A Novel Role Of GATA6 in Rhabdomyosarcoma

By

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ABSTRACT

One of the major events during embryogenesis is skeletal muscle formation. Over the years, this developmental process has been of great interest to many scientists. There’s no cancer in skeletal muscles. However, some cells are known to express differentiation markers such as *Myf5* and *MyoD*; these are the Rhabdomyosarcoma cells (RH30 and JR1). They harbor skeletal muscle characteristics, express early markers of differentiation but fail however in forming myotubes. The movement of cells from the proliferation to the differentiation stage is controlled by a specific group of myogenic factors. Deregulation of these transcription factors result in muscular defects and skeletal muscle diseases. *GATA6* is one of the myogenic factors that was found to be expressed in rhabdomyosarcoma cell lines and might play a role in down-regulating cellular proliferation and causing cell death. The objective of this study is to understand the role of *GATA6* in Rhabdomyosarcoma by assessing its expression at the transcriptional level and studying the effect of its down-regulating, by specific siRNA, on cell cycle and cellular proliferation. Real time PCR was used to assess the expression of *GATA6* in Rhabdomyosarcoma cell lines and Flow Cytometry was used to study the effect of
inhibition on the cell cycle. *GATA6* was shown to be expressed at the proliferation and differentiation stage. Down-regulation of *GATA6* by siRNA reduced the cellular proliferative potential and caused cell death. We conclude that the down-regulation of *GATA6* could represent a potential venue to develop drugs against rhabdomyosarcoma.

Key words: *GATA6*, Rhabdomyosarcoma, SiRNA, Real-Time PCR, Flow Cytometry.
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LIST OF ABBREVIATIONS

MRF: Myogenic regulatory factor

ENR: Engrailed repression

mRNA: Messenger ribonucleic acid

FOG: Friend of GATA protein

ES: Embryonic stem cells

RMS: Rhabdomyosarcoma

ARMS: Alveolar rhabdomyosarcoma

eRMS: Embryonal rhabdomyosarcoma

FKHR: Forkhead in human rhabdomyosarcoma

SiRNA: Small Interfering RNA

RISC: RNA inducing silencing complex

Bp: Base pair

(D1, D4): Differentiated cells

Prol: Proliferation stage

VSMCs: Vascular Smooth Muscle Cells
Chapter One

INTRODUCTION

Skeletal muscle development in vertebrates begin by the proliferation of the committed progenitor cells at the level of the somites (Buckingham, 2001). The proliferation process proceeds among myoblasts. This leads to fusion of myoblasts, and myotubes formation. Lastly, these myotubes leads to multinucleated muscle fibers formation (Buckingham, 2001). There’s no cancer in skeletal muscles, however some cells are known to express markers of differentiation; these are the Rhabdomyosarcoma cells.

Rhabdomyosarcoma (RMS) is a sarcoma, soft tissue cancer, originating from immature cells destined to become myogenic precursor cells. It is the most common type of sarcoma in children and the third most common extra-cranial solid tumor (Hicks & Flaitz, 2002). Rhabdomyosarcomas (RMS) harbor a morphological similarity to striated developing muscle (Sultan et al, 2010). Two distant histological subtypes of RMS exist: alveolar (ARMS) and embryonal (eRMS). ARMS affect most commonly the extremity muscles and the trunk while eRMS occurs in the head, neck region and genitourinary tract (Sultan et al, 2010).

Several genetic lesions (Table 1), such as translocations, loss of heterozygosity, amplifications, and mutational activations, have been linked to RMS (Hicks & Flaitz, 2002). In 20% of RMS cases, a chromosomal translocation disrupts the interaction between the 5’DNA-binding domain of Pax (a paired box transcription factor) and the trans-activation domain at the 3’end of FKHR fork-head/HNF-3 HNF-3 (Hepatocyte
This fusion alters the normal regulation of genes downstream of Pax (Anderson et al, 2001).

Table 1. Most common genetic lesions associated with human RMS. LOH: loss of heterozygosity; LOI: loss of imprinting (Hicks & Flaitz, 2002).

