Defining the anti-apoptotic function of the survival of motor neuron (SMN) protein and assessment of a novel therapy for the treatment of spinal muscular atrophy (SMA)

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BSc (Hons)

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ABSTRACT

Spinal muscular atrophy (SMA) is a neurodegenerative disorder primarily affecting motor neurons. This untreatable disease is caused by the absence of a functional survival of motor neuron 1 (SMN1) gene, which leads to a critical reduction in full-length survival of motor neuron (SMN) protein. The multifunctional SMN protein is important in the biogenesis of small nuclear ribonuclear proteins, pre-mRNA splicing and motor neuron viability. However, the precise functions of the SMN protein in promoting neuronal survival are yet to be completely understood. To this end, the aims of this project were to elucidate the pro-survival role(s) of the SMN protein and assess a novel therapeutic approach for the delivery of recombinant SMN protein into cells. Therefore, the hypotheses of this project stated that the SMN protein plays an important role in regulating apoptosis, and successful delivery of the SMN protein to cells can be achieved using a cell-penetrating peptide (CPP).

To elucidate the pro-survival functions of the SMN protein, a novel in vitro apoptotic cell death model was established. Using differentiated human SH-SY5Y neuroblastoma cells, a predominately apoptotic cell death was induced through the inhibition of the PI3-kinase/Akt survival pathway. Results from these experiments revealed that SMN over-expression protected SH-SY5Y cells from a caspase-dependent apoptotic cell death. Further, SMN over-expression blocked calpain-mediated activation and cleavage of the caspase-3 protein. The demonstration of this previously unknown mechanism has identified a pathway activated by the SMN to block apoptosis.
To further characterise how the SMN protein promotes survival, the relationship between the SMN and the anti-apoptotic protein Bcl-xL was investigated. Results from this study demonstrated a novel finding with therapeutic implications, whereby Bcl-xL over-expression increased SMN protein levels by approximately 2 fold in SH-SY5Y cells. Conversely, SMN over-expression increased Bcl-xL protein levels by approximately 6 fold in SH-SY5Y cells and 2.5 fold in the brains of transgenic mice over-expressing SMN (PrP-SMN mice). In addition, it was demonstrated that over-expression of both proteins produced an additive neuroprotective effect in the SH-SY5Y apoptotic cell death model. The search for an underlying mechanism for this regulation led to the identification of SAM68, a splicing regulator of both Bcl-xL and SMN1/2, which was markedly reduced following SMN and Bcl-xL over-expression, suggesting a feedback mechanism co-regulating levels of both proteins.

To conclude, a novel adenoviral expression vector was utilised for the generation and secretion of a TAT-fused SMN protein (TAT-SMN). Results from these experiments showed that the TAT-SMN protein was secreted and could be purified from adenovirally transduced cultured U251 glioma cells. However, whilst purified TAT-SMN protein was recovered, primary cortical neurons did not appear to be transduced with this protein. Nevertheless, this study led to the development of a novel method to generate full-length recombinant TAT-SMN protein, which may be applicable to the delivery of SMN as a treatment for SMA.

In summary, this thesis has identified novel mechanisms used by the SMN protein to promote cell survival. In agreement with the initial hypothesis, the in vitro and in vivo results have demonstrated that SMN expression prevents apoptosis and plays a key role
in regulating anti-apoptotic pathways and protein expression of SMN and Bcl-xL. It is anticipated that findings from this thesis will influence future studies on the regulation of SMN protein expression, strategies used to treat SMA, and the role of SMN in neuronal survival.
ACKNOWLEDGEMENTS

The completion of this thesis has been a long and rewarding journey. To those individuals I have shared this journey with, those I have met along the way, and those who have contributed to this thesis; I express sincere thanks and gratitude.

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I would also like to acknowledge my colleagues at ANRI. These individuals have made my time and experience completing this thesis as fun and enjoyable as possible. Finally, I would like to thank my mother, father and partner for their support and encouragement throughout my candidature.
DECLARATION

I hereby declare that this thesis is my own account of my research and that all other sources have been acknowledged and my contribution is clearly identified. Permission has been granted by co-authors for any work that has been co-published to be included in this thesis. This thesis and its main content have not previously been submitted or accepted for any other degree in this or any other institution.

Signed

Ryan Anderton

Signed

Bruno Meloni

Co-ordinating supervisor
PUBLICATIONS ARISING FROM THIS THESIS


CONFERENCE ABSTRACTS AND PRESENTATIONS ARISING
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>Akt</td>
<td>Also known as protein kinase B</td>
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<tr>
<td>AO</td>
<td>Antisense oligonucleotides</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CPP</td>
<td>Cell penetrating peptide</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's minimum essential medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<tr>
<td>FIB</td>
<td>Fibronectin signal peptide</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>HA</td>
<td>Human influenza hemagglutinin</td>
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<td>HDAC</td>
<td>Histone deacetylase inhibitors</td>
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<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Abbreviation</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PTD</td>
<td>Protein transduction domain</td>
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<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<td>QPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid ref Relative centrifugal force</td>
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<tr>
<td>RT-PCR</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>SAM68</td>
<td>Src substrate associated in mitosis of 68 kilodaltons</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>SMA</td>
<td>Spinal muscular atrophy</td>
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<tr>
<td>SMN</td>
<td>Survival of motor neuron</td>
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<tr>
<td>snRNPs</td>
<td>Small nucleotide ribonuclear proteins</td>
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<tr>
<td>TAT</td>
<td>Transcriptional activator protein</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Polyethylene glycol tert-octylphenyl ether</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylene-sorbitan monolaurate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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Chapter 1

General Introduction
GENERAL INTRODUCTION

Introduction

Spinal muscular atrophy (SMA) is an often fatal autosomal recessive genetic disease typified by the degeneration of motor neurons in the spinal cord and muscle atrophy. With an incidence of one in 6,000 live births, SMA is the most common genetic cause of infant mortality. The disease predominantly affects infants and results in neuronal degeneration, followed by a clinical decline in patient mobility, muscle strength and independence. In approximately 95% of SMA patients, the survival of motor neuron 1 (SMN1) gene is affected, causing severely reduced levels of the survival of motor neuron (SMN) protein (Lefebvre et al., 1995). The ubiquitously expressed SMN protein plays a role in spliceosome formation, small nuclear ribonucleic protein (snRNP) assembly, neuromuscular junction (NMJ) formation, pro-survival signalling and motor neuron development (Anderton et al., 2013). However, the precise molecular mechanisms by which SMN protein promotes survival are poorly understood.

