

**Characterization and Function of the ~70 kDa
Immunoreactive Species of Pannexin3 in
Rhabdomyosarcomas**

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Abstract

Rhabdomyosarcoma (RMS) is a skeletal muscle-derived tumour and is the most common soft tissue sarcoma of childhood. RMS tumours arise due to defects in differentiation, and as a result proliferate indefinitely. Pannexins consist of three members (pannexin1, 2, and 3) and are known to form single membrane channels. Reports have shown expression of pannexin3 (Panx3) as both the expected molecular weight species at ~43 kDa as well as a ~70 kDa immunoreactive species. While studies have begun to report on the function of the ~43 kDa form, the exact identity and function of the ~70 kDa immunoreactive species of Panx3 remains poorly understood. It has recently been reported that the ~70 kDa immunoreactive species of Panx3 is highly expressed in proliferative, non-differentiated human primary skeletal muscle myoblasts (HSMM), becoming drastically down regulated during differentiation. Indeed, knockdown of the ~70 kDa immunoreactive species of Panx3 inhibited proliferation without inducing differentiation in skeletal muscle myoblasts. We thus hypothesized that the ~70 kDa immunoreactive species of Panx3 would be upregulated in RMS supporting this proliferative phenotype. Here we now show that the ~70 kDa immunoreactive species of Panx3 is increased in RMS cell lines and tumours to a level similar to that seen in undifferentiated HSMM and fetal tissues, respectively. Further characterization of this species revealed that it is indeed a glycoprotein, an intrinsic characteristic of all pannexin members, it is recognized by two Panx3 antibodies targeting distinct epitopes, and it is reduced with Panx3 specific shRNA. Reduction of levels of the ~70 kDa immunoreactive species of Panx3 resulted in a significant decrease in proliferation of RMS cells without inducing differentiation. Taken together, these data suggest that the ~70 kDa immunoreactive species of Panx3 might be involved in keeping undifferentiated RMS cells in a proliferative state and that reduction of its levels or functions may be beneficial for RMS.

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List of Abbreviations

%	Percent
°C	temperature in degree Celsius
aRMS	Alveolar Rhabdomyosarcoma
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
BSA	Bovine Serum Albumin
cm	Centimeter
DMEM	Dulbecco's Modified Eagle Medium
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
HCL	Hydrochloric Acid
HEK	Human Embryonic Kidney cells
HSMM	Human skeletal muscle myoblasts
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
eRMS	Embryonal Rhabdomyosarcoma
FBS	Fetal Bovine Serum
kDa	Kilo Dalton
mM	Milli molar
MHC	Myosin Heavy Chain
MOI	Multiplicity of Infection
MRF	Muscle Regulatory Factor
NaCl	Sodium Chloride

Panx1	Pannexin 1
Panx3	Pannexin 3
PBS	Phosphate-Buffered Saline
PCNA	Proliferating cell nuclear antigen
RMS	Rhabdomyosarcomas
RPM	Revolution per minute
RFP	Red Fluorescent Protein
SDS	Sodium dodecyl sulfate
μM	Micro molar

Introduction

Background on Rhabdomyosarcomas

Rhabdomyosarcoma (RMS) is an aggressive form of childhood cancer with a poor prognosis and is the most common soft tissue sarcoma in children and adolescents ¹. It accounts for approximately 50% of all pediatric soft tissue sarcomas, and for 7-8% of all childhood malignancies ¹. RMS tumours arise as a result of differentiation defects during myoblast differentiation ². RMS can also originate from muscle satellite cells ³. These satellite cells are inactive muscle cells which have the potential to proliferate as a result of injury ^{3,4}. Like other cancers, RMS cells can proliferate indefinitely without terminally differentiating ⁵.

While RMS tumours develop in skeletal muscle, studies have shown that RMS tumours can originate from other non-muscle areas like salivary glands and genitourinary tracts ⁶⁻⁸. This indicates that these tumours are not limited to muscle tissue and can arise anywhere in the body ⁶⁻⁸. Current treatments for RMS involve chemotherapy, radiation and surgery ⁹⁻¹¹. However, these are associated with many side effects such as infertility, cardiac diseases and organ failure ⁹⁻¹¹. Even with these aggressive treatments, the survival rate for RMS has remained below 30% for the past 15 years ⁹. There is thus a need for a novel therapy for improved treatment of RMS with fewer side effects and better overall outcome.

Subtypes of Rhabdomyosarcoma and Their Origins

There are two major histological forms of RMS: embryonal (eRMS) and alveolar (aRMS) ^{12,13}. Embryonal RMS tumours are the most common form of RMS and are most frequently confined to the head and neck areas ^{2,14}. They are commonly seen in children below 10 years of age ^{2,13,14}. Unlike eRMS, alveolar RMS usually occur in people between 10-30 years of age and usually arise in the extremities ¹⁵. Alveolar RMS accounts for 25% of all RMS cases ¹⁵. While it is less common than eRMS, it is more aggressive and has a poorer prognosis ¹³.

Chromosomal translocations are very common in aRMS ^{7,16}. About 90% of cases of aRMS carry translocation between chromosome 2 and 13 or chromosome 1 and 13 resulting in fusion proteins, both of which are products of transcription factors ^{7,16}. The first transcription factor consists of Paired Box (PAX) transcription factor family members whereas the second class consists of Forkhead (FKHR) family members ⁷. PAX-FKHR suppresses differentiation by inhibiting the transcriptional activity of the myogenic regulatory factor MyoD ⁷. Therefore, chromosomal translocations between PAX3 and PAX7 with FKHR transcription factor genes (PAX3-FKHR & PAX7-FKHR) are the prime factors which lead to the origin of RMS tumors ^{2,7,13,17-20}. On the other hand, loss of heterozygosity, mutations in p53 gene and dysregulation in N-ras or K-ras signaling pathways are the leading causes for the progression of eRMS ^{2,13,17-20}. *In vivo* studies have

shown that ras activation is enough to initiate tumorigenesis suggesting that dysregulation of ras signaling is an important factor in the progression of eRMS ²⁰.

Skeletal Muscle Differentiation

The process of skeletal muscle terminal differentiation involves a sequence of organized events and cellular changes. There are various factors like expression of myogenin and muscle-specific proteins and fusion of myoblasts which play an important role in this process ^{5,21} (Figure 1). Proliferative myoblasts express muscle regulatory factors (MRFs) like myogenic factor 5 (Myf5), myogenin, myoblast determination protein (MyoD) and muscle regulatory factor 4 (Mrf4), but muscle-specific genes are expressed only after the formation of myotubes ^{6,21} (Figure 1). Skeletal muscle cell determination and differentiation are regulated by a network of four MRFs - Myf5, myogenin, MyoD and Mrf4/Myf6 ²¹. The transcription factors MyoD, Myf5, and Mrf4 are known as cell determination genes as they can direct muscle cell fate from somatic multipotent muscle progenitor cells ⁶. Indeed, mice triple mutants for these genes lack differentiated muscle fibers and myoblasts ^{15,22-24}. Double homozygous mutants lacking both Myf5 and MyoD were also shown to lose their capability to form muscles and did not express muscle markers and myogenin ^{22,24}. In the same study, homozygous mutation of myogenin in mice demonstrated inability of myoblasts to form myofibers ²². These reports suggested that myogenin was required for terminal differentiation of skeletal muscle cells ²². As members of the basic-helix-

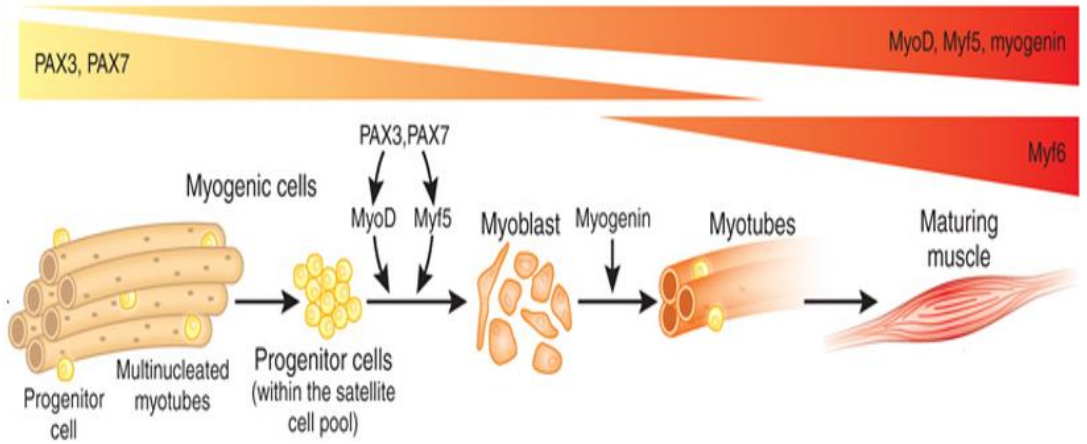
loop-helix (bHLH) transcription factor family, myogenin and MyoD activate muscle specific genes like myosin heavy chain (MHC) and support myogenesis by making cells differentiate from the proliferative phase ^{21,23}. MHC, MyoD, and myogenin can thus be effectively used as markers to study skeletal muscle differentiation ^{23,25–27}.

Differentiation Defects and RMS

RMS express MRFs like MyoD and myogenin, yet they are unable to complete the differentiation process ^{5,7,28}. Among all MRFs, MyoD is the main player controlling myogenesis ^{5,7}. Many studies have focused on investigating the role of MyoD in deregulation of differentiation in RMS. It has been shown that exit from the cell cycle is not achieved in RMS due to inhibition of MyoD activity ^{5,7}. PAX-FKHR proteins can suppress differentiation by inhibiting MyoD transcriptional activity, which is one of the main characteristics of RMS ^{5,7}. In addition, the suppression of PAX3 genes caused by the presence of the PAX3-FKHR results in the cells inability to exit cell cycle in RMS ⁷. As a result, PAX-FKHR proteins suppress terminal muscle differentiation thus promoting RMS development ^{7,27}. Hence, dysregulation in PAX-FKHR gene targets and MyoD function leads in part to the impaired differentiation in RMS ^{7,27,29}. As impaired differentiation in muscle cells promotes RMS and as RMS variants exhibiting a lesser degree of differentiation demonstrate greater malignant potential, it is

Figure 1. Schematic representation of mammalian skeletal myogenesis

Schematic representation of skeletal myogenesis from Hettmer and Wagers ³⁰. Muscle specification depends on myogenic transcription factors (such as PAX3, PAX7, MyoD, Myf5, myogenin and Myf6). These transcription factors are required for myogenic differentiation. Sequentially, this process involves the fusion of myoblasts to form myotubes, which are subsequently aligned to form muscle fibers. During skeletal myogenesis, expression of stem cells markers (PAX3, PAX7) reduces, whereas differentiation markers (MyoD, myogenin, Myf5, and Myf 6) increase.



commonly hypothesized that inducing differentiation in RMS tumors might prevent tumor progression and metastasis.

Pannexins

Recently, human genome analysis has revealed 3 genes with 25-35% sequence homology to the gap junction proteins of invertebrates, innexins and were subsequently called pannexins^{31,32}. This novel family consists of pannexin1 (Panx1), pannexin2 (Panx2), and pannexin3 (Panx3)³³⁻³⁵. Pannexin family did not share sequence homology with connexins, which are mammalian gap junction proteins was found, and were thus suggested to have distinct functions^{31,32,36}.

Expression Patterns and Distribution of Individual Pannexins

Studies have shown that, in humans, Panx1 transcripts can be detected in the heart, skeletal muscle, testis, ovaries, brain, colon, spleen, erythrocytes, pancreas, lung, liver, and skin³³⁻³⁵. However, at the protein level, Panx1 was detected in murine brain, kidney, liver, spleen and heart³³⁻³⁵. Panx2 transcripts have been detected in human adult brain^{34,35}. Panx3 transcripts were found in osteoblasts, fibroblasts and cartilages from inner ear of mouse^{33,34}. Western blotting analysis have shown two immunoreactive species for Panx3 at ~43 kDa and ~70 kDa^{33-35,37,38}. The ~43 kDa species of Panx3 corresponds to its expected molecular weight³³. Interestingly, the two forms were expressed in murine skin,

cartilage, and heart ventricles, while the ~70 kDa species was found in lung, liver, spleen, and thymus³³. The ~70 kDa species was also found in the male reproductive tract of adult rat³⁹. Recently, our laboratory has demonstrated that both Panx3 species, in addition to a form at ~51 kDa, were detected in human, mouse and rat skeletal muscle tissues³⁸.

Topology and Biochemistry of Pannexins

Pannexins exhibit a topology similar to connexins and innexins. They possess four α -helical transmembrane domains (TM), two extra cellular loops (EL) and one intracellular loop (IL)³³⁻³⁵. Variability in sequence is seen highest in the C-termini (CT) whereas the N-termini (NT) is an extremely conserved domain^{34,35}. Panx1 and Panx3 are homologous to each other³⁴⁻³⁶. Panx1 and Panx3 protein sequences reveal a 41% similarity at the amino acid level³⁴⁻³⁶. Moreover, Panx1 and Panx3 have similar CT domains and contains many glycosylation and phosphorylation sites³⁴⁻³⁶. Panx2 exhibits a larger CT domain resulting in the distinct and unique functions of Panx2³⁴⁻³⁶. Similar to innexins, the EL of pannexins contain two highly conserved cysteine residues^{34,40}. Site mutagenesis studies have shown that Panx1 forms channels via four cysteine residues^{34,41,42}. As opposed to Panx1, there isn't much known about the Panx3 channel formation^{34,41,42}. Panx3 releases ATP into the extracellular environment, thereby acting as a endoplasmic reticulum (ER) Ca²⁺ channel to promote chondrocyte and osteoblast differentiation^{34,35,43}. Panx3 has also been shown to reduce proliferation of

keratinocytes^{34,35,44}. Panx3 plays a key role in sperm transport and maturation in rats³⁹. Moreover Panx3 channels have been shown to regulate proliferation and differentiation of skeletal muscle myoblasts³⁸.