<table>
<thead>
<tr>
<th>GENE/Locus</th>
<th>Type of lesions</th>
<th>References</th>
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<tr>
<td>Pax3-FKHR</td>
<td>Translocations</td>
<td>Barr et al, 1993</td>
</tr>
<tr>
<td>Pax7-FKHR</td>
<td>Translocations</td>
<td>Davis et al, 1994</td>
</tr>
<tr>
<td>P53</td>
<td>LOH, missense mutation</td>
<td>Stratton et al, 1990</td>
</tr>
<tr>
<td>MDM2</td>
<td>Amplification/Overexpression</td>
<td>Meddeb et al, 1996</td>
</tr>
<tr>
<td>c-Met</td>
<td>Amplification/Overexpression</td>
<td>Ferracini et al, 1996</td>
</tr>
<tr>
<td>CDK4</td>
<td>Amplification/Overexpression</td>
<td>Khatib et al, 1993</td>
</tr>
<tr>
<td>N-Ras/K-Ras</td>
<td>Mutational activation</td>
<td>Chardin et al, 1985</td>
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</tbody>
</table>

RMS arise from progenitors cells that fail to finish the differentiation process and exit the cell cycle. This results in uncontrolled proliferation and defective myogenesis (Hicks & Flaitz, 2002). RMS cells share characteristics of skeletal muscles, express early differentiation markers (MyoD and Myf5), but cannot differentiate into myotubes (Hicks & Flaitz, 2002).

MyoD belongs to the MRF (Myogenic Regulatory Factors) family of transcription factors that control myogenesis by regulating the expression of specific muscle genes (Olson, 1990). MyoD is a monomer formed of 2α helices and a loop of short amino acid sequence (Massari & Murre, 2000). To study the role of MyoD in muscle formation, MyoD mutant mice were generated. These mice showed delayed limb muscle development compared to normal mice. This defect was not however compensated by other muscle regulatory factors (Kablar et al, 1997). To further characterize the role of MyoD, P19 cells (embryonic carcinoma cells isolated from teratocarcinoma derived embryos) over-expressing MyoD were generated. This was
associated with an increase in expression of pre-myogenic (Six1, Pax7) and myoblast
genes (Myf5, Mef2 and Myogenin), leading to skeletal muscle formation. However, when
a double negative MyoD/EnR stable clone (mouse engrailed repression domain replaced
the transcriptional activation domain of MyoD) mutant was used, the expression of the
pre-myogenic and myoblast genes was down-regulated and skeletal myogenesis was
inhibited (Venuti, Morris, Vivian, Olson, & Klein, 1995).

By its ability to terminate cellular proliferation and stimulate muscle specific genes,
MyoD, promotes differentiation in progenitor cells. It plays a major role in the
antagonistic relationship between the processes of proliferation and differentiation
(Tintignac, Leibovitch, & Leibovitch, 2001). The control MyoD has on normal
myogenesis implies that its absence in muscle stem cells could lead to the emergence of
Rhabdomyosarcoma which is characterized by both, the loss of MyoD activity and the
loss of control of cellular proliferation (Tintignac et al, 2001). Northern blot analyses
conducted on RMS cells showed expression of MyoD in these cell lines (Hosoi, 1992).

MRF family member, Myf5, plays a key role in the initiation of skeletal
myogenesis. Myf5 mutant mice show defects in the initial stages of muscle formation and
failure of ribs attachment to the sternum. These malformations are however compensated
by MyoD and no defects were detected at later stages (Braun, Rudnicki, Arnold &
Jaenisch, 1992). This was not the case with double homozygous mutant mice, where
mono-nucleated muscle precursor cells formation ceased to occur. In conclusion, Myf5
and MyoD, are both key players in controlling the myogenic fate of cells (Rudnicki et al,
1993). So just like MyoD, defects in Myf5 leads to RMS (Rudnicki et al, 1993). Northern
blot analyses in RMS cells detected as well an expression of Myf5 in these cells (Hosoi,
In addition to *MyoD* and *Myf5*, *GATA4* and *GATA6* transcription factors were shown to be expressed in Rhabdomyosarcoma cells (unpublished results from Professor Nemer’s laboratory). The objective of this study was to assess the role of *GATA4* in rhabdomyosarcoma. The results showed a decrease in the expression of *GATA4* upon switching of cells to the differentiated stage. However, when Real-time PCR and Gel Electrophoresis mobility Gel shift assay (EMSA) were conducted to assess the expression of all GATA members (*GATA1*, *GATA2*, *GATA3*, *GATA4*, *GATA5* and *GATA6*) in rhabdomyosarcoma cells, at the transcriptional and functional level respectively, *GATA6* was shown to be expressed. The role *GATA4* plays in regulating cell survival by regulating apoptosis as well as the role it plays in proliferation has already been established (unpublished results from Professor Nemer’s laboratory).

