells. In ARMS cells, depletion of TBX2 or expression of a dominant-negative TBX2 inhibited proliferation, migration, and anchorage-independent growth *in vitro* and tumourigenic growth *in vivo* (Zhu *et al*, 2014). Intriguingly, TBX2 has also been found to drive proliferation in breast cancer cells through repression of cystatin 6, a cysteine protease inhibitor that acts as a tumour suppressor (D'Costa *et al*, 2014).

TBX2 is also associated with two well-known cancer modifiers, the retinoblastoma tumour suppressor protein (pRB) and the Myc family. For pRB, TBX2 interacts with hypophosphorylated pRB and the interaction contributes to repression of p21 and affects the selectivity of TBX2 target genes (Vance *et al*, 2010). In skeletal muscle, pRB has a dual function in both mediating the cell survival of myogenic precursor cells and in the permanent withdrawal from the cell cycle (Zacksenhaus *et al*, 1996). Phosphorylation of pRB inactivates the protein. Typically, the inactivation of *RB1* contributes to the malignant progression of several major cancers. However, the role of *RB1* loss in RMS is controversial, with studies finding that *RB1* is largely unaffected in both ERMS and ARMS or that *RB1* is frequently lost, especially in ERMS (reviewed in Keller and Guttridge (2013)). Recent work has shown that the loss of *RB1* does not appear to initiate RMS, but is a disease modifier. The loss of *RB1* in an ERMS or an ARMS model modifies the tumour phenotype to mimic an undifferentiated pleomorphic sarcoma (Rubin *et al*, 2011) or a pleomorphic RMS identity (Kikuchi *et al*, 2013), respectively. The interplay between pRB and TBX2 in RMS or normal skeletal muscle is unknown. TBX2 collaborates with C-myc to immortalise cells and, in combination with additional oncogenes, can transform cells (Taghavi *et al*, 2008). Both C-myc and N-myc are highly expressed in RMS, with the highest expression observed in ARMS where N-myc is a transcriptional target of the PAX3-FOXO1 fusion (reviewed in Marshall and Grosveld (2012)).

Clearly, understanding the function and regulation of TBX2 in RMS will be important in interpreting the many known modifications in RMS including enhanced FGF signalling that may be an activator of TBX2, and the roles of pRB and the Myc family, which may share functional interactions with TBX2. TBX2 mediates repression by binding the HDAC1, which serves to target HDAC1 to promoters (Vance *et al*, 2005) and it was found that TBX2 recruits HDAC1 to target promoters, including p21, in RMS cells as well (Zhu *et al*, 2014). A summary of the known functions of TBX2 in RMS is shown in Figure 2. The HDAC-dependent mechanism of action of TBX2 is highly relevant in understanding the molecular effects of HDAC inhibitors (HDACi), which are known to inhibit RMS cell growth. Histone deacetylase inhibitors relieve the repression mediated by TBX2 and reactivate p21 and p14/19^{ARF} (Zhu *et al*, 2014), explaining at least one mechanism by which HDACi block RMS cell growth.

The identification of HDACi in the recent chemical screen for ERMS (Chen *et al*, 2014) and the novel identification of mutations in BCOR, a transcriptional repressor, which interacts with both class I and class II HDACs (Shern *et al*, 2014), highlight the importance of understanding how epigenetic modifications control the growth and oncogenic properties of RMS. The polycomb repressor complex (PRC2), which catalyses the methylation of lysine 27 of histone H3, a hallmark of gene repression, has been shown to be overexpressed in ARMS (Ciarapica *et al*, 2014). PRC2 expression in ARMS facilitates escape from programmed cell death, suggesting that PRC2 is a key factor in the proliferation and survival of ARMS cells (Ciarapica *et al*, 2014). Moreover, JARID2,

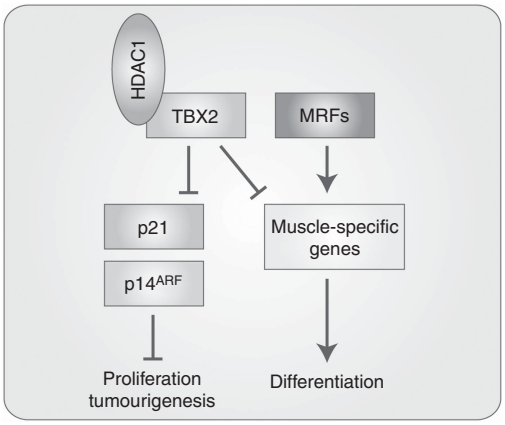

Figure 2. Model of the function of TBX2 in RMS. TBX2 interacts with the myogenic regulatory factors (MRFs) and represses MRF transcriptional activities through recruitment of the histone deacetylase, HDAC1, to target gene promoters to inhibit muscle-specific gene expression and to repress myoblast differentiation. TBX2 also represses cell-cycle regulatory factors, such as p21 and $p14^{ARF}$, to drive tumour cell proliferation and tumourigenesis.

a component of the PRC2 complex, which is important in recruiting PRC2 to target genes, has been found to be a direct target of PAX3-FOXO1 (Walters *et al*, 2014). Connecting signalling pathways and epigenetic modifications that regulate cell proliferation, survival, and myogenic differentiation will be key in fully understanding the cell biology of RMS, which will provide the foundation for developing novel therapeutic strategies for RMS treatment.

CONCLUSIONS

Recent studies have shed exciting new light on the signalling-pathway alterations that drive RMS cell growth and this insight offers new potential therapeutic targets. Taken together, these studies suggest novel connections on known alterations in RMS such as FGF signalling and p21 suppression and provide a new molecular understanding of how drugs can target these changes. The identification of new protein factors, which mediate cell growth in RMS, such as TBX2, may connect the deregulation of the PI3K pathway with histone deacetylation of the genes encoding pivotal cell-cycle regulators such as p21. These new findings suggest novel mechanisms for several drugs either approved or considered for use in RMS patients such as HDACi, which may work in part by activating p21; PI3K inhibitors, which may function in part by inhibiting TBX2; and GSK inhibitors, which may work by both repressing the canonical WNT/β-catenin pathway and reactivating myogenin. Continued study of the rapidly unfolding mechanisms of RMS cell growth will provide the necessary basic understanding to design powerful new combination therapies, which target the signalling pathway, the downstream effector molecules and/or the epigenetic modifications induced by these signals to improve therapeutic options for RMS patients.

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