EGFR AND AKT SIGNALING IN RHABDOMYOSARCOMA PATHOGENESIS

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EGFR AND AKT SIGNALING IN RHABDOMYOSARCOMA PATHOGENESIS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2018

Dedications

Life can be filled with many challenges and heartaches, but it is those beautiful unexpected moments of bliss, laughter, and accomplishments that make it all worth it in the end. I am truly blessed to have so many wonderful people in life, that have been there for me throughout all my many achievements, but there are three very important individuals that I would like to dedicate my dissertation to; my loving mom, supportive husband and precious daughter. All that I am and all that I hope to be, I owe it to them! It was during all those hard times and countless failed experiments that they always knew the right words to say to lift up my spirits and help get me through it all. To my sweet daughter Lillian Emma, I would like to add, that coming home to your precious smile always made all my worries and stresses of the day fade away. At the end of it all, you are and will always be my greatest achievement. May you always stay true to yourself and see the good within the world around you.

Be the change you wish to see in the world. - Gandhi

EGFR AND AKT SIGNALING IN RHABDOMYOSARCOMA PATHOGENESIS

Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2018

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Rhabdomyosarcoma is an aggressive soft-tissue malignancy comprised microscopically of neoplastic skeletal muscle-lineage precursors that fail to exit the cell-cycle and fuse into syncytial muscle - the underlying pathogenetic mechanisms for which remain unclear. We previously identified that misregulated myoblast fusion signaling via the TANC1 adaptor molecule promotes neoplastic transformation in RMS cells. As TANC1 is not presently pharmacologically targetable, here we have turned to our *Drosophila* RMS-related model to identify myoblast fusion-related elements potentially targetable in RMS. Genetic modifier screening against the fly model revealed that decreased Epidermal Growth Factor Receptor (EGFR) activity, which regulates myoblast fusion programming in flies, suppresses PAX-FOXO1 (PF)-induced lethality. As EGFR is pharmacologically targetable, we demonstrate that EGFR inhibitors antagonize RMS in a ERMS-RD cell line, but that other RMS cell lines are resistant. Further interrogation finds that EGFR inhibitor-sensitive cells exhibit marked downregulated activation of the Akt intracellular signaling transducer, but not MEK/MAPK or STAT3, suggesting that Akt promotes and/or sustains RMS. We then demonstrate that Akt

pharmacologic inhibition antagonizes RMS in vitro and in vivo, including RMS cells resistant to EGFR inhibition. We additionally find that sustained Akt1 activity promotes RMS cell terminal differentiation-arrest. Together, these findings point towards Akt activity as a broad RMS underpinning and therapeutic vulnerability.

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List of Abbreviations

RMS	Rhabdomyosarcoma
WHO	World Health Organization
ERMS	Embryonal Rhabdomyosarcoma
ARMS	Alveolar Rhabdomyosarcoma
PRMS	Pleomorphic Rhabdomyosarcoma
PAX3/7	Paired box gene 3/7
FOXO1a	Forkhead box 01
NCOA2	Nuclear receptor co-activator 2
MYOD1	Myogenic differentiation 1
VAC	Vincristine, dactinomycin cyclophosphamide
MET	Mesenchymal epithelial transition
BCL2L1	B-cell lymphoma 2-like 1
FGFR4	Fibroblast growth factor receptor
MHC	Myosin heavy chain
UAS	Upstream activating system
TANC1	Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1
EGFR	Epidermal growth factor receptor
RTK	Receptor tyrosine kinase
FC	Founder cell

Fcm	Fusion competent cell
HBEGF	Heparin-binding EGF-like growth factor
GFAP	Glial Fibrillary Acid Protein
HBEC	Human bronchial epithelial cell
IHC	Immunohistochemistry
MEK/MAPK	Mitogen-activated protein kinase- extracellular signaling-regulated kinase kinase/ mitogen-activated protein kinase
STAT	Signal transducer ad activator of transcription
JAK1/2	Janus Kinase 1 /2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
NSCLC	Non-small cell lung carcinoma
PTEN	Phosphatase And Tensin Homolog
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
sIL-6R	Soluble IL-6R
mIL-6R	Membrane bound

Chapter 1

Background

I. Rhabdomyosarcoma (RMS): A pediatric disease.

Rhabdomyosarcoma (RMS) is an aggressive soft tissue tumor that derives from primitive mesenchymal precursor cells and exhibits histological myogenic features. Soft tissue sarcomas account for 10% of all pediatric cancers, of which approximately 50% of those cases reported are RMS - making it the most prevalent [3] [5] (Figure 1). Approximately 600 new cases are reported each year in the United State. Of those cases, 59% occur in children and adolescents, in which the majority of patients diagnosed are children younger than 10 years of age [7]. RMS cases do arise in adults (i.e., Pleomorphic RMS), however, soft tissue sarcomas account for less than 1% of all adult malignancies, of which 3% are RMS, making the prevalence of RMS in all adult malignancies exceedingly rare [6].

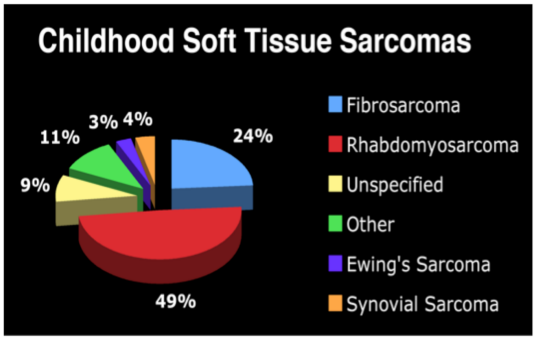

Figure 1: Prevalence of RMS cases in soft-tissue sarcomas. Adapted from SEERS program data, NCI

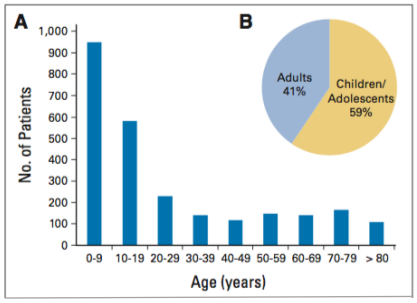

Figure 2: Age distribution of RMS patients. Adapted from [4], Fig. 1

RMS can develop in different anatomical regions all throughout the body, but predominantly arises in the following 3 sites; head and neck (35-40%), genitourinary tract (24%) and upper/lower extremities (19%) [9] [165]. Currently there are no clear predisposing risk factors involved in trigger the onset of the disease, although a slightly higher predominance of RMS

incidences have been reported in males - 1.4 times more common than in females [8]. In some cases, RMS has been shown to be associated with certain congenital abnormalities and familial cancer syndromes including neurofibromatosis type 1, Li-Fraumeni Syndrome, and Beckwith-Wiedemann Syndrome, though these rare inherited conditions only have accounted for a very small percentage of all RMS cases [10, 11]. Other studies have suggested prenatal exposure to x-rays [7] or the use of recreational drugs (e.g., cocaine and marijuana) by the mother during pregnancy, could increase the child's risk of developing RMS, but again only a few number of cases have been linked to these factors [12]. The majority of cases thus far seem to be attributable to sporadic triggers, but further investigation is ongoing to identify a common predisposing factor to RMS onset.

II. First incidence of Rhabdomyosarcoma reported: Cases of adult RMS

RMS was first described by a German physician named Weber C. in 1854, who reported a case of a 21 year-old man with a recurrent "localized enlargement" of the tongue muscle that was comprised of striated muscle cells in various stages of differentiation. Soon after this report several similar cases of these "striated muscle tumors", which all had various epidemiological characteristics, were described [13]. With more information available to physicians, more and more of these adult cases were now being reevaluated and properly diagnosed. In fact, during the 1930's and 40's, an increasing number of adult incidences were reported. These patients ranged from the ages of 50 -70 and most of them had tumor growths localized mainly in the lower extremities [165]. However, it was not until 1946 that RMS was officially classified as a distinct

diagnosis by Dr. Arthur P Stout. Dr. Stout was a surgical pathologist and is recognized as the first to characterize RMS [14]. In his 1946 publication titled *Rhabdomyosarcoma of the Skeletal Muscles*, he discussed Weber's work and clinically characterizes 121 cases; 14 of them new and 107 of them being previously reported cases. About 19% of those cases were patients under the age to 20 [13]. His work has been accredited with initiating further inquiry on RMS cases and eventually childhood sarcoma cases. diagnosed as round or spindle cell sarcomas, was recognized as RMS. Since then, several published reports have developed classification schemes that have vastly improved the way RMS is clinical identified and characteristics [165].

III. Histological and clinical characteristics of RMS subtypes

According to the most recent World Health Organization (WHO) classification, RMS is subdivided into four distinguished subtypes that are characterized with distinct histopathology, anatomical locations, genetic aberrations and clinical features [15] [5] (Figure 3 and Table 1). Traditionally, RMS had been subdivided into only three distinct groups, A. Embryonal RMS (ERMS), B. Alveolar RMS (ARMS), C. Pleomorphic RMS, however, D. Spindle cell/sclerosing RMS, which was once identified and categorized as an ERMS variant, was

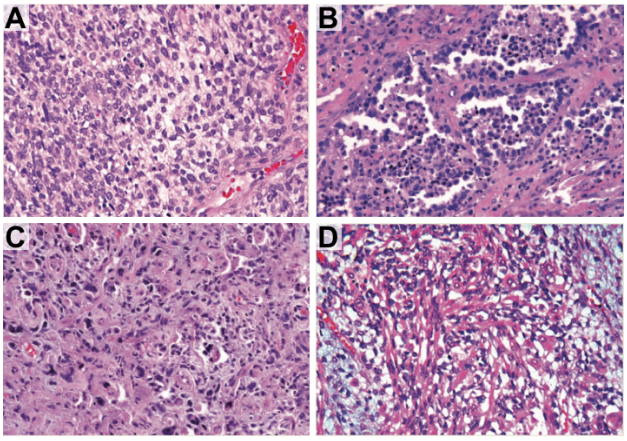

Figure 3: Histology images of RMS subtypes. A. Embryonal RMS, B. Alveolar RMS, D. Pleomorphic RMS, and G. Spindle Cell RMS. Provided by Dr. Galindo [4]

later characterized as a fourth RMS subtype due to its distinct clinical features (later discussed) [16, 17].

Table 1: Clinical Characteristics of RMS subtypes

RMS Subtypes	1. Embryonal	2. Alveolar	3. Pleomorphic	4. Spindle cell/ Sclerosing
Location	Head and neck, genitourinary tract, urinary bladder, prostate, biliary tract, abdomen, pelvis, and retroperitoneum	Upper/ Lower extremities, head and neck, chest, genital organs, abdomen, and anal area	Upper/ Lower extremities, chest and abdomen	Paratesticular, and head and neck (children); head and neck (adults)
Fusion Oncogene (PAX3/7-FOXO1)	-	+ (~20% Fusion-Negative)	-	-
~ % of all RMS cases	60%	30%	10%	<1%
Age Range (years)	<10	10-25	>45	<10 >40
Prognosis	Favorable	Unfavorable	Unfavorable	Favorable (Children) Unfavorable (Adults)

The table provides a general overview of the distinct clinical characteristics for each RMS subtype. The information on Table 1 was obtained from Kashi V. et. al., 2015; and Enzinger, Franz M., and Sharon W. Weiss. *Soft Tissue Tumors*. Mosby, 1995 [5, 165].

A. Embryonal Rhabdomyosarcoma

The most common of the four subtypes is ERMS, accounting for 60% of cases. ERMS primarily occurs in children younger than 10 years of age and typically is located in the head/neck and genitourinary system. Histologically, ERMS cells are small, round- to-spindle-shaped cells with alternating loose and dense cellularity; resembling embryonic muscle cells with 50-60% of the cases having discernible skeletal muscle-like cross striations [3]. ERMS is genetically characterized to be very heterogeneous. One of the most common genetic aberrations detected being loss of heterozygosity for chromosome 11p15.5, which is associated with gain of tumor-promoting networks and/or the loss of critical tumor surveillance genes [3, 18].

Patients diagnosed with ERMS typically have a favorable outcome with five-year survival rates reaching over 85% [3]. Moreover, some ERMS cases are further characterized into two ERMS variants; Botryoid and Spindle cell. Both variants are rare subtypes accounting for 6% and 3% of all RMS cases reported, respectively [15]. Botryoid RMS remains classified as a variant of ERMS, however, Spindle cell RMS, which was thought to primarily affect children, appeared in adults with different clinical prognosis; therefore it is now categorized as a distinguished RMS subtype (Spindle Cell/ Sclerosing RMS) and no longer as an ERMS variant [16]. Botryoid, which is derived from the Greek word “Grapes”, forms grape-like polypoid masses lined with epithelium, whereas Spindle cell RMS is comprised of elongated spindle cells with prominent nucleoli. Clinically, both Botryoid RMS and Spindle Cell RMS have a very similar favorable prognoses (only in children) to ERMS, where patients have a 5 year survival rate of 95% [15][165].

B. Alveolar Rhabdomyosarcoma

The second most distinguished subtype is ARMS, which accounts for 30% of cases and is the most aggressive form with high-risk metastatic features and poor clinical outcomes [9]. ARMS occurs more frequently in adolescents and young adults ranging from 10-25 years of age and has a much less favorable outcome. Primary ARMS tumors are largely located in the upper/lower extremities and within the trunk region [6, 19]. The 5-year survival rate for these patients is ~49%, however, the prognosis for patients presenting with high-risk metastatic ARMS drastically drops to 4 year survival rate of 8% [20][165]. ARMS cells have distinct alveolar architecture, hence their given name "alveolar". ARMS is typically comprised of aggregates of small round-oval poorly differentiated cells that are separated by dense hyalinized fibrous septa [3]. Interestingly, although ARMS cells poorly resemble mature or differentiated myogenic characteristics, they express much higher levels of myogenic differentiation markers (i.e. myogenin) in comparison to the more differentiated ERMS cells [15].

ARMS is also distinguished by the presence of a unique chromosomal translocation, t(2;13)(q35;q14) or t(1;13)(p36;q14), resulting in the corresponding expression of fusion oncoprotein PAX3-FOXO1 or PAX7-FOXO1. In both cases, the translocation is a fusion of a paired box (*PAX*) gene, either *PAX3* on chromosome 2 or *PAX7* on chromosome 1, to the 3' end of the forkhead box O1 (*FOXO1a*) gene on chromosome 13. The two translocation oncogene variants, *PAX3-FOXO1* or *PAX7-FOXO1*, are found in 70% or 10% of ARMS cases, respectively [15, 21]. Several other translocation variants have been reported in a few ARMS cases (ex. Fusion between *PAX3* with another FOXO family member, *FOXO4;* t[2:x]) nonetheless, these cases are exceedingly rare and are not well-studied [5]. Of note- not all ARMS cases are "fusion positive".

Around 23% of ARMS cases are identified as “fusion negative” and present with very different clinical outcomes in comparison to fusion-positive ARMS. Fusion negative ARMS share very similar favorable clinical and molecular characteristics to ERMS tumors [22].

C. Pleomorphic Rhabdomyosarcoma

The last two subtypes, Pleomorphic and Spindle cell/sclerosing RMS, are comparatively rare. Pleomorphic RMS (PRMS) occurs most often in adults over the age of 45 years and is exceedingly rare in children, whereas Sclerosing RMS affects both children and adults [23]. PRMS typically develops in the deep soft tissues of the extremities, predominantly in the thigh. Histologically, the cells found within PRMS tumors are loosely arranged, large anaplastic cells with enlarged, hyperchromatic nuclei and deeply eosinophilic cytoplasm [165]. It is notably characterized to be clinically highly aggressive, with most cases metastasizes early – within 5 years of diagnosis. The overall 5-year survival rate for these patients is approximately 40% [24, 25].

D. Spindle Cell/ Sclerosing Rhabdomyosarcoma

Spindle cell/ sclerosing RMS is a recently characterized subtype that accounts for <1% of all RMS and can affect both children and adults. The clinical diagnosis of spindle cell/ sclerosing RMS presents with some challenges, not only because of its rarity, but also due to some histology similarities it has to other mesenchymal malignancies such as angio- and osteosarcoma [17]. Spindle cell RMS tumors are composed of relatively differentiated elongated spindle-shaped cells with similar features of smooth muscle neoplasms, whereas Sclerosing RMS tumor are composed

of primitive round cells that are divided into small nests, lobules and microalveoli by an abundantly hyalinized matrix [165].

The primary location for these tumors varies; in children they predominantly develop in the paratesticular region, whereas, in adults, they are more commonly found in the head and neck region [16, 17]. Clinically, spindle cell/ sclerosing RMS have distinct characteristics depending on specific genetic features. Patients with Spindle cell RMS with rearrangements of the nuclear receptor co-activator 2 (*NCOA2*) gene typically have a favorable prognosis, in contrast, children and adults diagnosed with spindle cell/sclerosing RMS with recurrent myogenic differentiation 1 (*MYOD1*) mutations have a poor clinical outcome [5, 26].

IV. Treatment resources for Rhabdomyosarcoma patients

For the past three decades, the conventional forms of treatment for RMS have been limited to cytotoxic chemotherapies and surgery with or without radiation therapy [21, 27]. The use of this combination is also referred to as multidisciplinary therapy planning [18]. The prognosis and treatment option(s) for the patient is highly dependent on several factors including the health and age of the patient, stage of the disease, localization of the primary tumor and the histological subtype [28].

Surgical removal of the tumor is usually performed on all RMS patients. Once the tumor is removed, RMS patient will still be required to undergo chemotherapy. In some cases, RMS tumors are considered unresectable (complete removal of the tumor is unsuccessful) or inoperable as a result of medical concerns (e.g., the tumor size being too large, the location of the tumors, the

tumor is metastatic, and/or the patient health status) making surgery a health risk. In both instances, the patient must undergo chemotherapy and radiation therapy as part of their treatment regime [29-31].

The most common chemotherapy regimen used on patients is a 3-drug combination referred to as VAC (vincristine, dactinomycin, and cyclophosphamide). VAC combination has demonstrated to be effective in low- and intermediate-risk groups, unfortunately, it has not been shown to be successful in patients within the high-risk groups [27, 32]. Treatment option for advance stage RMS or high-risk RMS patients still remains limited, resulting in poor clinical outcomes and low survival rates [33]. Although, several advancements have been made to the current treatment options, one of the major causes for treatment failure in patients with high-risk metastatic RMS is the development of resistance to current chemotherapies [34]. This seems to also be a persistent issue for RMS patients with relapsed and/or recurrent tumors. Approximately one-third of RMS patients will experience a recurrence despite having achieved complete remission after treatments, in which many of them will experience a poor clinical outcome [35, 36].

Furthermore, patients considered to be "disease-free" remain at risk of developing treatment-related morbidities down the road. Studies have shown the invasive and harsh radio- and chemotherapy administered to these patients predisposes them to debilitating long-term effects that are associated with poor health outcomes that worsen with age [3]. RMS survivors have a much higher prevalence of organ dysfunctions (i.e. ovarian failure), heart diseases and development of second malignancies [37]. The severity of the side-effects that these patients undergo are dependent on the location of the tumor as well as the extent and type of treatment(s). In some incidences, now as adults, patients in remission from head and neck RMS have shown to have a high-risk of developing dentofacial abnormalities (e.g., facial asymmetry and jaw

hypoplasia) [38] hearing loss, visual/orbital problems [39] and neuroendocrine dysfunctions [40]. Other studies have reported exposure to chemotherapies (i.e., cyclophosphamide) during childhood increases the risk of infertility [41]. These findings strongly argue the need for new therapeutic approaches that would lead to the development of more advanced targeted therapies thus decreasing off-targeted responses and improving long-term health outcomes.

V. Recent reports in RMS: Where are we now?

Within the field of RMS, as stated previously, approaches by which to treat RMS remain limited. To date, we still don't have any effective targeted therapies approved to be used in the clinic to treat RMS patients [42]. Several research discoveries focusing on identifying specific genetic aberrations suitable as therapeutic candidates have pushed the field forward, advancing our understanding of RMS. Various molecular signatures involved in the progression of RMS have been detected such as, several PAX3-FOXO1 driven tumor-promoting genes [i.e receptor tyrosine kinase MET (mesenchymal epithelial transition) , anti-apoptotic protein BCL2L1 (B-cell lymphoma 2-like 1) and Fibroblast Growth Factor Receptor 4 – (FGFR4)] [43], activating Ras mutations (mainly identified in ERMS and associated with high risk cases) [44, 45] and misregulation of embryonic signaling pathways Notch and WNT [46].

Many of these factors have undergone further analysis and have demonstrated to be putative candidates for RMS gene therapy, unfortunately, for various reasons (i.e no available effective pharmacologic RAS inhibitors [47]) many of these factors haven't been tested clinically [18, 21, 28, 42, 48]. In order to be considered for clinical trial testing, there must be clear

understanding of their mechanism of action of the proposed novel agents and it must have undergone extensive in vitro and in vivo experimental analysis. Meeting all these requirements are necessary, for only a fraction of new agents that are considered the strongest candidates are selected mainly due to the fact that the number of eligible pediatric patients are very small and limited [42, 49].

Though published reports have identified some misregulated signaling networks in RMS, we still have an incomplete understanding of how molecular signatures influence RMS pathogenesis. This could be attributable to our unclear understanding of what specific mechanism(s) are responsible for driving the onset of RMS [50]. Within the literature, very little evidence of the tumorigenic mechanisms that influence the pathological process of RMS are available. Further investigation would not only advance our understanding of the genetic foundation behind RMS tumorigenic mechanisms but would also lead to the discovery of novel strategies that antagonize RMS and significantly improve patient outcomes.

VI. Uncovering dysregulated signaling networks utilizing RMS *Drosophila* model

The use of different animal modeling systems has been an incredible tool used in research to explore and enhance our understanding of human diseases such as cancer. There currently are a number of diverse RMS modeling systems that have revealed potential avenues by which to therapeutically treat RMS [5] - (*Myf6-driven Cre PAX3-FOXO1* transgenic mouse model [51] and *rag2-KRAS*^{G12D} Zebrafish model [52]. However, certain critical aspects of RMS remain undefined (i.e. cell of origin and disease onset in RMS remains debatable) [53, 54], thereby querying what

current models recapitulate the disease and pattern similar fundamental genetic and molecular networks involved in RMS?

Some studies strongly suggest RMS to be a disease of misregulated muscle development programs, being as RMS cells closely resemble skeletal muscle precursor cells and the PAX3/7 fusion oncogenes identified in ARMS are important transcription factors during skeletal muscle development, though more supportive evidence is needed [55, 56][. Therefore, one scientific approach to better study RMS, would be to utilize a RMS genetic modeling system by which to probe for disrupted myogenic signaling networks.

For these studies, we assessed the role of misregulated signaling pathways uncovered in an unbiased forward genetic screen utilizing a RMS *Drosophila* model generated in our lab [1]. *Drosophila* animal models provide researchers with distinct technical advantages and are ideal systems to use for performing genetic screens. Additional, as we investigate genetic abnormalities associated with myogenesis in RMS, *Drosophila* models are suitable platforms to use as both myogenesis and critical myogenic genes (i.e. human PAX3 and PAX7 orthologues of *Drosophila Gooseberry* and *Gooseberry-neuro*) [1] are evolutionary conserved [57] (Figure 4).

The RMS *Drosophila* model was generated by conditionally expressing the ARMS fusion oncogene PAX7-FOXO1 in terminally differentiated, Myosin Heavy Chain (MHC) expressing tissue, syncytial muscle fibers, utilizing the Gal4-UAS (Gal4-upstream activating systems) system. Misexpression of the PAX7-FOXO1 induced myoblast muscle patterning defects, which was visualized in real-time using fluorescent protein reporters (i.e., GFP) (Figure 4). The loss of muscle patterning resulted in larval semi-lethality which served as a suitable readout for a forward genetic screen analysis. The screen performed was a chromosomal deletion screen against the PAX7-FOXO1 model, in which genomic segments that modified PAX7-FOXO1 lethality were identified

and further probed for gene targets and cofactors [1]. Of note, though PAX7-FOXO1 is not as commonly expressed as PAX3-FOXO1 in ARMS patients, it was used in this model because human PAX7 displayed to have a higher sequence identity to *Drosophila* PAX3/7, therefore yielded a higher penetrance [58].

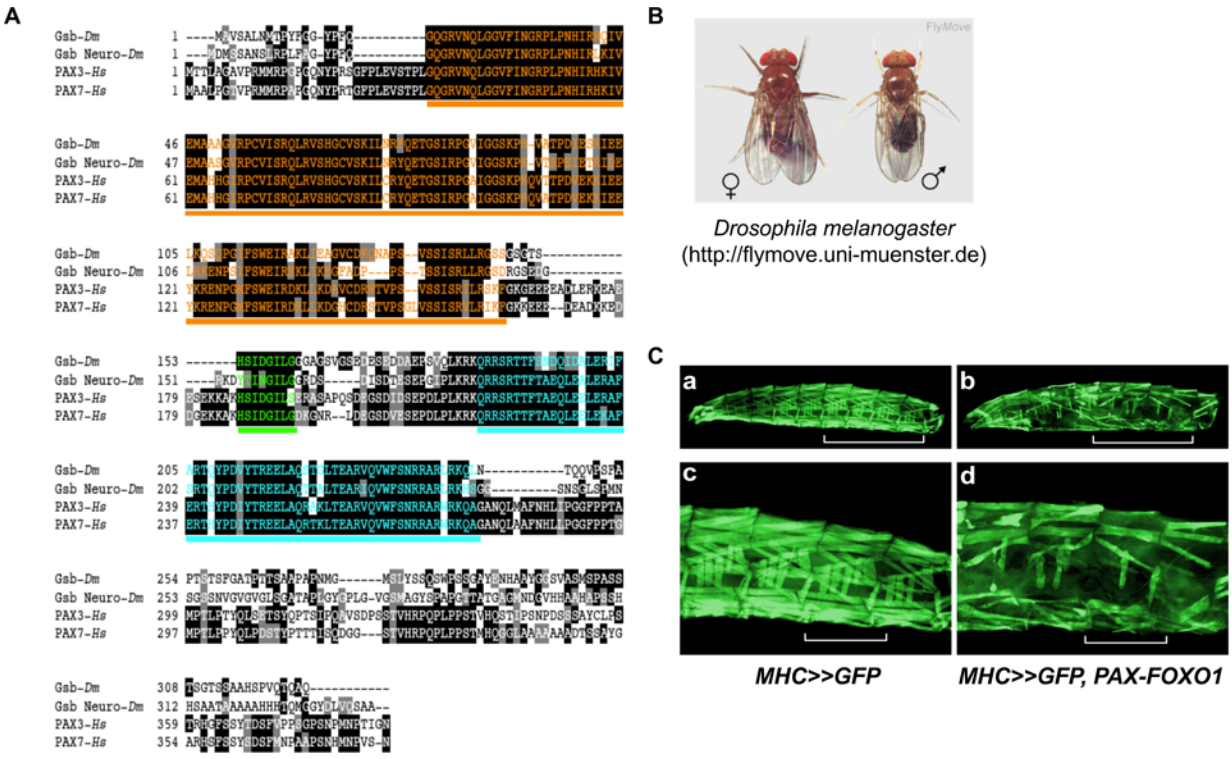

Figure 4: **PAX7-FOXO1 interferes with muscle development in *Drosophila*.**

These data are kindly provided by Dr. Rene Galindo's published report [1]. (**A**) Alignment of the gene sequences of human PAX3 and PAX7 and *Drosophila* orthologues Gooseberry and Gooseberry-neuro. The DNA binding motifs are shown in orange and blue. The octapeptide motif is shown in green. (**B**) A photographic representation of *Drosophila* males and females (http://flymove.uni-muenster.de) (**C**) Immunofluorescent image showing the muscle architecture of *Drosophila* larva. (**C-a**) Somatic musculature (striated muscles of the body wall) from a wild-type control *MHC-GAL4*, *UAS-GFP* larva. (**C-b**) A *MHC-Gal4*, *UAS-GFP*, *UAS-PAX7-FKHR Pax7-Foxo1* larva. (**C-c**) Representative hemisegments of wild-type somatic musculature. The four hemisegments indicated by the white bar in panel **a** are shown. (**C-d**) Representative abnormal hemisegments of PAX7-FOXO1 musculature. The four hemisegments identified by the white bar in panel **b** are shown.