GATA factors regulate cell survival in various cellular contexts. Several studies showed the correlation between GATA factors and apoptosis, whereby *GATA6* regulates cell survival by regulating apoptosis. *GATA6* knockdown leads to apoptosis. In a study conducted by Froese et al (2011) on human primary endothelial cells, down-regulating *GATA6* altered micro- and macro-vascular endothelial cells proliferation, migration and formation of capillary-like structures. On the other hand, over-expressing *GATA6* enhanced angiogenic activity, particularly in cardiac endothelial micro-vascular cells (Froese et al, 2011). Moreover, *GATA6* protected endothelial cells from undergoing apoptosis during growth factor deprivation. Mechanistically, the suppression of *GATA6* enhanced the expression of transforming growth factor TGFβ1 and TGFβ2. Enhanced *GATA6* expression on the other hand, decreased the activity of the Tgfβ1 promoter (Froese et al, 2011). Increased TGFβ1/β2 expression in *GATA6* null endothelial cells, up-regulated
the activity of the activin receptor-like kinase 5 (ALK5) and SMAD2. Inhibition of this signaling pathway restored angiogenic activity and survival of endothelial cells (Froese et al, 2011). Together, these findings show that GATA6 plays a major role in maintaining endothelial cells’ function and survival by partially inhibiting TGFβ autocrine expression and ALK5-dependent pathway. In another study conducted by Rong et al (2012), GATA6 was shown to promote cell survival by controlling the endodermal expression of BMP-2 and the basement membrane (BM). GATA6 ablation in mice stem cell-derived embryoid bodies (EBs) showed extensive apoptosis during EB differentiation (figure 5) (Rong et al, 2012). This increase in cell death was associated with decreased BMP-2 expression due to a direct interaction between GATA-6 and bmp2 promoters. SMAD1/5 activity was also inhibited (Rong et al, 2012). These results emphasize the importance of GATA6 in cell survival.

The role GATA6 plays in cell survival by regulating apoptosis makes it a key player in regulating cell growth and proliferation. A study conducted by Morrisey (2000), examined the potential of GATA6 in modulating cellular proliferation in vascular smooth muscle cells (VSMCs). The down-regulation of GATA6 expression in VSMCs and mouse embryonic fibroblasts (MEFs), inhibited the entry of cells into the S-phase (Morrisey, 2000). This shows the potential role GATA6 plays in regulation proliferation of cells. In a study conducted by Beuling et al (2012), GATA6 was conditionally deleted in adult mice from the small and large intestines. GATA6 deletion led to a massive decrease in the proliferative process of these cells. GATA6 knockdown in LS174T (colonic adenocarcinoma cell line) similarly resulted in massive down-regulation of the proliferation process (Beuling et al, 2012). These results show the necessity of GATA6 for normal proliferation in the colon. GATA6 was also shown to regulate the
proliferation renewal of peritoneal macrophages in mice (Rosas et al, 2014). Selective
GATA6 knockdown massively altered the proliferation of the macrophages during
homeostasis and inflammation, which led to delays in the resolution of the inflammatory
process (Rosas et al, 2014).

Taken together, those studies showed the importance of GATA6 for the survival and
proliferation in various cellular context.

In addition to its role in the regulation of proliferation and cell survival, GATA6
proteins are known play a crucial role in the regulating the differentiation program of some
cells. Zhao et al (2008) studied the role of GATA6 in controlling the onset of cardiac
myocyte differentiation. In Gata6+/− embryos, heart failed to form and differentiation of
progenitor cells into cardiomyocytes was blocked Zhao et al (2008). In addition, ES cells
derived from cardiac myocyte showed a decrease in expression of cardiac transcription
factors, cardiac myocytes markers (alpha cardiac actin [α-sarcomeric actin]), alpha
myosin heavy chain (αMHC), and smooth muscle actin (SMA)) (Zhao et al 2008).