The focus of this project is to characterise the cellular pathways by which the SMN protein promotes neuronal survival, and in particular, determine how SMN regulates apoptosis. Thus, the central hypothesis of this thesis is that the SMN protein exerts anti-apoptotic properties that help to maintain neuronal viability. A secondary hypothesis, therefore, is that direct delivery of recombinant SMN protein to neuronal cells (using a cell penetrating peptide), would prevent apoptosis. If exogenous SMN protein delivery is anti-apoptotic, such a novel approach would represent a feasible therapeutic avenue to treat SMA. To this end, it is anticipated that findings from this project will improve our
understanding of the pro-survival functions of the SMN protein, and determine if a recombinant SMN protein approach to treating SMA is feasible.

**Spinal Muscular Atrophy Background**

*Clinical classification*

Spinal muscular atrophy is characterised by the degeneration of motor neurons of the spinal cord, resulting in hypotonia and muscle weakness. Spinal muscular atrophy is divided into four clinical types on the basis of age of onset and motor function achieved, such as sitting or walking independently. These different types include a severe type I, intermediate type II, mild type III and adult onset type IV (Lunn and Wang, 2008). The SMA type I disease classification (Werdnig-Hoffmann disease) accounts for approximately 50% of all SMA cases. Type I SMA patients are distinguished by the earliest age of onset (<6 months) followed by death within the first 2 years of life. This form of the disease is characterized by profound hypotonia, the absence of head movement control, symmetrical flaccid paralysis, and an inability to sit up unaided (Russman, 2007).

The intermediate type II form of the disease is characterised by a disease onset between 7 to 18 months of age. Patients are unable to walk independently, but can maintain a sitting position unaided. These individuals frequently experience respiratory problems that usually contribute to their death in adolescence (Russman, 2007). Type III SMA (Kugelberg-Welander disease) patients show greater heterogeneity in their symptoms compared to other subtypes. For example, some patients may require wheelchair assistance in childhood, whereas other patients develop only minor muscle weakness
without any need for significant assistance (Lunn and Wang, 2008). Finally, in the adult onset form of the disease (type IV), muscle weakness occurs in the second or third decade of life, without the severe life-threatening complications of type 1 or 2 SMA (Russman, 2007).

**Epidemiology**

The incidence of SMA is approximately 1 per 6,000 live births making it the most common cause of genetically determined neonatal death. Neither physiological nor social factors show a correlation with SMA prevalence or age of onset (Pearn, 1978). Interestingly, type I SMA occurs at a higher frequency in males but does not influence age of onset or life expectancy (Hausmanowa-Petrusewicz et al., 1984; Pearn, 1978). In addition, a higher incidence of SMA has been linked to geographical locations. For example, in Saudi Arabia and many other Middle Eastern countries, the incidence of SMA can be as much as 50 times higher than in the western world (Al-Rajeh et al., 1992; Hasanzad et al., 2010). These high rates have been attributed to the cultural acceptance and popularity of consanguineous relationships.

**Molecular Genetics of SMA**

*Survival of motor neuron genes (SMN1/2)*

In SMA, approximately 95% of patients suffer a mutation or deletion in the *SMN1* gene. The *SMN1* gene lies on chromosome 5 and encodes the SMN protein (Lefebvre et al., 1995). Survival of motor neuron protein expression is ubiquitous throughout the body, with extremely high levels occurring in the brain and spinal cord. Interestingly, due to a
500 kilobase inverted duplication occurring during human evolution, several genes surrounding and including the \textit{SMN1} gene are duplicated (Lefebvre et al., 1995; Rochette et al., 2001). The two SMN genes have been designated telomeric \textit{SMN1} and centromeric \textit{SMN2}, and are separated by approximately 20 kilobases (Rossoll et al., 2003). The second, highly homologous copy of the \textit{SMN1} gene, termed \textit{SMN2}, differs by only five nucleotides. However, a single translationally silent nucleotide change (C \(\rightarrow\) T) in exon 7 of the \textit{SMN2} gene causes faulty splicing of the pre-\textit{SMN2} transcript (Lorson et al., 1999). This aberrant splicing causes the removal of exon 7 from approximately 80\% of all \textit{SMN2} transcripts, resulting in the formation of a truncated and unstable SMN (SMN\(\Delta7\)) protein product (Lorson et al., 1999).

The cause of \textit{SMN2} exon 7 skipping is due to the disruption of a splice modulator site/sequence which acts as an exon splicing enhancer. The \textit{SMN2} gene is unique to humans, making SMA a disease specific to humans. This situation occurs because without the small portion of functional SMN protein translated from \textit{SMN2}, the condition would be embryonically lethal. Correspondingly, the number of \textit{SMN2} gene copies (1-4) varies in SMA patients and this number invariably, but not always, predicts the SMA disease sub-types (McAndrew et al., 1997; Parsons et al., 1998). This is because \textit{SMN2} gene copy number correlates directly with full-length SMN protein levels and SMA disease severity, thus making copy number determination an excellent prognostic tool.