Post-Translational Modifications in Pannexins

Pannexins undergo considerable post-translational modifications. It has been reported that Panx1 is glycosylated, nitrosylated, and phosphorylated^{33,35,45,46}. Panx2 has been shown to be glycosylated and palmitoylated⁴⁷⁻⁴⁹. Glycosylation has also been observed in both species of Panx3^{33-35,37}. Our laboratory recently showed that, as opposed to its ~43 kDa species, the ~70 kDa immunoreactive species of Panx3 is also phosphorylated and sialylated³⁸. While the role of sialylation in pannexins is currently unclear, it may regulate its channel function since the gating of the voltage dependent channel has been shown to be regulated by sialic acid⁵⁰. The phosphorylation of the ~70kDa immunoreactive species of Panx3 has only been reported in skeletal muscle³⁸, and suggests that it may also possess characteristics that are distinct from the ~43 kDa form of Panx3³⁸.

Of all the pannexin post-translational modifications, glycosylation has been the best described. N-linked glycosylation is the most common and well-studied modification of all the pannexins³³⁻³⁵. Protein sequence analysis revealed that asparagine 254 and 71 are the N-linked glycosylation sites in the second and first extracellular loop of Panx1 and Panx3, respectively³³⁻³⁵. Moreover, Panx2

demonstrated N-linked glycosylation at asparagine 86 in the first EL domain ^{34,35} . Panx3 glycosylation plays a vital role in their proper trafficking and regulation of cellular localization ^{33-35,45,46} . In addition, pannexin glycosylation is important for intermixing between pannexin family members by regulating their channel function ³³⁻³⁵ . The tissue expression profile demonstrated that Panx1 was expressed in several mouse tissues such as brain, lung, kidney, liver, skin, spleen, heart ventricles and cartilages ³³⁻³⁵ . High variability in the degree of glycosylation in these tissues was observed ³³⁻³⁵ . High glycosylation of Panx1 was seen in murine brain and spleen as compared to murine skin and cartilage ³³⁻³⁵ . However, Panx3 was found below detectable levels in murine brain ³³⁻³⁵ . This variability in the degree of glycosylation suggest that the functions of pannexins might be differentially regulated by their state of glycosylation ³³⁻³⁵ . Moreover, murine skin and cartilage demonstrated the presence of both Panx1 and Panx3 suggesting that pannexins might exhibit distinct functions in the same tissues ³³⁻³⁵ . Both Panx1 and Panx2 were found to be highly expressed in brain ^{33-35,51} . It was shown that Panx1 and Panx2 acts as a tumor suppressor in C6 glioma cells ^{51,52} . Moreover, both Panx1 and Panx3 were found to be present as punctate and diffused staining in epidermis suggesting different functions of pannexins in skin ^{33-35,37} . Furthermore, it was demonstrated that both Panx1 and Panx3 were down regulated in human keratinocyte tumors ^{34,35,37} .

Channel Properties of Pannexins

Panx1 has been shown to form pannexin oligomers named pannexons⁴². Dye uptake assays have revealed that members of the pannexin family can interact to form intermixed single membrane channels^{34,35,45}. Intermixing between pannexins is dependent upon their glycosylation status as Panx2 interacts with Panx1^{33-35,45}. Furthermore, intermixing has been shown to occur primarily between Panx1 and Panx2. Interactions between Panx1 and Panx3 are rare^{33-35,45}. The intermixing between Panx1 and Panx2 results in regulation of single membrane channel function^{34,35,42,45}. It was shown that Panx2 plays a significant role in intermixing by inhibiting Panx1 channels^{33-35,42,45}. However the Panx1 and Panx3 intermixing did not lead to any change in the channel function properties^{34,42,45}. Recent studies have shown that pannexin channel properties are distinct from those formed by connexins^{34,53}. Indeed, pannexins do not form gap junctions but rather single membrane channels⁵³. As single membrane channels, Panx1 and Panx3 release ATP into the extracellular environment, thereby suggesting an important functional role for pannexins^{34,35,43}. Panx1 and Panx3 channels also participate in purinergic receptors (P2X and P2Y) signaling as well as acting as Ca²⁺ channels in the endoplasmic reticulum (ER)^{34,54}.

Cellular Functions of Pannexins

There have been multiple cellular functions associated with Panx channels to date. Panx1 has been shown to play a role in calcium wave propagation and vasodilation ^{54,55}. Panx1 also plays a vital role in apoptosis as caspases cleave Panx1 subunits inducing the release of ATP and UTP ^{34,43,56}. This ATP and UTP then acts as “find me” signals to attract phagocytes thereby clearing dead cells ^{43,56}. In addition, reports have also demonstrated that under stress conditions, Panx1 channels control blood flow by releasing ATP from erythrocytes ^{34,55}. Panx1 plays a role in vasodilatation ⁵⁵, inflammatory responses, ^{57,58} epilepsy ⁵⁹, ischemic death of neurons ⁶⁰, death of enteric neurons during colitis ⁶¹, and stabilization of synaptic plasticity and learning ⁶². Recent data in the literature has demonstrated that in organotypic cultures from rat epidermal keratinocytes (REK), Panx1 over expression disturbed the epidermis layer thickness, hence suggesting an important role of Panx1 in regulation of keratinocyte differentiation ^{34,44}. Our laboratory has recently shown that Panx1 plays an important role in the regulation of skeletal muscle cell differentiation ³⁸.

Contrary to Panx1, the physiological and cellular functions of Panx3 still remain poorly understood. It has been reported that the ~43 kDa species of Panx3 regulates osteoblast and chondrocyte differentiation by promoting intracellular ATP and cAMP release into the extracellular environment, ^{63,64}. Panx3 was also shown to act as an endoplasmic reticulum calcium channel to promote osteoblast

differentiation ^{34,63}. Regulation of proliferation of osteoprogenitor cells through Wnt and p21 signaling was also reported to be dependent upon Panx3 ⁶⁵. A new report has demonstrated that the nucleotide release and monocyte chemoattraction in skeletal muscle are dependent upon Panx3 channels ⁶⁶. Over-expression of ~43 kDa species of Panx3 has been shown to reduce keratinocyte proliferation, but did not alter the organotypic epidermis integrity ⁴⁴. In the same study, increases in the levels of the ~70 kDa immunoreactive species of Panx3 were detected during keratinocyte differentiation into organotypic epidermis, suggesting that it may play a role in this process ⁴⁴. Recently, our laboratory has shown that the overexpression of the ~43 kDa species of Panx3 inhibited the proliferation of human skeletal muscle myoblasts (HSMM) and induced their differentiation ³⁸. In addition, we have also demonstrated that the ~70 kDa immunoreactive species of Panx3 is regulated during skeletal muscle differentiation ³⁸. Indeed, the levels of the ~70 kDa immunoreactive species of Panx3 were very high in proliferative and undifferentiated HSMM and were significantly reduced during their differentiation ³⁸. Furthermore, the reduction of the ~70 kDa immunoreactive species of Panx3 using shRNA in HSMM significantly reduced their proliferation, but did not trigger their differentiation ³⁸.

Table 1. Tabular summary of the known functions of pannexins

Summary of the functions of the various pannexin species as reported to date.

Panx1	Panx2	Panx3
<ol style="list-style-type: none"> 1. Calcium waves ⁵⁴ 2. Vasodilation ⁵⁵ 3. Apoptosis ^{43,56} 4. Ischemic death of neurons ⁶⁰ 5. Inflammatory responses ^{57,58} 6. Stabilization of synaptic plasticity and learning ⁶² 7. Epilepsy ⁵⁹ 8. Carcinogenesis- C6 glioma cell and keratinocyte tumors ^{37,52} 9. Keratinocyte differentiation ⁴⁴ 10. Skeletal muscle differentiation ³⁸ 	<ol style="list-style-type: none"> 1. Neuronal differentiation ⁶⁷ 2. Carcinogenesis - C6 glioma cells ⁵¹ 	<ol style="list-style-type: none"> 1. Carcinogenesis- keratinocyte tumors ³⁷ 2. Chondrocyte differentiation ⁶⁴ 3. Osteoblast differentiation ⁶³ 4. Keratinocyte Differentiation ⁴⁴ 5. Human skeletal muscle differentiation and proliferation - ~43kDa species ³⁸ 6. Human skeletal muscle proliferation- ~70kDa species ³⁸ 7. Nucleotide release and inflammation in diabetes ⁶⁶

Role of Pannexins in Carcinogenesis

Pannexins have been shown to play a significant role in carcinogenesis. Indeed, tumor suppressor properties have been demonstrated for both Panx1 and Panx2^{34,37,51,52}. The over-expression of Panx1 and Panx2 reduced the proliferation of C6 glioma cells, as well as reduced the tumor growth in vivo^{51,52}. However, a contrary effect of Panx1 was observed in skin cancers⁶⁸. It was revealed that during progression of melanoma, Panx1 levels were increased and that Panx1 knockout lead to reduced melanoma tumor growth⁶⁸. On the other hand, it was shown that both Panx1 and Panx3 levels were significantly reduced in human keratinocyte tumors compared to normal skin, thus suggesting for the first time a role for pannexins in human carcinogenesis³⁷.

Differential Expression and Regulation of the ~70 kDa Immunoreactive Species of Panx3 during Skeletal Muscle Differentiation

Recent results from our laboratory indicate that the protein levels of the ~70 kDa immunoreactive species of Panx3 are modulated during skeletal muscle myogenesis³⁸. Proliferative undifferentiated skeletal muscle myoblasts exhibited high expression of the ~70 kDa immunoreactive species of Panx3, which became drastically down regulated during differentiation³⁸. In addition, high expression levels of the ~70 kDa immunoreactive species were found in fetal human skeletal muscle, as compared to the adult tissue³⁸. The successful knockdown of the ~70 kDa immunoreactive species of Panx3 using shRNA lead to a drastic reduction in

myoblast proliferation ³⁸. However, this was insufficient by itself to trigger the differentiation process of skeletal muscle cells ³⁸. Overall, these results suggest that the ~70 kDa immunoreactive species of Panx3 is a novel regulator of skeletal myoblast proliferation keeping cells in a proliferative state.

Rationale and Hypothesis

Recently, our laboratory has shown that both the expected molecular weight species (~43 kDa) and the ~70 kDa immunoreactive species of Panx3 are present in human skeletal muscle cells and tissues³⁸. It was revealed that the levels of the ~70 kDa immunoreactive species of Panx3 were highly expressed in fetal tissues as well as in proliferating, undifferentiated human skeletal muscle myoblasts. These levels were significantly reduced with the onset of myoblast differentiation³⁸. Indeed, shRNA knock down of the ~70 kDa immunoreactive species significantly inhibited skeletal muscle myoblast proliferation suggesting that it may play a role in keeping undifferentiated cells in a proliferative state³⁸. We thus hypothesize that the ~70 kDa immunoreactive species of Panx3 will be upregulated in RMS and that the restoration of its levels to that seen in adult differentiated skeletal muscle will suppress its malignant properties.

Objectives

The first objective of this study was to assess the levels of the ~70 kDa immunoreactive species of Panx3 in RMS cell lines and tumour samples. Next, we wanted to further characterize the ~70 kDa immunoreactive species of Panx3 detected in RMS cell lines. To achieve this, we assessed its immunoreactivity to two different antibodies against Panx3 and assessed the presence and degree of glycosylation. To provide further characterization and be able to assess function, we next wanted to establish a knockdown of the ~70 kDa immunoreactive species of Panx3 in RMS cell lines. To achieve this a shRNA approach was employed. Following successful knock down of the Panx3 ~70 kDa immunoreactive species, our final objective was to assess the changes in RMS malignant properties utilizing markers of myocyte proliferation and differentiation.

Materials and Methods

Cell Lines and Culture Condition

Rhabdomyosarcomas (RMS) cell lines were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 Units/ml penicillin, 100 µg/ml streptomycin and 2mM glutamine (Sigma-Aldrich, St Louis, MO, USA). The cells were cultured in a T-75 cm² flask (Corning Incorporated Coming, NY, USA) in a 5% CO₂ incubator at 37⁰C. After cells were confluent they were split into new T-75 cm² flask periodically. Embryonal RMS (eRMS) cell lines, Rd, Rh 18, Rh 36, and alveolar RMS (aRMS) cell lines, Rh28, Rh 30 and Rh 41, were a generous gift by Dr. Peter Houghton from St. Jude Children's Research Hospital with the exception of the Rd cell line, which was obtained from ATCC (American Type Culture Collection). HSMM (Primary Human Skeletal Muscle Myoblasts), SkGM media (containing of human Epidermal Growth Factor (hEGF)), Fetuin, Bovine Serum Albumin (BSA), Dexamethasone, Insulin, and Gentamicin/Amphotericin-B (GA) were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA).

Pediatric eRMS Tumor Sample

Human eRMS tumor tissues were obtained from the department of pathology at the Children Hospital of Eastern Ontario (CHEO), through collaboration with Dr. David Grynspan. Normal healthy fetal and adult skeletal muscle whole lysates were used as comparison, which were purchased from

Novus Biologicals (Littleton, CO, USA). These tissues were homogenized in lysis buffer containing 1% SDS in phosphate buffered saline (PBS) in the presence of protease inhibitors against serine, cysteine and acidic proteases (complete UltraMini, Roche Diagnostics Corp., IN, USA), phosphatase inhibitors against acid and alkaline phosphatases, serine/threonine phosphatases, and tyrosine phosphatases (PhosStop Roche Diagnostics Corp., IN, USA). Homogenization of tissues was carried at 4⁰ C, using an Omni Bead Ruptor Homogenizer, for 20 minutes using 2.38 mm stainless steel beads (Omni International, Kennesaw GA, USA).