Morrisey et al (1998) examined the role of GATA6 in regulating the differentiation of
visceral endoderm. GATA6 null (GATA6−/−) ES cells and mice were generated.
Differentiated embryoid bodies isolated from GATA6−/− ES cells lacked the visceral
endoderm covering layer and exhibited a decreased expression of the early and late
markers (HNF4, GATA4 HNF3β, α-fetoprotein (AFP)) (Morrisey et al, 1998). GATA6−/−
mice died at day E7.5 and suffered alterations in endoderm differentiation ranging from a
severe decrease in GATA6 expression to a complete loss of HNF4 gene expression
(Morrisey et al 1998). The regulation of differentiation by GATA6 occurs in some cellular
contexts and not in others. No studies so far linked GATA6 to the differentiation of
rhabdomyosarcoma cell lines.
Over the years, therapeutic methods were developed that aimed at silencing or remove disease causing genes. Double-stranded RNA-mediated interference (RNAi) has become widely used method for silencing target genes in an organism. The silencing results from the degradation of RNA into small RNA fragments. This activates ribonucleases that degrade homologous mRNA (Agrawal et al, 2003). In a study conducted by Lin et al (2012) on esophageal adenocarcinoma (EAC) cells, siRNA-induced silencing of GATA6 decreased the anchorage-independence and proliferation capacity of these cells. In another study conducted by Froese et al (2011) on endothelial cells, siRNA-induced silencing of GATA6 reduced the proliferation and migration capacity of these cells and inhibited the formation of capillary-like structures. Furthermore, Kwei et al (2008) and Zhong et al (2011) worked on pancreatic cell lines in Pancreatic Ductal Adenocarcinoma (PDAC) whereby siRNA induced silencing of GATA6 reduced cell cycle progression, cell proliferation and colony formation. Taken together, these results show the efficiency of siRNA in targeting specific genes and treating a range of disorders. Furthermore, SiRNA has been used in several studies to treat muscle diseases and showed great therapeutic potential. Kawakame et al (2013) assessed the effect of siRNA-targeted inhibition of myostatin (Mstn), a negative regulator of skeletal muscle development, on murine skeletal muscles in muscular dystrophy. A pronounced decrease in Mstn expression was detected in Mstn−/− mice. This could lead to hypertrophy of skeletal muscle and regain of motor disability in treated mice. Moreover, Mosler, Relizani, Mouisel, Amthor, and Diel (2014) used adult mice to study the effect of siRNA-induced inhibition of Mstn on skeletal muscle homeostasis. Mstn−/− mice were shown to exhibit skeletal muscle hypertrophy, which was correlated with an increase in satellite cells markers. This suggested that siRNA-
mediated silencing of Mstn triggered skeletal muscle growth. Noguchi, Ogawa, Kawahara, Malicdan and Nishino (2014) however, studied the effect of siRNA-mediated silencing on a point mutation in the collagen VI gene, COL6A1, in Ullrich congenital muscular dystrophy (UCMD). Mutation introduced into the gene, led to faulty collagen localization. However, when siRNA was applied, collagen IV localization around the fibroblasts was restored. These results showed the therapeutic potential of siRNA in treating muscle diseases.

To date, scientists lack a clear view on the transcription factors that regulate muscle development and disease. Rhabdomyosarcoma cells harbors muscle characteristics, express early markers of differentiation (MyoD and Myf5) as well as members of the GATA family (GATA4 and GATA6). The role of GATA4 in regulating muscle proliferation and differentiation in Rhabdomyosarcoma (Nemer et al, 2003) and since GATA6 plays key roles in regulating cell survival and proliferation, the objectives of this study were then to:

- Study the expression of GATA6 at the transcriptional level in two rhabdomyosarcoma cell lines (RH30 and J1).
- Investigate the effect of GATA6 inhibition (by siRNA) on the proliferation and differentiation potential of Rhabdomyosarcoma cell lines.
- Investigate the effect of GATA6 inhibition (by siRNA) on the cell cycle.
Chapter Two

Materials & Methods

2.1. Cell lines

Human Embryonal Rhabdomyosarcoma (JR1 cells) and Alveolar Rhabdomyosarcoma cell lines (RH30) were thankfully donated by Dr. Georges Nemer. These cells were maintained in DMEM culture media (Sigma) supplemented with 10% FBS (Sigma) and Penicillin streptomycin (P/S) (Sigma). All cells were incubated in a humid atmosphere of 95% air and 5% CO₂ at 37°C.