\textit{The SMN protein}

The \textit{SMN1} gene encodes a full-length protein with a predicted molecular weight of 32 kilodaltons, which is present in all tissues (Coovert et al., 1997). By contrast, the \textit{SMN2}
gene only encodes small amounts of full-length SMN protein (5-20% of the amount expressed by the SMN1 gene) due to aberrant splicing, as described previously. The vast majority of protein encoded by the SMN2 gene is truncated and lacks all of the carboxyl end amino acids encoded by exon 7. Full-length SMN protein is ubiquitously expressed and is found in the cytoplasm and nucleus. In the nucleus, the SMN protein localises to distinct structures called Gemini of coiled bodies (gems). Within neurons, SMN is present in axons, dendrites and the neuromuscular junctions of motor neurons (Broccolini et al., 1999; Francis et al., 1998; Pagliardini et al., 2000).

Functions of the SMN Protein

Developmental functions of SMN

Survival of motor neuron protein expression during development has been studied in numerous animal models and in the foetal tissue of SMA patients. Whilst high SMN expression occurs in early development (Burlet et al., 1998; Tizzano et al., 1998), its role during this crucial phase is largely unknown. In SMA patients, developmental expression of SMN is severely reduced, and this reduction has been correlated with elevated motor neuron cell death (Soler-Botija et al., 2002), thus illustrating SMN’s pro-survival role. Further, neuropathological features in SMA patients indicate that SMN is essential for NMJ synaptic formation and motor neuron differentiation (Fan and Simard, 2002; Simic et al., 2008). Outside the nervous system, SMN has also been linked to early bone remodelling (Shanmugarajan et al., 2007), which highlights its multifunctional role in human development.
The SMN complex

While many functions have been attributed to the full-length SMN protein, the most studied role of the protein is its involvement in the formation of snRNPs, via the SMN complex. Each snRNP is composed of small nuclear RNAs (snRNAs) bound to a common set of seven Sm proteins (Battle et al., 2006). The correct assembly of Sm proteins onto snRNAs is directly mediated by the SMN complex (Gubitz et al., 2004; Kolb et al., 2007; Pellizzoni, 2007; Raker et al., 1999), which consists of SMN and Gemin proteins (Gemins 2-8) (Baccon et al., 2002; Carissimi et al., 2006; Charroux et al., 1999; Charroux et al., 2000; Gubitz et al., 2002; Liu and Dreyfuss, 1996; Liu et al., 1997; Pellizzoni et al., 2002). As an important part of the spliceosome (Will and Luhrmann, 2001), snRNPs are involved in pre-mRNA splicing, a process essential for the generation of mature mRNA transcripts (Kolb et al., 2010).

Pro-survival role of SMN

Apoptosis, a form of programmed cell death, results in nuclear fragmentation, chromatin condensation and the formation of apoptotic bodies (Elmore, 2007; Kerr et al., 1972). This phenomenon is required during embryonic and central nervous system (CNS) development, and is commonly dysregulated in neurodegenerative diseases (Mattson, 2000). In addition to its spliceosomal function, SMN is also a key modulator of neuronal apoptosis (Kerr et al., 2000). Studies aimed at identifying a specific anti-apoptotic function have been lacking, but recent evidence suggests a role for SMN in regulating many anti-apoptotic proteins and pathways (Anderton et al., 2013).
Examination of SMA foetal tissue suggests immature motor neurons undergo a more prolonged period of apoptotic cell death than controls, implicating SMN in embryonic neuronal survival and brain development (Simic et al., 2000; Soler-Botija et al., 2002). In line with the foetal tissue findings, numerous *in vitro* studies in rat primary neurons, mouse NSC-34 cells, and human SH-SY5Y cells have demonstrated an anti-apoptotic role for SMN (Anderton et al., 2011; Kerr et al., 2000; Parker et al., 2008). Specifically, SMN has been shown to inhibit apoptosis by blocking the cleavage (Anderton et al., 2011) and activation (Parker et al., 2008; Vyas et al., 2002) of caspase-3, a potent end stage apoptosis inducer. Furthermore, interactions between the SMN protein and key regulators of survival, such as p53 and Bcl-2 proteins (Iwahashi et al., 1997; Young et al., 2002), imply a more complex role for SMN in regulating higher cell survival pathways.

**SMA Pathogenesis**

*Histopathological features*

The disruption of normal motor unit development leaves patients with a progressive paralysis and muscle atrophy due to denervation. While the molecular and genetic cause underlying neuronal degeneration is debatable, gross pathological changes are evident during disease progression. The predominant pathological feature of SMA is the loss of motor neurons in the spinal cord and brain stem motor nuclei. However, descriptions of ballooned neurons within Clarke’s nucleus of the spinal cord, the thalamus and dorsal root ganglia, also suggest the involvement of other neuronal types (Sumner, 2006).
SMN expression and motor neuron death

The sequence and stages of events in SMA pathogenesis remains unclear. The onset of SMA is variable, with most patients deteriorating over a period of days to weeks before a long period of slow decline (Sumner, 2006). Survival of motor neuron protein expression is elevated early during embryonic development and has been linked to a role in neuromuscular junction formation and neuronal migration. Studies in human foetal tissue have shown SMA patients contain heterogeneous populations of motor neurons, including normal, abnormal, apoptotic and migratory motor neurons, not seen in controls (Simic, 2008). Studies aimed at delineating neuronal cell death in genetically confirmed cases of human SMA foetal tissue have revealed that during embryonic development, immature motor neurons undergo a period of prolonged apoptotic programmed cell death, compared with motor neurons from non-SMA control human foetal tissue (Simic et al., 2000; Soler-Botija et al., 2002). Thus, these findings support the notion that during embryogenesis, loss of SMN leads directly to a higher than normal level of apoptosis, which itself, could be argued as being a very early event in the pathogenesis of SMA. Confounding the issue of SMA pathogenesis even further, studies have revealed that sub-regions within the brain and CNS, outside of the neuromuscular system, such as the neurons within the hippocampus and peripheral sensory network, also undergo changes when SMN levels are severely depleted (Wishart et al., 2010).
Current Therapeutic Approaches in Treating SMA