Western Blotting

Cells were lysed in 150mM NaCl, 10mM Tris-HCl (pH 7.4), 1mM EDTA, 1mM EGTA, 0.5% Nonidet P-40, 1% Triton X-100. Protease inhibitors against serine, cysteine, and acidic proteases (complete UltraMini, Roche Diagnostics Corp., IN, USA), and phosphatase inhibitors against acid and alkaline phosphatases, serine/threonine phosphatases, and tyrosine phosphatases (PhosStop Roche Diagnostics Corp., IN, USA) were added to it. The samples were lysed on ice and centrifuged for 10 minutes at 12000 RPM to remove cell debris. Protein concentration was measured using bicinchoninic acid (BCA) protein assay kit (Pierce, Biolynnx, Rockford, IL, USA). β -mercaptoethanol was added to 5X Laemmli Buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) and the samples were boiled for 5 min

and 30 µg of total protein were subjected to 10% SDS-PAGE. After separation by SDS-PAGE, proteins were transferred into Polyvinylidene fluoride (PVDF) membranes using the Transmembrane Turbo Blot (BioRad, Mississauga, Ontario, Canada). The membranes were blocked for 1 hour at room temperature with Odyssey blocking solution purchased from LiCor (Lincoln, NE, USA) followed by overnight incubation at 4⁰ C with anti-Pannexin 3 CT-379 antibody with dilution of 1: 1000 (Penuela et al., 2007, 2009), anti-Myosin Heavy chain antibody 1: 1000 (R&D systems, Minneapolis, MN, USA); or anti PCNA PC10 antibody (Dako Cytomation, Mississauga, ON, Canada). After 3 washes of 5 minute each with solution of PBS with 0.05% Tween20, the blots were incubated with secondary antibodies Alexa 680 (Molecular Probes, Eugene, OR) or infrared fluorescent-labeled secondary antibodies (1: 5000), for 45 minutes. After several washes, proteins were detected using the odyssey infrared imaging system (LiCor). Tubulin and GAPDH (Santa Cruz Biotechnology, CA, USA) were used as protein loading controls with 1: 5000 dilution. Precision plus Protein Dual Color Standards, (Bio-Rad Laboratories) were used as molecular weight standards and were depicted in kiloDaltons (kDa). Lysates from Human Embryonic Kidney cells (HEK293T) expressing Panx3 were used as positive controls. The blots were quantified using the Odyssey infrared-imaging system software (LiCor).

Deglycosylation Assay

Lysates from HEK 293T cells overexpressing Panx3 and Rd and Rh 30 RMS cell lines were subjected to deglycosylation. Briefly, lysates were treated with or without deglycosylation enzymes (20 μ l PNGase F Glycerol Free, 20 μ l *O*-Glycosidase, 20 μ l Neuraminidase, 20 μ l β 1-4 Galactosidase and 20 μ l β -N-acetylglucosaminidase), for 4 hours at 37⁰ C as suggested by the manufacturer (New England Biolabs, Whitby, ON, Canada). Samples were then boiled at 100⁰ C for 5 minutes and then separated on a 10% SDS-PAGE. After separation by SDS-PAGE, proteins were transferred into PVDF membranes using the Transmembrane Turbo Blot. Blots were then blocked with odyssey blocking buffer followed by overnight incubation at 4⁰C in anti-Panx3 CT- 379 antibody, followed by 1 hour incubation at room temperature with secondary antibodies Alexa 680 in 1:5000 concentration. Precision plus Protein Dual Color Standards (Bio-Rad Laboratories) were used as molecular weight standards and were depicted in kiloDaltons (kDa).

HSMM Differentiation

HSMM cells grown to 80-90% confluency were grown on collagen-coated coverslips in normal growth media. These cells were then induced to differentiate in differentiation medium composed of DMEM + 2% horse serum for 6 days. Cells were fixed with formaldehyde for 20 minutes at room temperature on days 0, 2, 4 and 6 respectively. Coverslips were then blocked in 2% BSA in PBS solution

+ 0.1% Triton X-100 for an hour. Immunolabeling was performed using anti-MHC antibody (R&D systems) in a dilution of 1:50 for 1 hour followed by 3 washes of 5 min each with PBS. Cells were then incubated in Alexa Fluor-488 conjugated secondary antibody (anti-mouse 1:250 dilution; Molecular Probes, Eugene, OR) for 45 min at room temperature followed by 3 washes of 5 minute each with solution of PBS with 0.05% Tween20. The coverslips were then counterstained with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, USA) to denote the nuclei and then mounted. Pictures were taken using a fluorescence microscope (20X objective, Olympus IX51 with Olympus DP72 camera, Cellsens Entry software).

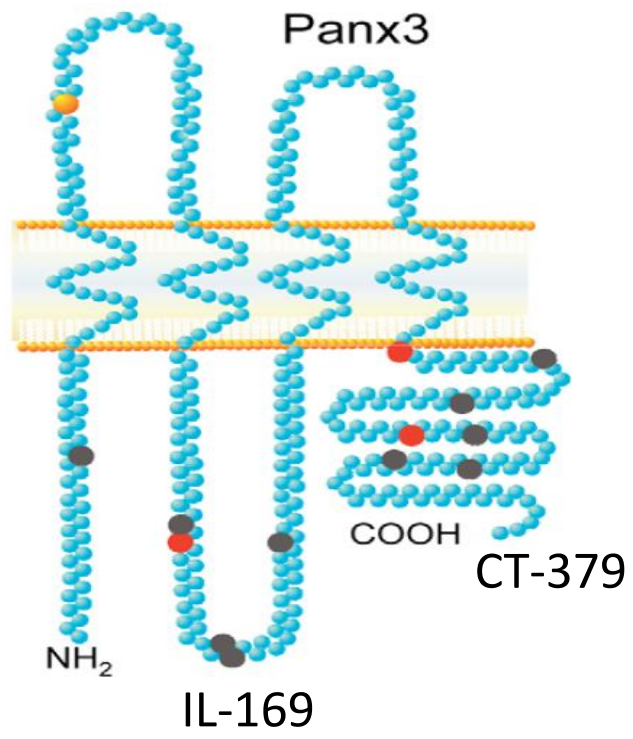
Detection of the ~70 kDa Immunoreactive Species of Panx3 by Two Different Antibodies

Five to six RMS cell lines were plated in 6 well plates. After reaching 85-95% confluency, cells were harvested in 100 μ l of lysis buffer (previously described). Thirty μ g of total protein from RMS cell lines as well as from undifferentiated and differentiated HSMM, were subjected to electrophoresis and blotted with antibodies targeting 2 different epitopes of mouse Panx3 (Panx3 CT-379 and Panx3 IL-169) through overnight incubation at 4^o C at a dilution of 1:1000. These antibodies were a generous donation by Dr. Dale Laird (University of Western Ontario). It was shown in his laboratory that these antibodies detect Panx3 by western blot and immunofluorescence, in both the intracellular compartments and at the plasma membrane ³³.

These antibodies were generated from two different epitopes of mouse Panx3 as illustrated in Figure 2^{33,45}. After 3 washes of 5 minute each, in solution of PBS with 0.05% Tween20, the blots were incubated with an anti-rabbit secondary antibody conjugated to Alexa 680 fluorophore (Invitrogen) and proteins were detected using the odyssey infrared imaging system (LiCor).

Figure 2. Schematic representation of the location of the two distinct epitopes used to generate Panx 3 antibodies

Schematic representation of the peptide structure of Panx3 modified from Penuela et al ^{33,34,45}. Putative N-glycosylation (red and orange residues) and phosphorylation (black residues) sites are shown. The carboxyl terminal (CT-379) and intracellular loop (IL-169) peptide sequence of mouse Panx3 were used to generate Panx3 specific antibodies are indicated.



Generation of stable RMS cell line expressing inducible Panx3 shRNAs using a lentiviral system

Four shRNA targeting Panx3 were obtained from Thermo Scientific (Waltham, MA, United States of America). The pTRIPZ lentiviral inducible vector is used to produce RNA in cells. The packaging components were separated into five plasmids consisting of pTLA1-PAK, pTLA1-ENZ, pTLA1-ENV, pTLA1-TOFF, pTLA1-Tat/Rev. Using the protocol provided by the manufacturer, the production of lentiviral particles was achieved after co-transfection of Trans-lentiviral packaging mixture (mixture of 5 plasmids with shRNAs against Panx3) in HEK293T packaging cells using calcium phosphate reagent from Thermo scientific System. Media containing virions were collected 64 hours post transfection. For removing floating cells and producing high titer viral particles, filtration was performed in which the supernatant was passed through a sterile, 0.22 - 0.45 μm low protein binding filter. 0.3 MOI was calculated according to the protocol suggested by the company to transduce the lentiviral vector into Rd and Rh 30 RMS cell lines in a 24 well plate for 48 hours. For the generation of stable cell lines, a puromycin kill curve was used to determine the minimum amount of puromycin required to kill non-infected cells. For this, 6×10^4 cells/well were plated in a 24-well plate and incubated overnight. The next day, media was applied to the cells containing different concentrations of puromycin in the range of 0-15 $\mu\text{g/ml}$

and incubated at 37⁰ C overnight. Every 2-3 days the media was replaced with freshly prepared selective media and the cells were monitored daily to assess the percentage of dead cells. Two µg/mL of puromycin was the lowest concentration that killed 100% of the cells in 2–4 days from the start of antibiotic selection. Thus puromycin at the concentration of 2µg/ml was added in fresh growth media to select for stably transfected cells in Rd and Rh 30 RMS cell lines. Selected cells from 24 well plates were then progressively expanded. In this way, a homogenous population of stably transfected cells was achieved for both Rd and Rh30 cell lines. Induction of Turbo RFP expression in transfected cells was achieved by the addition of doxycycline in the range of 0.5- 2µg/mL to cells 24 hours post-seeding. The presence of Turbo RFP expression was examined microscopically 96 hours after doxycycline addition. To demonstrate the efficiency of infection, representative photomicrographs were taken using a fluorescence microscope (20X objective, Olympus IX51 with Olympus DP72 camera, Cellsens Entry software). Cells were then harvested and reduction of the ~70 kDa immunoreactive species of Panx3 was assessed by Western blotting and compared to control shRNA, which consisted of a scrambled shRNA sequence. Quantification of blots was performed using the Odyssey infrared- imaging system software (LiCor).

Reduction of the ~70kDa Immunoreactive Species of Panx3 in Rh 30 RMS Cell Lines by Transient Transfections of shRNA

Rh 30 cells grown to 60-70% confluency were transiently transfected into Opti-MEM medium (Invitrogen, Burlington, ON) using Lipofectamine2000 (Invitrogen) and 1 μ g of plasmid DNA from four unique 29 mer shRNA constructs against Panx3 in a retroviral red fluorescent protein (RFP) vector (Origene technologies, Rockville, MD, USA). A non-targeting shRNA was used as a control. Transfection efficiencies were determined by assessing the percentage of RFP positive fluorescing cells at 4 days post transfection. Lysates were then collected and subjected to Western blotting, as described previously, using an anti-Panx3 antibody (CT-379).

Statistics

Experiments were performed a minimum of 3 times (exact numbers are given in the figure legends) and values obtained at each time point were expressed as mean \pm standard deviation. Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Student's t-test of multiple comparisons to establish differences between individual groups. Statistically significant difference was considered to be a p-value ≤ 0.05 . Data was analyzed using Prism 5.0 (Graph Pad Software, San Diego, CA).

Results

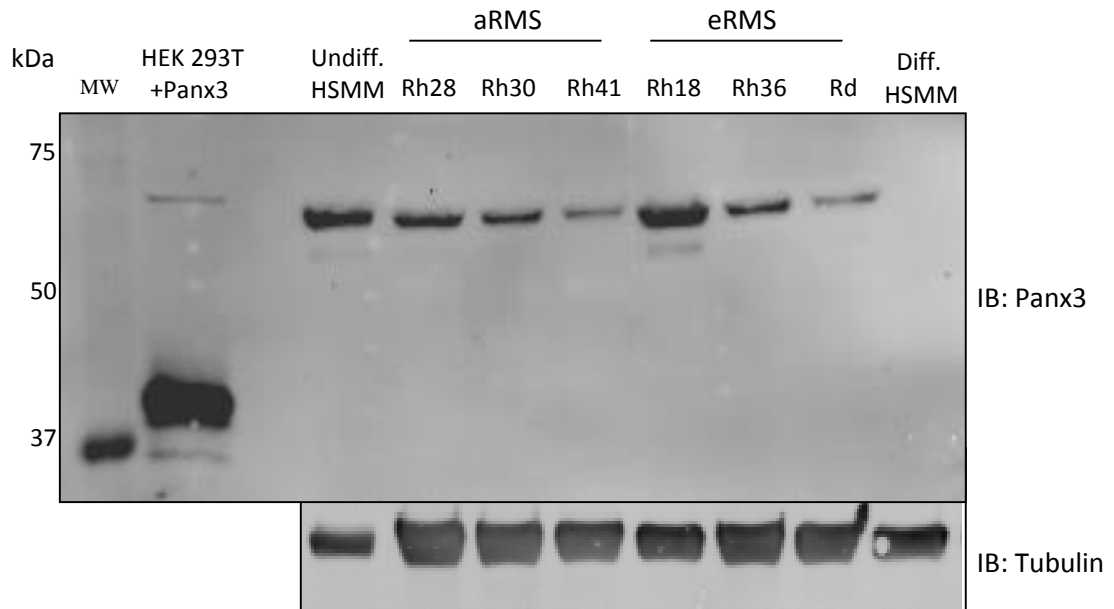
Assessment of the Levels of Pannexin3 in RMS Cell Lines and Tumours

Pannexin3 Expression Levels in RMS Cell Lines and Pediatric Tumours Are Similar to those in Undifferentiated Human Skeletal Muscle Cells

Our lab has previously demonstrated that the ~70 kDa immunoreactive species of Panx3 is highly expressed in proliferative, undifferentiated primary human skeletal muscle myoblasts (HSMM) and drastically down regulated during differentiation³⁸. As RMS tumors are thought to arise from a defect of skeletal muscle differentiation²⁸, I wanted to determine whether the levels of the ~70 kDa immunoreactive species of Panx3 are altered in RMS cell lines and tumours. In order to determine this, whole lysates from three aRMS (Rh28, Rh30, Rh41) and three eRMS (Rh18, Rh36, Rd) cell lines derived from pediatric patients were assessed. Lysates from undifferentiated and differentiated HSMM were used for comparison. As shown by western blotting, the ~70 kDa immunoreactive species of Panx3, which was found to be present in undifferentiated skeletal muscle cells but absent in differentiated myoblasts, was highly expressed in all six RMS cell lines (Figure 3). Hence, the results of this experiment suggest that the expression of the ~70 kDa immunoreactive species of Panx3 in eRMS and aRMS cell lines is upregulated compared to differentiated HSMM and is similar to that seen in undifferentiated, proliferative skeletal muscle myoblasts (HSMM).