2.2. Transfection

JR1 and RH30 cells were placed in 10 mm Petri dishes (Costar) at 70% confluency. Transfection was conducted on the second day using GATA6-targeted siRNA (1nmol) (Qiagen, Catalog numb: S104355008) and TransFectin Lipid Reagent (Biorad, catalogue 170-3351). For each plate, 10 µl of siRNA was added to 500 µl of serum free media and 10 µl of TransFectin was added to 500 µl serum free media. Both siRNA and TransFectin solutions were mixed and left for 20 min at room temperature (RT) to allow for complex formation. The resultant mixture was then applied on cells and medium was switched to differentiation stage when 90% confluency was reached.

2.3. Transcriptional expression

2.3.1. RNA extraction

RNA was extracted from RH30 and JR1 cells using QIAzol Lysis reagent (QIAGEN, Cat. No.79306). Briefly, after aspirating the media, 1 ml of QIAzol was added.
Cells were scraped and left for 5 min at RT for nuclear protein dissociation. Chloroform was then added and samples were left at RT for 10 min to allow for nucleic acid and protein separation. RNA was collected from the upper layer following centrifugation. Isopropanol 100% was used for RNA precipitation and ethanol was next used to wash the pellet. Following centrifugation, cells were re-suspended in 100 µl nuclease free water.

2.3.2. RNA quality control

RNA quality was evaluated by running the samples on a 1.5% TBE agarose gel (Invitrogen, Cat # 15510-027) and stained using ethidium bromide (Sigma, Cat # E8751). The detection of two bands corresponding to 28-S and 18-S ribosomal RNA was indicative of the quality. RNA concentration was assessed using the ND-1000 spectrophotometer NanoDrop; the 1.7-1.8 260/280 ratios were indicative of pure RNA.

2.3.3. Reverse Transcriptase- PCR (RT-PCR)

Complementary DNA (cDNA) was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. K1622) according to the manufacturer’s protocol using 3 micrograms of total RNA. The primers used to amplify the studied genes are presented in table 2. The PCR products were then loaded on a 1.5% TBE agarose gel and stained with ethidium bromide.
Table 2. Primers used to evaluate the expression of target genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Origin</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>GATA6</td>
<td>F:ttcccagactctcaactcc</td>
<td>Human</td>
<td>94°C for 30 sec</td>
</tr>
<tr>
<td></td>
<td>R:cgctatagtagagcccatct</td>
<td></td>
<td>58°C for 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C for 30 sec</td>
</tr>
<tr>
<td>GATA4</td>
<td>F:tggcctgtcatctcactac</td>
<td>Human</td>
<td>94º for 30 sec</td>
</tr>
<tr>
<td></td>
<td>R:aagaccaggctgttcaaga</td>
<td></td>
<td>60º for 30 sec</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>72°C for 30 sec</td>
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<tr>
<td>GAPDH</td>
<td>F:gtcagttggttgacgctacct</td>
<td>Human</td>
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<td>R:tcggctaagttgaagtcagagga</td>
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<td>60°C for 30 sec</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C for 30 sec</td>
</tr>
</tbody>
</table>

2.3.4. Real Time PCR

DNA expression was assessed using CFX96 Real Time PCR and Syber Green Supermix (BioRad, Cat Num: 170-8882) according to manufacturer’s instructions. Briefly, a 20µl reaction mixture was prepared with 2-3 µl of cDNA, 1 µl for each primer, 5-7 µl of water and 10 µl of Syber green. Cycling conditions included: denaturation at 95 °C for 5 min followed by 40 cycles at 95º C for 30 s, annealing for 30 s (temperature as
shown in table 6) and a final extension at 72° C for 45s. This was followed by a step of 40° C for 15 s with increments of 0.5° C to reach 95° C for the melting curve.