Background

The current goal of SMA research is to increase mean survival and successfully treat the progressive motor neuron loss observed in the spinal cord. At the forefront of current research is the goal to increase functional SMN protein. Restoration of SMN levels in animal models of the disease show an almost complete amelioration of the disease phenotypes (Foust et al., 2010; Narver et al., 2008; Passini et al., 2011). This has led research groups to pursue a range of methodologies targeting $SMN2$ exon 7 inclusion of mRNA transcripts, exogenous SMN expression and other SMN ‘restoration techniques’ as potential treatments for SMA. However, to date, despite extensive research, no therapy has been successfully applied to SMA patients, which provides a clinically beneficial long-term outcome.

Compounds that up-regulate $SMN2$

The presence of an adjacent $SMN2$ gene in humans presents a unique target for researchers aiming to elevate levels of SMN protein. In SMA patients, where the $SMN1$ gene is mutated or deleted, successfully manipulating expression of $SMN2$ would translate to a proportional increase in full-length SMN protein. The first compounds targeting $SMN2$ expression were the histone deacetylase (HDAC) inhibitors. Preclinical studies using valproic acid, a HDAC inhibitor, showed improvements in phenotype and SMN protein levels when administered to SMA mouse models (Tsai et al., 2008). Valproic acid and other chemically similar HDAC inhibitors can promote neuronal survival through the activation of survival pathways, potentially resulting in an
additional feed-forward up-regulation of SMN protein (Anderton et al., 2012). However, despite promising preclinical results, HDAC inhibitors have shown no benefit in SMA clinical trials (Kissel et al., 2011; Mercuri et al., 2007; Swoboda et al., 2010), suggesting treating the complex nature of SMA in humans may present further challenges.

*Exon 7 inclusion of survival of motor neuron transcript*

An alternative approach, also targeting the *SMN2* gene, involves the use of antisense oligonucleotides (AOs) to promote exon 7 inclusion of *SMN2* mRNA transcripts. Antisense oligonucleotide technology has previously been used to alter gene expression in different forms of cancer (Stahel and Zangemeister-Wittke, 2003) and has been used successfully to restore dystrophin expression in Duchenne muscular dystrophy patients (Cirak et al., 2011; van Deutekom et al., 2007). Many preclinical studies have demonstrated the effectiveness of AOs to promote exon 7 inclusion and increase SMN protein levels in patient fibroblasts (Hua et al., 2007) and show a near complete amelioration of disease phenotype (Passini et al., 2011). However, the effects of AO therapies in SMA patients are still unclear and require further investigation.

*Viral delivery of survival of motor neuron*

Considered to be the most promising of the current SMA therapeutic approaches, viral delivery of SMN presents an alternative method for systemic delivery of SMN protein. Azzouz et al first showed the potential for viral therapy, using a lentiviral vector to achieve strong expression in motor neurons in an SMA mouse model (Azzouz et al., 2004). Since these early studies, numerous improvements in gene delivery and viral
production have enhanced the efficiency of this technique. The most recent of these, the adeno-associated virus (AAV-9), successfully expresses SMN in the CNS and all peripheral tissues of SMA mouse models (Foust et al., 2010). Intravenous delivery of AAV-9 SMN to SMA mouse models improved survival and amelioration of the SMA phenotype (Dominguez et al., 2011; Foust et al., 2010). While promising, delivering live virus to humans is not without its risks, including potential viral reactivation, oncogenic formation and immunological disturbances.

Exogenous delivery of SMN

Another avenue previously investigated to treat SMA involves the delivery of recombinant SMN protein directly to mammalian cells. This concept is attractive as it avoids the need for genetic manipulations, viral vectors and the administration of drugs targeting the SMN2 gene. Francis et al took advantage of the specificity for motor neurons displayed by the tetanus toxic C-fragment (TTC), and generated a SMN fusion protein coupled with a membrane translocation toxin (DTx) isolated from Diptheria (Francis et al., 2004). However, this particular study found that internalization of the recombinant protein was minimal; suggesting that either the TTC fragment imposed upon the neurons ability to internalize the protein, or interference resulted from the moiety of the SMN fusion protein. Their research suggested that internalization of the SMN protein was a hurdle that needed to be overcome in order for this approach to succeed.
**Cell Penetrating Peptides**

*Background*

Cell penetrating peptides (CPPs) or protein transduction domains (PTDs) offer an alternative means of delivering therapeutic proteins to cells. These short peptide sequences, when fused/linked to different types of biological entities (e.g. a protein, peptide, or AOs) are capable of transporting these ‘cargoes’ across cell membranes, and importantly, across the blood brain barrier (BBB) (Schwarze and Dowdy, 2000; Schwarze et al., 1999). One of the first CPPs to be characterised was a peptide domain derived from the trans-activating transcriptional activator protein (TAT) of the human immunodeficiency virus type-1 (Fawell et al., 1994). Initial studies in mice using the 11 amino acid TAT peptide fused to β-galactosidase showed successful delivery to almost every tissue, including the brain (Schwarze et al., 1999). To this end, the TAT peptide has become one of the most widely used CPPs for both *in vitro* and *in vivo* studies.