Figure 3. Levels of the ~70kDa immunoreactive species of pannexin 3 are altered in rhabdomyosarcoma cell lines

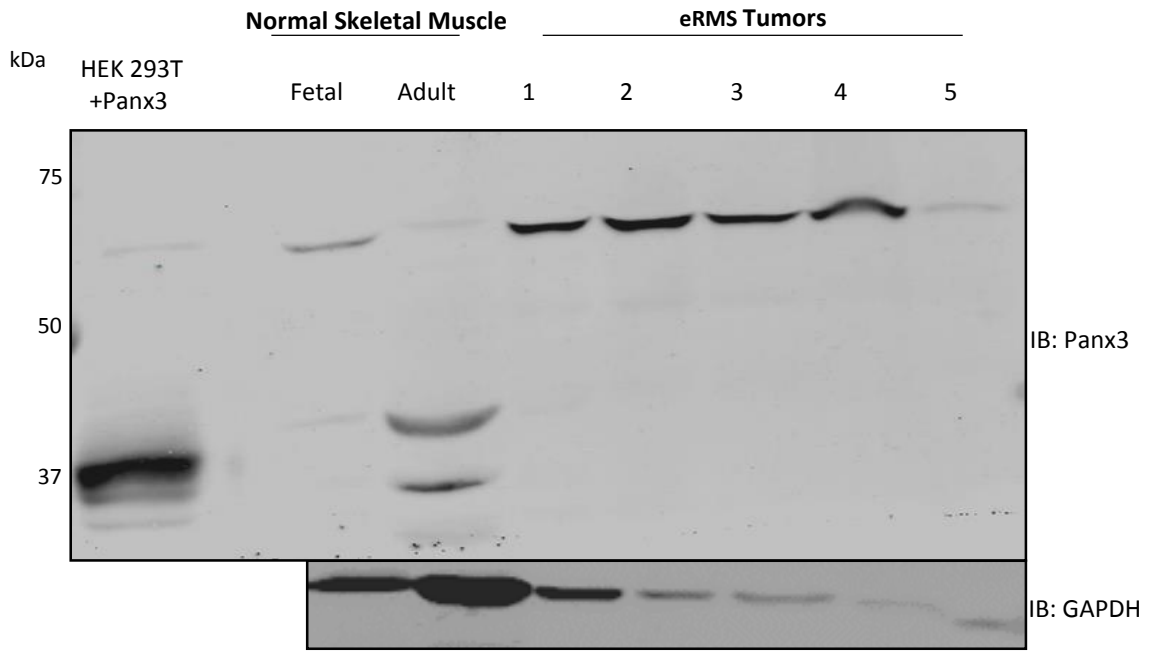
Total lysates from three aRMS (Rh28,Rh30, Rh41) and three eRMS (Rh18, Rh36, Rd) cell lines derived from pediatric patients, as well as from undifferentiated and differentiated HSMM as controls, were subjected to electrophoresis and blotted for Panx3. HEK 293T cells transfected with Panx3 were used as a positive control. Levels of the ~70 kDa immunoreactive species of Panx3 were up regulated in all RMS cell lines and were below detectable levels in differentiated HSMM.



We next wanted to determine whether the levels of this immunoreactive species would be similarly regulated in RMS tumours samples. In order to determine this, five eRMS tumour samples were lysed and subjected to Western blotting. Adult and fetal human skeletal muscle lysates were used as controls. The results of this experiment demonstrated that the levels of the ~70 kDa immunoreactive species of Panx3 were also highly expressed in RMS tumors compared to adult skeletal muscle cells and were similar to that found in fetal skeletal muscle tissue (Figure 4). This immunoreactive species was however expressed at very low levels in adult skeletal muscle tissue. In contrast, the classical species of Panx3, with an expected molecular weight of ~43 kDa, was found to be present in adult skeletal muscle but was absent or below detectable levels in fetal skeletal muscle and RMS tumours. Taken together, these results provide evidence that the levels of Panx3, including both the classical ~43 kDa form and the ~70 kDa immunoreactive species, in RMS tumours are similar to proliferative and partially differentiated fetal skeletal muscle tissues.

Figure 4. Pediatric embryonal rhabdomyosarcomas demonstrate expression of the ~70kDa immunoreactive species of pannexin 3 at levels similar to fetal skeletal muscle

Lysates from five eRMS tumour tissues derived from pediatric patients as well as fetal and adult normal skeletal muscle were subjected to electrophoresis and blotted for Panx3. Lysates from HEK 293T cells expressing Panx3 were used as a positive control. Expression of the ~70 kDa immunoreactive species of Panx3 in RMS tumours was similar to undifferentiated fetal skeletal muscle tissue. ~43 kDa species of Panx3 was found to be present only in adult skeletal muscle.



Identification and Characterization of the ~70 kDa Immunoreactive Species of Pannexin3

Recognition of the ~70 kDa Immunoreactive Species of Pannexin3 by Two Different Antibodies against Pannexin3

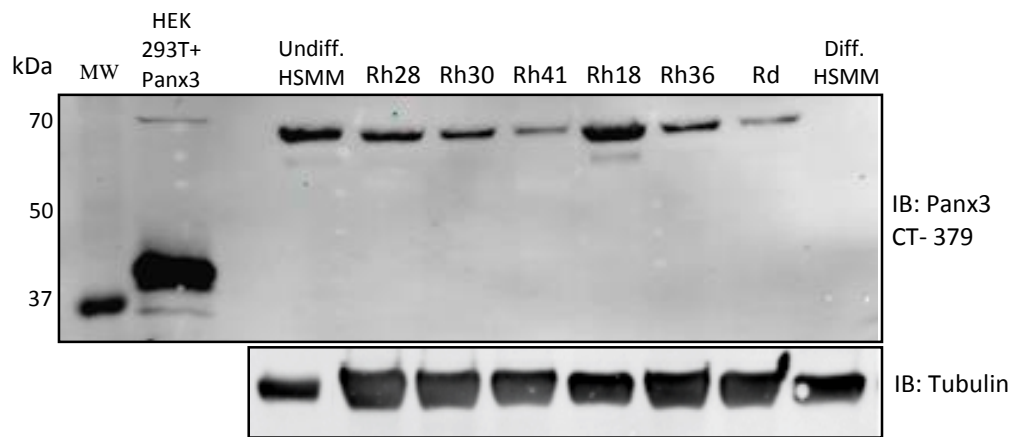
The levels of the ~70 kDa immunoreactive species of Panx3 have been previously shown to be downregulated during skeletal muscle myoblast differentiation. Moreover its suppression by shRNA inhibited the proliferation of undifferentiated myoblasts³⁸. We have now found that RMS cell lines and tumour samples have elevated levels of the 70 kDa immunoreactive species of Panx3 similar to undifferentiated myoblasts. Given that the specific identity of this species remains poorly understood, we next wanted to further characterize and confirm that it is indeed the same species that we have identified in our prior studies. In one such study, we have shown that the ~70 kDa immunoreactive species of Panx3 was detected by two different anti-Panx3 antibodies recognizing different epitopes of Panx3 in organotypic epidermis³⁷. Therefore, I wanted to confirm that the ~70 kDa immunoreactive species detected in RMS cell lines can also be detected by multiple Panx3 antibodies. To test this, lysates from five RMS cell lines were subjected to electrophoresis and probed with two antibodies targeted against two different epitopes of mouse Panx3 (Panx3 CT-379 & Panx3 IL-169) (Figure 2). Lysates from undifferentiated and differentiated HSMM cells were used for comparison. The results of my experiment demonstrated that the ~70 kDa immunoreactive species of Panx3 was detected in all five RMS cell lines as well

as in undifferentiated HSMM by both antibodies (Figure 5A & B). The ~70 kDa immunoreactive band was again absent in differentiated HSMM. Therefore, these results suggest that the ~70 kDa immunoreactive species of Panx3 present in RMS cell lines is similar in this respect to that identified in our prior studies.

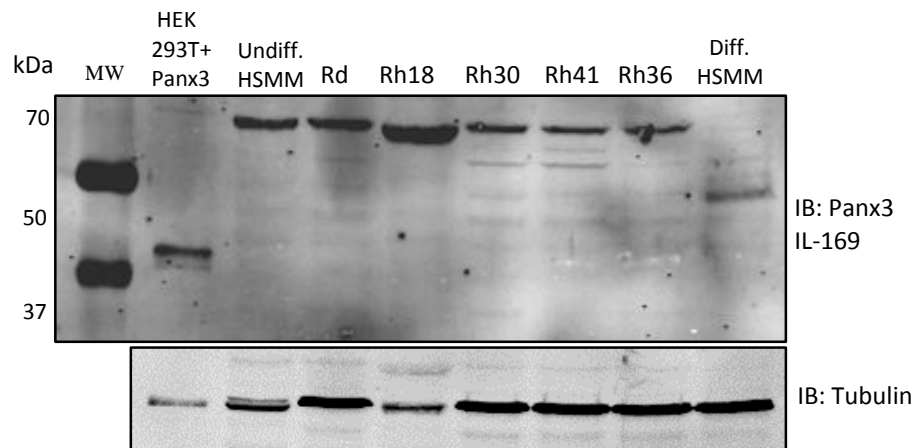
Figure 5. The ~70kDa immunoreactive species of pannexin 3 is recognized by two different antibodies directed against pannexin 3

Lysates from five RMS cell lines as well as from undifferentiated and differentiated HSMM were subjected to electrophoresis and blotted for Panx3 using two different antibodies shown in **A** (CT-379) and **B** (IL-169). Lysates from HEK 293T cells expressing Panx3 were used as positive controls. The ~70 kDa immunoreactive species of Panx3 was detected in all RMS cell lines as well as in undifferentiated HSMM by both anti-Panx3 antibodies.

A



B

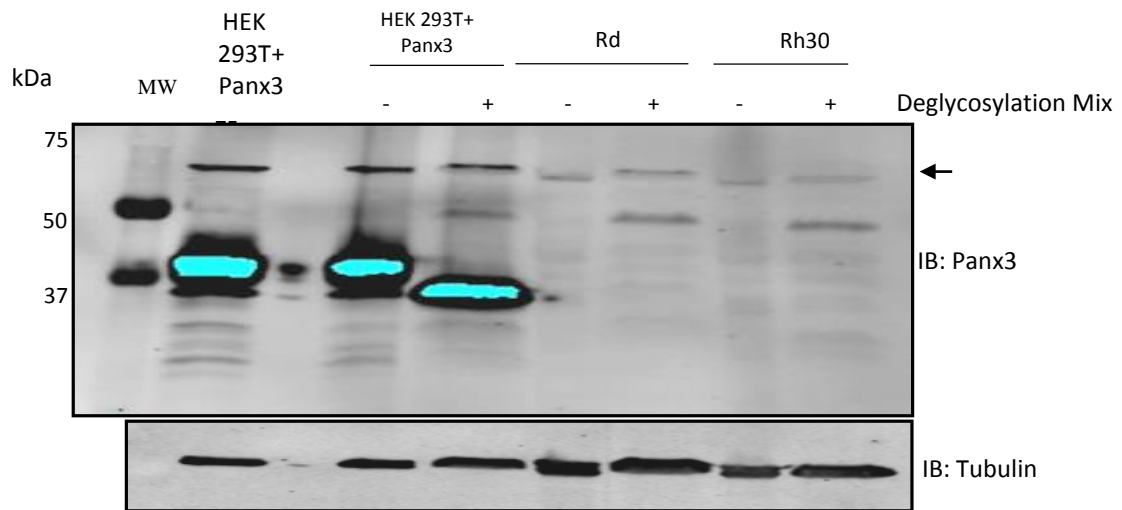


The ~70 kDa Immunoreactive Species of Pannexin3 is a Glycoprotein

Studies have shown that, similar to Panx1 and Panx2, the ~43 kDa species of Panx3 also exhibits N-linked glycosylation³³⁻³⁵. Panx3 glycosylation has been further shown to play an important role in Panx3 cellular localization, trafficking, and pannexin intermixing³³⁻³⁵. As glycosylation is a hallmark feature of pannexins, recent studies have also reported that similar to the ~43 kDa Panx3 species, the ~70 kDa immunoreactive species also undergoes glycosylation³⁷. Thus we next wanted to determine whether the ~70 kDa Panx3 immunoreactive species observed in RMS cell lines is also glycosylated to further confirm that it is the same species identified in our previous reports as well as to characterize any potential change in the degree of glycosylation that might occur in the malignant setting. In order to assess for the presence and degree of glycosylation, lysates from Rd and Rh30 RMS cell lines were treated with and without a mix of deglycosylation enzymes. As a positive control, HEK 293T cells transfected with Panx3 were similarly treated. The results demonstrate that deglycosylation treatment resulted in a shift in the electrophoretic mobility of the ~70 kDa immunoreactive species of Panx3 in both Rd and Rh30 RMS cell lines resulting in the presence of a band at ~50 kDa (Figure 6). These results suggest that similar to known species of Panx3 (~43 kDa)^{33,34} the ~70 kDa immunoreactive species identified in RMS cell lines is also glycosylated.