2.4. Flow Cytometry

Cells were fixed at first by spinning them down at 1500 rpm for 5 min, re-suspending the pellet in 1 ml ice-cold phosphate buffered saline (PBS), spinning them again as above, re-suspending the pellet in 1 ml ice-cold PBS and 4 ml absolute ethanol (100%) and storing them at −20° C. The next day, cells were stained using propiduim iodide (PI). They were brought down to room temperature by placing them in a 37° C water bath. Cells were then spun down for 5 min at 1500 rpm, re-suspended in 1 ml ice cold-PBS, spun down again, re-suspended in 500 µl PBS and transferred into polystyrene falcon tubes. In each tube, 30 µl PI stain was added (1 mg/ml prepared from a 10mg/ml stock in PBS) and vortexed. Cells were incubated in the dark for 10 min and read.

All data are presented as mean ± standard error. Two-tailed unpaired Student' s tests and ANOVA were used for statistical evaluation of the data. SPSS 18 statistical tool was used for data analysis. A p-value < 0.05 was considered significant.
Chapter Three

Results

3.1. Characterization of gene expression in Rhabdomyosarcoma

Rhabdomyosarcomal cell lines (RH30 and JR1) were plated in 10 mm petri dishes and left till 95% confluency was reached. RNA was collected at the proliferative stage for the first group of cells. The others were left to differentiate by serum-deprivation (media was switched from 10% FBS to 0.5% FBS). RNA was reverse transcribed to complementary DNA and Real-time PCR was carried out to study the expression of five target genes: GAPDH (house-keeping), GATA6, GATA4, Cyclin D1, Desmin, and Myf5. PCR was conducted as well and DNA was sequenced prior to real time PCR to make sure we have our target genes (not shown).

CFX96 Biorad software system was used to analyze the Real-Time PCR results. The results shown are from three different experiments worked in duplicates from three different RNAs. A similar pattern of expression was detected for both cell lines. GATA6 was shown to be expressed at the proliferation and differentiation stage in both cell lines (figure 1). Its expression decreased at Day 1 following serum deprivation. This shows that GATA6 plays an important role in the early stages of differentiation. GATA6 expression increases following day 1 reaching its peak at Day 3. This may be due to the effect of serum deprivation where afterwards there’s normalization of expression. A decrease in GATA4 expression (figure 2) was seen when cells were switched from the proliferation to differentiation stage in both cell lines (D4).
Figure 1. q-PCR showing GATA6 relative gene expression of JR1 and RH30 cells at the proliferation and differentiation stages (Day 1 till D4). GATA6 expression increased when cells were switched to the differentiation stage. (*) P value < 0.05.

Figure 2. q-PCR showing GATA4 relative gene expression of JR1 and RH30 cells at the proliferation and differentiation stages (Day 1 till D4). GATA4 expression decreased when cells were switched to the differentiation stage. (*) P value < 0.05.
To assess the proliferative potential of JR1 and RH30, *CyclinD1* was used. The results showed a high expression of *CyclinD1* at the proliferative stage followed by a decrease in the differentiation stage in both cell lines (figure 3).

![Real-Time PCR](image)

Figure 3.q-PCR showing CyclinD1 relative gene expression of JR1 and RH30 cells at the proliferation and differentiation stages (Day 1 till D4). CyclinD1 expression increased when cells were switched to the differentiation stage. (*) P value < 0.05.

To assess the differentiation potential of JR1 and RH30 cells, *Desmin* (figure 4) and *Myf5* (figure 5) were used. Unlike *CyclinD1*, the expression of these markers was minimal at the proliferation stage but increased at the differentiation stage.
Figure 4. q-PCR showing Desmin relative gene expression of JR1 and RH30 cells at the proliferation and differentiation stages (Day 1 till D4). Desmin expression increased when cells were switched to the differentiation stage. (*) P value < 0.05.

Figure 5. q-PCR showing Myf5 relative gene expression of JR1 and RH30 cells at the proliferation and differentiation stages (Day 1 till D4). Myf5 expression increased when cells were switched to the differentiation stage. (*) P value < 0.05.