*Mechanism of action*

Therapeutic protein and peptide delivery across the BBB by traditional cargo transport methods has been difficult due to the multiple limitations offered by the complicated physiological surroundings. Cell penetrating peptides can differ in basic peptide properties, and their mechanism of entry into cells is generally related to these properties. The TAT peptide belongs to the hydrophilic group of CPPs, which are able to deliver a variety of cargoes including peptides, proteins, oligonucleotides and plasmid DNA into mammalian cells (Dietz and Bahr, 2004). Furthermore, TAT has been reported to enter cells through a direct penetration energy independent pathway.
Such modes of entry mechanisms include inverted micelle formations, pore formation and membrane thinning models (Madani et al., 2011). However, the vast numbers of mechanistic studies have shown the nature of the cell line and the presence and type of the cargo may affect the specific mechanism used by the CPP.

Applications in neurological and neurodegenerative diseases

The ability of CPP-fused proteins to transduce the BBB presents an attractive therapy for many acute and degenerative neurological diseases. For example, the delivery of TAT-Bcl-xL in mouse models of stroke resulted in decreased cell death and neurological deficits (Cao et al., 2002; Kilic et al., 2002). Cell-penetrating peptides have also been used with positive results in pre-clinical models of Parkinson’s disease (Jeong et al., 2012) and Alzheimer’s disease (Lou et al., 2012). In addition, it has recently been demonstrated that the TAT peptide itself is neuroprotective (Meloni et al., 2013). The innate neuroprotective property of TAT, although limited in efficacy, supports its use as a delivery system for therapeutic proteins/compounds to treat neurological disorders such as SMA.

Secretion of CPP-fused recombinant proteins as treatments for SMA

Protein secretion from mammalian cells has been well characterised (Kelly, 1985) and represents a potential route for the engineering of therapeutic recombinant proteins. Indeed, several studies targeting neurological disorders have successfully incorporated a secretory signal peptide in expression vectors to facilitate the secretion of recombinant proteins (Haberman et al., 2003; McCown, 2006). Haberman et al showed that over-expression of a fibronectin (FIB) secretory signal fused to a green fluorescent protein
(GFP) resulted in transduced cells secreting GFP protein (Haberman et al., 2003). Further, an adeno-associated virus (AAV) expressing a FIB-galanin fusion peptide successfully attenuated seizures and neuronal death in an epilepsy animal model (Haberman et al., 2003; McCown, 2006). Galanin is a known neuroprotective peptide, and given its protective effects in the epilepsy studies described above, the FIB secretory system can produce functionally active peptides.

Engineering a cell to secrete TAT-fused proteins represents an attractive therapeutic option for many neurological and neurodegenerative disorders. This methodology was demonstrated by Flinterman et al, whereby transduced cells were engineered to secrete TAT-GFP (Flinterman et al., 2009). The TAT-GFP was then successfully used to transduce cells in culture, confirming the transduction domain and GFP remained intact. An important benefit is that secreted TAT-fused proteins are of mammalian cell origin and hence, are likely to possess the correct post-translational modifications necessary for optimal functionality.

Based on the above described studies, it appears feasible that a TAT-fused SMN protein could be used to deliver functional full-length SMN protein into cells. Furthermore, the ability of TAT-fused proteins to cross the BBB is an important additional feature for SMN delivery into motor neurons in vivo, thus making this approach potentially possible for the treatment of SMA.
Statement of Aims

The aim of my research project was to investigate the role of SMN in protecting cells from apoptotic cell death. The anti-apoptotic effects of SMN are not fully understood and elucidating the mechanisms responsible for promoting cell survival will greatly assist in understanding SMA pathogenesis. The project also aimed to develop a novel recombinant adenoviral vector capable of inducing host cells to express and secrete a TAT-SMN protein. Therefore, the data generated from this project has provided new insight(s) into the function of the SMN protein, and evaluated a potentially novel treatment approach for SMA.

Aim 1: Develop an in vitro apoptotic cell death model using differentiated human SH-SY5Y neuroblastoma cells.

Aim 2: Assess the pro-survival activity of SMN protein using the in vitro model (from Aim 1) and investigate the mechanisms used by the SMN protein to promote cell survival.

Aim 3: Generate a novel recombinant TAT-SMN protein and assess its functionality in the in vitro model (from Aim 1).

Aim 4: Develop a novel TAT-SMN secretory construct and assess the secretion and functionality of the resulting protein.
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Chapter 2

Spinal Muscular Atrophy and the Anti-apoptotic Role of Survival of Motor Neuron (SMN) Protein

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Spinal Muscular Atrophy and the Anti-apoptotic Role of Survival of Motor Neuron (SMN) Protein

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Signed...................................................... Signed......................................................
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Spinal Muscular Atrophy and the Antiapoptotic Role of Survival of Motor Neuron (SMN) Protein

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Abstract Spinal muscular atrophy (SMA) is a devastating and often fatal neurodegenerative disease that affects spinal motor neurons and leads to progressive muscle wasting and paralysis. The survival of motor neuron (SMN) gene is mutated or deleted in most forms of SMA, which results in a critical reduction in SMN protein. Motor neurons appear particularly vulnerable to reduced SMN protein levels. Therefore, understanding the functional role of SMN in protecting motor neurons from degeneration is an essential prerequisite for the design of effective therapies for SMA. To this end, there is increasing evidence indicating a key regulatory antiapoptotic role for the SMN protein that is important in motor neuron survival. The aim of this review is to highlight key findings that support an antiapoptotic role for SMN in modulating cell survival and raise possibilities for new therapeutic approaches.