VII. Further exploring identified misexpressed factors

From the RMS *Drosophila* screen, we have identified a panel of different misexpressed factors, one of which being TANC1 (tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1), human ortholog of the *Drosophila* intracellular adaptor molecule *Rolling Pebbles*. In one of our recent reports, we demonstrated that the misexpression of TANC1 elicited RMS pathogenicity and silencing its expression hindered both RMS oncogenicity and tumorigenicity. We also observed rescue in myoblast differentiation and fusion in TANC1 silenced RMS cells, indicated by the significant increase in Myosin Heavy Chain positive cells [maker for terminally differentiated cells [59]. These results suggested that dysregulated myogenic signaling networks identified in the screen, such as TANC1, influences RMS by impairing essential myoblast differentiation programs, thus uncovering a new approach by which to therapeutically target RMS. Unfortunately, TANC1 inhibitors are not available in order to be able to proceed into necessary preclinical testing, therefore we returned to the RMS-related PAX7-FOXO1 *Drosophila* model to identify pharmacologically targetable myogenic signaling networks.

For these studies, we focused on two *Drosophila* chromosomal deletions, *Df(2L)pr-A16* and *Df(2R)Excel*^{6076}, which dominantly suppress PAX7-FOXO1-based lethality (Figure 5). Within those chromosomal deletions, were gene sequences that transcribed and translated into *Drosophila Epidermal Growth Factor Receptor* (*EGFR*) and ligand *Epidermal Growth Factor* (which is *Drosophila*, is known as *spitz)*. *Df(2L)pr-A16* deletes chromosomal segments 37B2-12;38D2-5, which included the *Drosophila EGFR*, while *Df(2R)Excel*^{6076} deletes segments 57E1;57F3, which included *Drosophila Epidermal Growth Factor* (*spitz*). We next tested individual loss-of-function alleles in *EGFR* or *spitz*, which similarly suppressed PAX7-FOXO1-mediated lethality (Figure 5).

These results demonstrated that EGFR signaling could serve as a targetable network for RMS patients.

EGFR is a very well-studied receptor tyrosine kinase (RTK) involved in several essential cellular processes and is one of the most common signaling receptors pursued in different malignancies [60]. The availability of several clinically approved pharmacological EGFR inhibitors (i.e. Erlotinib and Cetuximab) allows for the necessary preclinical testing of this genetic pathway to be done. To date, no preclinical testing of EGFR inhibitors have been reported in RMS [42] and with little evidence regarding the connection between EGFR signaling and RMS, I aimed to investigate how EGFR activity impacts the pathobiology of RMS.

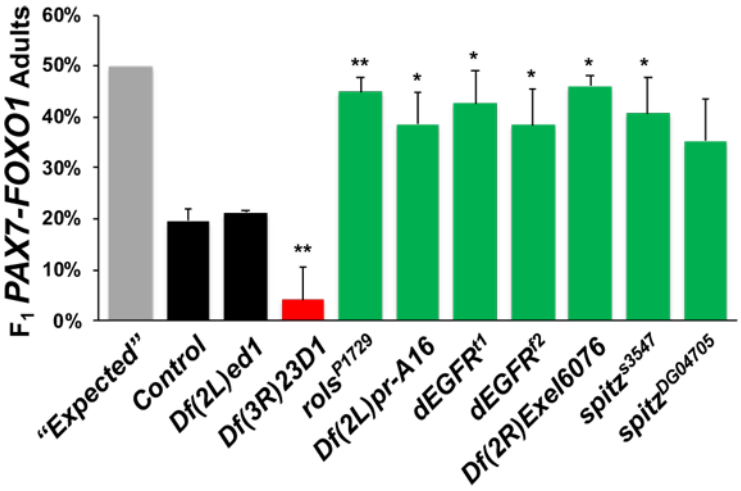

Figure 5: Mutational loss-of-function of *Drosophila EGF* or *EGFR* suppresses PAX7-FOXO1 pathogenicity in vivo.

Based on Mendelian ratios, the F_1 adult population should be comprised of 50% control and 50% PAX7-FOXO1-expressing adults ("*Expected*"). PAX7-FOXO1 causes semilethality, as PAX7-FOXO1 adults comprise only ~20% of F_1 adults ("*control*", n= 124). Chromosomal deletions *Df(2L)pr-A16* (n= 47) and *Df(2R)Excel*^{6076} (n=77) dominantly suppresses PAX-FOXO1 lethality, as do the *EGF* (names *spitz* in Drosophila) loss-of-function alleles, *spi*^{DG04705} (n= 55) and *spi*^{s3547} (n=66), and two EGFR loss-of-function alleles, *EGFR*^{f2} (n=66) and *EGFR*^{t1} (n=60) (though we note that *EGFR*^{t1} allele data showed a t-test value of 0.067 when compare to control). *Rolling Pebbles* (*rols*), the *Drosophila* ortholog of *TANC1*, and *rols*^{P1729} is the loss -of-function allele previously isolated as a PAX-FOXO1 suppressor. *Df(2L)ed1* (n=160) and *Df(3R)23D1* (n=74) are unrelated chromosomal deletions included as representative examples of a non-modifier and genetic enhancer, respectively. This figure was kindly provided by Dr. Rene Galindo (unpublished).

VIII. Investigating the mechanism(s) of RMS onset

For most malignancies, the most commonly sought out subjects are what are the underlining mechanisms that trigger cellular transformation and how do they elicit and promote tumor progression? Most sarcomas, such as RMS, typically are derived from primitive precursor cells that carry distinct differentiation programs, whereas carcinomas arise from intrinsically proliferative epithelial cells. Under normal conditions these endogenous programs initiate cell cycle arrest and promote cells to undergo cellular differentiation/ maturation [61, 62]. Acquired genetic lesions that specifically impair these differentiation networks could potentially result in cellular transformation contributing to tumor pathogenicity. Our TANC1 data, as well as other published reports [59] [56], support that dysregulated myogenic programs drive RMS progression, so we first questioned how EGFR activity influences myogenesis.

Myogenesis, is a tightly regulated multistep cellular process that involves committed mesenchymal precursor cells entering the myogenic cell lineage and differentiating into mature multinucleated muscle tissue [63]. Each step of myogenesis is controlled and coordinated by several different myogenic transcription factors and/or effector molecules (e.g., Pax3/7, Myf5 and MyoD) These transcription factors/ effector molecules facilitate and induce signaling transduction that stimulate step-specific morphology and cellular behavior changes [64]. Each stage can also be detected through the expression of specific markers such as MHC, which is typically expressed once myoblast cells have reached the later stages of myogenesis (i.e., myocyte differentiation and fusion). The stages of myogenesis begin with myogenic committed progenitor cells transitioning into highly proliferative myoblast cells and depending on the availability of particular growth factors/nutrients myoblasts will remain in this state. Once the concentrations of growth factors

begin to diminish, myoblast progress into differentiated myocytes by exiting the cell cycle and begin aligning with other myocytes. In the final stages, the aligned myocytes begin to fuse and mature into multinucleated myo-tubes/fibers eventually giving rise to bundled muscle tissue [2, 64, 65] (Figure 6).

As previously mentioned, specific signaling effectors facilitates the progression of myoblast differentiation, though the question still remains as to how is EGFR signaling involved in myogenesis? In order to further investigate this relationship, we first needed to understand the basic biology of myocyte differentiation/ fusion. Though there has been an extensive amount of investigation advancing our understanding of specific process and different stages of myogenesis, unfortunately, the specific mechanisms that regulate and facilitate differentiated myoblast fusion are poorly characterized in mammals [57, 66]. Therefore, we referenced the mechanism of fusogenic signaling (i.e muscle patterning) in a well-studied model, *Drosophila*.

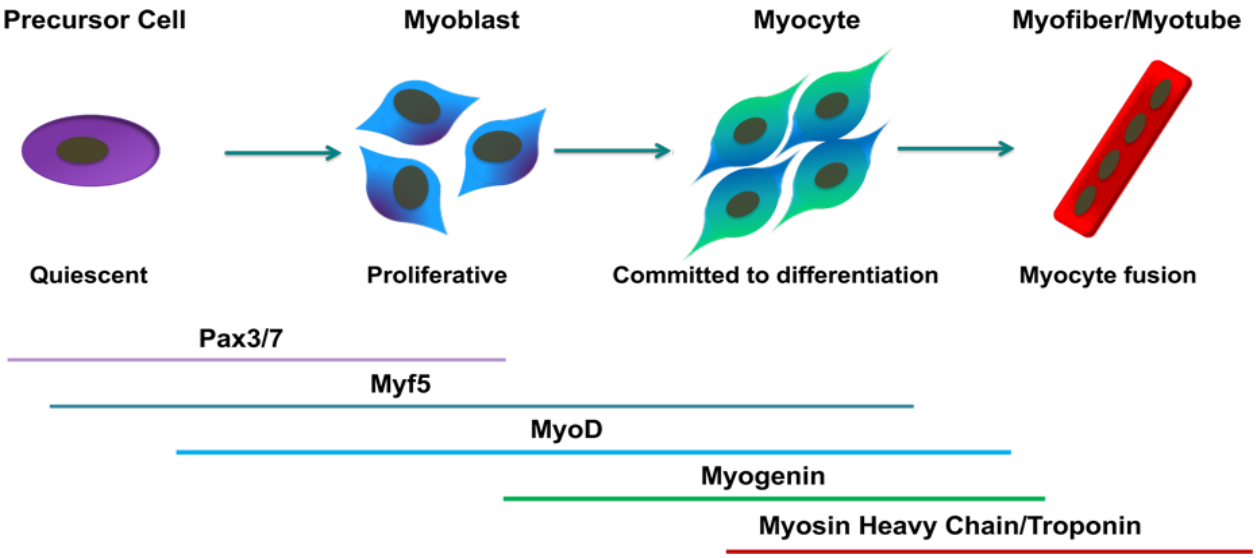

Figure 6: Myogenesis-The stages of myoblast differentiation.

Schematic representation of myoblast cell myogenesis and examples of common effector molecules and/or markers found within each stage. Adapted from [2, 3]

IX. Muscle patterning in *Drosophila* model: Linking EGFR

In *Drosophila*, the process of myoblast fusion is known as muscle patterning. Similar to mammalian myogenesis, *Drosophila* muscle patterning is also a tightly regulated process [67]. Muscle patterning requires two distinct myoblast populations, Founder Cells (FC) and fusion competent myoblasts (fcms), that are essential for somatic muscle formation to occur [68]. FCs are important for patterning, for they regulate and determine unique muscle physiology as well as the location and fusion events for each muscle through the expression of specific transcription factors. On the other hand, fcm's are considered a naïve population of myoblast that provide muscle mass by migrating out to locate and fusing with FCs until proper cellular/tissue mass is established [69, 70].

Muscle patterning begins by early myoblast progenitor cells undergoing cell fate specification, which is facilitated by specific patterns of gene expression (e.g., Notch [negatively regulates FC marker gene expression giving rise to fcms] and Myosin Heavy Chain [expressed in FCs]) that distinguishes myoblast cells into FCs or fcms fates. [71] Muscle formation is then initiated by a single FC that will begin fusing with neighboring fcms, seeding the development of a multinucleated muscle fiber (Figure 7). The identity of the two myoblast populations is critical for muscle patterning to occur. The loss of either population due to some type of genetic aberrations, would disrupt proper myoblast fusion, ultimately impairing normal muscle development [66].

Studies have identified a subset of gene products that mediate myoblast fusion and FC/fcm-fate specification (e.g. adaption/adhesion molecules Sticks and Stones in fcms and Dumbfounded/Kirre (Duf) in FCs [57]. Interestingly, EGFR activity has also been identified to

play a critical role in myoblast cell fate specification [72]. In *Drosophila*, EGFR is a critical Receptor Tyrosine Kinase (RTK) in fly embryos for FC-programming [70, 72, 73]. Initiation of EGFR signaling in uncommitted myoblasts drives FC differentiation, including the expression of downstream FC-fusion effectors (e.g., TANC1). Whereas the absence of EGFR activity in uncommitted myoblast causes these cells to exhibit a fcm cell fate. The regulation of EGFR expression is very critical in muscle patterning, for in the absence/loss of EGFR signaling, the FC population is lost resulting in an increase in fcms. In contrast, constitutive activation of EGFR drives the expansion of FC populations at the expense of fcm's. These findings demonstrated that EGFR activity plays a role in *Drosophila* muscle pattern and could similarly influence the mechanisms that regulate mammalian myoblast fusion [72] [71] (Figure 7).

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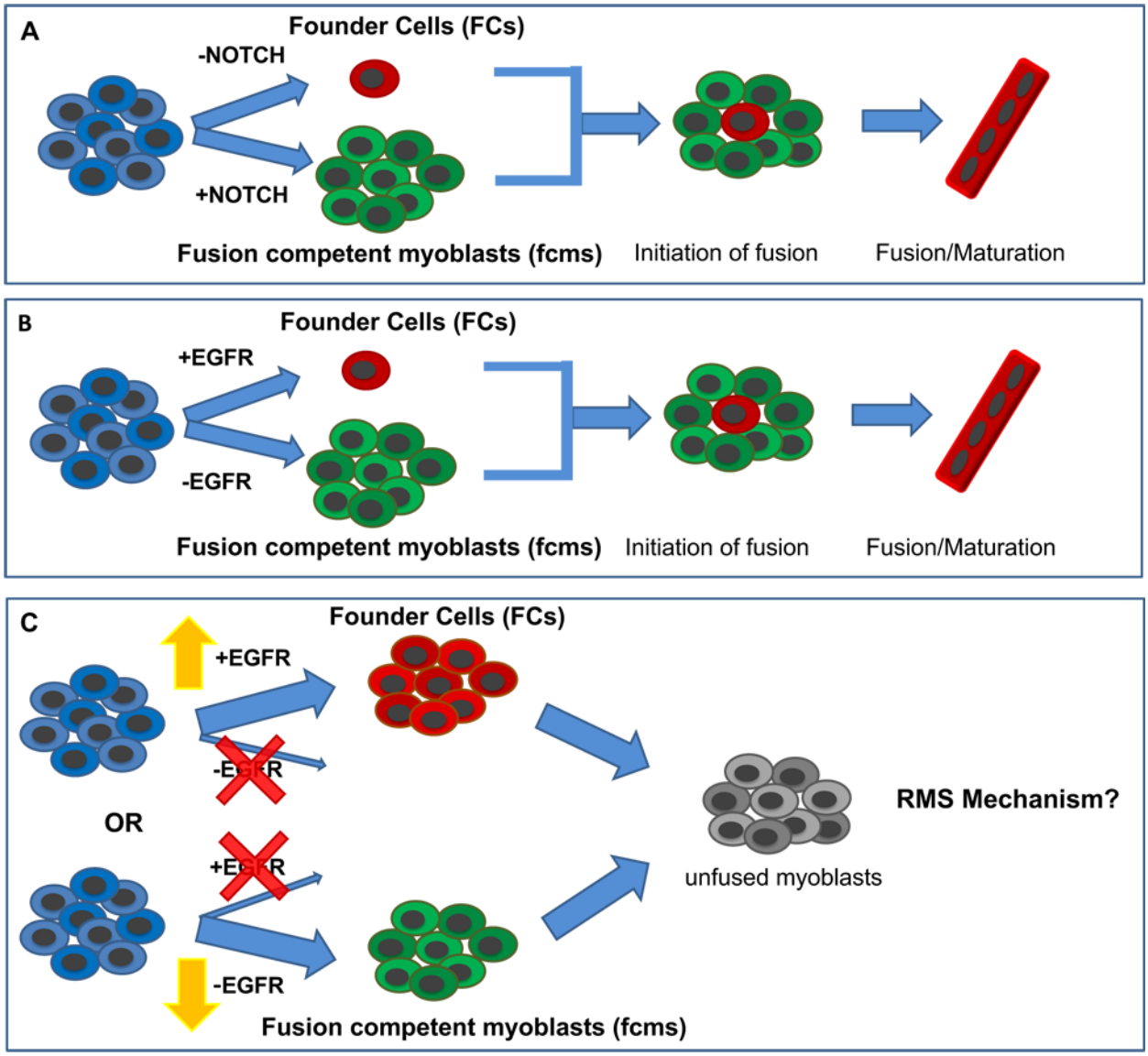


Figure 7: *Drosophila* Muscle Patterning.

(**A**) Schematic illustrations displaying myoblast muscle patterning in *Drosophila*. (**B**) EGFR expression found within muscle patterning. (**C**) Effects of abnormal expression of EGFR in muscle patterning. Over-expression of EGFR activity drives all myoblast population into a Founder cell fate, whereas loss of EGFR activity drives the entire population into becoming fusion competent cells. Yellow arrows indicate the increase (+) or loss (-) of EGFR activity in myoblast cells.

X. Dissertation Proposal

In *Drosophila*, EGFR signaling mediates FC/fcm- fate specification. Misexpression of EGFR leads to the loss of one of the two myoblast populations, obstructing muscle patterning and consequently resulting in a pool of unfused myoblasts. So, if the basic cellular events that take place during mammalian myogenesis are similar to *Drosophila* muscle patterning, then could the regulation of EGFR expression also influence mammalian myoblast differentiation and fusion? Could the misexpression of EGFR in mammals impede myogenesis and give rise to a pool of unfused myoblast, a similar phenotype to Rhabdomyosarcoma?

I hypothesized that if the regulation of EGFR signaling plays an essential role in myoblast differentiation and fusion, then its misexpression hinders normal mammalian myogenesis and drives RMS pathogenicity, which would point to EGFR as an RMS candidate therapeutic target.

Chapter 2

Investigating the role of EGFR signaling in Rhabdomyosarcoma pathogenesis

Introductions

EGFR: Targetable oncogene in cancer

Growth factors are essential in modulating critical biological activities, though an extensive amount of investigation has been put into further understanding their roles as oncogenes; the most well studied proto-oncogene being epidermal growth factor receptor (EGFR). EGFR is a transmembrane glycoprotein receptor that is a member of the ErbB family of receptor tyrosine kinases (RTKs)[74] [75]. It's expressed within tissues of neuronal, epithelial and mesenchymal origin and is involved in regulating and maintaining key processes of cell biology, such as proliferation, growth, survival and tissue homeostasis [76] [77, 78]. The activation of EGFR is initiated upon the binding of one of its seven growth factor ligands (e.g. epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HBEGF), which results in receptor homo- or heterodimerization, autophosphorylation and activation of specific downstream intracellular pathways [79, 80]. Under normal conditions EGFR signaling transduction is tightly regulated, though dysregulation of its activity leads to cellular transformation; driving the progression of several malignancies such as non-small cell lung carcinoma, breast, pancreatic and head and neck cancer [81-85]. EGFR gains its oncogenic characteristic through various mechanisms (e.g. constitutive activating mutations, gene amplification/ overexpression and failure to attenuate receptor signaling) [86]. Several studies have correlated EGFR expression levels with tumor

metastasis, resistance to chemotherapy and overall poor prognosis, making it an ideal target for cancer therapy [87].

EGFR expression in RMS

In our RMS-*Drosophila* screen, we identified EGFR has a misregulated signaling factor. Interestingly, though it has been the focus in several different cancers, its impact on RMS has not yet been studied. We first wanted to investigate if the results obtained from the PAX7-FOXO1 *Drosophila* screen were applicable to RMS patients, so we looked into the open-access NCI Oncogenomics RMS RNA-seq database to examine the expression levels of EGFR in a cohort of human RMS patient samples (compared with normal control tissues). We observed EGFR expression levels to be upregulated in RMS, though primarily in ERMS samples. As a control, we examined genes that were not associated with RMS (e.g. Glial Fibrillary Acid Protein –GFAP), which as expected showed to have no pattern of expression (Figure 8). These results indicated that overexpression of EGFR could be a potential mechanism by which EGFR elicits RMS progression, specifically in ERMS patients. To test this hypothesis, we took another look at the Oncogenomics data base and compared our RMS-EGFR expression analysis with that of Glioblastoma patient samples, a cancer in which EGFR gene amplification and overexpression serves as a mechanism that provokes tumor progression [88, 89]. The data analysis demonstrated comparable high EGFR expression levels in both Glioma and RMS patient samples, supporting our hypothesis that EGFR overexpression serves as a potential targetable mechanism driving RMS pathogenesis (Figure 8).

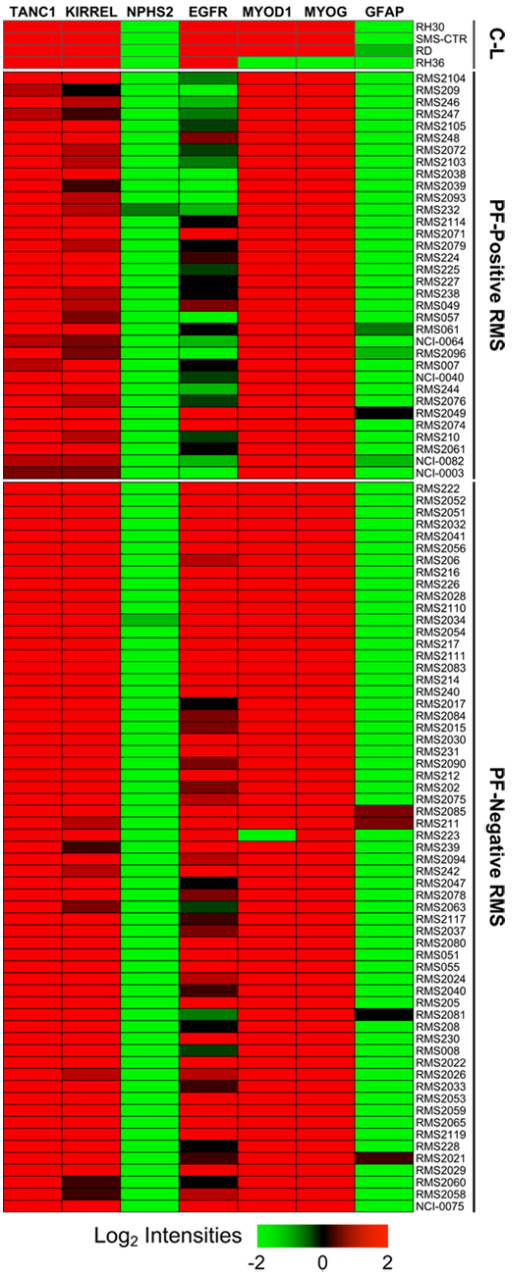


Figure 8: EGFR expression in RMS patient samples.

Heatmap of EGFR expression in a comprehensive cohort of human RMS tumor specimens profiled by RNA-seq. Red indicates upregulation and Green is downregulation (based on Log_2 values) as obtained from http://home.ccr.cancer.gov/oncology/oncogenomices. *MYOD1* and *MYOGENIN* (*MYOG*) are shown as known examples of RMS upregulated genes, while *GFAP* (*Glial Fibrillary Acid Protein*) is shown as an example of a non-RMS-related gene. Also shown are expression levels for *KIRREL*, human homolog of the *Drosophila* FC myoblast-specific fusion receptor; *NSPH2*, human homolog of the fly fcm-specific fusion receptor; and EGFR. C-L = Cell Line. Of note, three PAX3-variant fusion specimens are included in the Oncogenomics database, but are not shown. This figure was kindly provided by Dr. Rene Galindo.

Results

Investigating EGFR activity during C2C12 myoblast differentiation

As we aimed to further explore the role of EGFR signaling in RMS, we first wanted to investigate the biological role of EGFR activity during myogenesis by examining EGFR signaling during myoblast differentiation in a wild-type mammalian system. For these studies, we utilized C2C12 cells, an immortalized murine myoblast cell line that under serum-starved conditions will undergo differentiation and form multinucleated myotubes (Figure 9A), and first assessed if EGFR signaling could be detected throughout wild-type myoblast differentiation. We probed for EGFR activity (P-EGFR) and expression levels (total EGFR) in cultured C2C12 myoblasts undergoing differentiation over the span of 6 days. We observed EGFR activity increased over days 0-2 of differentiation and then decreased over days 2-6, suggesting that EGFR signaling is active and regulated during C2C12 myoblast differentiation (Figure 9B).

In order to further investigate these findings, I examined if EGFR signaling plays a critical role in initiating myoblast differentiation and if terminal differentiation/fusion is dependent on the regulation of EGFR activity. I first tested if EGFR signaling is necessary for myoblast to undergo differentiation, by blocking its activity in C2C12 cells undergoing differentiation using an EGFR inhibitor, Erlotinib. The concentration used for this study was determined by testing a series of different doses, to be able to identify the lowest concentrations that would effectively block EGFR activity, which was observed via immunoblot analysis (Figure 10A). C2C12 were treated with Erlotinib (two different doses were tested – shown in figure) either at the initiation of differentiation (day 0) or on day 2 – when we observed the highest levels of EGFR activity during differentiation. C2C12 cells treated with Erlotinib on either day 0 and day 2 of differentiation, both

exhibited a marked decrease in their ability to differentiate and form multinucleated myotubes in comparison to their controls (Figure 10B and C). These results suggest that EGFR signaling is required in the early stages of myoblast differentiation and loss of its activity disrupts myoblast differentiation/fusion.

Next, I assessed if the regulation of EGFR expression is essential for myoblast terminal differentiation/fusion. In order to test for this, I treated C2C12 with exogenous EGF ligand daily in order to maintain elevated levels of EGFR signaling throughout the entire six-day span of myoblast differentiation (Figure 11A). I observed that continuous activation of EGFR signaling resulted in a loss of myoblast differentiation and fusion, suggesting that the regulation of EGFR activity is an important process that mediates proper terminal differentiation/ fusion to occur (Figure 11B). Overall, I demonstrated that the inhibition and/or dysregulation of EGFR activity impedes myoblast differentiation/ fusion, indicating that EGFR signaling plays a critical role in mediating mammalian myoblast differentiation/ fusion, though further testing is still needed to elucidate the specific intracellular mechanisms behind how EGFR influences myoblast differentiation.