Figure 6. The ~70kDa immunoreactive species of pannexin 3 is glycosylated

Rd and Rh30 RMS cell line lysates were treated with and without a mixture of deglycosylation enzymes and subjected to electrophoresis and blotted for Panx3. HEK 293T cells transfected with Panx3 were used as positive controls to show the deglycosylation of the known species of Panx3. A shift in electrophoretic motility (arrow) of the ~70 kDa immunoreactive species of Panx3 in both Rd and Rh30 RMS cell lines was detected



Generation of Rhabdomyosarcoma Cell Lines Displaying Reduced Levels of the ~70 kDa Immunoreactive Species of Pannexin3

Stable Generation of Rd and Rh30 RMS Cell Lines Expressing Pannexin3 shRNA using a Lentivirus Inducible System

A recent report from our group has shown that the ~70 kDa immunoreactive species of Panx3 plays an important role in reducing skeletal muscle myoblast proliferation³⁸. Furthermore, our previous results have demonstrated an upregulation of the levels of the ~70 kDa immunoreactive species of Panx3 in RMS cell lines and RMS tumours relative to differentiated skeletal muscle cells and tissues. We next wanted to assess whether suppression of this species would reduce proliferation of RMS cell lines. To test this, a knockdown of the ~70 kDa immunoreactive species of Panx3 was performed using a TRIPZ shRNA doxycycline-inducible lentivirus system with an RFP reported to create stable RMS cell lines. For this purpose, two RMS cell lines, Rd and Rh30, were chosen to represent both eRMS and aRMS, respectively. Four unique shRNA targeting Panx3 were used as well as one non-specific control shRNA. Following infection and sequential selection, inducible expression of the construct was confirmed following the addition of doxycycline by the presence of RFP positive fluorescence (Figures 7 & 8). RFP fluorescence demonstrated that our construct was expressed in the presence of 2µg/mL of doxycycline in both Rd and Rh30 RMS cell lines. No fluorescence was observed in the absence of doxycycline. These findings indicate the successful generation of stable RMS cell lines expressing our constructs.

Figure 7. Creation of a stable Rd rhabdomyosarcoma cell line expressing pannexin 3 shRNA

Representative photomicrographs showing Rd cells stably expressing one of four specific inducible pannexin3 shRNAs or the non-specific control shRNA with an RFP reporter in the presence and absence of the inducing agent doxycycline. While no cells were RFP+ive in the absence of doxycycline, the presence of 2 µg/mL of doxycycline resulted in expression of the construct as indicated by the RFP+ive fluorescence. The total number of cells are evident in the visible light photomicrographs. Bars=50 µm

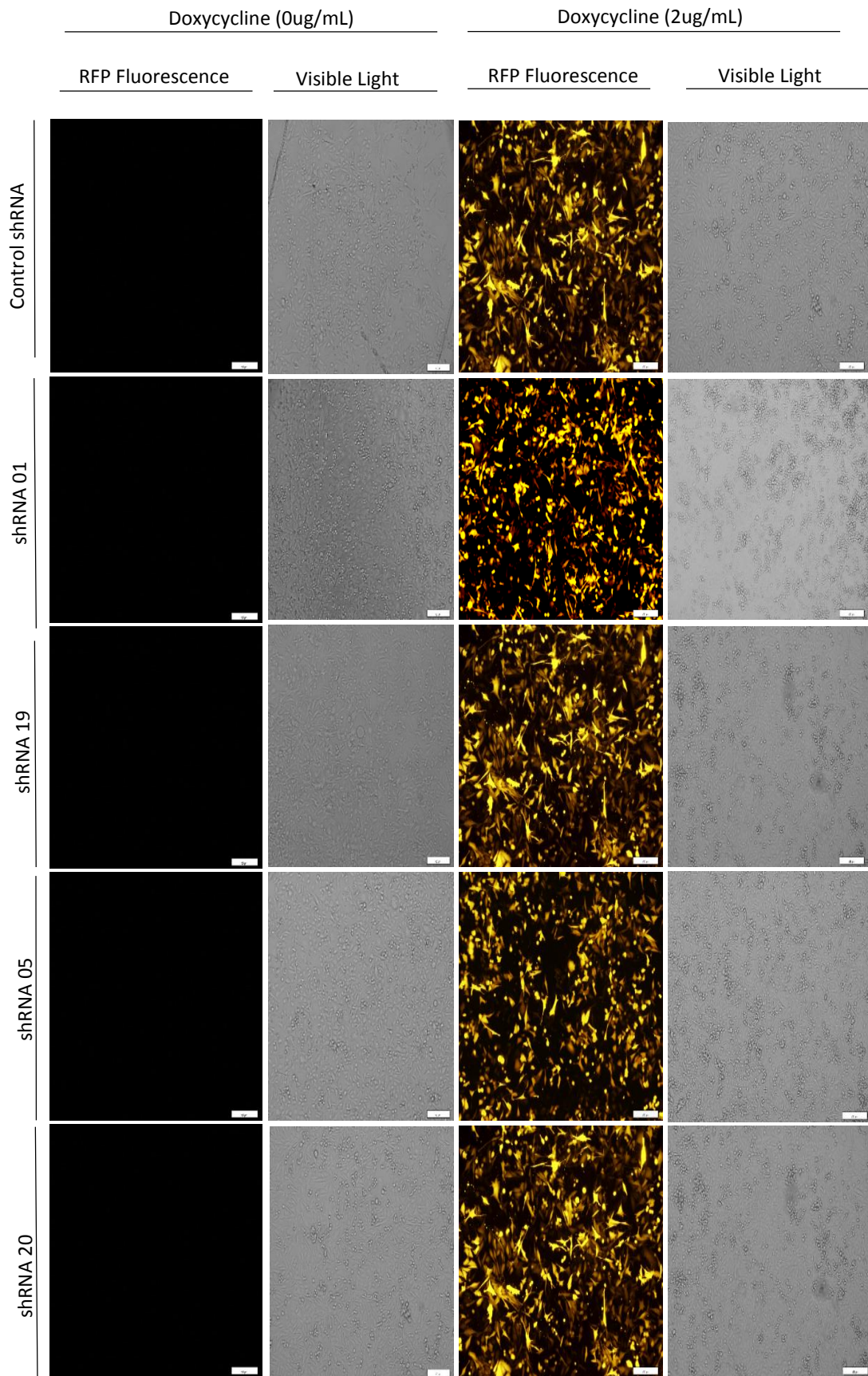
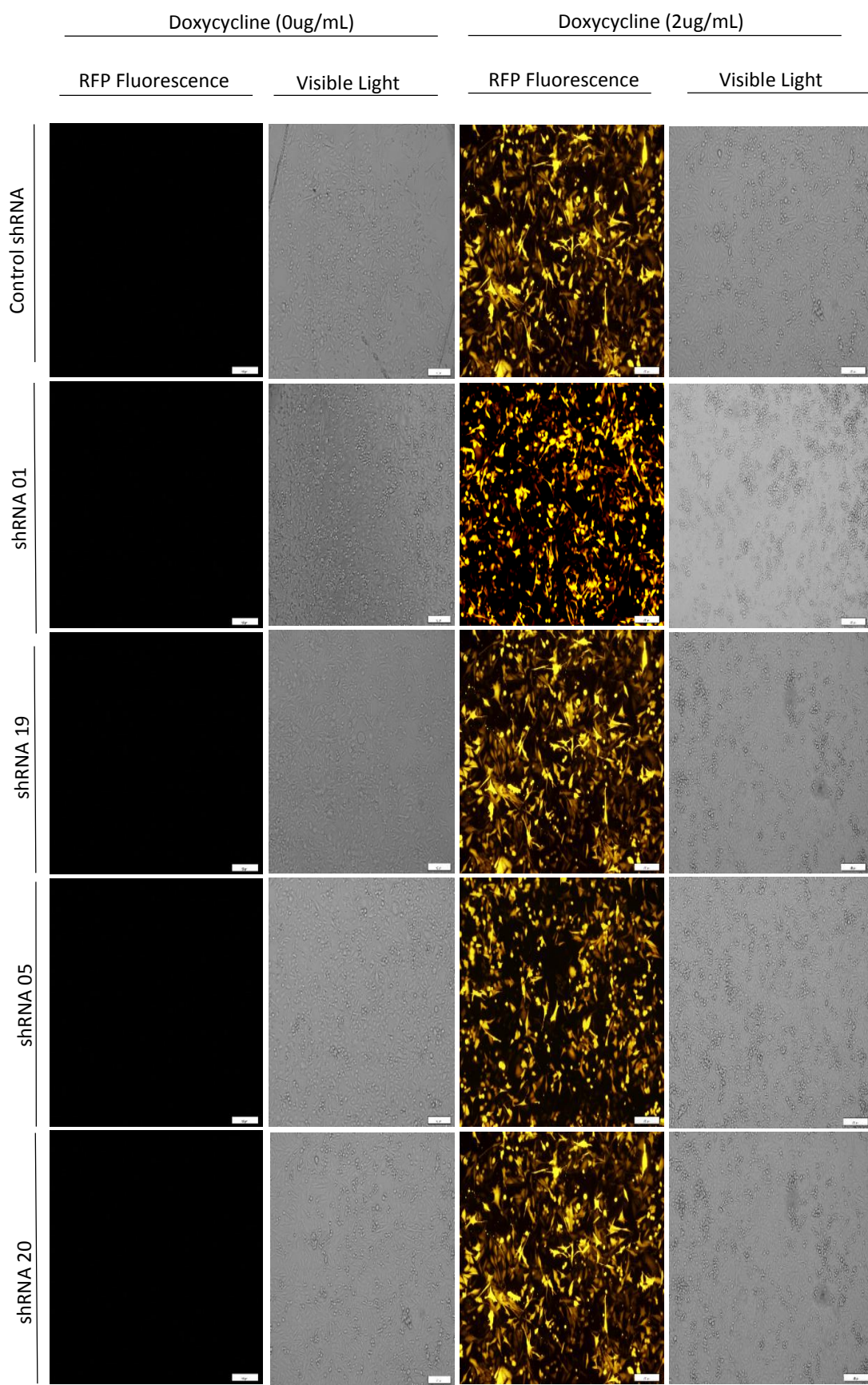


Figure 8. Creation of a stable Rh30 rhabdomyosarcoma cell line expressing pannexin 3 shRNA

Representative photomicrographs showing Rh30 cells stably expressing one of four specific inducible pannexin3 shRNAs or the non-specific control shRNA with an RFP reporter in the presence and absence of the inducing agent doxycycline. While no cells were RFP+ive in the absence of doxycycline, the presence of 2 µg/mL of doxycycline resulted in expression of the construct as indicated by the RFP+ive fluorescence. The total number of cells are evident in the visible light photomicrographs. Bars=50 µm



Reduction of the ~70 kDa Immunoreactive Species of Pannexin3 was not Achieved Using the shRNA Lentiviral Constructs in Both Rd and Rh30 Rhabdomyosarcoma Cell Lines

As we had previously demonstrated the successful creation of stable Rd and Rh30 RMS cell lines expressing one of four Panx3 shRNA constructs as well as a control shRNA construct, we next wanted to assess whether these shRNAs were able to reduce the ~70 kDa immunoreactive species of Panx3. In order to achieve this, the above stable Rd and Rh30 RMS cell lines were treated with (2 μ g/mL) and without (0 μ g/mL) doxycycline and compared to wild type Rd and Rh30 cells. Cell lysates were then subjected to electrophoresis and blotted for Panx3. The results indicated that while the ~70 kDa immunoreactive species was present in both the wild type and doxycycline absent controls, none of the Panx3 specific shRNA were able to reduce the levels of this species in either Rd or Rh 30 RMS cell lines (Figure 9 & 10 respectively). These results demonstrate that the current shRNA knockdown approach was unsuccessful at targeting the ~70 kDa immunoreactive species of Panx3.

Figure 9. Levels of the ~70kDa immunoreactive species of pannexin 3 were not reduced by any of the shRNA in the Rd rhabdomyosarcoma cell line

Lysates from wild type Rd cells or those stably expressing doxycycline-inducible pannexin3 shRNA or the control shRNA in the presence or absence of 2 μ g/mL doxycycline were subjected to electrophoresis and blotting for Panx3. HEK 293T cells transfected with Panx3 were used as a positive control. No reduction of the ~70 kDa immunoreactive species of Panx3 was seen under any condition.

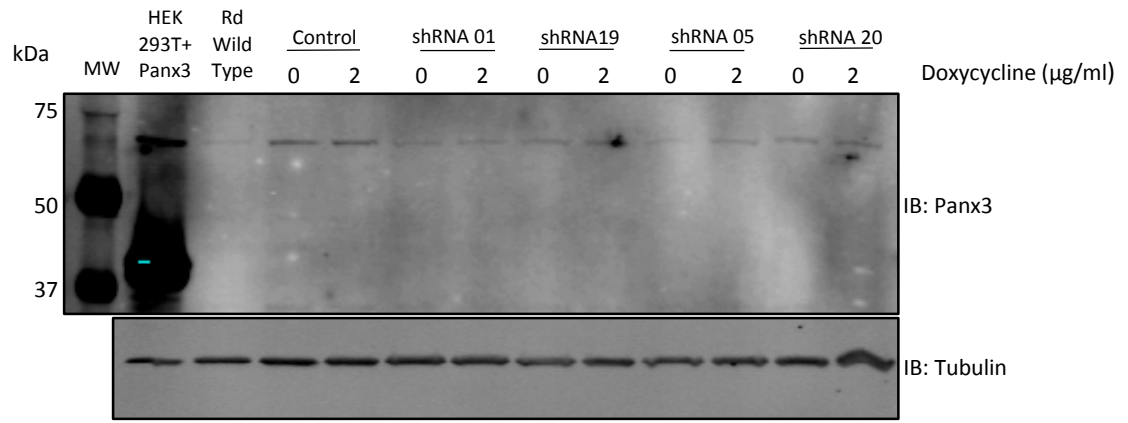
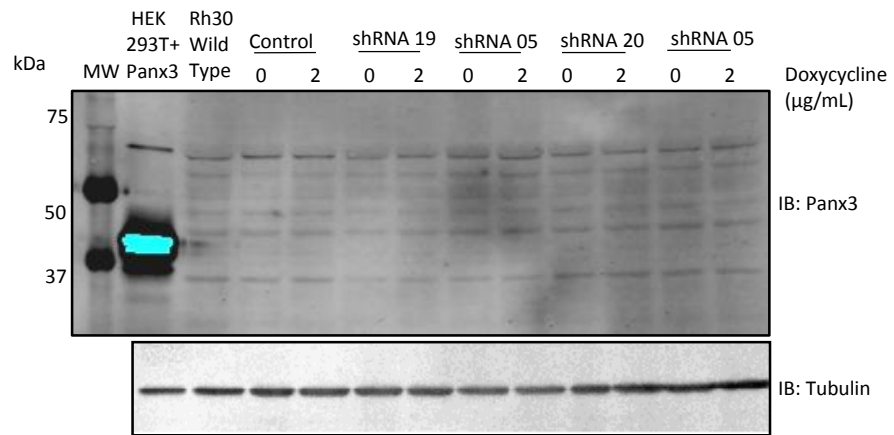


Figure 10. Levels of the ~70kDa immunoreactive species of pannexin 3 were not reduced by any of the shRNA in the Rh30 rhabdomyosarcoma cell line

Lysates from wild type Rh30 cells or those stably expressing doxycycline-inducible pannexin3 shRNA or the control shRNA in the presence or absence of 2µg/mL doxycycline were subjected to electrophoresis and blotting for Panx3. HEK 293T cells transfected with Panx3 were used as a positive control. No reduction of the ~70 kDa immunoreactive species of Panx3 was seen under any condition.