Keywords Survival of motor neuron (SMN) protein · Spinal muscular atrophy (SMA) · Antiapoptotic · Apoptosis · Prosurvival

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive infantile neurodegenerative disease that has an incidence of one in 6,000 live births. SMA is characterized by the progressive degeneration of motor neurons of the anterior horn of the spinal cord. Clinically typified by profound muscle weakness, hypotonia, and trunk paralysis, SMA is classified into three main subtypes (I–III) based on age of onset and disease severity [1]. Type I SMA accounts for ≈50 % of SMA cases, manifests within 6 months of birth, and is usually fatal before the age of four. Patients with type II SMA develop muscle weakness before 18 months of age, often develop severe orthopedic and pulmonary complications [2], and may survive into adolescence [3]. Patients with type III SMA, typically display a later onset and suffer from a milder but still debilitating phenotype.

Approximately 95 % of SMA patients have a deletion or mutation in the survival of motor neuron 1 (SMN1) gene [4] that encodes the survival of motor neuron (SMN) protein. A second highly homologous copy of this gene, SMN2, differs by only a single translationally silent base change within exon 7. However, this base change causes aberrant splicing (exon 7 exclusion) in ≈90 % of SMN2 transcripts and when translated, results in truncation, instability, and reduced activity of the SMN protein (Fig. 1). Full-length SMN protein is ubiquitously expressed and occurs in the cytoplasm and nucleus of most cells. Among other functions, SMN plays an important role in the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) and pre-mRNA splicing. SMN also modulates apoptosis by directly blocking caspase activation and by affecting other key regulators of cell survival such as Bcl-2, p53, ZPR1, and Bcl-xL [5–9]. However, a clear understanding of SMA pathogenesis and the full functions of the SMN protein is still to be determined. In this review, we summarize the evidence that antiapoptotic and prosurvival pathways are affected by reduced SMN protein levels and explain the importance of targeting apoptosis as a potential therapeutic approach for SMA.

Genetics of Spinal Muscular Atrophy

In 1995, Lefebvre et al. [10] discovered that mutations in the SMN1 gene caused SMA. The functional SMN1 gene

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encodes the SMN protein that is necessary for motor neuron development and survival. During human evolution, a second, almost identical, copy of the SMN gene, termed SMN2, arose following a 500-kb inverted duplication of the region containing SMN1 on chromosome 5 [11]. Both the SMN1 and SMN2 genes contain identical promoter sequences and display enhanced neuronal expression [12]. Although the two genes differ by only five nucleotides, a critical translationally silent single C → T nucleotide base change in exon 7 of the SMN2 gene causes exclusion of this exon in 90 % of SMN2 RNA transcripts [13] (Fig. 1). Exclusion of exon 7 occurs due to disruption of a splice modulator site/sequence described as acting as an exon splicing enhancer (ESE) or an exon splicing silencer (ESS) [14, 15]. The C terminus of the SMN protein, which contains the region encoded by exon 7, is essential for survival, and when deleted, the mutant non-functional SMN protein (SMNΔ7) fails to reduce cell death following injury [16]. The importance of exon 7 is further exemplified in conditional exon 7 knockout mice that demonstrate similar phenotypes to different SMA mouse models [17].

The Survival of Motor Neuron Protein and Spinal Muscular Atrophy Pathogenesis

The Survival of Motor Neuron Protein

The SMN1 gene encodes a 38-kDa full-length and functional protein. In contrast, while encoding the same protein, alternative splicing of the SMN2 transcript results in the expression of relatively small amounts of full-length SMN protein (10–20 %; Fig. 1). The vast majority of protein encoded by the SMN2 gene is missing the 16 carboxyl end amino acids encoded by exon 7, causing the protein to be less active and unstable [18, 19]. Ubiquitously expressed and developmentally regulated [20], full-length SMN protein is found in both the cytoplasm and nucleus. In the nucleus, SMN protein localizes to structures called Gemini of coiled bodies (gems) that coincide with Cajal bodies in most cell lines but are distinct from Cajal bodies in fetal tissues [21–23]. Cajal bodies and gems colocalize to some degree, and this relationship may be partly mediated by the interaction of SMN with the Cajal body protein coilin [24, 25]. The SMN protein is also present in axons and dendrites [26, 27] and independently localizes to the neuromuscular junction (NMJ) [28].

Spinal Muscular Atrophy Pathogenesis

While not all cell types are equally affected by reduced SMN levels, SMA is characterized by the degeneration of alpha motor neurons of the anterior horn of the spinal cord [29]. The increased vulnerability displayed by motor neurons may be due to a motor neuron-specific SMN2 splicing inefficiency, whereby reduced SMN levels further exacerbates SMN exon 7 exclusion in motor neurons [30]. It is clear that reduced levels of oligomerization-competent intracellular SMN initiate the pathogenic cascade, but the sequence of events in SMA pathogenesis is debatable [31]. The most-studied function of the SMN protein is in forming a complex containing Gemin proteins (Gemin 2–8) and
small nuclear ribonucleoproteins (snRNP) assembly [21, 32–38]. The SMN complex specifically binds to and assembles Sm proteins (ribonucleoproteins) onto small nuclear RNAs (snRNAs), generating an active snRNP complex [25, 39–41]. As a component of the spliceosome [42], snRNPs play a role in pre-mRNA splicing that is essential for the expression of mature mRNA [43]. An important question is to what extent is SMN's role in RNA processing fundamental to SMA disease? Theoretically, the SMN protein impacts many mRNAs processed, suggesting defective splicing is playing an important role in the pathophysiology of SMA. Burghes and Beattie [44] suggest that disruption of snRNPs alters the splicing of genes critical to motor neuron function. Recently, Lotti et al. [45] identified an SMN-regulated U12 intron-containing gene, named Stasimon, responsible for normal synaptic transmission and motor neuron function. Restoration of Stasimon in a zebrafish SMA-like model rescued SMN-dependent motor neuron deficits [45], suggesting dysregulation of Stasimon plays a key role in the motor neuron-specific pathology of SMA.