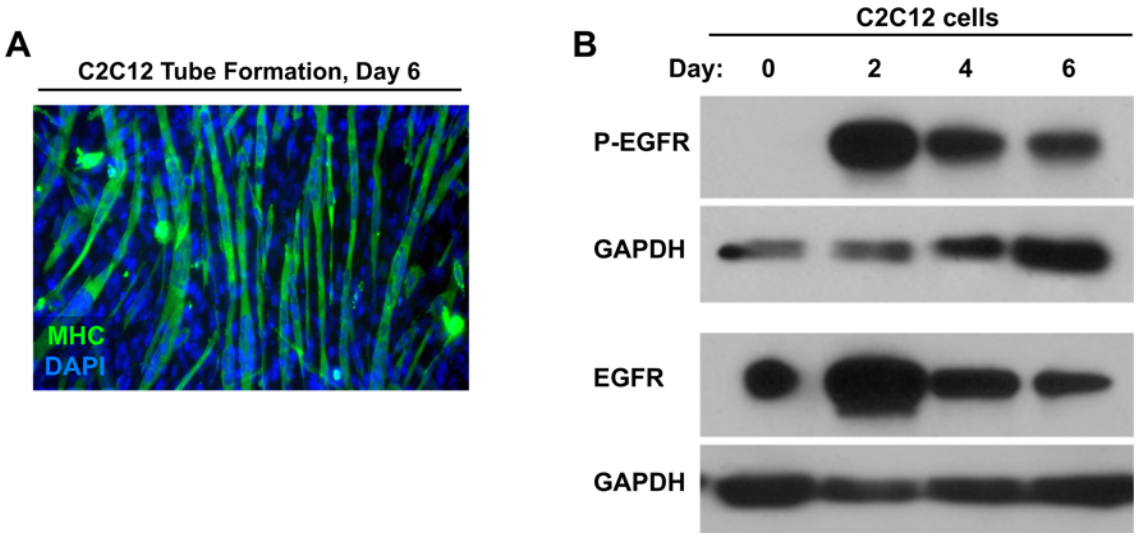


Figure 9: EGFR expression profile during C2C12 differentiation.

C2C12 myoblasts were seeded in differentiation media (low serum media) and incubated for a total of 6 days. (**A**) Immunofluorescent staining against MHC (shown in green) was performed on D6 of differentiation to assess for C2C12 tube formation. DAPI nuclear stain shown in blue (**B**) Immunoblot analysis were performed on generated C2C12 lysates (D0, D2, D4, and D6) and probed for the steady-state phosphorylated levels of EGFR (P-EGFR) and total EGFR protein levels during the time course of differentiation (6 days). GAPDH was used as the loading control. Immunofluorescent images taken at 20x magnification. Western Blot kindly provided by our fellow technician Pooja Dalal.

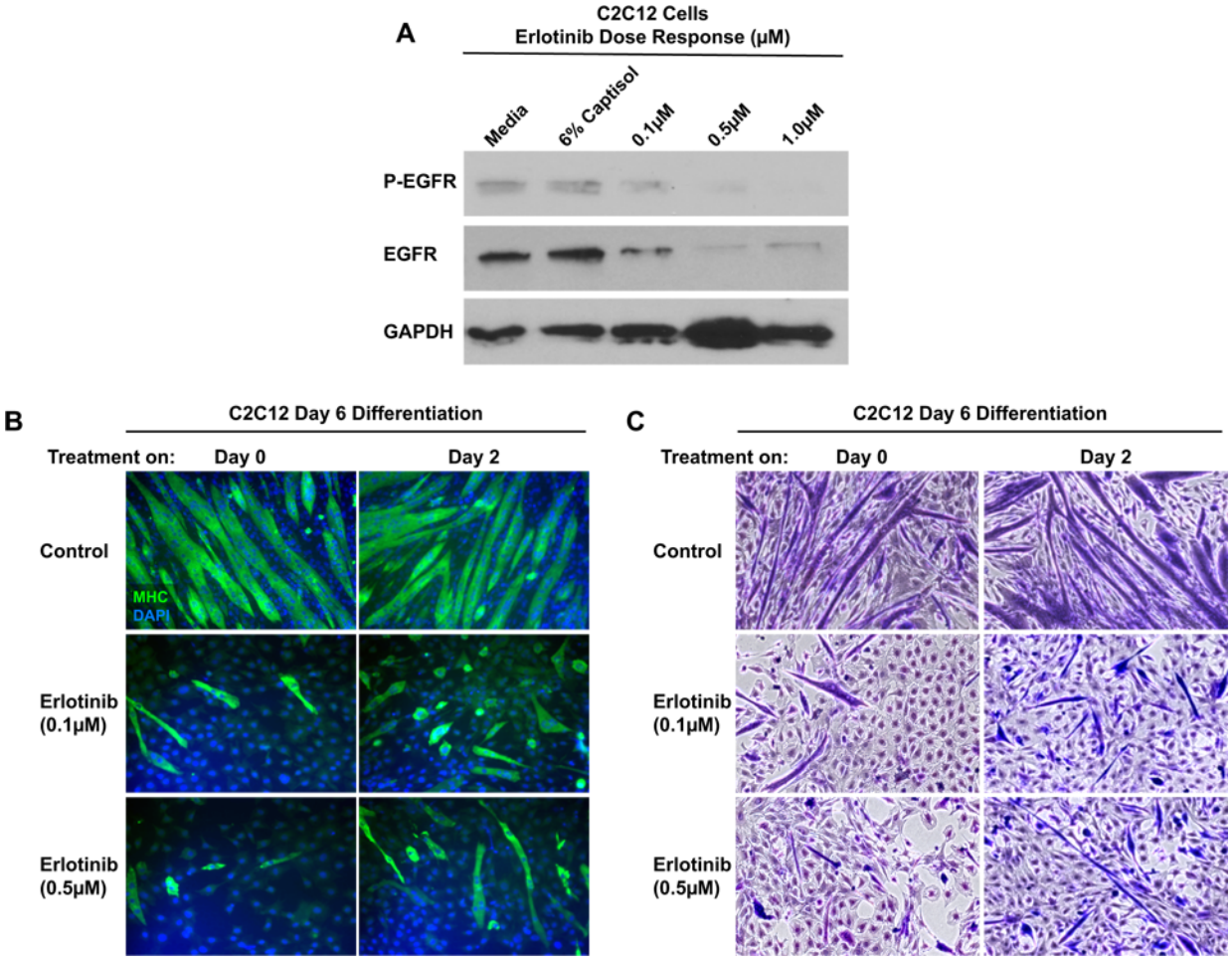


Figure 10: Blocking EGFR signaling in C2C12 hinders myoblast differentiation.

C2C12 cells seeded in differentiation media (low serum media) were treated with Erlotinib to test the effects of EGFR blockage on myoblast differentiation. (**A**) Different concentrations of Erlotinib were tested in C2C12 to asses for EGFR activity (P-EGFR) blockage by immunoblot analysis. Immunoblot analysis was performed on Erlotinib treated C2C12 lysates generated on day 6 of differentiation. Lysates were probed for anti-P-EGFR and anti-EGFR . GAPDH was used as the loading control. (**B and C**) C2C12 were seeded in differentiation media and treated with Erlotinib on either day 0 or day 2 of differentiation. Anti-MHC immunofluorescent staining (shown in green) and Crystal violet (purple images) were performed on day 6 of differentiation. DAPI nuclear stain shown in blue. Immunofluorescent (IF) images at 20x magnification and crystal violet (CV) images magnification at 10x. 6% Captisol was used as the vehicle control, labeled as control.

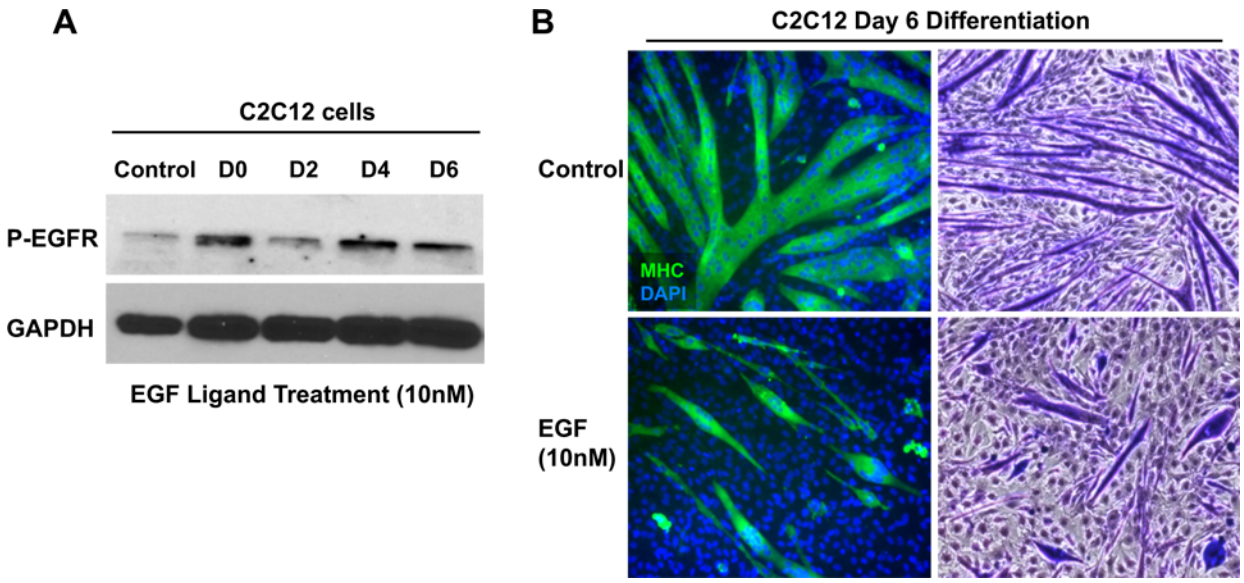


Figure 11: Prolonged activation of EGFR in C2C12 results in loss of myoblast differentiation and fusion.

C2C12 seeded and incubated in differentiation media were treated with 10nM of EGF ligand daily throughout a 6 day time period. (**A**) Cell lysates were generated on day 0, 2, 4, and 6 of differentiation and protein expression of P-EGFR was assessed via immunoblot staining using anti-PEGFR antibodies. GAPDH was used as the loading control. (**B**) Tube formation was observed to asses for the effects of continuous exogenous activation of EGFR on C2C12 myoblast differentiation and fusion. Crystal violet (purple images) and anti-MHC immunofluorescent staining (shown in green) were performed on day 6 of differentiation. DAPI nuclear staining shown in blue. PBS was used as control for EGF. Immunofluorescent (IF) images at 20x magnification and crystal violet (CV) images magnification at 10x.

Targeting EGFR in RMS: shRNA analysis

Having shown that EGFR signaling is misexpressed in RMS (Oncogenomics database), we next wanted to verify its expression in different RMS cell lines (both ERMS and ARMS). Immunoblot analysis showed several RMS cells (ERMS- RD, RH36, SMS-CTR and ARMS-RH30) to exhibit EGFR signaling. Human bronchial epithelial cell lines (HBEC) where used as a positive control since these cells are known to express activated levels of EGFR (Figure 12A). We next analyzed if EGFR signaling influences RMS pathogenicity by silencing its activity through the use of EGFR-shRNA. We began these studies by testing two different EGFR-shRNA constructs (F8 and F9) in RD cells (commonly used ERMS cell line) and observed reduced levels of EGFR signaling; F9 inducing the strongest silencing of the two (Figure 12B). Stable shRNA-RD cell lines were then generated and further assessed. We observed that EGFR silencing markedly reduced anchorage independent growth- assessed by colony formation analysis (Figure 12C). These data provided evidence that EGFR signaling was a oncogenic driver so we next wanted to test if EGFR misexpression plays any role in the disruption of myoblast myogenic programs. We tested this using our stable shRNA-RD cells and observed that EGFR silencing significantly increased myoblast differentiation and fusion, indicated by MHC-positive cells (Figure 12D and E). These results provided evidence that EGFR activity in RD cells not only drives oncogenicity but that its misexpression somehow also impedes cellular programs involved in myoblast differentiation for silencing EGFR signaling is sufficient to significantly counter oncogenic activity and rescue myogenic processes.

Moving into in vivo studies, we tested EGFR-shRNA RD cells in mouse xenograft models which exhibited significant decrease in tumor burden in EGFR-shRNA tumors compared to their GFP-shRNA controls (Figure 13A). Further analysis of tumor sections showed EGFR silencing in vivo increased differentiation, similar to our in vitro data, as well as decreased mitotic index (Figure 13B, C and D). These results suggest that EGFR is involved in RMS pathogenicity and silencing its activity hinders both oncogenicity in vitro and tumorigenicity in vivo.

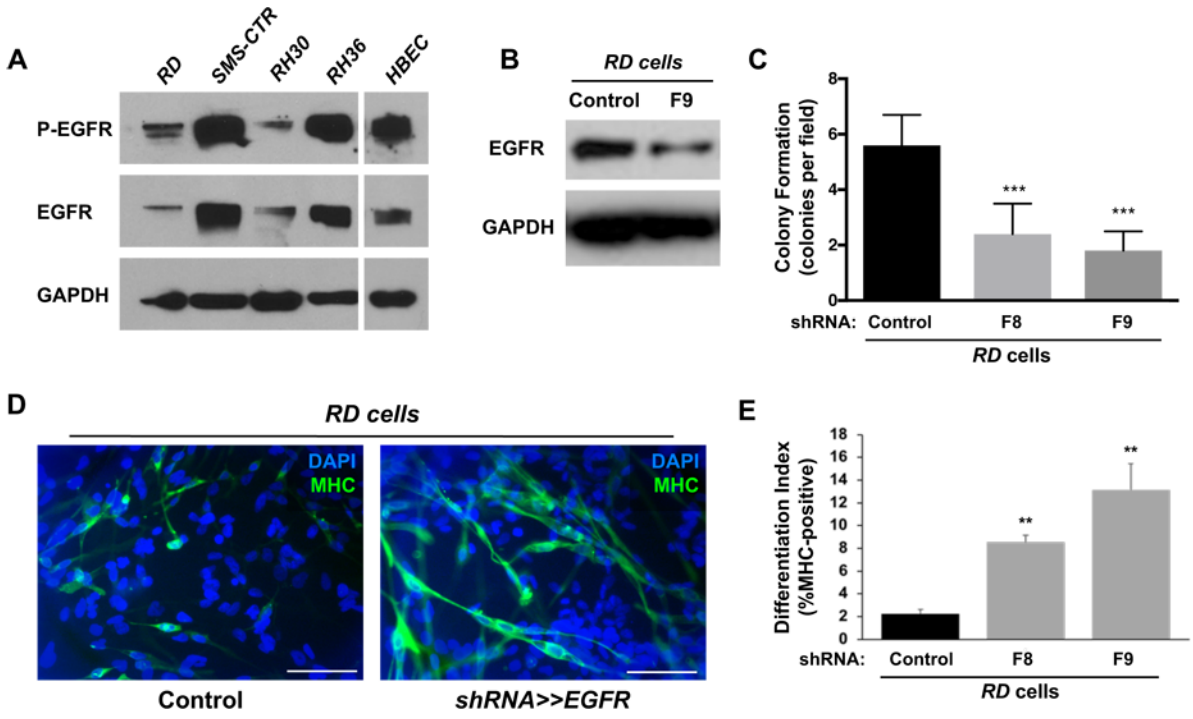


Figure 12: Silencing of EGFR activity decreases oncogenicity and rescues rhabdomyoblast differentiation.

Silencing of EGFR activity was tested in E-RMS cell line, RD. (**A**) Immunoblot analysis detecting EGFR activity (P-EGFR) and expression (total EGFR)in a panel of 4 different RMS cells lines (E-RMS: RD, SMS-CTR and RH36 and A-RMS: RH30). (**B-E**) RD cells stably transfected with shEGFR- F8 and shEGFR-F9 hairpins, or "control" GFP hairpin. (**B**) Confirming silencing of EGFR expression in RD cells via immunoblot analysis. GAPDH was used as the loading control. (**C**) EGFR-shRNA RD cells show decreased colony formation in soft agar. Shown are average number of colonies per 20×-objective field. (**D**) Representative images (see **Methods**) for RD cells expressing EGFR hairpins ("F8", "F9") or control GFP hairpin, stained with anti-MHC antibody (green) and DAPI (blue) and observed using immunofluorescent microscope. Images are at a 20X magnification. (**E**) Graphical representation of MHC percent index differentiation counts taken in shEGFR RD cells and control. . MHC = Myosin Heavy Chain. Scale bar = 100 mm. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$, $**P < 0.01$ versus Control. Figures B-E are kindly provided by our fellow technician Pooja Dalal.

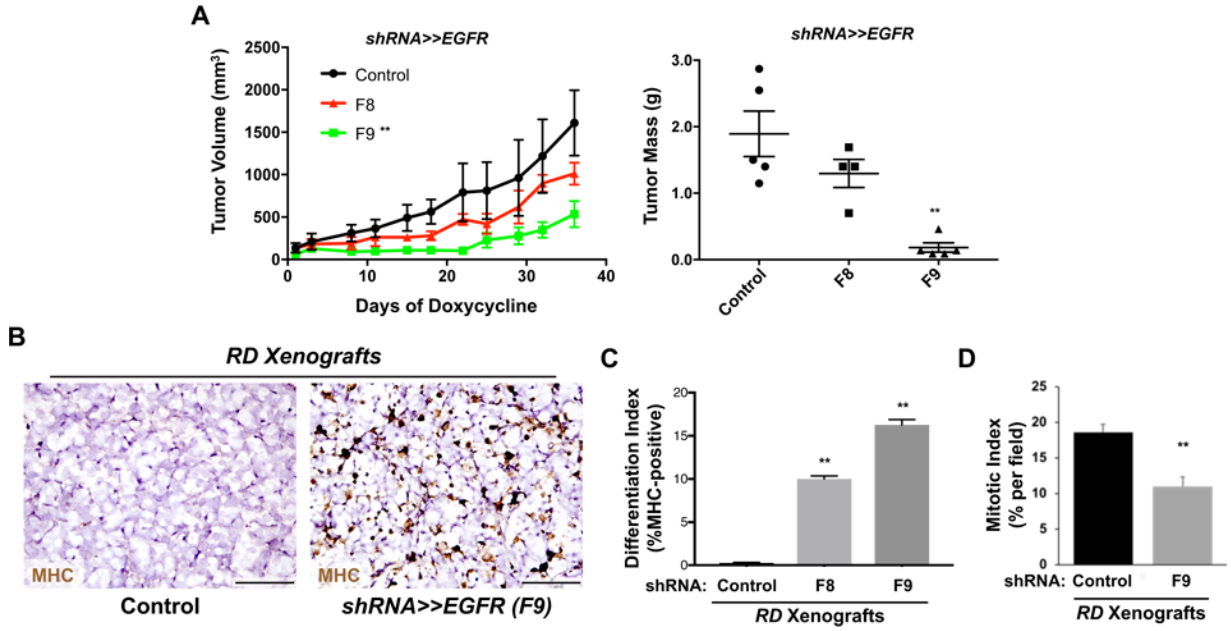


Figure 13: Silencing EGFR signaling reduces tumor burden in RD xenografts.

Testing the effects of silencing EGFR activity utilizing RD mouse xenograft model. (**A**) EGFR-shRNA RD xenograft tumors demonstrate a blockage in tumor progression and final tumor weights. Shown are growth plots for "Control" (shRNA-GFP) (n = 5) and EGFR shRNA-expressing ("F8", "F9") (F8, n = 4; F9, n = 5) xenograft tumors. (**B**) MHC immunohistochemistry (IHC) shows enhanced differentiation within EGFR-shRNA xenografts. Representative images of Control and F9 xenografts are shown. (**C**) Graphical representation of IHC images showing differentiation index counts. (**D**) Mitotic index of RD shEGFR and control samples were calculated based on counts done using IHC H&E stained tumor sections. MHC = Myosin Heavy Chain. Scale bar = 100 mm. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$, $**P < 0.01$ versus Control. These figures kindly provided by fellow technician Pooja Delal.

Targeting EGFR in RMS: Pharmacological EGFR inhibitors

With the availability of several pharmacological EGFR inhibitors, I wanted to extend these studies into one that was more preclinical and assess if blocking EGFR activity with different FDA-approved inhibitors would antagonize RMS. I tested two clinically available EGFR inhibitors, Erlotinib (a selective small molecule tyrosine kinase inhibitor) and Cetuximab (a humanized monoclonal antibody) [74] [90, 91] in three EGFR positive RMS cell lines (ERMS - RD and RH36 and ARMS – RH30). Before performing any experimental analyses, I tested for cell viability for each cell line against Erlotinib and Cetuximab, to confirm working concentrations used were not over IC_{50} values (Figure 14A and B), and verified working concentrations blocked EGFR activity which was observed by immunoblot analysis (Figure 15A). Similar to our shRNA results, I observed that treatment with either Erlotinib or Cetuximab significantly reduced cell proliferation and soft agar colony formation in culture in RD cells (Figure 15B and D). EGFR blockage also increased both MHC-positive differentiation and fusion indexes (Figure 15C). I next wanted to test if treatment with either of the EGFR inhibitors induced programmed cell death, since published studies have reported to detect cytotoxic cell death when assessing these inhibitors in other cancer cell lines [92]. I observed via TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) analysis, no detectable evidence of drug-induced apoptotic cell death (Figure 15E).

After testing pharmacological inhibitors in culture, I wanted to investigate if EGFR inhibitors hinder RMS progression in vivo. From these studies, I observed RD-xenografts treated with either Cetuximab or Erlotinib exhibited a significant decrease in tumor burden over time and final tumor weight measurements (Figure 16A and F), as well as, an increase in event-free survival

(Figure 16B and G). Immunoblot analysis showed significant reduction in EGFR activation (P-EGFR) in treated tumor lysates compared to vehicle controls (Figure 16C and H). Further examination of tumor sections from EGFR treated xenografts exhibited an increase in MHC-positive differentiated tissue (detected by IHC- immunohistochemistry) and a decrease in mitotic activity in comparison to vehicle control tumor sections (Figure16D, E, I and J). Of note, both RH36 and RH30 cell lines demonstrated to have no change in cell proliferation or rescue of differentiation in culture despite detecting EGFR activity(Figure 17A and B), which I will further discuss in the next chapter- Chapter 3: Blocking Akt signaling, an effective target in RMS.

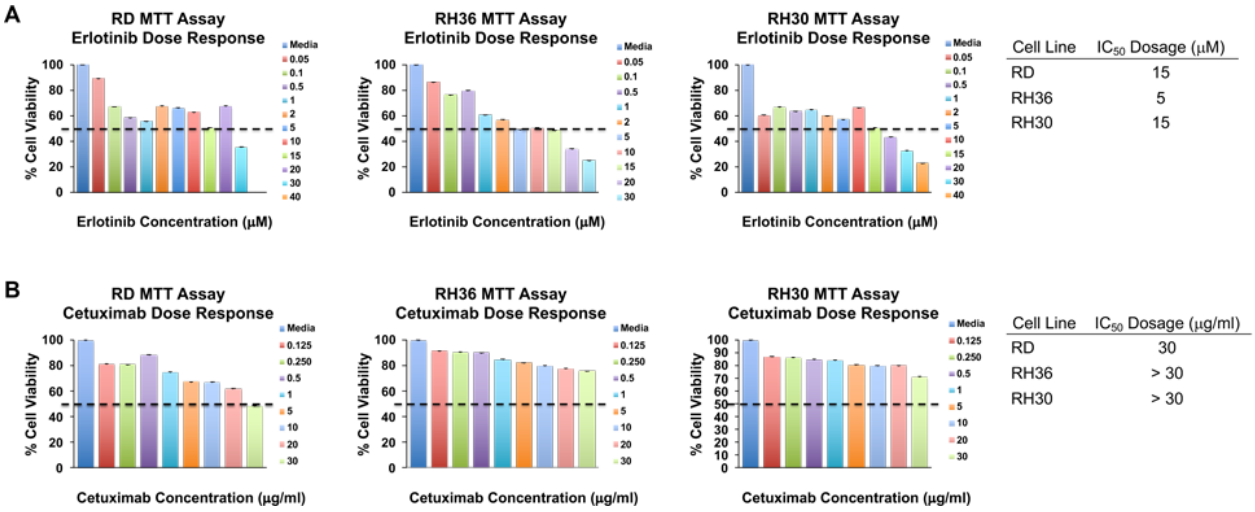
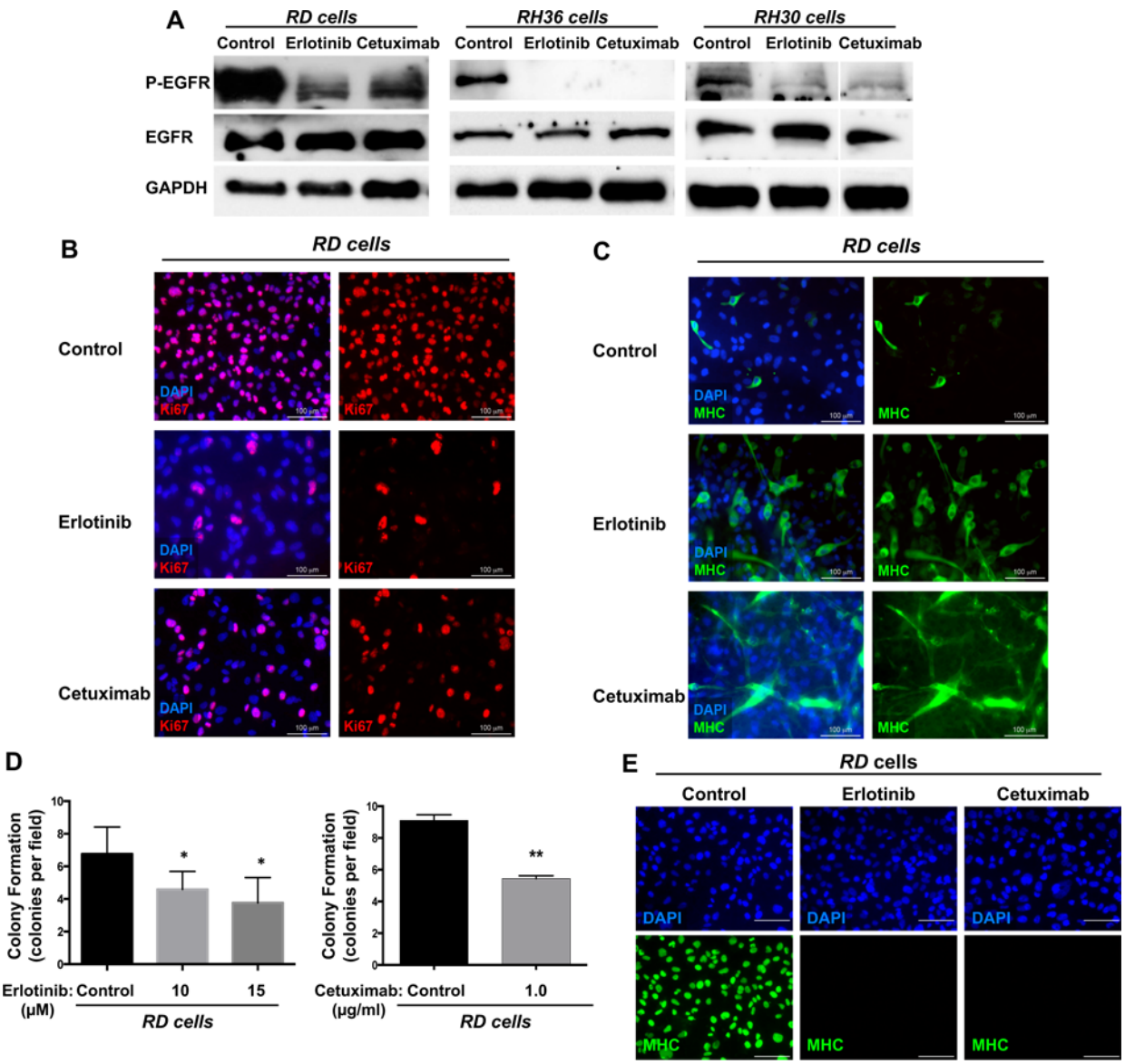


Figure 14: Viability of RMS cells when treated with EGFR inhibitors.