Taken together, these results show that GATA6 is expressed in rhabdomyosarcoma cells and show proliferative and differentiation potential. As it has been already established, GATA6 plays a key role in cellular proliferation. To assess this in JR1 and RH30 cells, GATA6 specific siRNA was used next.
3.2. Down-regulation of GATA6 inhibits proliferation

To study the effect of GATA6 down-regulation on the proliferation and differentiation of cells, JR1 and RH30 were plated in 10 mm petri dishes and transfected with GATA6-specific siRNA. RNA was collected at the proliferative stage for the first group of cells. The others were left to differentiate by serum-deprivation (media was switched from 10% FBS to 0.5% FBS). RNA was reverse transcribed to complementary DNA and Real-time PCR was carried out to study the expression of four target genes: GAPDH (house-keeping), GATA6, Cyclin D1, Desmin, and Myf5. PCR was conducted as well and DNA was sequenced prior to real time PCR to make sure we have our target genes (not shown).

CFX96 Biorad software system was used to analyze the Real-Time PCR results. The results shown are from three different experiments worked in duplicates from three different RNAs. A similar pattern of expression was detected for both cell lines. Day 3 (D3) was used as a reference since at Day 4 (D4) cells will start to die which might affect the analysis of the results. A decrease in the expression of all four genes (GATA6 (figures 6), CyclinD1 (figures 7), Desmin (figures 8) and Myf5 (figure 9) following down-regulation of GATA6 in both cell lines. The decrease was however more important in this case at the proliferation stage in both cell lines. These results shows the efficiency and specificity of siRNA deducted from the decrease in GATA6 and highlights once again the necessity of GATA6 for cellular proliferation (deduced by the decrease in CyclinD1 expression following GATA6 down-regulation).
Figure 6. q-PCR showing the relative expression of GATA6 in JR1 and RH30 cells following down-regulation by siRNA. GATA6 expression decreased upon transfection, compared to the control, at both stages. This decrease was more pronounced at the proliferation stage. (*) P value < 0.05.

Figure 7. q-PCR showing the relative expression of CyclinD1 in JR1 and RH30 cells following down-regulation of GATA6 by siRNA. CyclinD1 expression decreased upon transfection, compared to the control, at both stages. This decrease was more pronounced at the proliferation stage. (*) P value < 0.05.
Figure 8. q-PCR showing the relative expression of Desmin in JR1 and RH30 cells following down-regulation of GATA6 by siRNA. Desmin expression decreased upon transfection, compared to the control, at both stages. This decrease was more pronounced at the proliferation stage. (*) P value < 0.05.

Figure 9. q-PCR showing the relative expression of Myf5 in JR1 cells following down-regulation of GATA6 by siRNA. Myf5 expression decreased upon transfection, compared to the control, at both stages. This decrease was more pronounced at the proliferation stage. (*) P value < 0.05.
3.3. Down-regulation of GATA6 leads to cell death

In order to assess the effect of GATA6 down-regulation on the cell cycle, flow cytometry was conducted. Cells were at first fixed using cold-ice PBS and absolute ethanol (100%), and then stained using PI. The results obtained showed that 13.26 % of transfected cells were detected at the G0/G1 phase compared to 43.98% cells in the control, 13.26% compared to 16.66% in the control at the S phase, and 14.85% compared to 23.35% in the control at the G2/M phase (Table 3). This decrease in cell number, proves again that GATA6 down-regulation reduces the proliferative potential of JR1 and RH30 cells. Since this decrease was detected in the 3 phases, we conclude that GATA6 down-regulation leads to cell death.

Table 3. Flow cytometry results showing the effect of GATA6 down-regulation on cell cycle.

<table>
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<th>S percent for R2 gated by PO1.R1 (%)</th>
<th>G2/M percent for R2 gated by PO1.R1 (%)</th>
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Chapter Four

Discussion

Most studies done on GATA genes were either done in the cardiac or hematopoietic systems where they are highly enriched. So far no published studies have shown the role of the GATA family in muscle development and rhabdomyosarcoma. Our primary goal was to assess the expression of GATA6 in rhabdomyosarcomal cell lines and to study the effect of down-regulating GATA6 on the proliferation and differentiation processes. SiRNA is known to modulate gene expression by cleavage of the mRNA. The assessment of the inhibition effect, involved characterizing GATA6 expression at proliferation and differentiation stages following inhibition. Our results revealed a significant role of GATA6 in the proliferation process.