Another suggestion is that NMJ formation and axonal function is affected in SMA, leading to the degeneration and eventual cell death of the motor neurons [31]. This is supported by the axonal location and axonal transportation of the SMN protein [27, 46]. To this end, knockdown of SMN expression causes defects in motor neuron axonogenesis, branching, and NMJ formation independent of snRNP biogenesis [47–49]. In addition, anterograde transport of the truncated SMNΔ7 protein does not occur and overexpression of SMNΔ7 results in axonal defects [46].

While SMN appears important to motor neuron function, pathogenic models of SMA suggest a general requirement for the SMN protein in many other tissues. This review will delineate the antiapoptotic functions of the SMN protein and the contribution made by apoptosis to the pathogenesis of SMA. In addition, we will discuss the current and future therapeutic approaches aimed at achieving neuroprotection in SMA.

Apoptosis in Spinal Muscular Atrophy

Apoptosis, or programmed cell death, is a specific and deliberate cellular mechanism that results in nuclear fragmentation, chromatin condensation, and the formation of apoptotic bodies [50, 51]. This phenomenon is required during embryonic development and for the homeostatic maintenance of proliferative tissues [52, 53]. Apoptosis is essential for normal development in the Central Nervous System (CNS) and functions to rapidly remove redundant neurons and nerve cells that fail to establish the appropriate synaptic connections [54]. Apoptosis during development and later in adult life is strictly regulated by a balance of antiapoptotic and proapoptotic signals. Dysregulation of apoptosis due to a disturbance of this balance can be associated with the development of cancer, autoimmune diseases, ischemia-related injuries, and neurodegenerative disorders [51].

There is increasing evidence that neurodegeneration in motor neuron disorders such as SMA and amyotrophic lateral sclerosis (ALS) may also involve apoptosis [55–57]. In genetically confirmed cases of SMA, examination of fetal tissue has revealed that during embryonic development, immature motor neurons undergo a more prolonged period of apoptotic programmed cell death, compared with motor neurons from control human fetal tissue [58, 59]. Furthermore, it is considered that the apoptotic loss of motor neurons in SMA patients after birth has been significantly underestimated due to the rapid elimination of these cells from spinal cord tissue [31]. Elevated levels of proapoptotic genes and apoptosis have also been observed in the CNS of SMA mouse models [60, 61]. Taken together, these human and animal studies provide strong evidence that the excessive loss of motor neurons by apoptosis plays an important role in the early stages of SMA.

The important function of SMN in regulating cell survival and apoptosis is supported in experimental studies showing that SMN levels are proportional to cell death rates following apoptotic stimuli [62]. For example, the SMN protein can protect various cell types against a range of apoptotic stimuli, including growth factor deprivation, PI 3-kinase inhibition, and camptothecin- and staurosporine-induced apoptosis [62–65]. In addition, SMN knockdown in neuronal-like NSC-34 cells results in reduced cell survival and elevated levels of apoptosis [62]. Therefore, irrespective of its other functions, it is clear that SMN can reduce apoptosis in normal cells and in cells induced to undergo apoptosis.

Proposed Antiapoptotic Mechanisms Used by Survival of Motor Neuron

Studies aimed at identifying SMN's specific antiapoptotic mechanisms are limited. Kerr et al. [66] first postulated that SMN may play a crucial role in modulating neuronal-specific apoptotic mechanisms. To date, the most well-defined antiapoptotic mechanism of action of SMN is through inhibition of active caspase-3 subunit formation. Caspase-3 is a key mediator of apoptotic cell death and is usually activated by the apoptosome complex consisting of cytochrome c, caspase-9, and Apaf-1. The activation of procaspase-3 (32 kDa) involves a two-stage process: calpain-mediated removal of the 3-kDa prodomain resulting in a 29-kDa subunit (p29), followed by spontaneous proteolytic cleavage of p29 into large and small subunits (17/12 kDa) by caspase-9 [67–69]. Caspase-3 is also activated by caspase-8 through the extrinsic pathway. This
pathway involves the activation of death receptors such as Fas and tumor necrosis factor (TNF), the recruitment of death receptor-associated molecules such as Fas-associated death domain containing protein (FADD), and the subsequent activation of the caspase-8 (reviewed in [51]). The ability of SMN to block calpain-mediated activation of procaspase-3 [65] and thereby reduce caspase-3 activation [62–65, 70] provides strong support for the view that SMN functions in an antiapoptotic caspase-3-dependent manner (Fig. 2). In support of this, human SMA motor neurons derived from induced pluripotent stem cells (iPSCs) exhibit increased levels of caspase subunits and caspase-3 cleavage [71].

What makes the involvement of caspase-3 and calpain potentially interesting is their ability to directly cleave full-length SMN protein following injury [66, 72, 73]. Caspases are predicted to cleave SMN at amino acid Asp-252, generating a ≈29-kDa truncated protein that is present in cells undergoing apoptosis in brain tissue [66]. Not surprisingly, mutating the SMN caspase cleavage site results in an SMN protein with enhanced antiapoptotic properties [66]. However, it is unclear whether caspase cleavage of SMN abolishes its intrinsic antiapoptotic properties or if the resulting SMN cleavage products have their own unique proapoptotic functions.