The viability of RMS cells at increasing concentrations of the EGFR inhibitors Erlotinib and Cetuximab (**A-B**) MTT assays for RD, RH36, and RH30 cells treated with Erlotinib (panel **A**), Cetuximab (panel **B**) Three replicate experiments were done for each concentration tested.


A
RD cells
RH36 cells
RH30 cells
Control Erlotinib Cetuximab
Control Erlotinib Cetuximab
Control Erlotinib Cetuximab
P-EGFR
EGFR
GAPDH
B
RD cells
Control
Erlotinib
Cetuximab
DAPI
Ki67
C
RD cells
Control
Erlotinib
Cetuximab
DAPI
MHC
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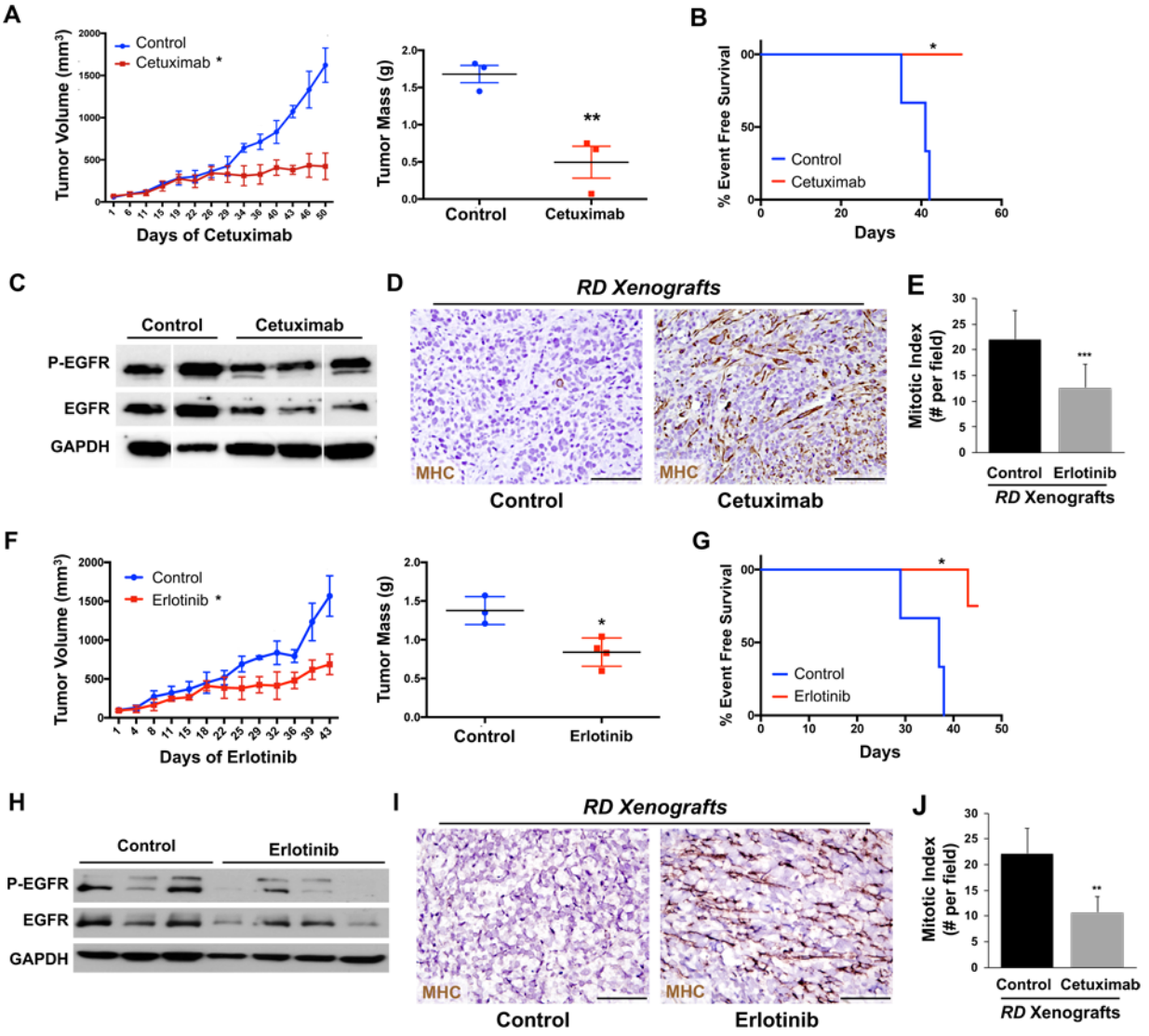
Colony Formation (colonies per field)
Erlotinib: Control 10 15
(μM)
RD cells
Cetuximab: Control 1.0
(μg/ml)
RD cells
E
RD cells
Control
Erlotinib
Cetuximab
DAPI
MHC

Figure 15: Pharmacological EGFR inhibitors significantly reduce oncogenicity.

The EGFR pharmacologic inhibitors Erlotinib and Cetuximab block oncogenicity in RD cells. (**A-E**) Erlotinib- or Cetuximab-treated RD cells. (**A**) Immunoblot analysis demonstrated EGFR Inhibitors, Erlotinib and Cetuximab, blocked EGFR activity (P-EGFR) in lysates generated from RMS treated cells. (E-RMS RD, RH36 and A-RMS RH30) (**B-C**) RD cells treated with EGFR inhibitors displayed a marked decrease in proliferation (Ki67- shown in red) and enhanced MHC-expression compared to vehicle controls (shown in green) detected via Immunofluorescent staining. DAPI nuclear stain shown in blue and images are at a 20x magnification. Representative images are shown. (**D**) EGFR blockage significantly hindered anchorage independent growth in RD cells assessed for colony formation in soft agar. Shown are average number of colonies per 20×-objective field. (**E**) TUNEL assay detected no evidence of drug induced cell death in RD cells treated with Erlotinib or Cetuximab. 6% Captisol was used for vehicle control in Erlotinib studies, whereas PBS was used as vehicle control for Cetuximab studies. For cell culture studies, Erlotinib concentration = 10 μM, Cetuximab concentration = 1 μg/mL. MHC = Myosin Heavy Chain. Scale bar = 100 mm. *P* values were calculated by two-tailed Student's *t* test: $^*P < 0.05$ versus Control.


A
Tumor Volume (mm³)
Control
Cetuximab *
Days of Cetuximab
Tumor Mass (g)
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% Event Free Survival
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Cetuximab
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Cetuximab
RD Xenografts

Figure 16: Pharmacological EGFR inhibitors antagonizes RMS tumorigenicity.

Treatment with pharmacological inhibitors Cetuximab or Erlotinib markedly decreases tumorigenicity in RD xenograft models.(**A-B and F-G**) Shown are assessment of tumor burden represented by tumor volumes ([L*W2]/2) measured over time, final tumor weight, and event-free survival (see **Methods**). Vehicle controls used were PBS and 6% Captisol for Cetuximab and Erlotinib experiment, respectively. Achieved Statistical Power for "tumor volume" and "event-free survival" analyses were 0.99 and 0.82, respectively. Erlotinib experiment: Vehicle control used was 6% Captisol. (**C and H**) Immunoblot analysis demonstrated lower expression levels of phosphorylated EGFR (P-EGFR) and total EGFR in Cetuximab/Erlotinib treated RD-xenograft tumor lysates in comparison to vehicle control. GAPDH used as loading control. (**D and I**) MHC immunohistochemistry shows enhanced differentiation within Cetuximab- and Erlotinib-treated xenografts. (**E and J**) Mitotic index were calculated based on counts done using IHC H&E stained tumor sections. Representative images of "Control" and Cetuximab/Erlotinib xenografts are shown. MHC = Myosin Heavy Chain. Scale bar = 100 mm. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

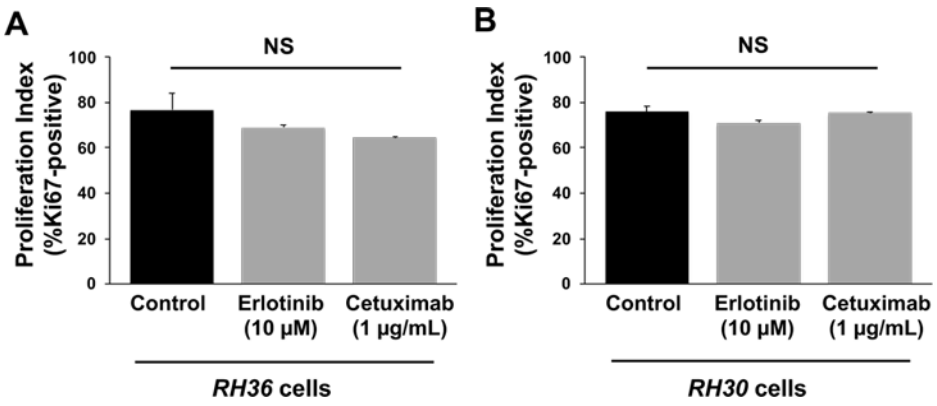


Figure 17: EGFR expressing RMS cells are non-responsive to EGFR inhibition.

RMS cell lines, RH30 and RH36, demonstrated no significant alteration in proliferation **(A and B**) Shown are the graphical representations of the proliferation indexes of both RH36 and RH30 cell treated with either Erlotinib or Cetuximab. Cells were then stained with anti-Ki-67 (Proliferation marker) and Images were captured using an immunofluorescent microscope. Vehicle "controls" for Erlotinib and Cetuximab are 6% Captisol and PBS, respectively. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

Probing to identify intracellular EGFR-pathway driving RMS pathogenesis

Having shown that blockage of EGFR activity hinders RMS pathobiology both in culture and in xenograft models, what remained unclear was what specific downstream pathway(s) were involved in EGFR driven RMS pathogenesis? To further elucidate the mechanism by which EGFR influences RMS pathogenesis, I probed for common EGFR intracellular signaling effectors: MEK/MAPK (mitogen-activated protein kinase- extracellular signaling-regulated kinase kinase/ mitogen-activated protein kinase), STAT3 (signal transducer ad activator of transcription) and Akt. It's important to note, that the RD cell line used for these studies carry an oncogenic N-RAS mutation [NRAS_183A>T (Q61H)], alluding that another EGFR- signaling arm is involved other than the RAS→MEK→MAPK axis. I performed immunoblot analysis on RD cells treated with EGFR inhibitors, Erlotinib or Cetuximab, and observed significant downregulation in AKT activity (Figure 18A). No changes in activity were detected in either MEK/MAPK or STAT3, suggesting that Akt signaling is involved RMS progression (Figure 18B-D).

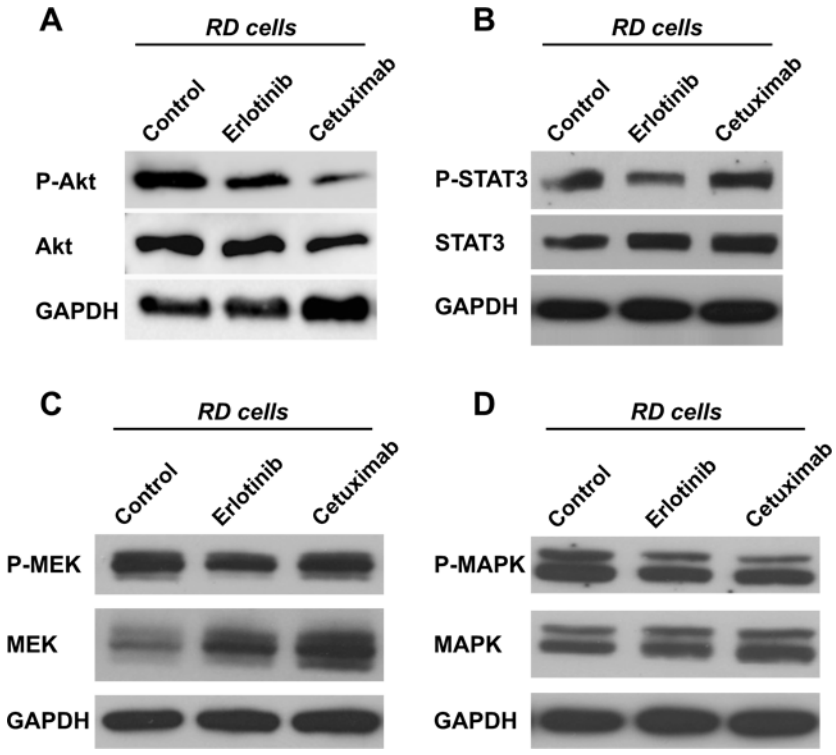


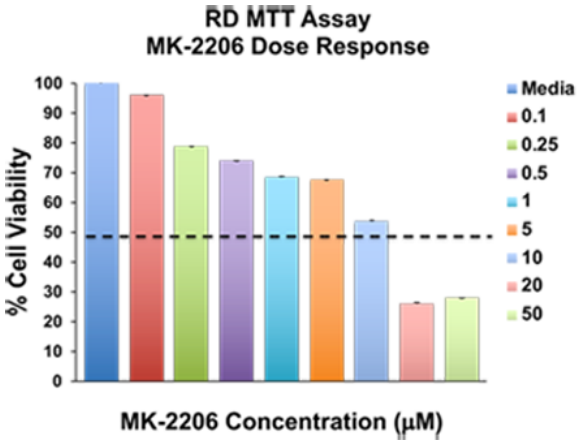
Figure 18: Akt activity identified as a signaling pathway involved in EGFR-driven RMS.

Treatment with pharmacological inhibitors Erlotinib or Cetuximab blocks Akt activation in RD cells. (**A-D**) Immunoblot analysis probing for intracellular pathways downstream of EGFR demonstrated lower expression levels of phosphorylated Akt (P-Akt) in lysates generated from RD cells treated with either Erlotinib or Cetuximab in culture, but no changes were observed in STAT3 or MEK/MAPK signaling. GAPDH used as loading control. Vehicle "controls" for Erlotinib and Cetuximab are 6% Captisol and PBS, respectively.

Validating Akt signaling as a critical pathway involved in RMS

To further interrogate the putative role of Akt activity in RMS, I treated RD cells with MK-2206, an allosteric pan-Akt inhibitor. I assessed for cell viability in RD cells treated with MK-2206 to verify working concentrations were not over IC_{50} values, (Figure 19) and confirmed RD-treated cells exhibited blockage of Akt activation (Figure 20A). Similar to my EGFR inhibitor results, MK-2206 treated cells significantly reduced cellular proliferation and significantly hindered colony formation in culture (Figure 20B and C). TUNEL analysis verified there to be no detection of MK-2206-induced apoptotic cell death (Figure 20D).

Finally, I extended these studies in vivo and tested MK-2206 in RD-xenografts. I observed Akt inhibition significantly reduced tumor burden, final tumor weights and also increased overall event-free survival (Figure 21A and B). In order to examine the tumors further I generated tumor lysates and probed for Akt signaling. Lysates from MK-2206 treated tumors exhibited a decrease in Akt activity in comparison to vehicle control lysates (Figure 21C). I also observed, when compared to vehicle control tumors, a diminish in Ki67 positive cells within tumor sections of MK-2206 treated mice (Figure 21D). It is important to note, in both my MK-2206 in vitro and in vivo analysis, I was not able to detect significant levels of rescue of myoblast differentiation in culture or in vivo, as was observed in the EGFR treated experiments, which I address in the next coming experiments (Figure 22A and B). Taken together, these results support the notion that Akt activity is a key intracellular effector involved in EGFR-driven RMS.



Cell Line	IC_{50} Dosage (μM/ml)
RD	10

Figure 19: Viability of RD cells when treated with MK-2206.

The viability of RMS cells at increasing concentrations of Akt inhibitor MK-2206. MTT assays for RD cells treated with MK-2206. Three replicate experiments were done for each concentration tested.

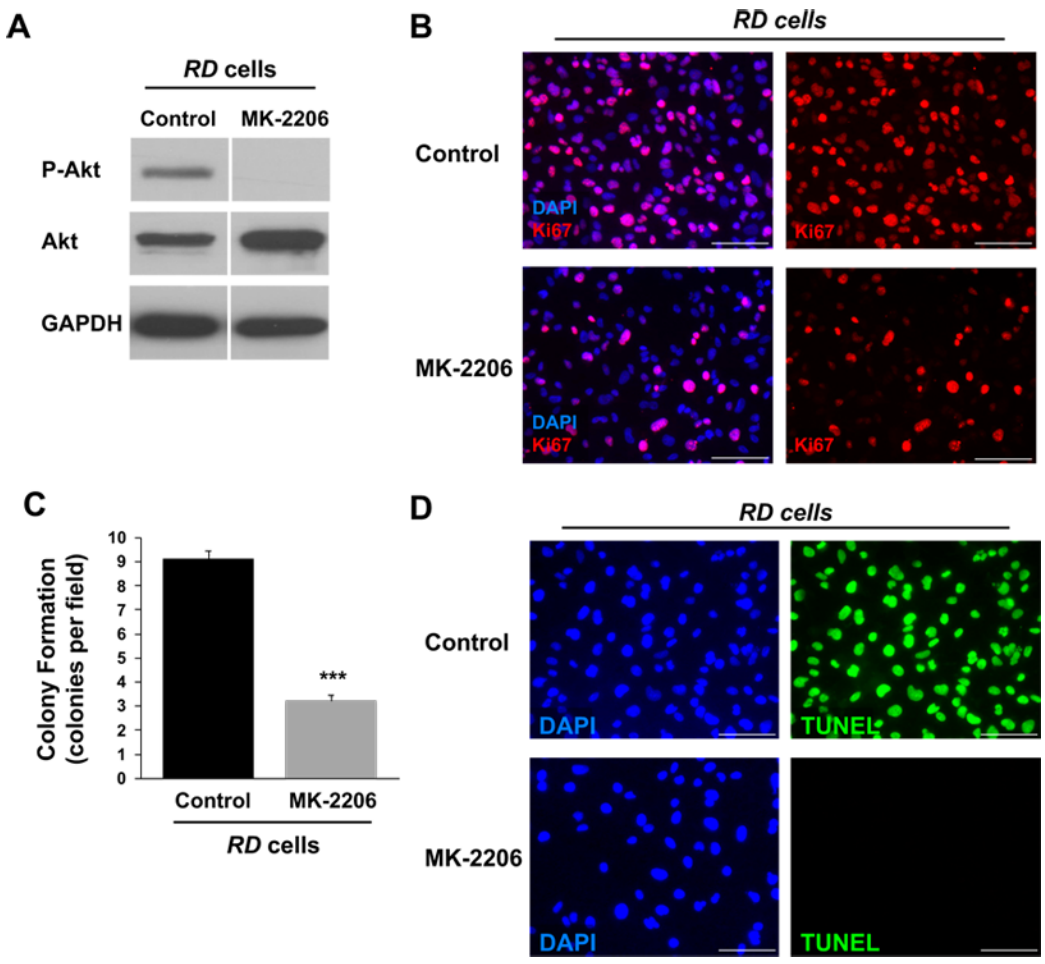


Figure 20: Pan-Akt inhibitor MK-2206 hinders RD pathogenicity in culture.

Inhibiting Akt activity is sufficient in blocking RD cell oncogenicity. (**A**) Immunoblot analysis confirming treatment with MK-2206 in RD cells blocks Akt activation (P-Akt). GAPDH used as loading control. (**B**) Immunofluorescent images of RD cells stained with proliferative marker anti-ki67 (red) showed MK-2206 treatment markedly reduced the number of proliferating cells. Representative immunofluorescent images of Control and MK-2206 treated RD cells are shown. Dapi nuclear stain shown in blue. Images taken at 20x magnification. (**C**) Anchorage independent growth was significantly decrease in RD cells treated with MK-2206 which was measured by the number of colonies formed per field in soft agar. Shown are average number of colonies per 20x-objective field. (**D**) There was no evidence of detectable MK-2206 induced cell death in RD cells tested using TUNEL assay. DMSO used as Vehicle "Control" for MK-2206. Scale bar = 100mm. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

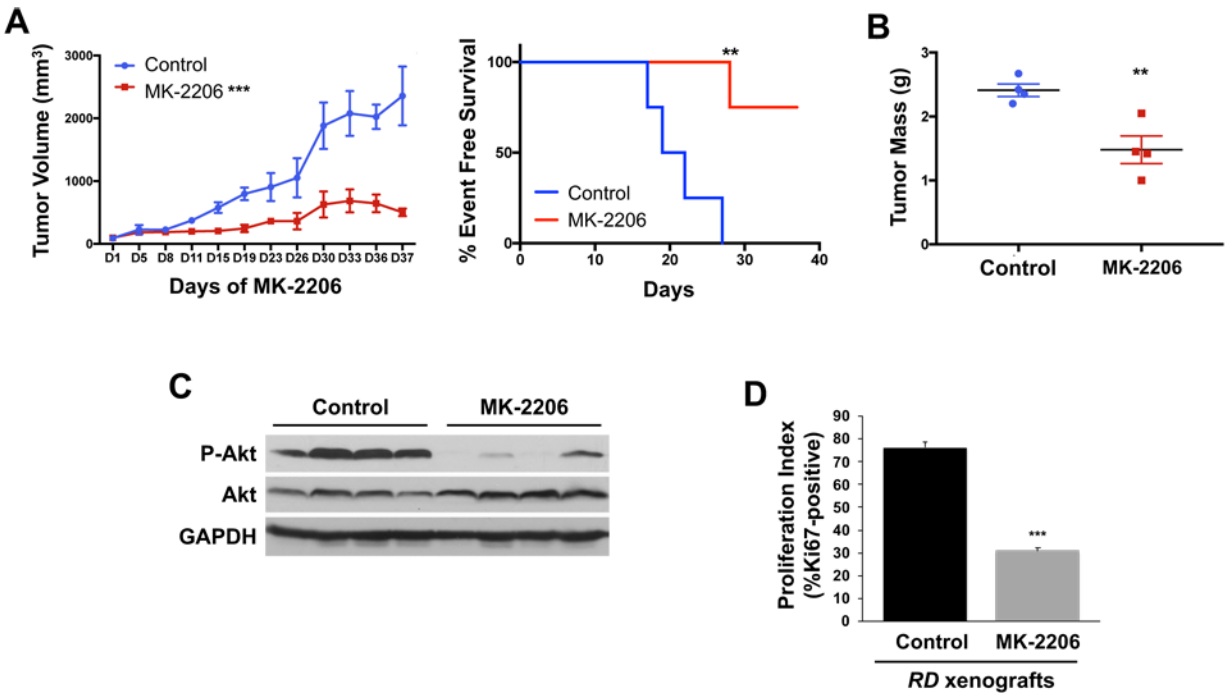


Figure 21: Blocking Akt activity significantly decreases tumorigenesis in RD xenografts.

Treating mice with MK-2206 significantly blocked tumorigenicity in RD xenograft model. Inhibiting Akt activity is sufficient in blocking RD cell oncogenicity. **A and B**) Shown is the assessment of tumor burden represented by tumor volumes ([L*W2]/2) measured over time, final tumor weight, and event-free survival (see **Methods**) plots for RD mouse xenograft models treated with MK-2206 (180mg/kg, oral gavage, 3 times/week). 30% Captisol was used as vehicle "control" in these experiments. (**C**) Immunoblot analysis demonstrated lower expression levels of phosphorylated Akt (P-Akt) in lysates generated from fresh MK-2206 treated RD-xenograft tumor samples in comparison to vehicle control tumor lysates. GAPDH used as loading control. (**D**) Shown is the graphical representation of Ki67 immunohistochemistry done on RD-xenograft tumor sections. MK-220 treatment demonstrated to significantly decrease cell proliferation (decrease Ki67 positive cells) within the tumor sections of MK-2206 RD-xenografts in comparison to their vehicle controls. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

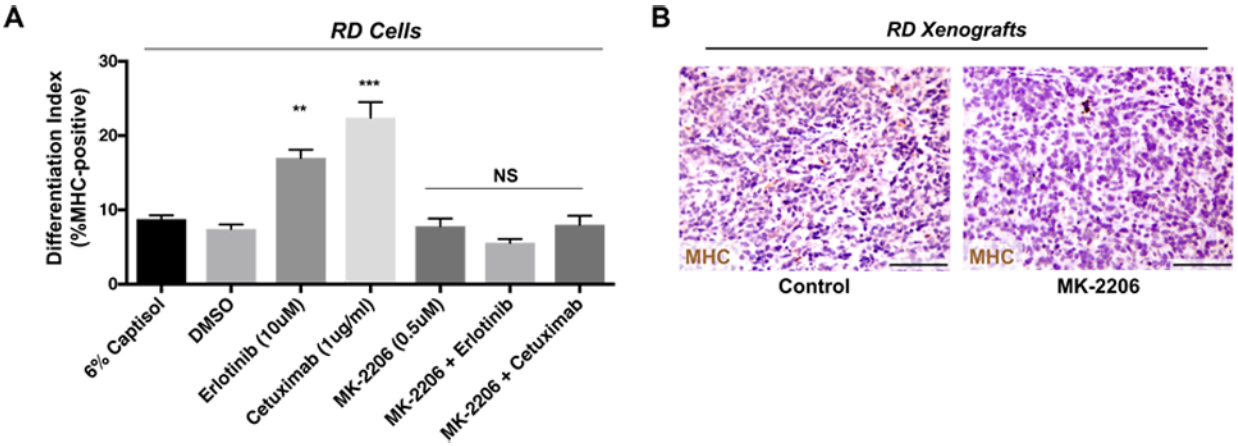


Figure 22: Akt signaling inhibition fails to rescue rhabdomyoblast differentiation.

No significant differences in rhabdomyoblast differentiation was observed in MK-2206 treated RD cells in culture or in RD xenograft models. (**A**) Graphical representation of MHC percent index differentiation counts demonstrated MK-2206 treatment demonstrates no significant increase in MHC positive expressing rhabdomyoblast in comparison to the vehicle controls. Counts were taken from MHC-stained immunofluorescent images of RD cells treated with EGFR inhibitors (Erlotinib or Cetuximab), or MK-2206 as well as various combinations. MHC = Myosin Heavy Chain. (**B**) MHC immunohistochemistry shows little to no differentiation within MK-2206-treated xenograft tumor sections. Representative images of "Control" and mk-2206 xenografts are shown. Vehicle control used for MK-2206 in culture and in the xenograft studies were DMSO or 16% Captisol, respectively. Scale bar = 100 mm. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$, $**P < 0.01$ versus Control.

Elucidating the role of Akt isoforms in RMS cells

Studies investigating the role of Akt in different malignancies have further identified specific individual Akt isoforms driving cancer progression. However, this remains unclear if a specific Akt isoforms influences RMS. Within the EGFR studies, I have shown that blocking EGFR-Akt signaling antagonized RMS pathogenicity, though only EGFR inhibition was able to rescue myoblast differentiation/ fusion. In order to better understand these results, I began to dig a little deeper into the literature and found that Akt comprises of three different isoforms Akt 1, 2, and 3. Interestingly, there has been several published reports demonstrating the role of Akt isoforms 1 and 2 during myogenesis. The expression levels of Akt 1 and 2 have been detected in skeletal muscle tissue, though recent reports have identified Akt2 expression as the predominant isoform expressed [93]. Most reports have primarily focused on Akt isoforms 1 and 2, for levels of Akt3 measure to be undetectable in skeletal muscle tissue [93]. In these reports, they identified Akt isoforms 1 and 2 to have explicit separate roles during the stages of myoblast differentiation; Akt 1 primarily functions in the earlier stages of myogenesis regulating cellular proliferation, whereas Akt 2 is involved in promoting cell cycle arrest and initiating myoblast differentiation [94]. These findings indicated that in order for myoblast to undergo myogenesis the activation of Akt isoforms 1 and 2 are essentail, for the loss of their activity would result in impaired myoblast differentiation. Thus, I hypothesized that treatment with pan-Akt inhibitor MK-2206 failed to induce rescue of myoblast differentiaton due to the inhibition of essential Akt isoforms required during myogenesis.