Reduction of the ~70 kDa Immunoreactive Species of Panx3 was not achieved throughout a Range of Doxycycline Doses in Both Rd and Rh30 RMS Cell Lines

As the level of the ~70kDa immunoreactive species of Panx3 was not reduced in the stable RMS cell lines expressing doxycycline-inducible shRNAs for Panx3 at the prior doxycycline dose, we next wanted to assess whether suppression could be attained at a more optimal concentration of doxycycline. To test this possibility, a range of doxycycline dosages (0.5 μ g- 2.5 μ g/mL) were chosen, based upon the manufacture's specifications, and assessed in stable Rd and Rh30 RMS cell lines for their ability to reduce the ~70 kDa immunoreactive species of Panx3. Wild type cells as well as those stably expressing the non-specific shRNA were used as control. Cell lysates were again subjected to electrophoresis and blotted for Panx3. While the ~70 kDa immunoreactive species of Panx3 was present in wild type and control shRNA conditions, the presence of Panx3 shRNA at the range of doxycycline doses failed to induce a reduction in its level (Figure 11 & 12). Furthermore, to exclude the possibility of an insufficient duration of doxycycline treatment, the stable Rd and Rh30 RMS cells were exposed to doxycycline for up to a week as opposed to the initial 4 days of treatment. Even after the prolonged doxycycline exposure, none of the shRNA were successful at reducing the levels of the ~70 kDa immunoreactive species of Panx3 (data not shown). Taken together, these results suggest that the current shRNA knockdown approach was unsuccessful at targeting the ~70 kDa immunoreactive species of Panx3.

Figure 11. Levels of the ~70 kDa immunoreactive species of pannexin3 were not reduced by any of the shRNA throughout a range of doxycycline doses in the Rd rhabdomyosarcoma cell line

Lysates from wild type Rd cells were compared, by electrophoresis and blotting for Panx3, with those from Rd cells stably expressing the doxycycline-inducible pannexin3 shRNAs or the control shRNA in the absence or presence of increasing doses of doxycycline in the range of 0.5 μ g- 2 μ g/mL. HEK 293T cells transfected with Panx3 were used as a positive control. No reduction of the ~70 kDa immunoreactive species of Panx3 was seen under any condition.

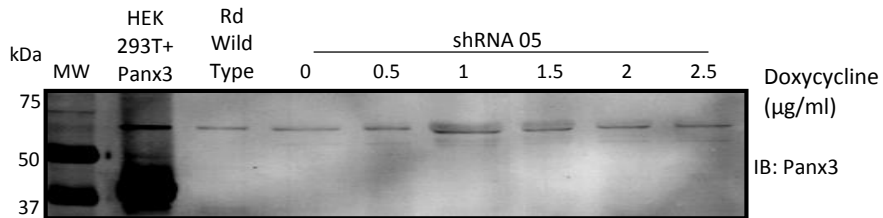
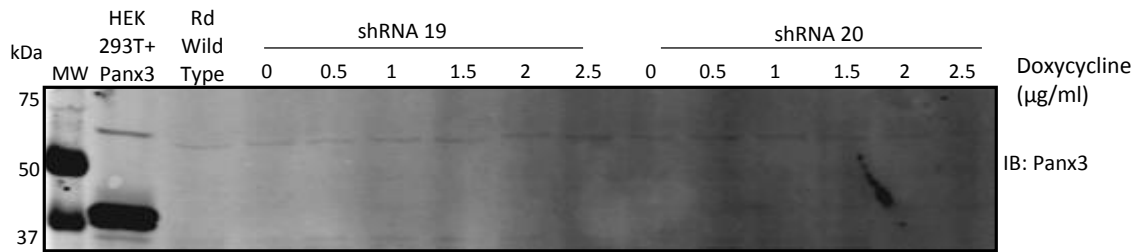
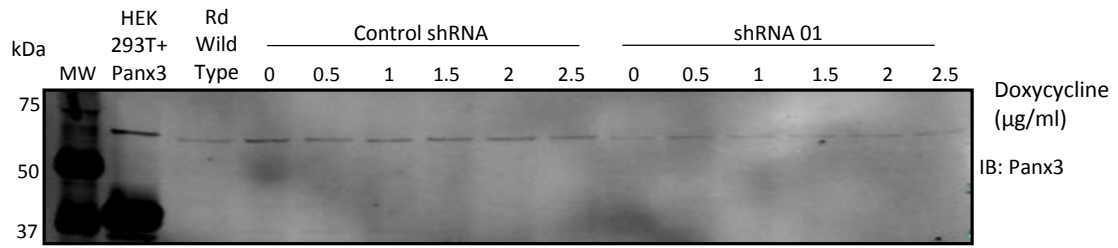
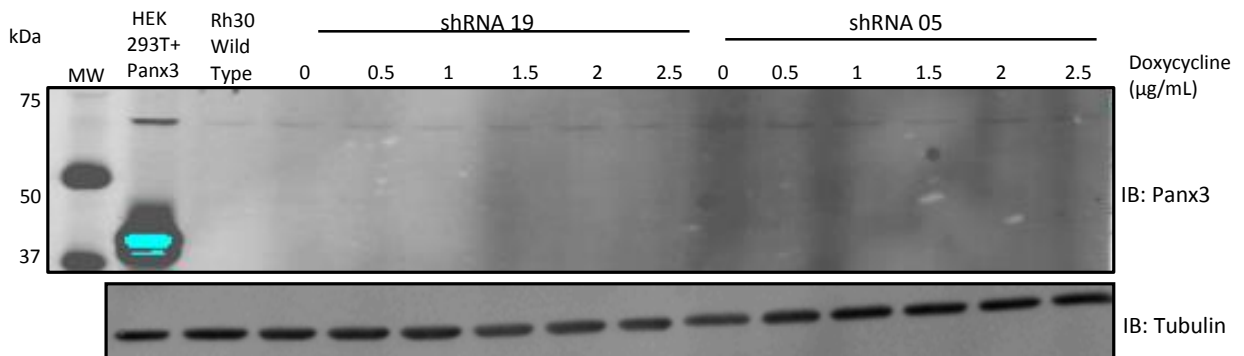
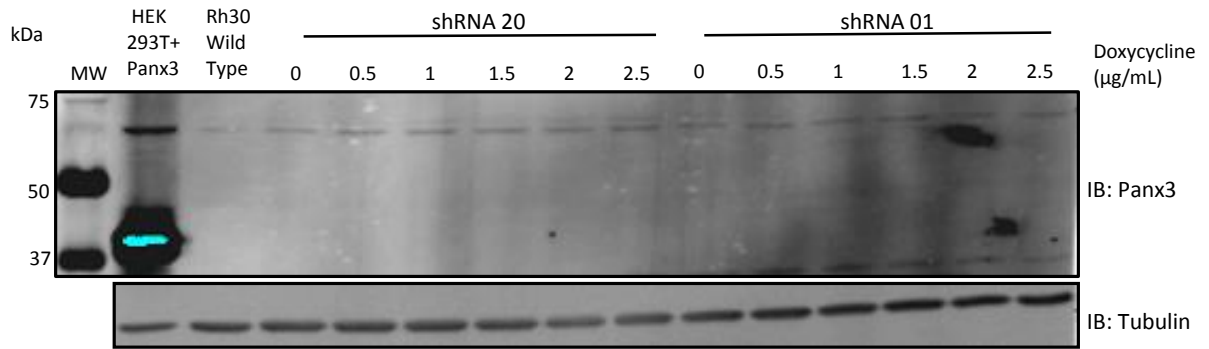
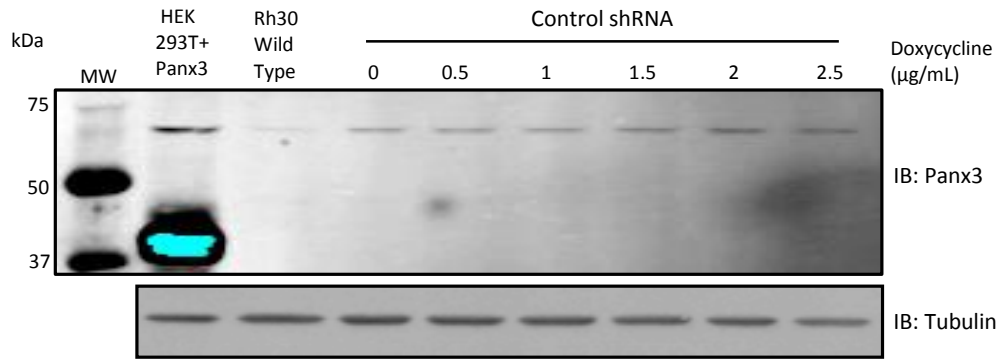


Figure 12. Levels of the ~70 kDa immunoreactive species of pannexin3 were not reduced by any of the shRNA throughout a range of doxycycline doses in the Rh30 rhabdomyosarcoma cell line

Lysates from wild type Rh30 cells were compared, by electrophoresis and blotting for Panx3, with those from Rh30 cells stably expressing the doxycycline-inducible pannexin3 shRNAs or the control shRNA in the absence or presence of increasing doses of doxycycline in the range of 0.5 μ g- 2 μ g/mL. HEK 293T cells transfected with Panx3 were used as a positive control. No reduction of the ~70 kDa immunoreactive species of Panx3 was seen under any condition.



Reduction of the ~70 kDa Immunoreactive Species of Pannexin3 through Transient Transfection of shRNAs in the Rh30 Rhabdomyosarcoma Cell Line

Given the inability of the prior lentiviral Panx3 shRNAs to reduce the ~70 kDa immunoreactive species of Panx3 and in light of our recent report demonstrating that in a separate set of Panx3 specific shRNAs, two out of four shRNAs were able to reduce the expression of this species in HSMM by transient transfection ³⁸. Thus, we next wanted to investigate whether this different set of Panx3 shRNAs could be successful in reducing the levels of the ~70 kDa immunoreactive species of Panx3 in RMS cell lines. To test this, all four shRNAs in addition to control shRNA were transiently transfected in the Rh30 RMS cell line. As these constructs contained an RFP reporter, efficiency of transfection was assessed as cells displaying RFP positive fluorescence and compared to wild type Rh30 cells as a control. All constructs including the control shRNA showed high transfection efficiencies as indicated by RFP fluorescence (Figure 13). Next, to assess whether transient transfection of the new set of Panx3 specific shRNAs could induce a reduction in the ~70 kDa immunoreactive species, cell lysates from wild type Rh30 cells as well as those transfected with either the control shRNA or one of the four Panx3 specific shRNAs and subjected to electrophoresis and immunoblotting for Panx3. While no reduction in the ~70 kDa band was detected with either shRNA61 62, or 63 as compared to wild type and control shRNA, shRNA64 showed a significant reduction in the ~70 kDa immunoreactive species of Panx3

Figure 13. Efficiency of transient transfection of pannexin3 specific shRNA in the Rh30 rhabdomyosarcoma cell line

Representative photomicrographs showing wild type Rh30 cells or those transiently transfected with one of four pannexin3 specific shRNAs or the non-specific control shRNA with an RFP reporter. While no RFP+ive cells were seen in the wild type control, efficiency of transfection of the respective shRNAs is indicated by the RFP fluorescence. The total number of cells are evident in the visible light photomicrographs. Bars=50 μ m