GATA genes are known to play a key role in embryogenesis by activating and repressing a set of transcription factors (Buckingham, 2001). Prior to transfection, GATA6 was shown to be expressed at both the proliferation and differentiation stages in JR1 and RH30 cells. This goes hands in hands with the results obtained from Professor Nemer research (unpublished results). CyclinD1, marker of cellular proliferation, was highly expressed at the proliferation level in both cell lines compared to the differentiation stage as expected. Upon cellular transfection, GATA6 expression decreased showing the specificity of siRNA in targeting and silencing specific genes. It must be noted however that not all cells were silenced in the process. Furthermore, cyclinD1 expression decreased in both cell lines. These results show the potential role that GATA6 plays in regulating proliferation. Our study goes hand in hand with what is
already known about the relationship between GATA6 and proliferation. Froese et al (2011) assessed the role of GATA6 in the ability of endothelial cells (HUVECs and HCMECs) to proliferate following transfection. Down-regulation of GATA6 dramatically reduced the proliferative potential of these cells compared to the control siRNA. The fact that GATA6 regulates proliferation and can be down-regulated by specific siRNA makes it a possible treatment approach for rhabdomyosarcoma.

Since following GATA6 down-regulation, the proliferation potential of cells is dramatically reduced, these latter have two fates: differentiation or cell death. To assess the differentiation potential of cells, Desmin and Myf5 were used. Prior to transfection, Myf5 and Desmin showed the highest level of expression at the differentiation stage in JR1 and RH30 as expected. Following GATA6 down-regulation, a minimal decrease in the expression of Desmin and Myf5 was detected in JR1 and RH30. This suggests that GATA6 might have no effect or minimal effect on differentiation. No studies were published so far that linked GATA6 to differentiation. The main focus of this study was on proliferation, since the role role of GATA6 in this context has already been established.

Since cells are not differentiating, cell death was postulated. To study the effect of GATA6 down-regulation on the cell cycle, flow cytometry was conducted. A decrease in the number of transfected cells was detected at the three stages compared to the control. This decrease in number of cells means that GATA6 down-regulation, inhibits proliferation and leads thus to cell death. Our study goes hand in hand with what is already known about the relationship between GATA6 and cell death (apoptosis). In Vascular Smooth Muscle Cells (VSMCs), GATA6 down-regulation inhibited cells entry into the S-
phase. This was associated with an increase in the expression of the cyclin-dependent kinase (Cdk) inhibitor p21 (Perlman, Suzuki, Simonson, Smith & Walsh, 1998). This data further verifies our assumption of cell death. However, Tunnel Assays must be conducted to further study this hypothesis.

Finally, and unlike GATA6, GATA4 expression increased upon serum deprivation in JR1 (figure 14) and RH30 (figure 15). The importance of GATA4 in promoting cellular differentiation has already been established. A study conducted by Dr Nemer and his team (Nemer et al, 2003), showed how overexpressing GATA4 inhibited skeletal muscle differentiation of C2C12 cells (muscle cells) by inhibiting myotubes fusion. Down-regulation of GATA4 however enhanced the differentiation potential of JR1 and RH30 cells (Nemer et al, 2003). Northern blot was conducted on stable clones overexpressing GATA4 and compared to the control (GATA4). The clones exhibited a decrease in differentiation markers (E-box, MyoD and Myogenin). Analysis of the PCR bands using image J quantification software showed an increase in microRNA (mir-659) (down-regulates GATA4) expression upon serum deprivation. Taken together these results show that GATA4 functions as a negative regulator of muscle cells differentiation.

The results obtained from this research provide new treatment insights for rhabdomyosarcoma. Till today rhabdomyosarcoma is one of the most common sarcoma diseases and is still treated by means of surgery or radiation or a mix of both which places the patients’ life at risk. Several studies have shown the role GATA6 plays in promoting cell proliferation. Our results showed that siRNA-mediated silencing of GATA6 decreased the proliferative potential of these cells and might act as a potential treatment approach.
Furthermore, we speculated cell death. This needs to be further evaluated by doing Tunnel Assays.

Finally, *in-vitro* studies face several limitations, it is thus important to study this process using *in-vivo* models by subcutaneously injecting xenograft mice with rhabdomyosarcoma cells and GATA6-specific siRNA, and measuring the tumor diameter. This will provide further information on the inhibitory effect on siRNA on tumor development.
BIBLIOGRAPHY