To test this hypothesis, I used shRNA constructs specific for either Akt 1 or 2 to further characterize the explicit role of each isoform in RMS (Figure 23A). Silencing of Akt1 in RD cells

resulted in a significant rescue of myoblast differentation whereas, Akt2 silencing showed little resuce of differentiaton and was comparable to control levels (Figure 23B and C). The data from these studies demonstrated that Akt2 activity is important for rhabdomyoblast differentiation, where as Akt1 acitivity seems to be dispensable, supporting distinct roles for Akt1 and Akt2 activity during myogenesis. These results suggests that Akt 1 activity influences the inablilty of RMS to differentiate and silencing of Akt1 is sufficient to rescue rhabdomyoblast differentiation.

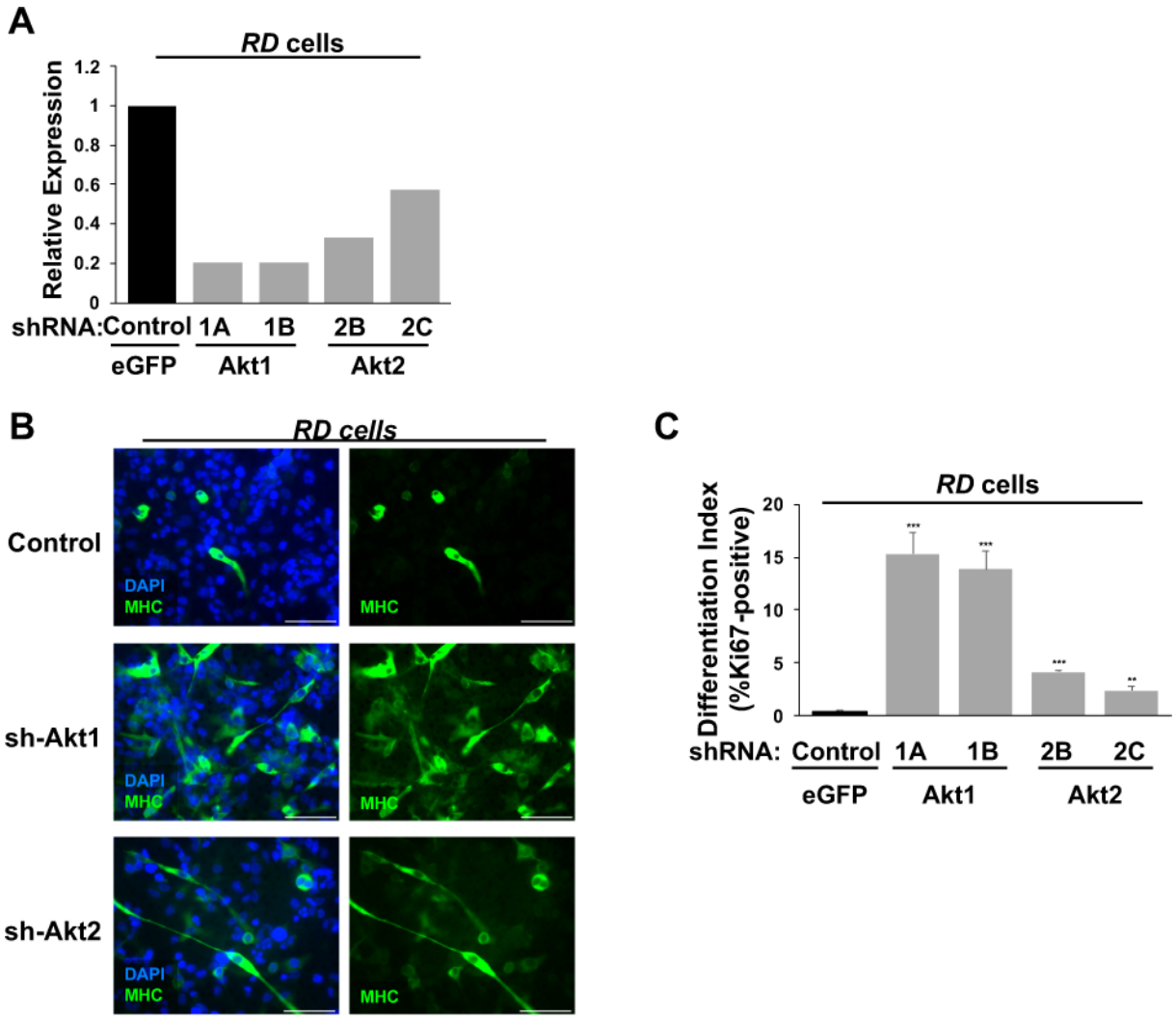


Figure 23: Silencing of Akt1 activity rescues rhabdomyoblast differentiation.

Silencing of Akt1 preferentially rescues RMS cell MHC-positive terminal differentiation-arrest. (**A**) Relative expression levels of Akt1 and Akt2 in control, shRNA-Akt1, and shRNA-Akt2 cells. (**B**) Akt1-silenced RD cells exhibited a marked rescue of rhabdomyoblast differentiation when compared to control. RD cells expressing shRNA against GFP ("Control"), Akt1, or Akt 2 are shown stained with MHC (Green) antibody. DAPI nuclear stain shown in blue. Representative images taken at 20x magnification are shown. (**C**) Graphical representation of MHC+ differentiation indexes from immunofluorescent images. *P* values were calculated by two-tailed Student's *t* test: $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control.

Summary

Through our PAX-FOXO1 *Drosophila* models we identified EGFR signaling to be misexpressed and verified elevated expression in both RMS patients samples and in tested RMS cell lines. Mammalian wild-type studies demonstrated that EGFR activity is involved in mediating myoblast differentiation and fusion. When I tested EGFR inhibitors, Erlotinib and Cetuximab, I observed a significantly decreased in both oncogenicity and tumorigenicity in RMS cells. EGFR inhibition also rescued RMS-myoblast differentiation, providing evidence that disrupted myogenic networks is a mechanism that triggers RMS pathogenesis. Further analysis, verified Akt signaling as a key intracellular signaling pathway involved in EGFR driven RMS progression. Additionally, I also uncovered that targeting of isoform Akt 1 is sufficient to induce RMS myoblast differentiaton.

Chapter 3

Blocking Akt signaling, an effective target in RMS

Introduction

EGFR resistance in RMS cell lines

Within the literature, several cases of intrinsic or acquired resistance to EGFR inhibitors in EGFR misexpressed tumors are reported (e.g. Non-small cell lung carcinoma [NSCLC], breast cancer and colon cancer) [95-97]. Under these circumstances, treatment for patients becomes problematic resulting in unfavorable results. This in turn has become one of the most common challenges faced with EGFR inhibitors in the clinic. EGFR inhibitor resistance can occur in tumors bearing either a constitutive EGFR-activating mutation or misexpression of EGFR due to gene amplification/ over-expression. In most cases the molecular heterogeneity found within tumors is the mechanism of resistance [97]. Resistance of EGFR inhibitors can also be observed in culture, as some cancer cell lines do not respond to treatment as those sensitive to EGFR inhibitors. Within these EGFR studies I came across two RMS cells lines (RH30 and RH36) that did not respond to either RTK inhibitor Erlotinib or human monoclonal antibody Cetuximab (Reference Figure 17A and B), irrespective of their expression of EGFR signaling observed. (Reference Figure 15A) As previously stated, treatment with either inhibitor significantly reduced EGFR signaling in all tested cell lines, ruling out this as a possible factor. (Reference Figure 15A) So, in order to investigate the mechanism of EGFR- resistance, I next investigated possible alternative pathways that could be mediating cellular oncogenicity in RH30 and RH36 RMS cell lines.

Alternative signaling pathways in EGFR inhibitor resistant cell lines

As I aimed to elucidate the mechanism of EGFR inhibitor resistance in these RH30 and RH36 RMS cell lines, I further examined these cell lines to identify any other misexpressed signaling networks that could be activated and utilized as an alternative pathway to bypass EGFR inhibition. I discovered that both RH30 and RH36 to be very heterogeneous cell lines. In recent publications, genomic sequencing analysis detected a number of different genetic aberrations in both RH30 and RH36, though the one that caught our interest was the dysregulation of another RTK- Fibroblast Growth Factor Receptor 4 (FGFR4). Genomic sequencing data displayed ERMS cell line, RH36, to possess a FGFR4 activating mutation [FGFR4_1648G>C (V550L)] whereas in fusion gene positive ARMS cells, RH30, FGFR4 is a direct gene target of PAX3-FOXO1 leading to its misexpression [98-100].

FGFR4 is another member of the RTK family and similar to EGFR, has also been shown to advance the progression of several different malignancies such as breast cancer, non-small cell lung carcinoma, and even in a subset of RMS cases [101-103]. Oncogenic FGFR4 signaling alterations have been reported in both ARMS and ERMS. Some of the mechanisms involved in driving the dysregulations of FGFR4 signaling in RMS include, direct targeted transcription by fusion protein *PAX3-FOXO1* [104], ERMS localized gene amplification [105] or acquired missense activating mutation - 7.5% of cases reported [103, 106]. Studies testing blockage of FGFR4 activity in RMS cells (e.g., FGFR4 inhibitor ponatinib), observed antitumor responses both in vitro and in vivo, though cell lines carrying a FGFR4 mutations displayed a much more significant sensitivity level than those with wild-type FGFR4, limiting the number of RMS patients eligible for anti-FGFR4 inhibition [106, 107]. Reports similar to these, indicate FGFR4 as a

potential therapeutic target, however, results from early clinical testing of several pan-FGFR4 on other FGFR4-dependent malignancies entail that these inhibitors could have low efficacy due to selectivity concerns, ultimately delaying RMS-FGFR4 clinical testing [108].

RTK mutation are very common in cancer and are known to be one of the most frequently mutated gene families, though gene amplification/ protein overexpression has also been detected [109, 110]. RTK members are involved in regulating parallel cellular processes and do so by activating common downstream signaling pathways, so it's possible that the inhibition of one could be bypassed and compensated by the activation of another. As both of our EGFR-inhibitor resistant cell lines tested, RH30 and Rh36, carry a form of dysregulated FGFR4 expression, could this be the signaling mechanism of resistance?

Akt signaling, critical downstream pathway in RTKs.

RTKs are responsible for initiating downstream signaling transductions that regulate cell survival, growth and proliferation upon ligand-receptor binding. Common pathways activated are the following, RAS>MAPK, JAK>STAT3, PI3K> Akt [111-113]. Each of these intracellular signaling arms have demonstrated to play critical roles in several malignancy transformation and have under gone an extensive amount of investigation as potential targetable candidates [4, 114]. In the previous chapter, I observed that inhibition of EGFR activity in RD cells only effected the activity level of Akt, but had no effect on the either the RAS>MAPK or JAK>STAT3 networks, suggesting Akt signaling served as a critical downstream pathway mediating EGFR driven RMS pathogenicity. (Reference Figure 18A) This was further demonstrated testing Pan-Akt inhibitor,

MK-2206, in both cell culture and mouse xenograft studies. (Reference Figure 20 and 21)

As these data give us insight to the intracellular networks that influence RMS, I further tested EGFR-inhibitor resistant cell lines in order to identify the signaling pathway(s) responsible for mediating inhibitor resistance. I first probed for Akt signaling in EGFR inhibitor treated RH30 and RH36 cells as I previously identified Akt as a key pathway downstream of EGFR. Interestingly, I detected no visible reduction in Akt activity in both RH30 and RH36 cells when treated with either Erlotinib or Cetuximab, even though a reduction of EGFR signaling was observed (Figure 24A and B). A possible explanation for this, could be due to the misexpression of FGFR4 signaling identified in both RH30 and RH36 cell lines; a potential alternative pathway utilized to compensate for EGFR inhibition that also mediates the activation of Akt signaling. From these studies, I hypothesized that since RMS cells lines that are non-responsive to EGFR inhibitors are driven by multiple RTKs (EGFR and FGFR4), then targeting a key downstream pathway that is common between the two transmembrane receptors and also demonstrated to influence RMS, Akt signaling, could be a testable method to overcome EGFR resistance.

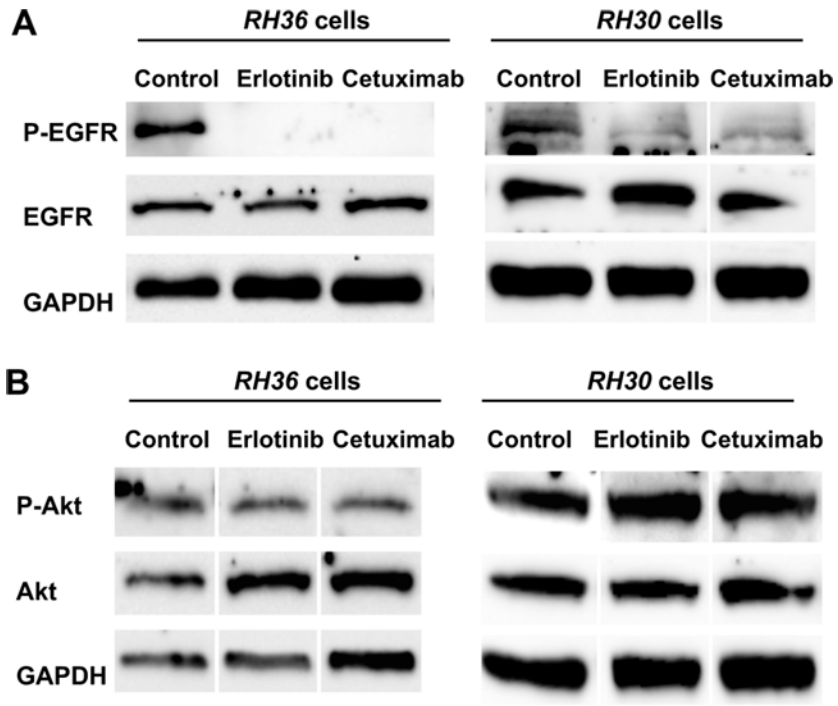


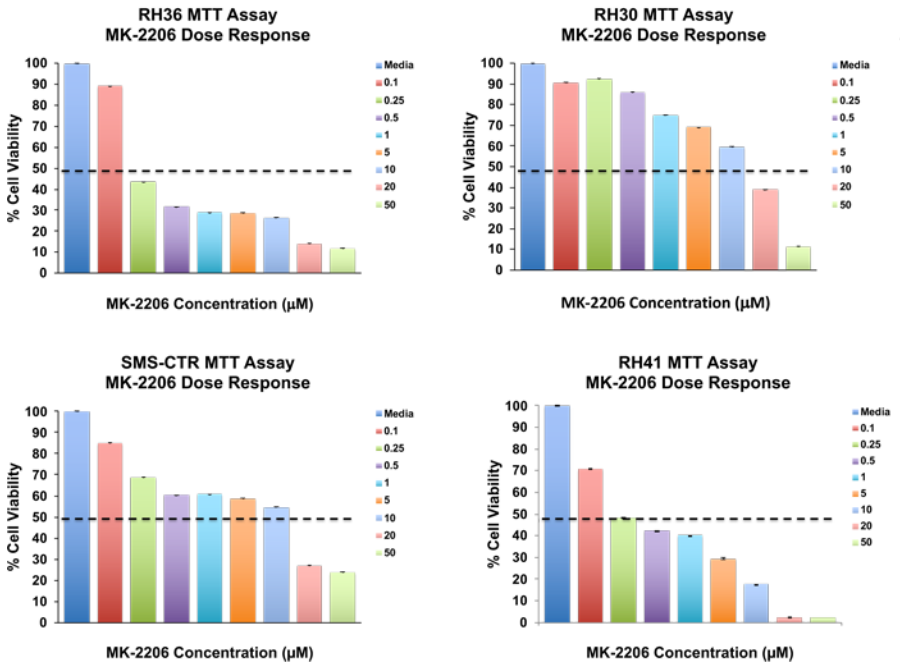
Figure 24: RMS cells non-responsive to EGFR inhibitors exhibit no reduction in Akt activity.

Treatment with pharmacological inhibitors Erlotinib or Cetuximab does not block Akt activation expression levels in EGFR inhibitor non-responsive RMS cells lines Rh36 and RH30. (**A and B**) Immunoblot analysis probing for EGFR and it's downstream intracellular pathway Akt, exhibited no visible reduction in expression levels of phosphorylated Akt (P-Akt) when treated with either Erlotinib or Cetuximab. Cell lysates were generated from Erlotinib or Cetuximab treated RD cells in culture. GAPDH used as loading control. Vehicle "controls" for Erlotinib and Cetuximab are 6% Captisol and PBS, respectively.

Results

EGFR inhibitor resistant/sensitive RMS cell lines driven by Akt signaling

For these studies, I wanted to assess if the failure to diminish levels of Akt activity in non-responsive EGFR inhibitor RMS cells, RH30 and RH36, which are driven by another RTK-FGFR4, could be the mechanism of EGFR-inhibitor resistance? In order to test for this, I treated EGFR- inhibitor resistant cell lines with the pan-Akt inhibitor, MK-2206, and examined the effects Akt inhibition had on oncogenicity in culture. In addition to these studies, I included two additional RMS cell line, EGFR positive SMS-CTR (ERMS) and RH41(ARMS). I first verified working concentrations of MK-2206 were not over IC50 values for each cell line (assessed for cell viability using MTT analysis) (Figure 25) and observed that MK-2206 treatment significantly blocked Akt signaling in comparison to vehicle controls and EGFR inhibitor treatments in all four cell lines, RH30, RH36 and SMS-CTR and RH41 (Figure 26A-D).



Cell Line	IC_{50} Dosage (μM)
RH36	0.25
RH30	15
SMS-CTR	10
RH41	0.5

Figure 25: Viability of RMS cells when treated with MK-2206.

The viability of RMS cells at increasing concentrations of Akt inhibitor MK-2206. MTT assays for RH36, RH30, SMS-CTR, and RH41 cells treated with MK-2206. Three replicate experiments were done for each concentration tested.

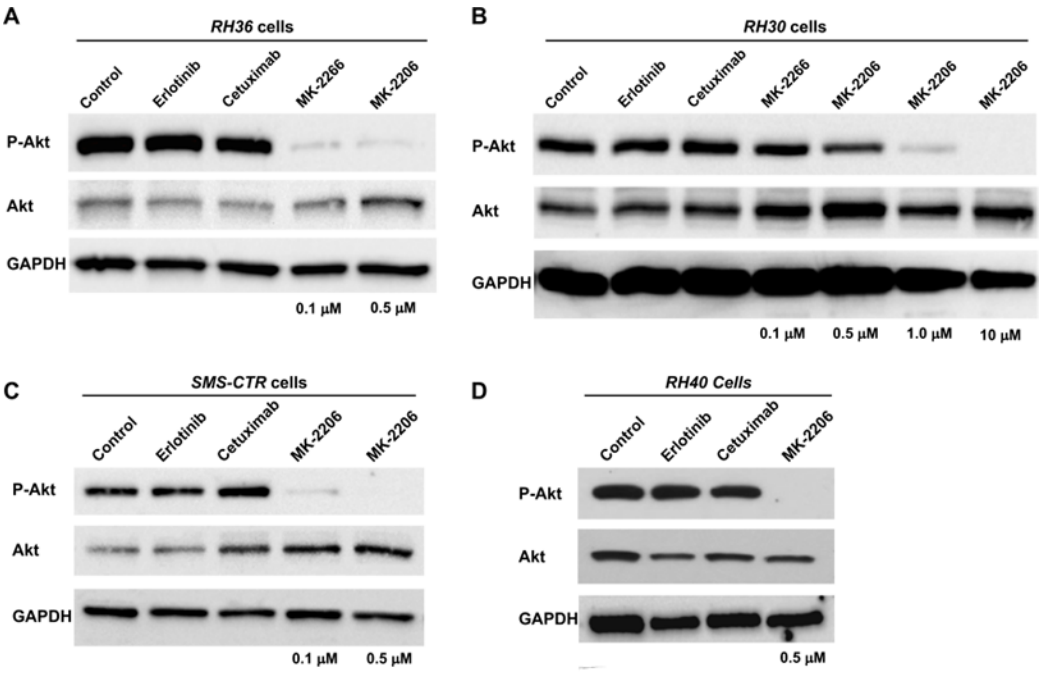


Figure 26: MK-2206 treatment effectively inhibits Akt activation in all ERMS and ARMS tested.

Treatment with pan-Akt inhibitor, MK-2206, blocks Akt activation in all 4 cell lines tested (RH36, RH30, SMS-CTR, and RH40. (**A-D**) Immunoblot analysis probing for activated and total Akt detected a significant decrease in the expression levels of phosphorylated Akt (P-Akt) when treated with MK-2206. Different doses of MK-2206 were used in some cell lines to verify dose dependent blockage of inhibitor. GAPDH used as loading control. Vehicle "controls" for MK-2206 used was DMSO.

Targeting Akt activity blocks RMS cell oncogenicity

In culture, treatment with MK-2206 in all three EGFR-inhibitor resistant cell lines (RH30, Rh36 and SMS-CTR), as well as the tested ARMS cell line RH41, exhibited a marked decrease in cell proliferation and in anchorage-independent growth in RH30 and SMS-CTR cells (soft-agar colony formation assay) – Note RH36 and RH41 cells do not form adequate colonies so no colony formation experiments were performed using these cell lines (Figure 27A and B). Additionally, having tested MK-2206 in RH30 cells and observing significant reduction in proliferation and colony formation, I wanted to take this opportunity and further evaluate the effects of Akt inhibition on cellular metastasis/migration. RH30 cells are derived from a patient diagnosed with metastatic ARMS, making this an ideal cell line to further examine the effects of Akt inhibition on metastasis [115]. In order to assess for metastasis/migration, I performed a scratch assay, also referred to as wound healing assay, that tests the effects of cell migrations. RH30 cells treated with MK-2206 for 48 hours, displayed to have a reduction in cell migration in comparison to vehicle controls (Figure 28). Though this assay was not quantified, it provided preliminary data supporting that Akt blockage inhibits RH30 cell metastasis by decreasing cell migration. Overall, I detected that all three RMS cell lines, which were not responsive to EGFR inhibitors, now displayed a significant reduction in oncogenicity upon inhibition of Akt signaling in vitro.

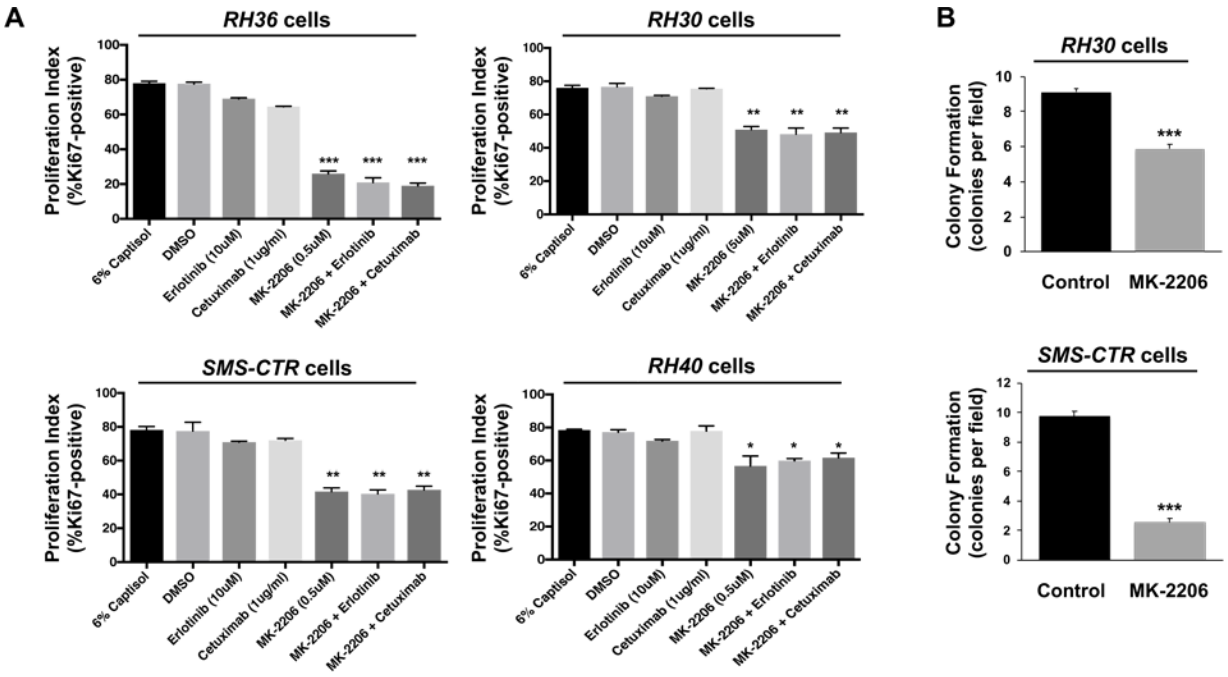


Figure 27: MK-2206 antagonizes oncogenicity in ERMS and ARMS cells.

Inhibition of Akt activity significantly reduced cell proliferation and colony formation in tested RMS cell lines. (RH36, RH30, SMS-CTR and RH40). (**A**) Graphical representation of percent proliferation index counts demonstrate marked reductions in RMS cells treated with MK-2206. Combo treatments of EGFR inhibitor Cetuximab or Erlotinib with MK-2206 showed no difference in proliferation counts in comparison to MK-2206 treatment alone. RMS cells were stained with anti-Ki67 and pictures were captured using an immunofluorescent microscope for counting. (**B**) Mk-2206 treatment significantly decreased colony formation in RH30 and SMS-CTR cells. Shown are the average number of colonies per 20x-objective field. Vehicle control used for MK-2206 was DMSO. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

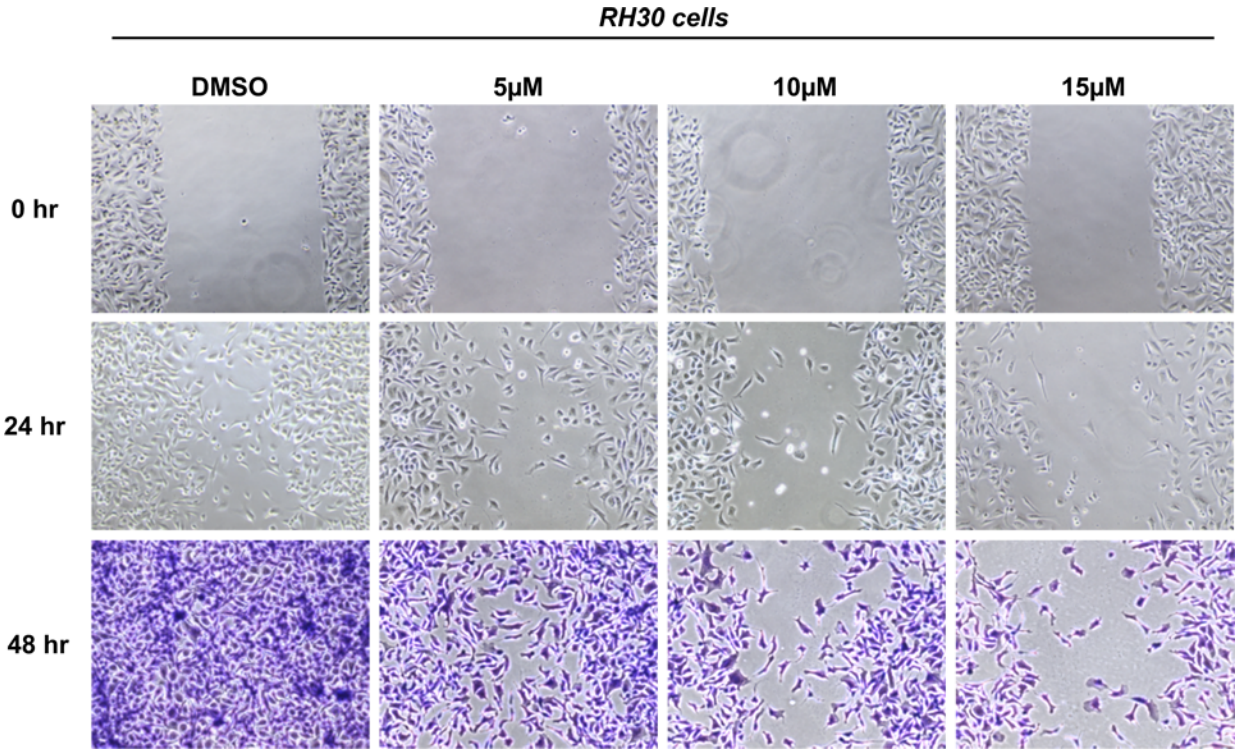


Figure 28: Inhibiting Akt activity in RH30 cells decreases cell migration.