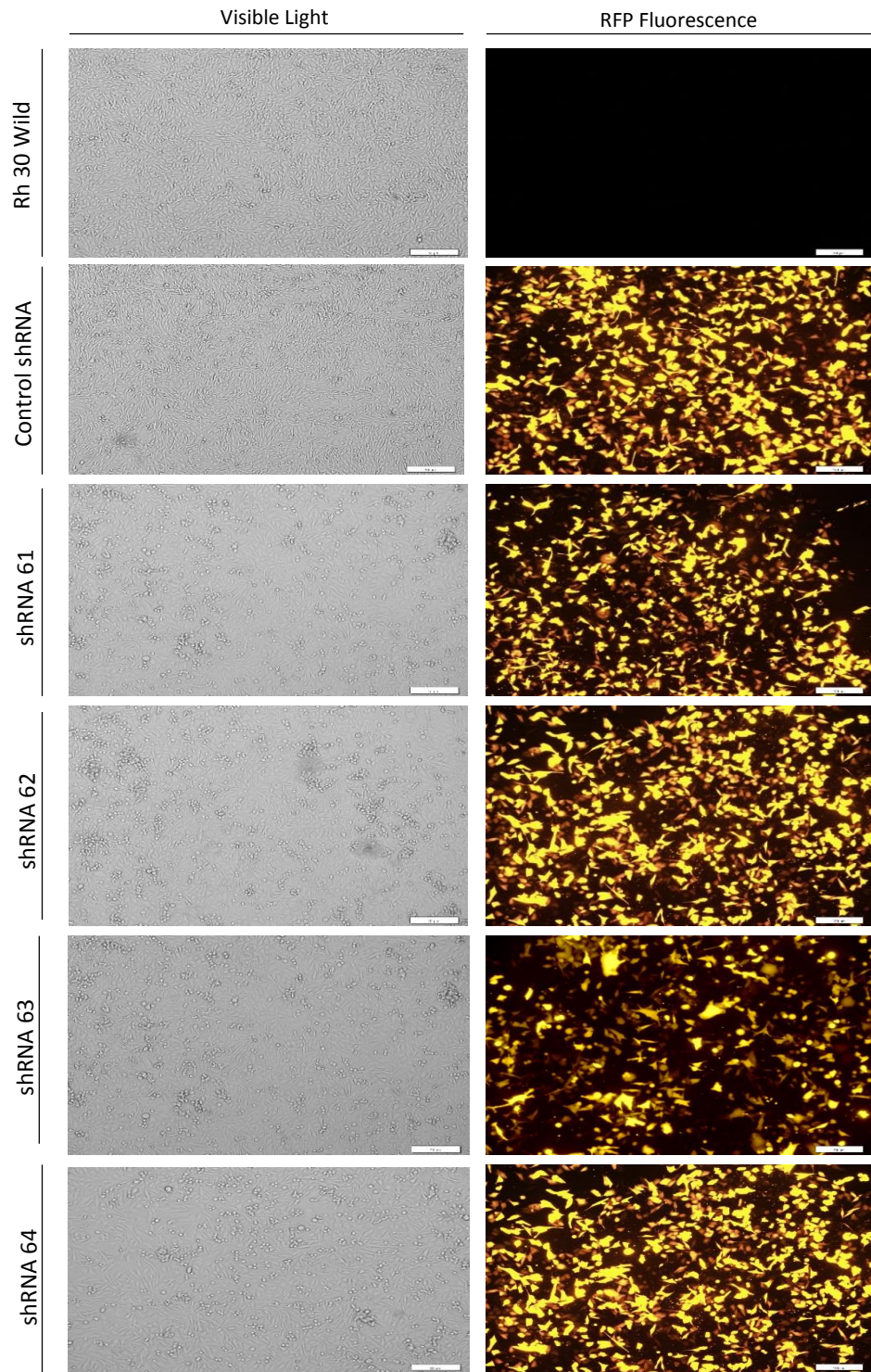
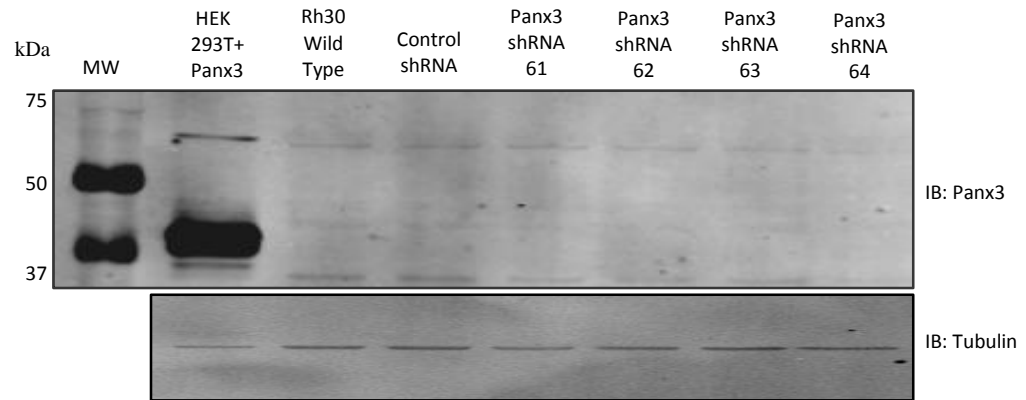


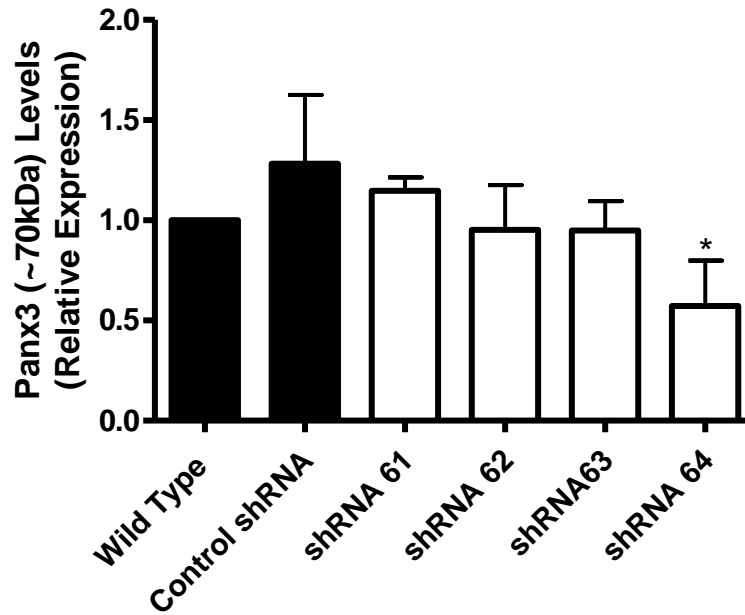
Figure 14. Reduction of the ~70 kDa immunoreactive species of pannexin3 in Rh30 rhabdomyosarcoma cells using targeted shRNAs

Rh30 RMS cells were transiently transfected with four shRNAs against human Panx3, as well as scrambled control shRNA. Lysates were collected and probed for Panx3 as shown in **A** and quantified in **B**. Only shRNA64 significantly reduced the expression of the ~70 kDa immunoreactive species of Panx3. HEK 293T cells transfected with Panx3 were used as a positive control. Results shown are representative of three independent experiments. Data is presented as mean +/- standard error. * represents a P value <0.05 relative to control shRNA.

A



B



immunoreactive species of Panx3 (Figure 14 A and quantified in 14B). These results indicate that the knockdown of the ~70 kDa immunoreactive species of Panx3 can be attained with specific Panx3 shRNA in an RMS cell line, lending further support to the notion that this immunoreactive band is indeed a species of Panx3.

Assessment of Changes in Rhabdomyosarcoma Malignant Properties Following Knock Down of the Panx3 ~70kDa Immunoreactive Species

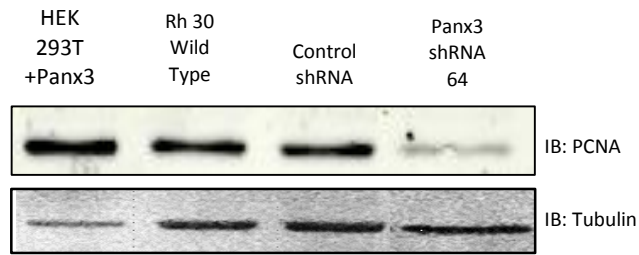
Knockdown of the Panx3 ~70 kDa Immunoreactive Species Inhibits Rh30 Rhabdomyosarcoma Cell Proliferation

Previous reports have shown that knockdown of the ~70 kDa immunoreactive species of Panx3 inhibited skeletal muscle myoblast proliferation³⁸. As our results thus far have indicated an upregulation of the ~70kDa Panx3 immunoreactive species in both RMS cell lines and tumors similar to levels seen in undifferentiated myoblasts, we next wanted to assess whether a reduction of this species could inhibit RMS cell proliferation. To test this, Rh30 RMS cells were transiently transfected with the Panx3 specific shRNA64, compared to wild type Rh30 cells or those transfected with the control shRNA, and the levels of the proliferating cell nuclear antigen (PCNA), as a marker of cell proliferation, were assessed by Western blotting. Myoblast proliferation, as indicated by the presence of a PCNA specific band, was abundantly present in both wild type and control shRNA cells, while those expressing the Panx3 specific shRNA showed a significant reduction in the levels of proliferation (Figure 15A and quantified in 15B). Thus, the ~70 kDa immunoreactive species of Panx3 is important in maintaining the proliferative phenotype of RMS cells.

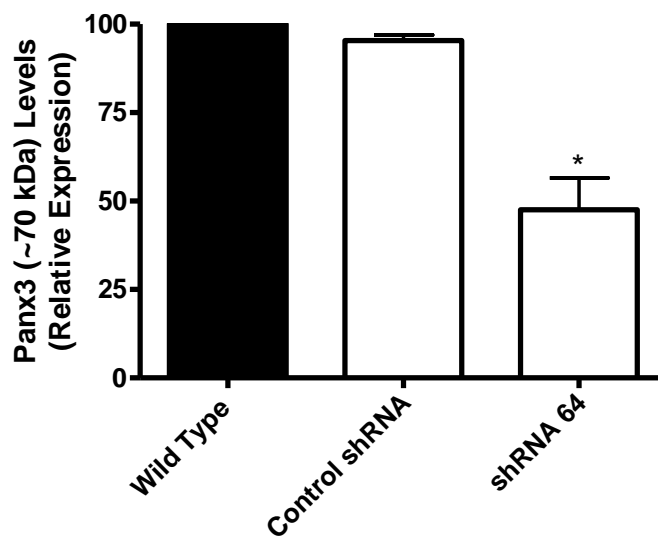
Figure 15. Knockdown of the ~70 kDa immunoreactive species of pannexin3 inhibits proliferation of Rh30 Rhabdomyosarcoma cells

Lysates from wild type Rh30 cells or those transiently transfected with either the control shRNA or the pannexin3 specific shRNA64 were subjected to electrophoresis and blotting for proliferating cell nuclear antigen (PCNA) as shown in **A** and quantified in **B**. A significant reduction in PCNA was observed with the pannexin3 specific shRNA64 as compared to controls. HEK 293T cells transfected with Panx3 were used as a positive control. Results shown are representative of three independent experiments. Data is presented as mean +/- standard error. * represents a P value <0.05 relative to control shRNA.

A



B



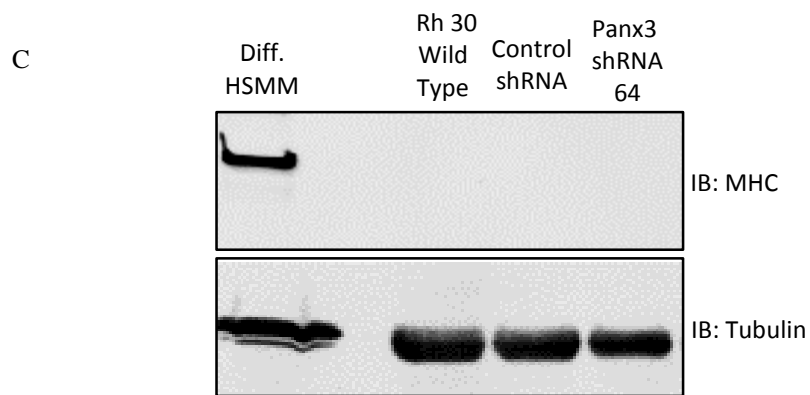
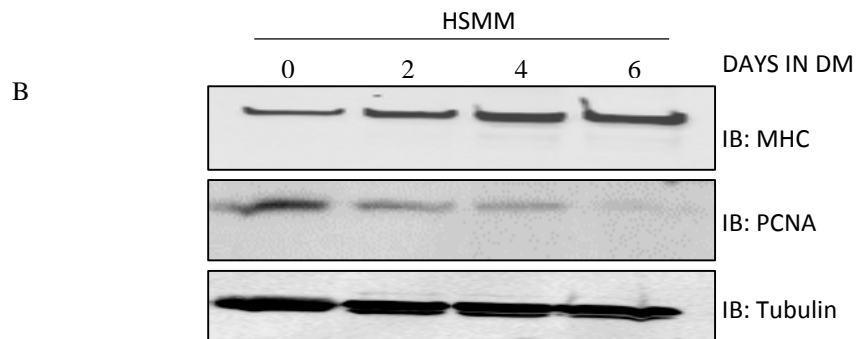
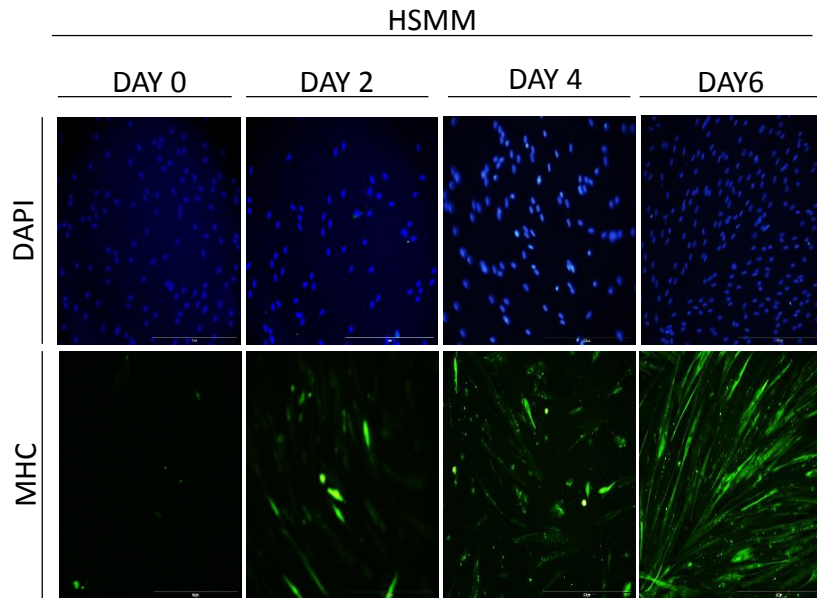
Knockdown of the Panx3 ~70 kDa Immunoreactive Species does not Induce Rh30 Rhabdomyosarcoma Cell Differentiation

It has been previously shown that the onset of differentiation is associated with an abrupt downregulation of the ~70 kDa Panx3 immunoreactive species³⁸. In addition, as we have now shown that suppression of the ~70 kDa immunoreactive species of Panx3 inhibits RMS cell proliferation, and as growth arrest is often linked with initiation of differentiation, we next wanted to investigate whether suppression of this species could induce differentiation in RMS cell lines. To address this, we first wanted to ensure that we had a viable model of skeletal muscle myoblast differentiation using myosin heavy chain (MHC) as a marker of differentiation. To test this, HSMM were used in a differentiation assay over the course of 6 days in culture as previously described³⁸. A progressive increase in HSMM differentiation over the time course in culture was noted by increases in both MHC immunolabelling and Western blotting (Figure 16A & 16B, respectively). The increase in differentiation was mirrored by a corresponding decrease in myoblast proliferation, as indicated by a reduction in PCNA by Western blot over the same time course (Figure 16B). Having established the differentiation model, we next compared differentiated HSMM to wild type and control shRNA Rh30 cells or those transfected with the Panx3 specific shRNA64 (Figure 16C). In contrast to the differentiated HSMM, MHC was below detectable levels in all Rh30 conditions suggesting that knockdown of

Figure 16. Knockdown of the ~70 kDa immunoreactive species of pannexin3 does not induce differentiation in the Rh30 rhabdomyosarcoma cell line

A. HSMM were induced to differentiate for 6 days in differentiation medium and images were taken every 2 days to assess MHC expression (labeled in green)(blue = Dapi nuclear stain). The images depicts that over the course of differentiation, MHC expression in HSMM was increased. **B.** Differentiation and proliferation within HSMM cells were studied by Western blot for MHC and PCNA respectively (numbers represent days in differentiation medium). During the differentiation process, MHC levels were increased while PCNA levels were decreased. **C.** Lysates from wild type Rh30 cells or those transiently transfected with either the control shRNA or the pannexin3 specific shRNA64 were subjected to electrophoresis and blotting for MHC. Differentiated HSMM were used as a positive control. No increase in MHC was observed with the pannexin3 specific shRNA64 as compared to controls. Results are representative of three independent experiments.

A



the ~70 kDa immunoreactive species of Panx3 did not result in the induction of differentiation in the Rh30 RMS cell line.

Taken together, this data suggests that the ~70 kDa immunoreactive species of Panx3 plays a role in regulating RMS cell proliferation but not differentiation.

Discussion

Failure of differentiation of muscle precursors into mature skeletal muscle is one of the leading hypothesis for the origination of RMS ²⁸. Since terminal myogenesis is known to switch off cell proliferation and migration, it is expected that promotion of RMS differentiation would antagonize tumor growth and metastasis ^{1,2,28}. Recently our laboratory demonstrated that the ~ 70 kDa immunoreactive species of Panx3 is glycosylated, sialylated and phosphorylated in normal skeletal muscle myoblasts and is regulated during skeletal muscle differentiation ³⁸. Indeed, the ~70 kDa immunoreactive species of Panx3 was highly expressed in proliferative undifferentiated myoblasts and drastically downregulated during differentiation ³⁸. Furthermore, reduction of this species using Panx3 shRNAs inhibited myoblast proliferation suggesting that it regulates this process. Since our findings indicate that the ~70 kDa immunoreactive species of Panx3 is tightly regulated during differentiation and regulates myoblast proliferation, it was tempting to speculate that its levels may be altered in RMS, in which differentiation properties are compromised. The results of my thesis demonstrate that the ~70 kDa immunoreactive species of Panx3 expressed in RMS also corresponds to a glycoprotein that is recognized by two different anti-Panx3 antibodies. Furthermore, reduction of this species by Panx3 shRNA resulted in a significant reduction of PCNA in RMS cells without inducing the levels of MHC, suggesting an inhibition of proliferation without inducing cell differentiation. Taken together, these results further suggest that this ~70 kDa immunoreactive

species corresponds to another form of Panx3 that may play a role in the proliferative status of RMS.

The Levels of the ~70 kDa Immunoreactive Species of Pannexin3 are Upregulated in Rhabdomyosarcoma Tumours and Cell Lines

While the exact identity of this protein is still unknown, findings from our laboratory indicate that it is highly expressed in proliferative and undifferentiated skeletal muscle myoblasts and is involved in keeping these cells in a proliferative state ³⁸. It was thus tempting to speculate that the levels of this species may be deregulated in RMS, compared to normal skeletal muscle. Remarkably, my results show that the levels of the ~70 kDa immunoreactive species of Panx3 were high in pediatric RMS cell lines and were similar to that found in undifferentiated human skeletal muscle myoblasts. As previously reported, the ~70 kDa immunoreactive species of Panx3 was mainly detected in fetal skeletal muscle tissue and in undifferentiated human skeletal muscle cells ³⁸. As RMS cell lines arise from differentiation defects, the possibility exists that these RMS cell lines might have properties that are similar to that of undifferentiated and proliferative cells. In order to confirm the data obtained using cell lysates, the levels of the ~70 kDa immunoreactive species of Panx3 were evaluated in pediatric eRMS specimens and compared to normal skeletal muscle tissue. Accordingly, the levels of the ~70 kDa immunoreactive species of Panx3 in eRMS were similar to that found in the partially differentiated fetal skeletal muscle tissue. Furthermore, the ~43 kDa species of Panx3 was highly expressed in adult skeletal muscle but very low or

below detectable levels in the fetal tissue and eRMS tumour specimens. It has been suggested that this ~43 kDa species of Panx3 is expressed further along the differentiation process and is expressed in non-proliferative and fully differentiated skeletal muscle tissue. Only one study so far has investigated the levels of Panx3 in human cancer using immunohistochemistry and reported that Panx3 levels are reduced in human keratinocyte tumours as compared to normal skin ³⁷. While the Panx3 antibodies used in that study are known to recognize both Panx3 species by Western blotting ^{33,37}, it is unclear whether both species or only the ~43 kDa form of Panx3 was detected by immunohistochemistry. While the aRMS cell lines also showed high levels of the ~70 kDa immunoreactive species of Panx3, it would be valuable to also confirm these results in aRMS tumour specimens in order to determine whether this upregulation is observed in both of the main RMS subtypes.

The ~70 kDa Immunoreactive Species of Pannexin3 Corresponds to a Another Form of Pannexin3

In addition to human and rodent skeletal muscle ³⁸, expression of the ~70 kDa immunoreactive species has been reported in murine lung, thymus, spleen, liver, skin ³³, as well as in rat organotypic epidermis ⁴⁴ and the male reproductive tract ³⁹. Since the exact identity of this protein is unknown, experiments were undertaken to better characterize it. I have now shown that the ~70 kDa immunoreactive species of Panx3 also corresponds to a glycoprotein, which is an intrinsic characteristic of the pannexin family of proteins and in agreement with

data obtained using human skeletal muscle myoblasts ³⁸. In addition, similar to what was shown using rat organotypic epidermis ³⁷, the ~70 kDa immunoreactive species of Panx3 expressed in RMS cell lines was recognized by two different anti-Panx3 antibodies. Since our laboratory has shown that this species does not correspond to a dimer of Panx3 ³⁸, it is possible that its higher molecular weight may be due to additional post translational modifications or another isoform. Based on findings now presented, the ~70 kDa immunoreactive species of Panx3 also exhibits some glycosylation in RMS cell lines. However, the degree of glycosylation of the ~70 kDa immunoreactive species of Panx3 may differ from that of the lower molecular weight form. Our laboratory has shown that in HSMM, additional post-translational modifications like phosphorylation and sialylation were present in the ~70 kDa immunoreactive species of Panx3. However, whether this form has additional post-translational modifications and whether these are also present in the ~70 kDa immunoreactive species of Panx3 expressed in RMS remains to be investigated.

Knockdown of the ~70 kDa Immunoreactive Species of Pannexin3 Inhibits Rhabdomyosarcoma Cell Proliferation without Inducing their Differentiation

The next aim was to begin assessing the possible functions of the ~70 kDa immunoreactive species of Panx3 in RMS. To this end, RMS stable cell lines were generated as opposed to transient knockdowns, as I planned to use these cell lines in future *in vivo* xenografts studies. Unfortunately, the Panx3 shRNA constructs provided by thermoscientific were not successful at reducing the expression of the

~70 kDa immunoreactive species of Panx3 in both Rd and Rh30 RMS cell lines despite attempts at optimization. However, during that time, it was shown in our laboratory that two other Panx3 shRNAs resulted in successful knockdown of the ~70 kDa immunoreactive species of Panx3 in HSMM³⁸. These shRNAs were then utilized in RMS cells and subsequently, I was able to show that one of them (shRNA 64) was successful at significantly reducing the levels of the ~70 kDa immunoreactive species of Panx3 in Rh30 RMS cells.

Rh30 RMS cells with the successful knockdown of the ~70 kDa immunoreactive species of Panx3 were then used to start assessing its possible function in regulating RMS malignant properties. One of the promising novel therapeutic strategy for RMS includes inducing their redifferentiation into normal skeletal muscle cells²⁸. As pannexins have been previously shown to regulate skeletal muscle cell proliferation and differentiation, we therefore wanted to next investigate whether the modulation of pannexin levels may be beneficial for RMS. Our laboratory has shown that the levels of the ~70 kDa immunoreactive species of Panx3 are regulated during myogenic differentiation and that its reduction using shRNAs led to an inhibition of skeletal myoblast proliferation without inducing differentiation³⁸. My results showed that the knockdown of the ~70 kDa immunoreactive species of Panx3 significantly reduced the levels of the proliferation marker PCNA in Rh30 cells thus suggesting inhibition of RMS cell growth. However, the knockdown failed to enhance the levels of MHC, suggesting

that it did not induce the differentiation of Rh30 cells. Differentiation of skeletal muscle cells is a highly ordered and complex process involving many mechanisms, transcription factors and muscle specific proteins^{28,29,69,70}. These processes thereby led to myoblast fusion resulting in the formation of myotubes^{6,14}. As only MHC was used as a differentiation marker, the assessment of early differentiation markers may be helpful in determining whether a partial differentiation has been induced in these conditions. The effect of the ~70 kDa immunoreactive species of Panx3 in RMS is currently being confirmed and the mechanisms involved will be investigated. However, the data obtained so far may suggest that this higher molecular weight species of Panx3 might be involved in keeping the undifferentiated RMS cells in a proliferative state and that reduction of its levels or functions may be beneficial for RMS.

Concluding Remarks, Future Directions, and Significance

In conclusion, my results suggest that the ~70 kDa immunoreactive species of Panx3 corresponds to another form of Panx3 that may be involved in the regulation of RMS proliferation.

Our laboratory has previously shown that this species is not a dimer of Panx3 and is glycosylated, sialylated and phosphorylated in normal skeletal muscle myoblasts ³⁸. While I have shown that the levels of this species are also modulated in RMS, it would be interesting to determine whether it is also sialylated and phosphorylated. This is important as it would provide further information as to whether other post-translational modifications like sialylation are present in the ~70 kDa species of Panx3 expressed in RMS cells and whether they are altered when compared to those observed in normal myoblasts such as HSMM. Better characterization of the glycosylated status of this species can be performed by treating RMS cells with glycosylation enzymes individually and compared that to what was found using HSMM ³⁸. Moreover, treatment of RMS cell lysates with dephosphorylation assays consisting of calf intestine phosphatase (CIP) might predict the phosphorylation status of the ~70 kDa immunoreactive species of Panx3 in RMS. Glycosylation, sialylation, and phosphorylation of this species might play a role in regulation of its function. Importantly, isolating the ~70 kDa immunoreactive species of Panx3 by immunoprecipitation by using different anti-Panx3 antibodies coupled with mass spectrometry in both Rd and Rh30 RMS cells

may reveal the exact identity of this protein and identify possible additional post-translational modifications that might be present.

In order to further study the functions of the ~70 kDa immunoreactive species of Panx3 in RMS malignant properties, confirmation of its effect on proliferation, by performing a bromodeoxyuridine incorporation assay, and comparing this to parental and control cells using both Rd and Rh30 cell lines is required. While no increase in MHC was detected in Rh30 cells that are reduced in the ~70 kDa immunoreactive species of Panx3, it is possible that only a partial state of differentiation was achieved. In order to assess this possibility, I would next evaluate the levels of other differentiation markers, such as MyoD and myogenin ^{28,29,69}. Evidence of early fusion could also be assessed by counting the number of nuclei per cell ⁷¹. In order to assess for any other potential functions of the ~70 kDa immunoreactive species of Panx3 in regulating RMS malignant properties, migration and invasion assays using transwell chambers in the absence or presence of matrigelTM could be performed. Furthermore, *in vitro* tumor growth and the metastatic potential of these cells could be determined using soft-agar colony formation assays ⁷². Finally, the *in vitro* data would be confirmed *in vivo* using established mice models of RMS tumour xenografts ⁷³.

Determination of the exact identity of the ~70 kDa immunoreactive species of Panx3 would be very useful as it would possibly also allow over-expressing its cDNA to further investigate its functions in RMS in comparison to normal skeletal muscle myoblasts. The identification of its post-translational modifications when

expressed in RMS, compared to normal skeletal muscle myoblasts, as well as its function in regulating RMS malignant properties, will reveal whether this species is a potential therapeutic target for RMS. Further studies involving the molecular mechanisms regulating the expression of the ~70 kDa immunoreactive species of Panx3 may also identify possible direct or indirect future approaches targeting pannexins in RMS patients.

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Figure 1. Schematic representation of mammalian skeletal myogenesis

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Figure 2. Schematic representation of the location of the two distinct epitopes used to generate Panx 3 antibodies

Dale Laird wrote:

That would be fine
Regards
Dale Laird

Kushal Gill wrote:

Dear Dr. Dale,

I am writing to request permission to use one of your figures in my Master's thesis. It is Figure 1.b (Structure of Pannexin 3) from your paper titled "Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins". I came across your paper while writing the introduction to my thesis which is based on Pannexin3 and Rhabdomyosarcomas and was hoping to include your figure in my thesis.

Thank you

Regards
Kushal Gill