RH30 cells treated with MK-2206 had a marked reduction in cell migration. Cell migration was assessed by performing a wound healing assay (scratch assay). RH30 cells were treated with different concentrations of MK-2066 and imaged after 24 and 48 hours of treatment. At 48 hours cells were stained with crystal violet. Shown are the representative images of vehicle control DMSO and MK-2206 treated cells. Images taken at 20x magnification.

MK-2206 treatment hinders tumor burden in EGFR resistant RMS cell lines

Moving into more preclinical studies, I next wanted to investigate if Akt inhibition would block tumorigenicity in EGFR-inhibitor resistant cell lines. For these studies, I utilized RH30 cells for this xenograft study, not only because these cells are ideal for in vivo studies, but also to extend the in vivo MK-2206 studies, testing its effect on an ARMS cell line. (Previously mentioned our studies tested MK-2206 in ERMS RD-studies) From the RH30-xenograft studies, I observed that MK-2206 treatment significantly reduced tumor burden survival in comparison to the vehicle control treated animals (Figure 29A). MK-2206 treated mice also exhibited an increase in their event-free survival (Figure 29A). Further analysis of tumor tissue harvested revealed MK-2206 treated tumors displayed to have a significant decrease in proliferation (detected by IHC Ki67 staining) (Figure 29B). From these studies, I have shown that blocking Akt signaling significantly hindered oncogenicity in both EGFR-inhibitor resistant cell lines and ARMS cell line RH41, as well as blocked tumorigenicity in RH30 – xenografts. These results, suggest that Akt inhibition antagonizes RMS pathogenesis in both ERMS and ARMS cell lines, including those tested to be non-responsive to EGFR inhibitors, and could be a much more ideal intracellular therapeutic target.

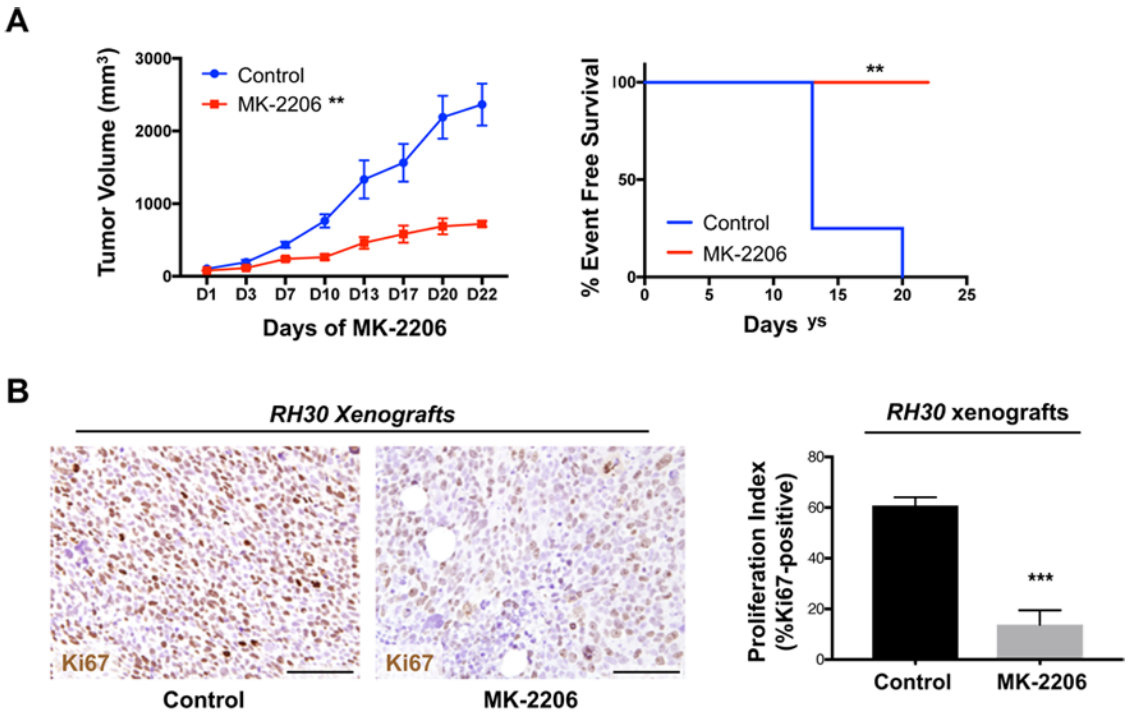


Figure 29: MK-2206 treatment hinders tumor burden in ARMS RH30 xenograft models.

Treating RD xenograft models with MK-2206 significantly blocks tumorigenicity. (**A**) Tumor burden, represented by tumor volumes ([L*W2]/2) measured over time, was significantly reduced in MK-2206 treated RD xenografts (180mg/kg, oral gavage, 3 times/week). Additionally, mice treated with MK-2206 had an increase in event-free survival (see **Methods**). 30% Captisol was used as vehicle "control" in these experiments. (**B**) Immunohistochemistry Ki67 staining of xenograft tumor sections demonstrated marked decrease in proliferation in comparison to the vehicle control tumor sections. Representative images of Control and MK-2206 xenografts are shown as well as graphical representation of Ki67 positive proliferation indexes. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

Summary

From our previous studies, I identified EGFR-inhibitor resistant and sensitive RMS cells. RMS cells lines non-responsive to EGFR inhibition, were uncovered to have misexpression of another RTK, FGFR4. Further probing verified that the intracellular signaling pathways, Akt was the mechanism responsible for both EGFR- inhibitor sensitivity and resistance. EGFR-inhibitor resistant cell lines, RH30 and RH36, treated with pan-Akt inhibitor, MK-2206, now demonstrated to have a marked decrease in both RMS oncogenicity and tumorigenicity (RH30 xenografts). These studies were expanded by the addition of two new cell lines, SMS-CTR (ERMS) and RH41 (ARMS), in which treatment of MK-2206 also exhibited a reduction in cellular oncogenicity. Irrespective to the genetic aberrations identified, Akt inhibition was sufficient in antagonizing the progression of RMS in all cell lines tested.

Chapter 4

IL-6R signaling, another avenue by which to antagonize RMS : A pilot study

Introduction

Uncovering alternative pathways to overcome therapeutic resistance to EGFR inhibitors

Within the field of cancer, molecular targeted therapies have become a major form of medical treatment for patients. Unlike the hormone or cytotoxic chemotherapies, which usually target all rapidly dividing cells within the body, targeted therapies are designed to intervene with specific molecules required for tumor progression [116]. As targeted therapies have become the focus for anticancer drug development, several pharmacological agents have been designed and FDA approved [117]. One of the most commonly used targeted therapies for treating cancer patients being those that antagonize the tyrosine kinase receptor, EGFR.

Over the years, EGFR inhibitors have been successful in treating patients with different malignancies such as breast and non-small cell lung cancers. Though recently, there has been a significant increase in patients developing innate or acquired resistance to EGFR inhibitors, which has become a challenging problem to overcome in the clinic [96, 97]. Several different resistance mechanisms have since then been identified, including the development of secondary activating mutations, aberrant expression of downstream pathways (loss of PTEN [phosphatase and tensin homolog]) and the activation of alternative pathways [95] Many research groups have focused on advancing our understanding of the different resistant mechanism, such as these, in order to develop methods by which to overcome EGFR resistance and improve patient outcome.

These findings are of interest to me, as one of my research focuses is investigating how EGFR facilitates myoblast transformation and influences RMS tumor progression. Within my studies I have demonstrated pharmacological EGFR inhibitors, Erlotinib and Cetuximab, to effectively hinder RMS progression both in vitro and in vivo. Although these results provide ensuring evidence that support EGFR inhibitors as a suitable candidate for clinical trial studies, concerns of RMS patients developing similar resistance to the treatment remain. Emerging studies focus on using combination therapies, rather than mono-targeted agents, as a potential method to prevent the occurrence of therapeutic resistance [118] [119]. Wanting to identify another targetable candidate, I began surveying the literature for alternative pathways that have been linked with EGFR resistance in other cancer and uncovered the signaling pathway of Interleukin 6 (IL-6) and its corresponding receptor, Interleukin 6 receptor (IL-6R).

Exploring IL-6 signaling in cancer

IL-6 is a pleiotropic cytokine produced by multiple different cell types and is involved in regulating a various of distinct biological functions (e.g. angiogenesis, innate and adaptive immune responses and hematopoiesis) [120, 121]. IL-6 signals via a hexameric signaling complex composed of the dimerization of two of its receptors, IL-6R (comes in two forms, membrane bound [mIL-6R] and soluble form [sIL-6R]), and two individual transmembrane signaling-transducing glycoprotein, GP130 [122]. IL-6 is most commonly associated as a key mediator of both pro- and anti-inflammatory responses within the body, however, recent reports have discovered IL-6 signaling to also be involved in driving tumor initiation and progression in diverse cancer types

including colorectal, ovarian, breast and lung cancer [123] [124]. In fact, patients presenting with high serum concentrations of IL-6 are associated to have very poor outcomes, therefore it has become a highly sought out effector in the field of cancer [125] [126] [127] [128].

Furthermore, studies have also demonstrated an association with both IL-6 and EGFR misexpression in cancers such ovarian, glioblastoma and non-small cell lung carcinoma. In some patients, EGFR misregulated activation was shown to promote and initiate the expression of both IL-6 and its receptor components in transformed cells [129-131]. IL-6/IL-6R has been identified as an alternative mechanism in EGFR resistant cancers in published reports, in which inhibition of EGFR is bypassed through the upregulation of IL-6/IL-6R expression. (e.g. lung, and head and neck carcinomas) [132-134]. These findings reveal IL-6 signaling as an ideal candidate that is also associated with the oncogene EGFR, however the question remains if IL-6 signaling plays any role in RMS progression?

IL-6 - Its role as a myokine.

As mentioned previously, IL-6 plays a central role in modulating several biological processes, one of which being its role as an essential myokine during muscle development. IL-6 has been recognized to be involved in stimulating hypertrophic muscle growth and mediating early myoblast proliferation as well as display promyogenic functions during myogenesis [135, 136]. Similar to my wild-type EGFR studies, activation of IL-6 is an important signaling factor within the initial stages of myoblast differentiation - mediating the expansion of early myoblast cells. This led to the question if any form of misregulation of IL-6 activity could potentially facilitate the

progression of myoblast like RMS cells? These findings indicate IL-6 signaling as a prospective testable pathway, however further profiling of IL-6 signaling in RMS is required. Another benefit to pursing these studies is the availability of an existing FDA approved humanized anti-IL6R monoclonal antibody (Tocilizumab®), a necessary component for pre-clinical testing. Thus, I aimed to test whether IL-6 signaling influences RMS progression and if targeting both EGFR and IL-6 signaling concurrently, represents an efficient novel method by which to therapeutically treat RMS.

Preliminary Results

Profiling IL-6 signaling complex expression in wild-type myoblast

Having identify IL-6 as a potential pathway to target in tandem with EGFR, I first wanted to validate its expression in wild-type C2C12 cells. In a published report done by Weigert and her colleagues, they tested for the expression analysis of IL-6 throughout the course of C2C12 differentiation. Similar to the expression profile observed in our EGFR studies, they detected both mRNA and protein IL-6 levels to steadily increase during C2C12 differentiation. Though in contrary to the EGFR levels which slowly begins to decrease after it peaks around Day 3, IL-6 expression continued to increase and remained high even during the late stages of myogenesis [135]. Within published reports it was evident that Il-6 expression influenced myoblast differentiation, though date analyzing the expression profile of the signaling complex of IL-6 – transmembrane or soluble IL-6R and glycoprotein signaling transducer, GP130 had not been evaluated. To examine IL-6 signaling complex expression levels, I probed for both IL-6R and

GP130 in differentiating C2C12s and observed the expression of both the soluble and transmembrane form of IL-6R to gradually increase over the 6 day differentiation time course. In contrast, the expression of GP130 rapidly peaked around D3 and remained at an elevated level of expression throughout the final stages of differentiation (Figure 30). Reports on IL-6 activity involvement during myogenesis have demonstrated its activity to be important throughout the beginning and final stages of myoblast differentiation/ fusion [137] [138]. From these results, I validated the expression of the transmembrane signaling complex of IL-6 in wild-type myoblasts and detected it to not only be expressed in the early stages, but also the late stages of differentiation, providing evidence that IL-6 signaling occurs throughout myoblast expansion and differentiation.

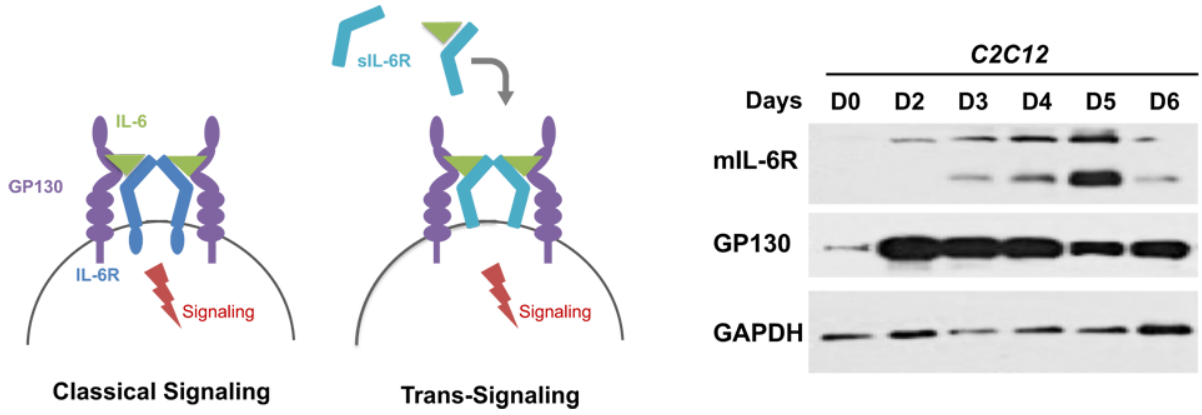


Figure 30: IL-6/IL-6R signaling complex expression in C2C12 myoblast.

IL-6 signaling complex is expressed in differentiating C2C12. (**A**) Simple schematic representation of both membrane bound (classical signaling) and soluble (Trans-signaling) forms of IL-6 signaling complex. (**B**) Immunoblot analysis detecting IL-6 signaling complex, IL-6R and GP130, in differentiating C2C12. Levels of IL-6R steadily increase over the course of differentiation until day 6 where the levels decrease. In contrast, GP130 expression levels quickly rise on Day 2 and remain high throughout differentiation. GAPHD is used as a loading control.

Interrogating IL-6 signaling influence in RMS progression

After demonstrating that the components within the IL-6 signaling complex are expressed in wild-type myoblast, I next tested for their expression in multiple different RMS cell lines: ERMS cell lines RD, SMS-CTR and RH36 and ARMS cell line, RH30. RT-PCR analysis detected all components of the signaling complex - IL-6, soluble IL-6R (sIL-6R) membrane bound (mIL-6R) and GP130 in all four tested RMS cell lines (Figure 31). I began these preliminary studies testing one ERMS (RD cells) and ARMS (RH30 cells) cell line due to the limited reagent available (neutralizing antibody – anti-IL6R antibody). I first investigated if IL-6 signaling was involved in RMS progression by blocking its activity in both RD and RH30 cells using a neutralizing anti-IL-6R antibody. Blockage of IL-6 signaling showed a significant decrease in proliferation in both RD and RH30 cells assessed by Ki67 immunofluorescence staining (Figure 32A and B). As these studies provided evidence that IL-6 influences RMS cell progression in vitro, I then went forth to testing the effects of duel treatment with both EGFR inhibitor, Erlotinib and neutralizing anti-IL6R antibody. RMS cells treated with Erlotinib and anti-IL-6R antibody, in tandem, demonstrated to have a significantly lower decrease in RMS proliferation in comparison to RMS cells treated with either Erlotinib or anti-IL-6R alone, suggesting combination treatment to have an additive/synergistic effect on RMS oncogenicity - which would need to be further tested (Figure 32A and B).

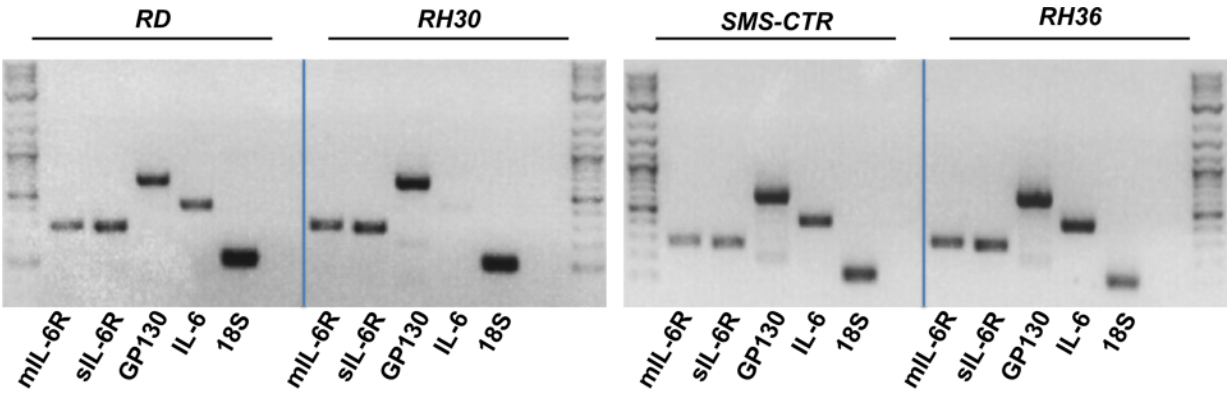


Figure 31: RMS cells express the IL-6/IL-6R signaling complex.

Semi-quantitative RT-PCR detected the expression of the IL-6 signaling complex in RMS cells. The expression of human (h) IL-6 signaling complex [membrane bound IL-6 receptor (hmIL-6R), soluble IL-6 receptor (hsIL-6R), glycoprotein signal transducing receptor (hGP130) and IL-6 ligand (hIL-6)] were tested in RD, RH30, SMS-CTR and RH36 cell. Primers specific for each component within the complex was The expression of IL-6 signaling complex, membrane bound IL-6R (mIL-6R), RT-PCR product separated by 1% agarose gel electrophoresis. Corresponding primers were used (see methods for primer sequence) in untreated RMS cells. DNA ladder in first and last wells. Primers against 18S used as internal control.

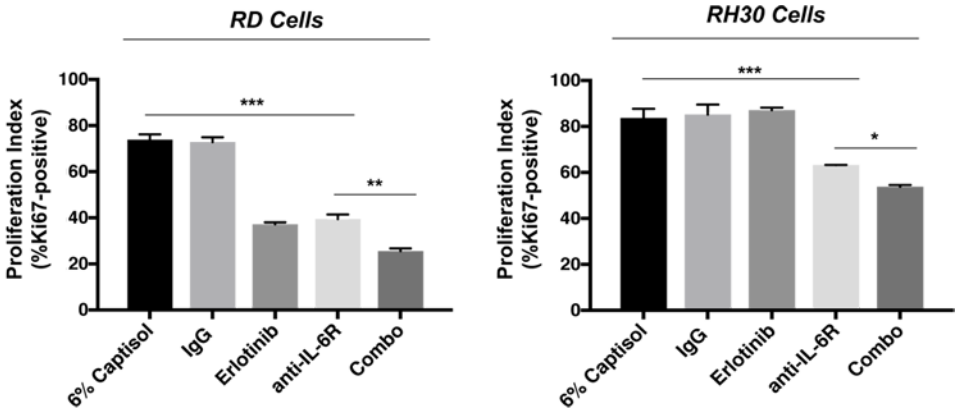
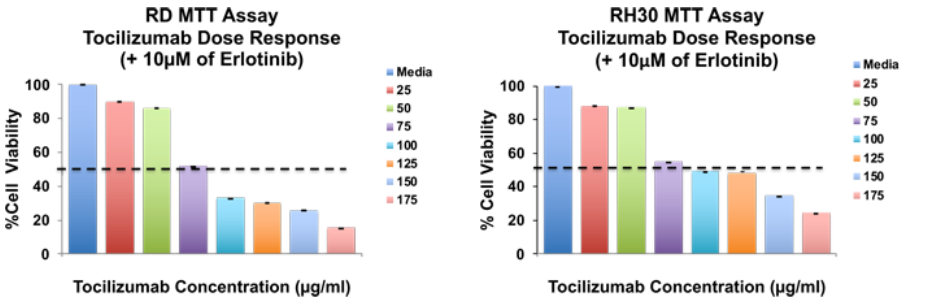


Figure 32: Blocking IL-6/IL-6R signaling hinders RMS proliferation.

Blocking the activity of EGFR and IL-6/IL-6R, in tandem, shows to significantly hinder proliferation in comparison to single inhibition of EGFR or IL-6R. (**A and B**) Shown are the graphical representations of the proliferation indexes of both RD and RH30 cell treated with Erlotinib and anti-IL-6R neutralizing antibody separately or in tandem. Cells were stained with anti-Ki-67 (Proliferation marker) and images were captured using an immunofluorescent microscope and counted. Vehicle controls for Erlotinib and anti-IL6R neutralizing antibody are 6% Captisol and IgG isotype, respectively. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

Testing the anti-tumor effects of IL-6R inhibitor, Tocilizumab, in RMS cells

Moving forward into a more pre-clinical relevant study I began testing the humanized monoclonal anti-IL-6R antibody, Tocilizumab, in combination with EGFR inhibitor, Erlotinib. Before testing the duel effects of Tocilizumab and Erlotinib, I assessing cell viability in both RD and RH30 cells, in order to assess for the optimal working concentrations to use for these studies that were well beneath IC50 concentrations (Figure 33). Similar to the results observed using the neutralizing antibody anti-IL-6R, combination treatment with Tocilizumab and Erlotinib resulting in a significantly lower reduction in RMS proliferation when compared to cells treated with Tocilizumab or Erlotinib alone (Figure 34A). Combination treatment of both inhibitors also demonstrated an additive/synergistic effect in soft agar colony formation (both RD and Rh30 cells tested) (Figure 34B) and cell migration (only RH30 - tested by wound healing assessment) (Figure 35). Further probing of downstream signaling nodes involved in both EGFR and IL-6R activity, revealed both Akt and STAT3 activity to be decreased when treated with either Erlotinib or Tocilizumab, however, combination treatment resulting in better blockage of their activity. No detectable changes in MEK/MAPK activity were observed in neither individual or combo treated cells (Figure 36). Overall, in these studies I have detected that IL-6 signaling alone does influence RMS progression and when both IL-6 and EGFR activity are blocked RMS cells exhibited a much greater reduction in oncogenicity, suggesting duel treatment with EGFR inhibitors, such as Erlotinib, in combination with IL-6R inhibitor, Tocilizumab, could represent a new therapeutic approach by which to treat RMS patients.



Cell Line	IC_{50} Dosage (μg/ml)
RD	75
RH30	100

Figure 33: Viability of RMS cells when treated with Tocilizumab.

The viability of RMS cells at increasing concentrations of IL-6R inhibitor, Tocilizumab. MTT assays for RD, and RH30 cells treated with Tocilizumab. Three replicate experiments were done for each concentration tested.

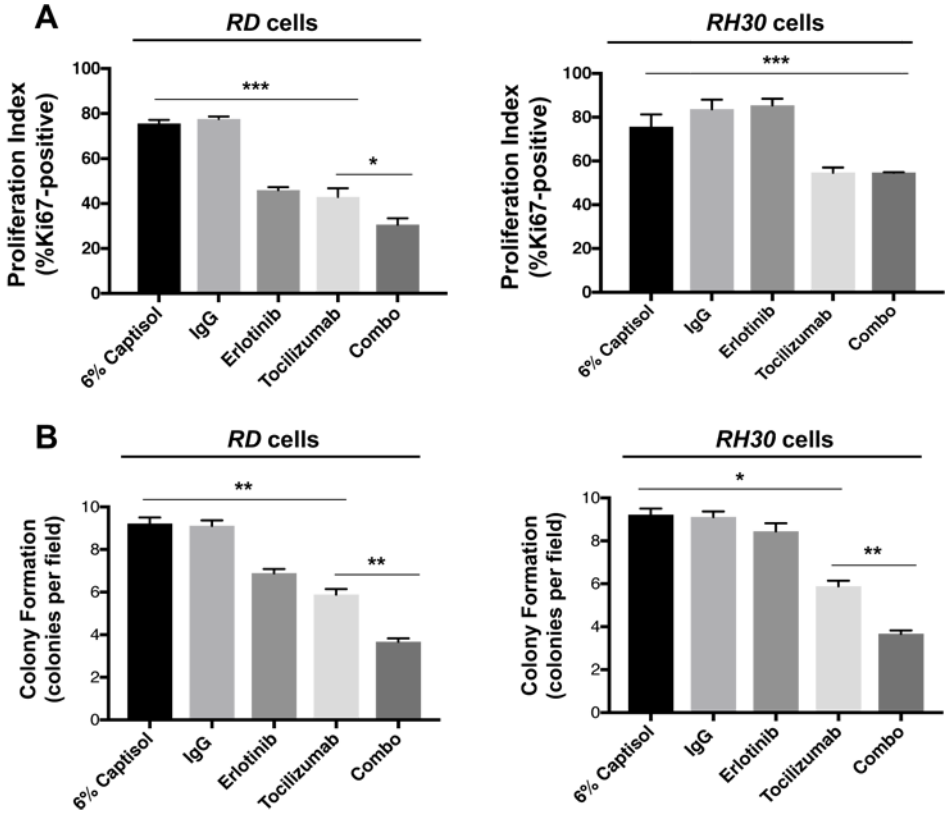


Figure 34: Duel treatment with Erlotinib and IL-6R inhibitor, Tocilizumab, has an additive/ synergistic antagonistic effect on RMS oncogenicity.

Targeting EGFR and IL-6R, in tandem, markedly decreases RMS proliferation and colony formation. **(A)** Shown are the graphical representations of the proliferation indexes of both RD and RH30 cells treated with Erlotinib and humanized anti-IL-6R antibody, Tocilizumab, separately or in tandem. Cells were stained with anti-Ki-67 (Proliferation marker) and images were captured using an immunofluorescent microscope counted. (**B**) Colony formation was significantly reduced in RD and Rh30 cells treated with Erlotinib and Tocilizumab. Shown are the average number of colonies per 20x-objective field. Vehicle controls for Erlotinib and Tocilizumab are 6% Captisol and IgG isotype, respectively. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

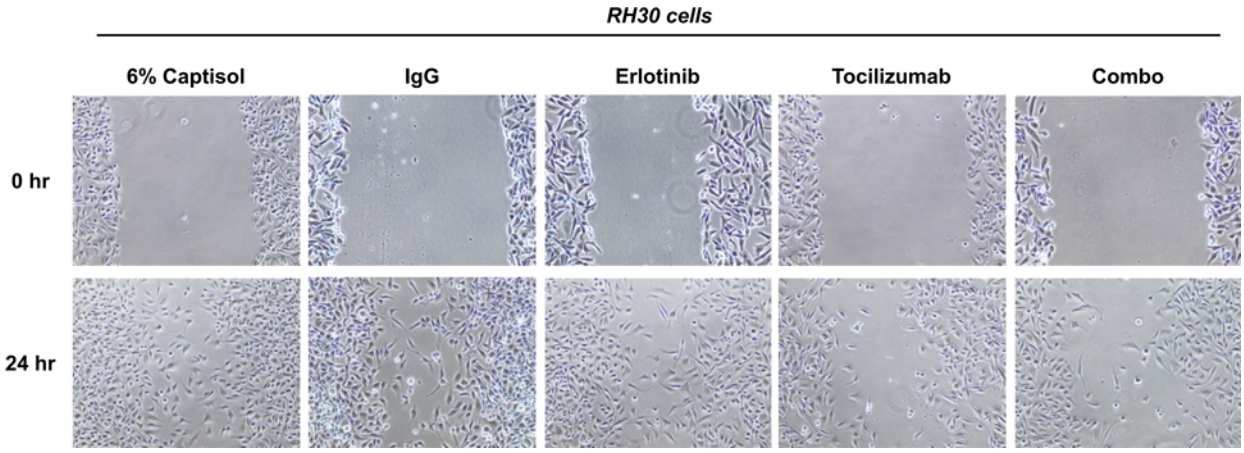


Figure 35: Cell migration is impeded in RH30 cells treated with Tocilizumab alone and in combination with Erlotinib.

RH30 cells treated with Tocilizumab alone or in combination with Erlotinib demonstrate a reduction in cell migration. Wound healing assay (scratch assay) was done to asses for cell migration. RH30 cells were treated with Erlotinib and Tocilizumab separately or in tandem for 24 hours. Images were taken prior treatment (0hr) and 24 hours after treatment (24hr). Shown are the representative images of 3 total experiments. Images taken at 20x magnification. IgG was used as an isotype control for Tocilizumab.

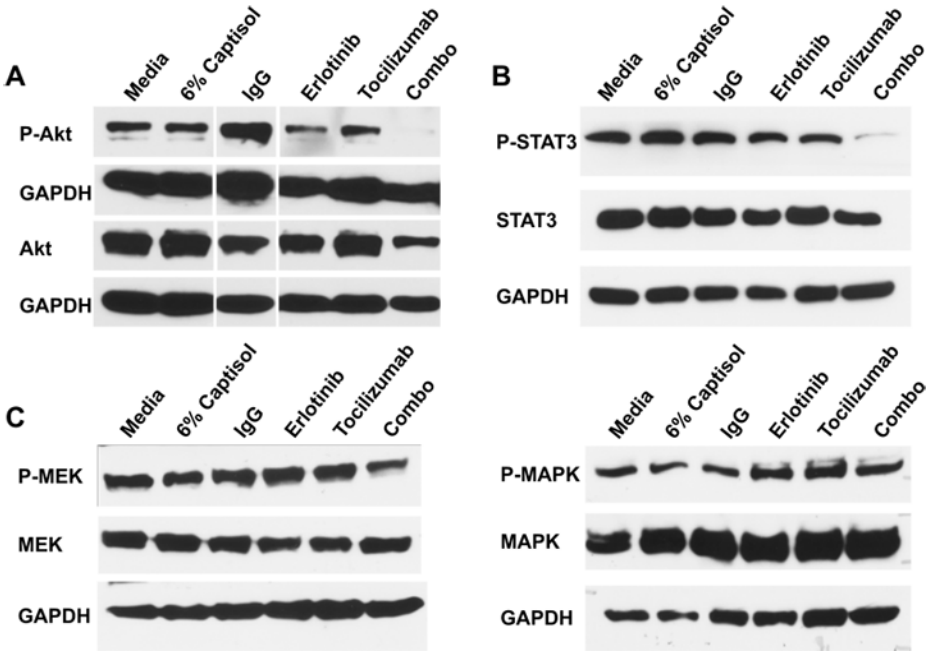


Figure 36: Combination treatment with Erlotinib and Cetuximab has a greater effect on inhibiting Akt and STAT3 signaling.

Treatment with inhibitors Erlotinib and Tocilizumab blocks activation of Akt and STAT3 in RD cells. (**A-C**) Immunoblot analysis probing for common intracellular pathways downstream of EGFR and IL-6R (Akt, STAT3 and MEK/MAPK) demonstrated lower expression levels of phosphorylated Akt (P-Akt) and STAT3 (P-STAT3). Lysates generated from RD cells treated with either Erlotinib and Tocilizumab in culture. No changes were observed in MEK/MAPK signaling. GAPDH used as loading control. Vehicle controls for Erlotinib and Tocilizumab are 6% Captisol and IgG isotype control, respectively.

Summary

These preliminary studies reveal that IL-6 signaling is a critical pathway involved in RMS pathogenicity. In wild-type C2C12 myoblast, the expression of its signaling complex was observed during both the early and later stages of differentiation. Blockage of IL-6R pathway utilizing a neutralizing anti-body demonstrated IL-6 activity to influence RMS progression. When combined with EGFR inhibitor, Erlotinib, this led to a significantly greater reduction in RMS oncogenicity. Moving forward into a much more pre-clinical investigation, treatment with IL-6R inhibitor, Tocilizumab, in tandem with Erlotinib markedly decreased RMS pathogenicity in tissue culture in comparison to RMS cells treated with each individual inhibitor alone, recapitulating our earlier studies. Further investigation revealed both Akt and STAT3 intracellular effectors involved in both EGFR and IL-6R signaling in RMS. Based on these analyses, I hypothesize that duel targeting of EGFR and IL-6/IL-6R might represent an effective form of therapy against RMS with a decreased acquired resistance often seen from monotherapy.

Chapter 5

Disscussion

I. Misexpressed myogenic network, EGFR influences the pathogenicity of RMS

The discovery and characterization of EGFR activity in malignant transformations has vastly impacted the field of molecular oncology. It is one of the most extensively studied genes reported in a wide range of malignancies and its involvement in tumorigenesis has led to the development of multiple different target specific cancer therapeutics [139, 140]. Since then, several EGFR inhibitors have been tested and approved to be used in the clinical and have resulted in promising prognosis [74].

A. Gene amplification/ overexpression mechanisms of EGFR oncogenic phenotype in RMS

Within these studies, we were able to identify EGFR and its ligand EGF as potential misexpressed signaling factors that could be influencing RMS progression from an unbiased forward genetic screen done using our PAX7-FOXO1 *Drosophila* model [1] [58]. We further verified EGFR expression in a cohert of RMS patients using open-access NCI Oncogenomics RMS RNA-seq database. We observed both ERMS and ARMS patient samples had elevated levels of EGFR expression in comparison to control tissue, though high EGFR levels were mainly associated with ERMS patient samples. These findings suggested EGFR activity as a potential oncogenic driver in RMS, though its tumorigenic mechanism remained unclear. There has been no evidence reporting any identified EGFR mutations in RMS, suggesting gene amplification and

misexpression as the mechanism promoting RMS pathogenicity, similar to several other cancers (Glioblastoma, head/neck squamous cell carcinoma) [141] [88].

Within the literature, published reports such as one done by members in our lab, suggests RMS transformation to be due to impaired myoblast differentiation/fusion (i.e. misexpression of TANC1) [59] [56]. So, we questioned whether misregulated expression of EGFR could influence myoblast differentiation resulting in the progression of RMS pathogenicity. As I referred to studies done in *Drosophila* that have uncovered the mechanism of myoblast differentiation (i.e. muscle pattern), I learned that this process is a tightly controlled system that is dependent on the regulated expression of several genes, one of which being EGFR [71] [57]. If EGFR expression is either lost or constitutively active, this leads to the loss of the two discrete pools of myoblast, FC's and fcms, which are essential for muscle patterning to occur. These studies support that the mechanism driving RMS is loss of differential properties due to the misexpression of EGFR activity, making it a targetable therapeutic candidate.

B. Inhibition of aberrant expression of EGFR impedes RMS progression in vitro and in vivo

I initially verified EGFR signaling was expressed in RMS patient's samples through open-access NCI Oncogenomics database, as well as in tested RMS cell lines, observed by immunoblot analysis. As I learned how critical EGFR signaling is during myoblast fate specification in *Drosophila* muscle patterning, we next sought out if similar mechanisms occur during mammalian myoblast differentiation. In order to test this, we analyzed myoblast differentiation using an immortalized mouse myoblast cell line, C2C12. We were able to detect EGFR activity during myoblast differentiation and observed EGFR expression to peak at the early stages, around day 2,

and decrease over time, as differentiation progressed towards its final stages (experiment was terminated at Day 6). Additional, loss of function and gain of function analysis revealed that the expression and regulation of EGFR signaling plays an important role for differentiation/ fusion to occur. Similar to *Drosophila* muscle patterning, mammalian myoblast differentiation seems to be influenced by EGFR activity, though further analysis still needs to be done to establish how its expression influences differentiation and identify possible downstream mechanisms that mediate myogenesis by directly interacting with EGFR. Another aspect that remains unclear is whether, such as in *Drosophila* muscle patterning, two distinct populations of myoblast are needed for differentiation to occur in mammals? These findings would allow us to improve our understanding of RMS pathobiology and might uncover novel intracellular processes that trigger the onset of RMS which could be directly or indirectly involved with EGFR.

These studies suggest that misexpression of EGFR signaling could be an ideal target in RMS, thus we tested the effects of silencing its activity in RMS cells as a proof of concept. EGFR silencing significantly decreased RMS progression, which was observed in both in vitro and in vivo. Moving into a more preclinical study, I next tested the effects of pharmacological inhibitors, Erlotinib and Cetuximab, in RD cells. Both in vitro and in vivo studies demonstrated that EGFR inhibition markedly decreased RD cell oncogenicity and tumorigenicity. Within these studies, both EGFR silencing and pharmacological inhibition rescued myoblast differentiation and fusion. From these results, I observed that not only does misexpression of EGFR influence RMS progression but it also facilities the disruption of differentiation properties in myoblast. These findings, provides supporting evidence that blockage of EGFR is sufficient in restoring myogenic networks in transformed RMS myoblasts resulting in the loss of malignant characteristics tested in RMS cells. It's important to note that only one of the three tested cell lines, RD cells, demonstrated to

have an effect when EGFR signaling was hindered. In all three cell lines EGFR expression was detected and treatment with either Cetuximab or Erlotinib did effectively reduce EGFR activity, so the question remained as to why RH30 and RH36 cell lines did not respond as RD cells did? I further investigate these findings chapter 3.

C. Identifying Akt as the intracellular pathway mediating EGFR-driven RMS pathogenicity

Moving forward, I next wanted to further interrogate what intracellular mechanisms downstream of EGFR facilitates the progression of RMS? In these studies, I identified Akt activity as a critical pathway influencing EGFR-driven RMS pathogenicity. Upon EGFR inhibition, I observed a reduction in Akt signaling, whereas other pathways probed for (i.e. MEK>MAPK and JAK>STAT3), no significant changes were detected. I further validated the role of Akt signaling in RD cells by testing the effects of blocking Akt activity using the pan-Akt inhibitor, MK-2206. These studies demonstrated that inhibition of Akt signaling effectively decreased both oncogenicity and tumorigenicity, recapitulating the data obtained from our EGFR- inhibition studies and validating that RMS progression is driven by the EGFR>Akt pathway. As previously mentioned EGFR blockage also demonstrated to rescue myoblast differentiation and fusion, though, this was not observed when RD cells were treatment with MK-2206. I question if there could be another missed intracellular effector downstream of EGFR involved in disrupting myoblast differentiation other than Akt pathway. This would explain why EGFR inhibition rescued differentiation whereas sole targeting of Akt does not.

D. Distinct roles for Akt isoforms 1 and 2

Published reports demonstrated that the activity of Akt is a critical myogenic factor, but more specifically that its isoforms, Akt1 and Akt2 play critical roles in regulating different stages during myogenesis [142, 143]. Currently, though Akt1 and Akt2 are known to regulate various aspects of myogenesis, studies testing the different functions for Akt1 or -2 have not been previously explored in RMS. In these studies, I uncovered that Akt1 preferentially promoted RMS cell differentiation at higher levels in comparison to shAkt2 and control RD cells. Targeting Akt1 was sufficient to induce RMS rhabdomyoblast terminal differentiation rescue, whereas silencing Akt2 alone does not recapitulate strong results, suggesting the activity of Akt2 may be necessary during myogenesis. Recent publications revealed the activation of Akt2 as an important myogenic factor involved in impairing the stability of the mRNA complex Pitx2/HuR/Ccnd1 resulting in the arrest of myoblast proliferation and initiation of differentiation [144]. These findings provide evidence as to why blockage of Akt activity failed to rescue differentiation in MK-2206 treated RD cells, a pan-Akt inhibitor. A couple of questions that arise from these results are if Akt1 activity is the main isoform mediating the progression of EGFR-driven RMS? If so, then could clinical inhibition of its activity be sufficient in hinder RMS pathogenicity?

Furthermore, within these studies when RD cells were treated with EGFR inhibitors I detected a significant decrease in Akt activity but not a complete loss of signaling as I did when RD cells were treated with MK-2206. Could these results indicate that partial diminished levels of Akt activity is sufficient in hindering RMS progression but a small sustained amount of activity, perhaps that of Akt2, is necessary for the rescue of differentiation and fusion in transformed RMS cells? Perhaps treatment with EGFR inhibitors reduces the activity of Akt1 more selectively

resulting in partial activation of Akt2? In order to test for this, stable cell lines silencing Akt1 and Akt2 isoforms can be generated to assess the effects silencing has on RMS oncogenicity and tumorigenicity. I have now generated RD shAkt1 and shAkt2 stable cell and have done some preliminary testing for anchorage independent growth. I observed that silencing of both Akt1 and Akt2 significantly decreased the number of colonies formed in comparison to shEGFP control cells, though silencing of Akt1 had a much stronger reduction in comparison to shAkt2 cells (Figure 37). This suggests that Akt1 plays a much stronger role in the progression of RMS in comparison to Akt2, at least when it comes to testing colony formation, though these studies need to be repeated and further investigated to validate these observations.

Recently published reports have demonstrated specific Akt isoforms mediating tumor progression in selective cancers (i.e. Akt1 activating mutation found in non-small cell lung cancer) [145, 146] [147]. By further pursuing these studies, we can advance our understanding on how Akt isoforms 1 and 2 influence RMS progression and interrogate their mechanisms that underlie RMS pathogenicity. Of note, 3 Akt isoforms have been identified (Akt1, 2 and 3). AKT3 expression is much higher in testes and brain tissues in comparison to other tissues throughout the body. AKT3 is not highly expressed in skeletal muscles thus little information regarding its role in mammalian myogenesis remains unclear. Nonetheless, it would still be intriguing to assess if levels of Akt 3 expression can be detected in either RMS patient samples and test if it's activity plays any role in RMS.

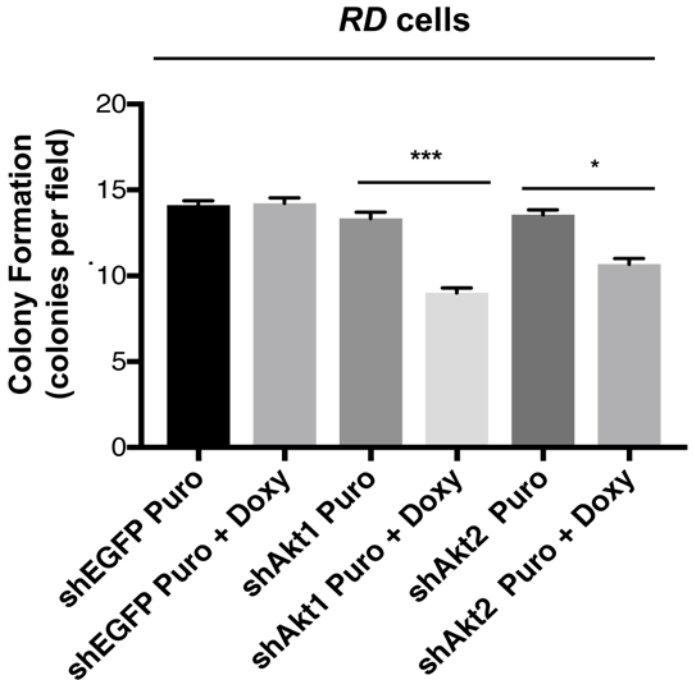


Figure 37: Silencing of Akt 1 has a greater effect in hindering colony formation in RD cells.

Stable inducible shAkt transfected cells demonstrated that silencing of Akt activity significantly reduced colony formation in RD cells. Of note, shAkt1 RD cells showed to have a much lower decrease in colony formation in comparison to shAkt2 RD cells. Shown are graphical representation of the average number of colonies per 20x-objective field. shEGFP use as control, puromycin inducible vectors. *P* values were calculated by two-tailed Student's *t* test: $*P < 0.05$ versus Control.

E. Loss of endogenous differentiation programs- the mechanisms of RMS disease onset

Our data illustrated how preclinical inhibition of EGFR activity was sufficient in impeding RMS pathogenicity both in culture and in tumor xenograft models. What was most intriguing, was the observed rescue of myoblast differentiation. These results provided evidence supporting our hypothesis that the inability of myoblast to undergo differentiation through the loss or misregulation of factors (i.e. EGFR) leads to the progression of RMS, as well as indicate the promising effects EGFR inhibitors have as a form of differentiation therapy against RMS.

Differentiation therapy is an alternative approach to the traditional chemo- or radiotherapies which aims to reactivate hindered cellular differentiation programs rather than eliciting cytotoxic cell death [148] [61]. By coaxing cancer cells out of their transformed stage and reverting them back into their "normal" state, they lose their malignant properties and fall into a state of remission. Differentiation therapies are typically less toxic (*e.g.* IKKalpha in Nasopharyngeal Carcinoma and retinoic acid in Acute Promyelocytic Leukemia) and better tolerated with less long-term effects than standard cancer treatment which could be advantageous for the much younger patient cohort that is afflicted by RMS [149] [150].

II. Akt, a broader targetable intracellular pathway

Within our EGFR studies, I identified both EGFR-inhibitor sensitive and resistant cell lines and questioned what mechanism(s) of resistance could be accountable for these results. In other malignancies, EGFR resistance can occur when cancer cells acquire random point mutations

blocking EGFR inhibitor(s) receptor binding sites [96] [95]. Though, this mechanism seems unfavorable, since no EGFR activated mutations have been identified in RMS patients thus far. So, in order to investigate why some RMS cell lines are not responding to EGFR inhibition, I probed for intracellular mechanisms that could contribute to EGFR-inhibitor resistance/sensitivity. Having identified Akt signaling as a critical pathway involved in EGFR driven RMS pathogenesis, I first probed for Akt activity in EGFR-inhibitor resistant cell lines. In these cell lines, I observed there to be no diminished levels of Akt signaling, even though blockage of EGFR signaling was detected. Whereas in cells sensitive to EGFR blockage, Akt levels were decreased, suggesting the EGFR resistant mechanism could be due to the inability to perturb Akt signaling. I questioned if there could be other genetic aberrations that could be functioning as alternative pathways to compensate for EGFR blockage.

A. RMS cells bypass EGFR inhibition by misregulated FGFR4 signaling; an alternative RTK pathway.

To further elucidate what mechanisms could be a contributing factor to EGFR-inhibitor resistance, I reexamined our cell lines to identify other oncogene drivers. I found within published genomic sequencing data, that both RH30 and RH36 non-responsive cell lines were also driven by another RTK, FGFR4 (FGFR4 activating mutation in RH36 [FGFR4_1648G>C (V550L)] and PAX3-FOXO1-induced misexpression of FGFR4 in RH30) [98] [99] [100]. The identification of FGFR4 in RMS is not novel to the field for its role in this disease has been explored. Detection of FGFR4 expression has been exhibited in both ERMS and ARMS, though higher expression is detected within ARMS samples [107]. Blockage of FGFR4 activity demonstrated an anti-

tumorigenic response in only RMS cells lines driven by activated mutations, which are mainly found in ERMS cells [106, 107]. Within these studies, they demonstrated FGFR4 does in fact influence RMS, however what has not been explored is its role in EGFR- inhibitor resistance.

Interestingly, within the literature, identified malignancies, which have developed resistance to EGFR inhibitors, also have dysregulated expression of other RTK family members such as FGFR family proteins [151, 152]. Additionally, several studies have detecting some malignancies are driven by multiple RTKs [110] [153]. These findings provide supporting evidence that the activation of another RTK member could be the mechanism of resistance in RMS cells; bypassing EGFR signaling blockage. So, I hypothesized that FGFR4 activity could be driving Akt signaling, since RTKs share similar downstream intracellular pathways, thus RMS cells evade EGFR inhibition through this alternative pathway and acquire a resistant phenotype.

B. Inhibition of RTK Intracellular downstream pathway, Akt, antagonizes EGFR resistant RMS cells both in vitro and in vivo

To test if the absence of response to EGFR inhibitors in certain RMS cell lines (RH30 and RH36) is due to the inability to downregulate Akt signaling, I directly targeting intracellular Akt activity using pan-Akt inhibitor, MK-2206, in RMS cell lines non-responsive to both EGFR inhibitors, Erlotinib and Cetuximab. I also extended these studies by testing two additional cell lines, SMS-CTR – an ERMS cell line also resistant to EGFR inhibitors and RH41- an ARMS cell line to expand our ARMS- MK-2206 studies. Overall, I observed that once Akt activity was blocked, MK-2206 treatment significantly blocked oncogenicity in all cell lines tested in culture (i.e. MK-2206 treatment significantly decreased cellular proliferation, reduced colony formation

in RH30 and SMS-CTR and impeded cell migration/metastasis in RH30 cells) and also demonstrated to be effective in vivo, reducing tumorigenicity in our RH30-xenograft model.

C. Future studies investigating EGFR/ FGFG4 activity in RMS

From these studies, I have identified RMS cell lines that were not responsive to EGFR inhibitors to also be driven by some form of dysregulated expression of FGFR4 (activating mutation or gene amplification/ over expression) and demonstrated that blocking Akt activity is sufficient to antagonized RMS progression in all tested ERMS and ARMS cell lines. I hypothesized that the misexpression of FGFR4 in RMS cells could serve as an alternative pathway by which to evade EGFR inhibition, however in my studies I only focused on the downstream intracellular pathway of EGFR and FGFR4, Akt. Another key experiment to consider would be to assess if EGFR-inhibitor sensitivity could be restored when FGFR4 signaling is blocked. By blocking FGFR4 activity, I would be able to test if FGFR4 signaling is the mechanisms by which RMS cells acquire their resistance to EGFR blockage. I would hypothesize that if FGFR4 misexpression functions as an alternative pathway, then duel blockage of the two RTKs driving RMS would decrease Akt signaling and subsequently hinder RMS oncogenicity and tumorigenicity. I could also then question if duel inhibitions of multiple RTKs (EGFR and FGFR4) could be a new therapeutic method for RMS patients? The rate of treatment failure due to acquired drug resistance to monotherapies with first-generation RTK inhibitors continues to increase, making duel therapy regimens a better options by which to prevent these incidences. However, the challenges and risks that arise when testing multiple agents concurrently in patients should be highly considered.

D. Precision pre-clinical testing of MK-2206 treatment

Currently, based on the Children's Oncology Group – Soft Tissue Sarcoma Section, the standard procedure for testing new agents is to test them in combination with current therapeutic protocols, thus enhance the efficacy of the treatment. So, moving forward with these Akt studies, I would want to asses MK-2206 treatment in combination with chemotherapeutic agents given to RMS patients (i.e. Vincristine) in both ERMS and ARMS xenograft models. Testing MK-2206 in combination with current chemotherapies in vivo, would be much more accurate form of pre-clinical testing and would provide evidence as to how effective duel treatment will be in patients.

E. Mono-targeting of Akt is sufficient in blocking both RMS oncogenicity and tumorigenicity

Irrespective to the genetic aberrations that drive each individual RMS cell line, these studies demonstrated Akt signaling as a critical intracellular network in RMS pathogenesis. Of note, other studies have looked into investigating the role of the PI3K>Akt>mTOR pathway in RMS though in the context of examining the effects of duel inhibitors of both the PI3K>mTOR and MEK pathways in selected RMS cells. They found that some of the RMS cell lines tested didn't respond strongly to monotherapies but duel inhibition of the two pathways either triggered drug-induced cytotoxic cell death in culture or had greater effect on tumor growth in vivo [154] [155]. Though these results display to have identified putative therapeutic candidates for RMS treatment, early- phase clinical trials testing PI3K>mTOR and MEK inhibitors in solid tumors, exhibited to show low activity in combination [156]. Other reports have had more promising outcomes testing duel agents in patients with advanced malignancies though at the expense of

patients developing much greater toxicity effects [157]. As with any new agent(s), testing multiple new agents concurrently in a clinical trial remains highly challenging [156]. In contrast to these reports, our studies provide evidence that solely targeting Akt activity is sufficient in decreasing RMS pathogenicity in both in vitro and in vivo, thus MK-2206 treatment represents a highly more suitable, less toxic, therapeutic approach to treat RMS.

III. RMS cells exploiting EGFR/IL-6R signaling networks

A. Exploring IL-6/ IL-6R signaling in RMS

As the number of EGFR resistant cases continues to increase, duel therapies are considered to be the new solution to overcome these challenges within the clinic. In one of my previous studies discussed, I have shown EGFR to be a potential targetable candidate by which to treat RMS, however the rising incidences of acquired resistance to anti-EGFR monotherapies is a cause for concern that this may occur overtime with RMS patients. Therefore, as a pilot study, I aimed to identify another key pathway associated with EGFR resistance that could also be targeted concurrently with EGFR inhibitor treatment as a preventive measure to avert the development of resistance from occurring. Here, I have uncovered IL-6 signaling as an ideal pathway to target in conjunction with EGFR signaling as a novel form of treatment for RMS patients.

B. Uncovering a novel approach by which to therapeutically antagonize RMS.

Within the literature, several reports indicating that in other malignances such as lung carcinoma, patients whom have developed a resistance to anti-EGFR therapy is a result of the upregulation of the alternative pathway IL-6/IL-6R signaling [132] [133] [134]. From these studies, I questioned if perhaps IL-6/IL-6R could also be exploited in RMS cells, hence serve as an alternative mechanism in the development of EGFR resistance. As RMS cells present to be myoblast like transformed cells, I first probed if IL-6 signaling plays any role in muscle development. According to recent published data, IL-6 is also termed as a myokine for its role in regulating skeletal muscle growth and repair [138] [137]. Utilizing wild-type myoblast, C2C12, I verified the expression of the IL-6 signaling complex throughout the early and late stages of differentiation. Then, moving on to testing if IL-6 expression can be detected in RMS cell samples, I detected all elements of the complex (IL-6, sIL-6R, mIL-6R and GP130) to be present in several tested RMS cells.

Having confirmed the expression of IL-6 signaling, I next tested the effects of IL-6 activity blockage in RMS cells using a neutralizing anti-IL-6R antibody. I was able to show that blocking of IL-6R activity decreased RMS progression in cultured cells. When tested in tandem with EGFR inhibitor, Erlotinib RMS oncogenicity resulting in a much greater reduction in proliferation and cell migration. From these studies, I was then able to move forward into a more pre-clinical investigation, testing the humanized monoclonal antibody against IL-6R, Tocilizumab, which displayed to have similar results. What remains to be tested is if these effects are either synergistic or additive, a critical experiment when testing multiple therapeutic agents. These studied provided

evidence that not only does IL-6 activity influence RMS oncogenicity but duel blockage of the IL-6 and EGFR pathway provides a more effective form of targeted therapy.

Moving forward, I would next want to test the duel effects of Tocilizumab and Erlotinib treatments in vivo, utilizing mouse xenograft models. Could duel treatment significantly block tumorigenicity and perhaps even lead to tumor regression? I would also want to expand these studies by testing more RMS cells lines in order to have a better representation as to how effective duel treatment will be when tested in the clinic. As mentioned earlier I was able to detect all elements of the IL-6 signaling complex in two other cell lines (SMS-CTR and RH36) which were not tested. Aside from testing more cell lines, it would also be interested to test other EGFR inhibitors in my Tocilizumab studies, more specifically the EGFR monoclonal antibody Cetuximab. Cetuximab use in the clinic has exhibited to work more effectively in patients where tumorigenesis is driven by the misexpression of EGFR activity as result of gene amplification/ over expression [158]. Results from my study as well as data obtained from open-access NCI Oncogenomics RMS RNA-seq database suggested EGFR overexpression to be the driving mechanisms of RMS pathogenicity. In my RMS- Cetuximab studies I did detect a slightly enhanced blockage of both oncogenicity and tumorigenicity in tested RD cells, suggesting duel treatment with both monoclonal antibodies, Tocilizumab and Cetuximab, could represent a better therapeutic option. (Data can be found in Chapter 2: Investigating the role of EGFR signaling in Rhabdomyosarcoma Pathogenesis)

C. Interrogating the oncogenic mechanisms of IL-6 in RMS

Another key experimental assessment that is necessary for these studies, is to better interrogate how IL-6 is involved in RMS pathogenesis? Does IL-6 signaling play any role in RMS

tumor initiation? I have demonstrated its potential role in mediating RMS progression though the mechanism as to how it influences RMS is unclear. The oncogenic mechanisms of IL-6 signaling vary between different malignancies. Its expression could be driven by another dysregulated effector such as EGFR or by P53 mutations seen in renal cell carcinoma cases [159]. In some cancers, IL-6 signaling is dysregulated is due to a STAT3 activated mutation that facilitates this continuous autocrine and paracrine signaling cascade [160]. Though, RMS, another possible mechanism that needs further exploring could be one related to its role as a myokine.

IL-6 signaling is sometimes labeled as the double edge sword for its unique duel role in myogenesis; mediating both the expansion of myoblast populations and maturation/ differentiation into fused multinucleated myotubes [136, 138]. It does so through the activation of two distinct downstream signaling pathways, JAK1>STAT1>STAT3 and JAK2>STAT2>STAT3. Signaling via JAK1 (Janus kinase 1) promotes myoblast proliferation and actually prevents early differentiation, whereas JAK2 (Janus kinase 2) signaling is necessary for myoblast differentiation [161, 162]. This raises the question if the dysregulation of specific intracellular effectors involved in regulating the activity of either one of the JAK/STAT pathways mentioned, could then result in the continuous activation of the JAK1>STAT1>STAT3 and/ or loss of the myogenic pathway JAK2>STAT2>STAT3, hence driving RMS pathogenicity? Further testing would need be done in order to uncover the oncogenic mechanism of IL-6 in RMS.

Chapter 6

Conclusions and Future Directions

Conclusion

To conclude, here I have explored the role of different misregulated signaling networks in mediating RMS progression, 1. EGFR and 2. Akt. These studies were initiated by the identifications of EGFR, a misexpressed factor uncovered in a forward genetic screen utilizing a RMS PAX7-FOXO1 *Drosophila* model. The mechanisms that underlie RMS pathogenicity continues to remain debatable within the field, thus the need for mechanistic focused studies is greatly emphasized. Within the literature, some studies have demonstrated that the loss or impairment of myoblast differentiation programs leads to the development and progression of this disease [56, 59]. Our RMS model allowed for us to discover dysregulated myogenic signaling pathways that mediated RMS pathogenicity. Upon further characterize of these networks, I demonstrated how these mechanism(s) influence RMS progression and could serve as targetable therapeutics by which to treat RMS patients.

I began this studies with the hypothesis that the loss or disruption of intracellular differentiation programs drives the transformation of early myoblast and/or precursor myoblast cells, so the identification of these networks could not only aid in improving our understanding of the disease but could also serve as candidate targets. As I mentioned earlier, the misexpression of EGFR signaling was uncovered in our *Drosophila* RMS genetic screen, and since within these studies I aimed to assess if these networks contribute to the disruption of myogenesis, I first explored its role as a myogenic effector. Myogenesis has been a very extensively studied process,

however the actually mechanism involved in mediating myoblast cell-to-cell fusion are poorly understood within the mammalian system, hence I turned my focused to the *Drosophila* system in which terminal differentiation/fusion, known as muscle patterning, is better understood. Through these published reports, I found that fusion during muscle pattering is dependent on the presence of two distinct myoblast population, FCs and fcms. Interestingly, fate specification of these two populations is driven by the expression of EGFR, where FCs are EGFR positive and fcms are EGFR negative, and any misexpression of its activity (loss/absence or constitutive activations) results in a pool of unfused myoblast – hindering differentiation/ fusion. Seeing that several aspects of muscle development are highly conserved between *Drosophila* and mammals (e.g. myogenic factors- Pax3/7), I enquired if a similar process as EGFR-myoblast fate specification could also influence mammalian myoblast differentiation/fusion. This lead to my hypothesis that if the regulation of EGFR signaling plays a critical role in myoblast differentiation/fusion, then its misexpression impedes normal mammalian myogenesis and drives RMS pathogenicity, thus making EGFR activity a candidate therapeutic target.

Within my EGFR studies, after expression was verified within wild-type mammalian system (C2C12), I observed that its activity was regulated during myoblast differentiation and misregulation of its activity resulting in the loss of myoblast differentiation/ fusion, suggesting the importance of the regulation of EGFR signaling in myoblast. In RMS gene amplification/ overexpression of EGFR exhibited to be the oncogenic mechanisms utilized in RMS cells, which was displayed within the oncogenomics RMS RNA-seq database provided by NCI. Further testing of EGFR signaling in RMS cell lines, demonstrated that EGFR inhibition using pharmacological agents, Erlotinib and Cetuximab, significantly reduced RMS oncogenicity and tumorigenicity.

Additional, EGFR downregulation in RMS cells also resulting in a rescue of myoblast differentiation/fusion, providing evidence that disrupted differentiation networks play a significant role in RMS pathogenicity. Probing of the intracellular signaling nodes that act upon EGFR activation lead to the identification of Akt. Further investigation, testing Akt inhibitor, MK-2206, revealed Akt activity as a critical pathway mediating RMS progression not only in EGFR driven RMS cells, but in EGFR-inhibitor resistant and non-EGFR related RMS cell lines. In RMS cell lines that displayed no response to EGFR blockage, now demonstrated a marked decrease in pathogenicity both in culture cells and tested tumor xenograft models.

From these studies, I have assessed the role of EGFR activity in RMS cells lines, which lead me to uncovering the molecular target, Akt. Currently, both EGFR and AKT inhibitors have undergone phase 1 testing in pediatric cancers and were well tolerated making them potential suitable agents by which to treat RMS [21, 163, 164]. Though these results did display that EGFR inhibition reduced RMS pathogenicity, only RD cells had an effect from all other ERMS and ARMS cell lines tested. I later discovered non-responsive RMS cell lines carried another RTK genetic aberration, dysregulaiton of FGFR4, rendering EGFR inhibition to be non effective. However, after observing that Akt signaling remained active in these cells lines, further analysis treating these cell lines with Akt inhibitor, MK-2206, were done. The data demonstrated that in multiple RMS cell lines tested, both ERMS and ARMS carrying diverse identified oncogeneic mutations and/or misexpression, Akt inhibtion displayed to have a marked decrease in RMS pathogenesis in much more broader tested cohert of cell lines.

In addition to these studies, I have also explored and done some preliminary work testing the significance of IL-6/IL-6R signaling in RMS. Its misexpression as been shown to be associated with EGFR driven cancers, therefore making it an intriguing pathway to further pursue. As it turns

out, within the literature, IL-6 is referred to as a myokine as it plays a very important role regulating myogenesis. Similar to EGFR, the regulation of IL-6 activity is central in order to maintain proper muscle differentiation. In regards to RMS, preliminary studies I have done demonstrated that blockage of IL-6 signaling through the use of IL-6R neutralizing antibodies or FDA approved anti-IL-6R monoclonal antibody, Tocilizumab, significantly decreases RMS oncogenicity. When combined with EGFR inhibitor, Erlotinib, RD cells demonstrated a much greater decrease in oncogenicity in comparison to cells treated with either Tocilizumab or Erlotinib alone, suggesting a possible synergistic/ additive effect.

So overall, I have assesed the function of 3 druggable signaling mediators, EGFR-Akt, Akt and IL-6/IL-6R pathways. All 3 pathways were found to mediate myoblast differentiation processes supporting the notion that a loss or misregulation of intracellular myogenic networks, serves as a mechanism of cellular transformation, resulting in RMS pathogeniciy. It's important to note that since EGFR inhibition only had an effect on RD cells, it might only be effect in a much smaller subset of patient. Though it was through these studies that I was able to identify Akt acitivity as a better suited targetbale candidate to move forward into clinical testing. My work investigating Tocilzumab treatment in RMS indicated some promising data in culture, though much more preclinical assessment is still needed to be done. By testing these agents, this work has provided the field with new theraputic avenues that could improve RMS patient outcomes.

Future Directions: Examining the specific mechanism(s) that trigger RMS tumor initiation

The discovery and development of biomolecular strategies for therapeutic interventions against different malignances has been one of the main focuses in cancer research. Malignancies, such as Rhabdomyosarcoma, benefit from studies such as the one explored here, for it introduces much needed treatment options for RMS patients. Though, this is an essential issue to address within the field of RMS, another necessary area of focus is that of advancing our understanding in the biomolecular mechanisms that drive RMS tumor initiation. As mentioned previously, the cellular mechanisms that are thought to influence RMS pathogenicity remain debatable, however within the literature compelling evidence demonstrates that the loss or disruption of important myogenic networks involved in differentiation leads to cellular transformation. Within my work, I have observed blockage of oncogenic factors, such as EGFR, not only results in the arrest of cellular proliferation but also rescues myoblast differentiation, providing evidence that misregulated factors involved in normal differentiation programs are key effectors in RMS. Further investigation as to the cause or "trigger" leading up to the disruption/loss of these networks, whether they be environmental or genetic, is a critical next step necessary to advance our knowledge of this disease.

To date, through the use of genetically engineered animal models, several advancements within the field have provided some insight on potential pathways that drive RMS onset [5]. Unfortunately, there has been little focus on connecting how misregulated myogenic networks could be a major contributor to RMS pathogenicity. This in part could be attributed to the fact that there is a lot of uncertainty as to what the cell of origin is (cell of origin may depend on specific

RMS subtype), however given the evidence that some cases of RMS do possess myogenic markers (e.g., myogenin), misregulation of myogenic processes could a mechanism driving tumor onset. Future work will be aimed at further examining the role of identified myogenic pathways (e.g. EGFR, AKT1 and IL-6) during normal mammalian myogenesis and address the potential oncogenic mechanisms that trigger the misregulation of these pathways.

As it has been previously mentioned, the biology behind mammalian myoblast fusion is a system that remains to be unclear, so moving forward I would aim to extend my studies further characterizing the identified molecular myogenic networks (EGFR, Akt and IL-6/IL-6R) as well as profile other key pathways that facilitate myogenesis, in order to uncover the exact mechanisms by which these signaling pathways influence this highly conserved process. For example, when it comes to the activation of Akt isoforms 1 and 2, little is understood regarding the specific intracellular mediators that facilitate the regulations of one isoform versus the other. As it has been shown, Akt2 activation seems to promote differentiation, whereas Akt1 activity not only facilitates cell proliferation but recent reports have also suggested its potential role in inhibiting myogenin gene transcription. It is speculated that perhaps post transcriptional modification are at play in regulating activation specification, though this would need to be further tested [142].

As future directions, I find it critical to further profile these myogenic networks that govern the biological cellular processes of mammalian myoblast differentiation and fusion, for it could advance our understanding of RMS itself. Even though, from my studies I obtained compelling data demonstrating how targeting key myogenic factors effectively impedes RMS pathogenicity, the more broadly effective inhibitor being against Akt activity (MK-2206), several clinical challenges still remain and could potential delay the approval of its use in patients. Ideally, the discovering of a targeted therapy that benefits a high percentage of RMS patients is what the field

is aiming for, though realistically with the complexity that comes with patient's high tumor heterogeneity, the development of a treatment that would serve as the "magic bullet" for all RMS patients presents with several challenges down the road. Within the field, drug target discovery and development is still a much valuable and needed subject to continue on in research, however studies aimed to further elucidate how specific biomolecular mechanism(s) initiate RMS tumor progression could have a greater scientific impact.

Chapter 7

Materials and Methods

Genetics, expression profiling, and statistics.

In a screen for PAX7-FOXO1 suppressors, the *UAS-PAX7-FOXO1* and muscle-specific *Myosin Heavy Chain-Gal4* transgenes were used, and rescue of lethality assessed as previously described (14, 16). All stocks were obtained from the Bloomington *Drosophila* Stock Center. Data represent mean ± SEM; unpaired two-tailed Student's tests with P-values < 0.05 considered significant.

Cell culture, transfections, and cell lines.

C2C12 cells (ATCC) were grown in DMEM (Sigma-Aldrich) with 20% FBS (Atlas Biolabs). For differentiation, DMEM was supplemented with 2% horse serum (Sigma-Aldich). ERMS cells (RD, RH36 and SMS-CTR) were cultured in DMEM (Sigma-Aldrich) with 10% FBS and ARMS (RH30 and RH41 cells) (ATCC) were cultured in RPMI 1640 (Sigma-Aldrich) with 10% FBS. Transfections were done using Lipofectamine (Invitrogen) following standard protocol. Constructs for the shRNA studies were obtained from Open Biosystems. For stable cell lines, cell lines were generated by transduction of lentivirus generated in HEK293T (ATCC) cells for each individual shRNA construct into the desired cell line. Puromyosin selection was added to stable cell lines to maintain positive selected cells. Constructs for shRNA against AKT isoforms were doxycycline inducible. Concentrations of doxycycline and puromycin used were 0.5ug/ml and 2ug/ml, respectively. Cells were tested for knock down by qRT-PCR and/or immunoblot analysis.

Reagents

Erlotinib, Cetuximab, MK-2206, and Tocilizumab (Actemra®) were provided by LC Laboratories, the UTSW Campus Pharmacy, Selleck Chemicals (Houston, Tx, USA), and Genentech respectively. Mouse EGF ligand (#E5160) used was provide by Sigma-Aldrich. The concentrations used in cell culture experiments were the following: 10uM of Erlotinib, 1ug/ml of Cetuximab, 0.5uM of MK-2206, 75ug/ml Tocilizumab and 10nM of EGF ligand. For cell culture studies, vehicles used were Captisol (Cydex, Pharmaceuticals) [Erlotinib], PBS (Cetuximab), and Dimethyl Sulfoxide (DMSO – Sigma #D2660) [MK-2206]. Mouse IgG isotype control (G3A1) was used for Tocilizumab related studies (Cell Signaling #5415S) Neutralizing anti-IL-6R antibody (R&D Systems, #7506)

Cell Viability Analysis

MTT (3-[4,5-Dimethylthythiazol-2-yl]-2,5- Diphenyltetrazolium Bromide) assays were performed to assess the sensitivity of cells to drugs using Vibrant™ MTT Cell Proliferation Assay Kit (V-13154) provided by Molecular Probes/Invitrogen. RMS cells (10,000 cells/well) were cultured in triplicate in flat-bottomed 96-well plates at 37°C with 5% CO_2. Cells were treated with a dilution series of tested drugs for 72 hours and absorbance was read at 540mm. Final results were represented as values normalized to a vehicles-treated control.

Cell Death Analysis: TUNEL

TUNEL Assays were performed to analyze the detection and quantification of apoptosis using Deadend Fluorometric TUNEL System which measures fragmented DNA by catalytically incorporating fluorescein-12-dUTP at 3’ – OH DNA ends. RMS cells were seeded in 4 chamber

slides and treated with 10uM of Erlotinib, 1ug/ml of Cetuximab or 0.5uM of MK-2206 for 24 hours. TUNEL assays were done following the kits standard protocol. Detection of fluorescein-12-dUTP-labled DNA was done by fluorescence microscopy. Images were taken and the number of positive cells were counted.

Immunofluorescence, immunoblotting and immunohistochemistry.

Immunofluorescence staining was performed on cells seeded in 4 chamber slides treated with 10uM of Erlotinib, 1ug/ml of Cetuximab (EGFR inhibitors) or 0.5uM of MK-2206 (Akt inhibitor) for 72 hours (Ki67-Proliferation) or 5 days, stable cells expressing shRNA were treated with 150ng/ml doxycycline in differentiation media 24hrs after seeding for 4 or 6 days. C2C12 EGF ligand / Erlotinib wild-type studies were treated with 10nM of mouse EGF ligand daily or with 0.1uM Erlotinib (0.5uM also test) every other day for the entire 6 day differentiation period. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde at room temp for 5 min, permeablized and blocked with 0.1% Triton X-100 and 3% BSA in PBS for 15 min. Antibodies used: mouse MF-20 (1:1; Developmental Studies Hybridoma Bank; University of Iowa; Iowa City, IA), rabbit Ki67 (1:200; #MA5-14520, Invitrogen), Alexa-488 goat anti-mouse IgG, and Alexa-568 goat anti-rabbit IgG (1:5000; Invitrogen). Cells were mounted using vectashield with DAPI (Vector Laboratories). Fusion, differentiation and proliferation indices were calculated from three independent experiments. For each experiment, four random fields were counted. For fusion, the number of nuclei in bi- or multinucleated myotubes were scored. For differentiation, the number of nuclei present in MHC-positive cytoplasmic tissue were scored. For proliferation, the number of mitotic figures or Ki67-positive cells were scored.

Immunoblotting analysis were done on whole-cell lysates generated in RIPA buffer. For the C2C12 time course and both EGF and Erlotinib wild-type studies, cells were harvested at time points of Day 0, 2, 4 and 6 after the switch from growth media to differentiation media. EGF and Erlotinib wild-type differentiation studies, C2C12s were treated with 10nM EGF daily or with 0.1uM (also tested 0.5uM) of Erlotinib every other day. All RMS cells (RD, RH36, SMS-CTR, RH41 and RH30) were harvested 72 hours after appropriate drug treatments. RD EGFR-shRNA (F9) or RD GFP-shRNA control cells were harvested 72 hours after transient transfection, as were stable cell lines. For in vivo xenograft tumor tissue analysis, sections of fresh tumor tissues were harvested from xenograft models at the end of the experiment and lysates were generated using a high-pressured homogenizer to break up the tissue in RIPA buffer. Primary antibodies were from the following suppliers: phospho-EGFR$^{(Tyr1068)}$ (#3777), total EGFR (T43, #2963), phospho-Akt$^{(Ser473)}$ (#4058), total Akt (#9272), phospho-Mek1/2$^{(S217/221)}$ (#9154), total Mek1/2 (D1A5, #8727), phosphor-MAPK$^{(T202/Y204)}$ (#4370) and total MAPK (Erk1/2 137F5, #4695) [Cell signaling]; mouse-IL-6R (CD126, #46-1269-42)[Invitrogen]; GP130 (M-20, #H1814)[Santa Cruz Biotechnology]. All primary antibodies were used at 1:1000. Secondary antibodies (Sigma-Aldrich) were used at 1:5000.

Immunohistochemistry's were done following the standard protocol for paraffin embedded tissue sections and counterstained with hematoxylin; primary antibodies used were mouse MF-20 (1:1; Developmental Studies Hybridoma Bank; University of Iowa; Iowa City, IA); and rabbit Ki67 (1:200; #MA5-14520, Invitrogen).

Crystal Violet Staining

Cells were seeded in 60 mm petri dishes and treated with Media in treated cells seeded in a 60 mm petri dish was gently aspirated. The plates were then rinsed using 3mls of chilled (4°) PBS, 3 times. Excess liquid was gently aspirated as to not disrupt any of the bottom layer of cells. 3mls of crystal violet fixing/staining solution (0.5 g Crystal Violet (0.05% w/v) 27 ml 37% Formaldehyde (1%) 100 mL 10X PBS (1X) 10 mL Methanol (1%) 863 dH20 to 1L) was added to each plate and stained at room temperature for 10 mins. The stain was then carefully removed and properly disposed of (Formaldehyde waste) and the cells were then gently washed with water. Excess liquid was then gently aspirated and allowed to air dry before pictures were taken.

Soft agar colony assays

Base Agar layer had 0.5% agar (BD Difco Agar Noble) with 20% DMEM or RPMI-no phenol red (Hyclone). RMS cells (RD, RMS13 and SMS-CTR) and RD shRNA stable cells were trypsonized, counted and resuspended in 20% RPMI-no phenol red + 0.7% agarose (Mercury reagents) (5,000 cells/ plate). Top agarose layer was a final concentration of 0.35%, plated over the set 0.5% base agar. Plates were incubated at 37^0C. Cells were fed with fresh 20% DMEM or RPMI media treated with either 10uM Erlotinib, 1ug/ml Cetuximab or 0.5uM MK-2206 every other day. RD stable shRNA cells were treated with puromyocin (2ug/ml) each time the media was replenished. Colonies were counted 3-4 weeks after treatment. Colonies were stained with 0.005% crystal violet prior to analysis. Replicate plates were scored independently, and four random 20×-lens fields were scored for each plate.

Wound Healing Assay: Scratch Assay

Rh30 cells were seeded onto a 60m petri dish (70% confluence) and allowed to adhere overnight. Four representative vertical lines where thin scraped on each dish, disrupting the signal monolayer of cells, using the end of a pipette tip. Images were taken at each of the "wound sites" and treated with 15uM of Erlotinib, 0.5uM of MK-2206 and/or 75ug/ml Tocilizumab for 48 hours. Images were then re-taken. Images prior to treatment were compared with images taken 24 and 48 hours after treatment. 6% Captisol and DMSO treated cells were used as a vehicle control for Erlotinib and MK-2206, respectively. IgG as used an isotype control for Tocilizumab. The data is representations of n=3 with similar results.

qRT-PCR Analysis

RNA was isolated from RMS cell lines (RD, RMS13, SMS-CTR, RH36, RD shAkt1A, RD shAkt1B, RD shAkt2B and RD shAkt2C) using Rneasy extraction kit (Qiagen). Reverse Transcription (RT) was performed using 4ug of total RNA (QuantiTect Reverse Transcription, Qiagen). PCR was then performed on each of the cDNA samples using Phusion Hot Start DNA Polymerase (Finnzymes) using primer sets. 18S was used as an internal control. Experiments were repeated 3 times for each cell line. Primers used were specific to human (h).

shAkt Knock-down Study (K. Nakatani et al. JBC 1999)

Primers	Forward	Reverse
hAkt1	5'-TAATACGACTCACTATAGGGCCAAG GAGATCATGC-3′	5'-GATTTAGGTGACACTATAGCTC CAAGCTATCGTCC-3′
hAkt2	5'-ATGAATGAGGTGTCTGTCATCAAAG AAGGC-3'	5'-TGCTTGAGGCTGTTGGCGACC-3'
18s	5′-ACAGTCAGCCGCATCTTCTT-3′	5′-TTCTAGACGGCAGGTCAGGT-3′

IL-6 Preliminary Study (T. Ara et al. cancer research 2009)

Primers	Forward	Reverse
hIL-6Ra	5′-CATTGCCATTGTTCTGAGGTTC-3′	5′-GTGCCACCCAGCCAGCTATC-3′
hsIL-6R	5'-CAGCAGTTCAAGAAGACGTGG AAGCT-3'	5'-GTGCCACCCAGCCAGCTATC-3'
hGP130	5'-GCAAGATGTTGACGTTGCAGAG ACTTG-3'	5'-GGGCATTCTCTGCTTCTACCCAGAC-3'
hIL-6	5'-TAGCCGCCCCACACAGACAG-3′	5′-GGCTGGCATTTGTGGTTGGG-3′
18s	5′-ACAGTCAGCCGCATCTTCTT-3′	5′-TTCTAGACGGCAGGTCAGGT-3′

Mouse Xenograft Models

All the experiments involving animals were performed following IACUC approved guidelines. Genetic background was Fox ChaseSCID Beige Mice (Charles River Laboratories). The shRNA-TANC1 xenografts were performed in 10-12 week-old pups, while all other xenografts were performed in 4-5 week-old pups. Xenografts were prepared by subcutaneously injecting 5 x 10^6

cells suspended in either Matrigel (shRNA-TANC1) or 20% DMEM no-phenol red medium (all remaining studies) into the right flank of each mouse. Once tumor size was approximately 100 mm^3, the following were performed: (i) for shRNA studies, expression was induced by supplementing drinking water with 1 mg/mL doxycycline; (ii) for Erlotinib, drug was administered 50 mg/kg (6% Captisol) daily by oral gavage (45); (iii) For Cetuximab, drug was administrated 1 mg/kg (PBS), three times per week by IV (46); & (iv) For MK-2206, drug was administered 180 mg/kg (15% Captisol), three times per week by oral gavage (42). Tumor growth was measured manually twice a week using calipers, and the tumor volume was calculated using the formula (Volume = L*(W2)*0.5) (45). Tolerability of the drugs was estimated by monitoring body weight and general health over the course of each study. Once the tumor volume reaches 2cm in either length or width, animals were sacrificed following IACUC guidelines and tumors were harvested for further studies. All plots were generated using Prism 7 (GraphPad). All animal studies were performed in accordance with UTSW IACUC guidelines.

Statistics

Type I error was evaluated by the two-tailed Student's *t* test, a *P* value of less than 0.05 was considered significant. Type II was evaluated by Achieved Statistical Power analysis (post hoc analysis), a Power value of greater than 0.80 was considered significant. All data are mean ± SEM. An event for the inhibitor studies was based on PPTP criteria (42): a quadrupling of tumor volume from a base volume (here, 200 mm^3 for RD cells, 250 mm^3 for RH30 cells). Software programs used were Excel 2011/2016 (Microsoft), Prism 7 (GraphPad), and G*Power 3 (Heinrich-Heine-Universität Düsseldorf).

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