Cell Death and Prosurvival Proteins Affected in Spinal Muscular Atrophy

The Role of the Bcl-2 Protein

As a consequence of its multifunctional roles, the SMN protein impacts a variety of pathways and genes implicated in SMA pathogenesis. Of particular interest are the relationships and interactions between SMN and key proteins that are

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**Fig. 2** Proposed cell signaling pathways linking SMN expression to the regulation of cell survival. Neurotrophic factors commonly signal through the PI 3-kinase pathway, phosphorylating Akt and preventing BAD sequestration of Bcl-2 and Bcl-xL. SMN interacts with Bcl-2 and regulates the expression of Bcl-xL, both of which prevent cytochrome c release from the mitochondria. The SMN protein also interacts with the tumor suppressor protein, p53, and when bound, colocalizes across the nuclear membrane. Under normal conditions, the p53 protein tightly regulates Bax activation, and the interaction between Bcl-2 and SMN synergistically prevents Bax-mediated apoptosis. In SMA patient cells, levels of the p53 modulator, MDM2, are reduced and unable to prevent p53 activation of Bax. Prolactin and erythropoietin signal via the Jak/Stat pathway. Prolactin increases SMN levels by stimulating Stat5, a transcription factor known to increase Bcl-xL levels. Activation of this pathway by prolactin is also known to activate Fyn, a tyrosine kinase involved in the activation of the prosurvival PI 3-kinase pathway and the phosphorylation of the RNA binding protein, Sam68. Phosphorylated Sam68 splices Bcl-x and SMN transcripts, promoting the long Bcl-xL isoform and inclusion of exon 7 in SMN transcripts. End-stage apoptosis begins with the activation of caspase-9, via cytochrome c-mediated activation of Apaf-1 or by activation of cell surface death receptors. Activation of caspase-3 requires cleavage of the 3-kDa procaspase-3 prodomain, a step blocked by SMN. Expression of ZPR1 and NAIP are correlated with SMA severity and SMN protein levels, and both proteins can directly regulate caspase-3 activation. The overall inhibition of caspase-3, directly or indirectly by SMN, prevents cleavage of death substrates and apoptosis. [5, 6, 9, 65, 81, 92, 93, 101, 131, 159]
directly or indirectly involved in regulating cell survival (Table 1). For example, SMN is known to interact with Bcl-2, a well-characterized member of the Bcl-2 family that negatively modulates apoptosis by several mechanisms [74, 75]. The interaction of SMN with Bcl-2 provides a synergistic antiapoptotic action against Bax- and Fas-mediated apoptosis [5, 76]. Moreover, the truncated SMNΔ7 protein is unable to produce a similar antiapoptotic effect, further supporting the importance of exon 7 to SMN protein functionality [5]. Finally, in SMA model mice, the transcription factor WT-1, a key regulator of Bcl-2 expression and apoptosis, is down-regulated [77]. The WT-1 protein modulates apoptosis by upregulating Bcl-2 expression and by directly interacting with p53 to inhibit apoptosis [78, 79].

The altered expression of Bcl-2 in SMA is likely to have a significant impact on neuronal development. During murine embryogenesis, Bcl-2 expression peaks at embryonic day 11 and plays an essential role in regulating developmental apoptosis [53]. Interestingly, at 15 weeks of gestation, in the spinal cord tissue of SMA patients, expression of Bcl-2 is downregulated, a factor which is likely to lead to increased total motor neuron cell death [8, 58].

An Interaction with ZPR1

Another protein involved in negatively regulating caspase activation and known to interact and colocalize with the SMN protein is ZPR1 [7]. Like Bcl-2, expression of ZPR1 is reduced in SMA patients [80] and is also thought to play a role in SMA pathogenesis [81]. Similar to SMN, complete knockout of ZPR1 is embryonically lethal, supporting an essential role for ZPR1 in cell survival [81]. Indeed, ZPR1 deficiency causes Cajal body defects, axonal abnormalities, defective embryonic growth, mislocalization of SMN, and increased apoptosis [7, 81, 82]. Interestingly, ZPR1 knockdown can induce caspase-3 activation and play a significant role in motor neuron degeneration in mice [82]. Taken together, these findings suggest that ZPR1 may play an important role in determining the extent of neuronal apoptosis in SMA and thereby contribute to disease pathogenesis.

Table 1 List of proteins affected in SMA that play a role in regulating cell survival

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Relationship</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Anti-apoptosis. Prevents Bax-mediated apoptosis, cytochrome c release and sequesters Apaf-1</td>
<td>Interacts, downregulated</td>
<td>[70, 71, 75]</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Anti-apoptosis. Prevents cytochrome c release and can modulate SMN levels</td>
<td>Down/coregulated</td>
<td>[75–77]</td>
</tr>
<tr>
<td>Beta-synuclein</td>
<td>Protects against caspase activation in a TP53/p53-dependant manner [78]</td>
<td>Downregulated</td>
<td>[79]</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Mediates many processes such as apoptosis</td>
<td>Downregulated</td>
<td>[79]</td>
</tr>
<tr>
<td>DP-1</td>
<td>Forms a E2F-1/DP-1 complex that can mediate apoptosis</td>
<td>Downregulated</td>
<td>[72]</td>
</tr>
<tr>
<td>Hsp60</td>
<td>Necessary for cell survival following toxicity and stress</td>
<td>Downregulated</td>
<td>[79]</td>
</tr>
<tr>
<td>Htra2-beta</td>
<td>Binds to and blocks the action of inhibitor of apoptosis proteins (IAPs) [80]</td>
<td>Downregulated</td>
<td>[81]</td>
</tr>
<tr>
<td>Ku70</td>
<td>Suppresses mitochondrial translocation of Bax, inhibiting apoptosis [82]</td>
<td>Downregulated</td>
<td>[72]</td>
</tr>
<tr>
<td>MDM2</td>
<td>Inhibits TP53/p53-mediated apoptosis [83]</td>
<td>Downregulated</td>
<td>[72]</td>
</tr>
<tr>
<td>NAIP</td>
<td>Blocks apoptosis and the activation of caspase-3</td>
<td>Modifying gene</td>
<td>[84, 85]</td>
</tr>
<tr>
<td>P21</td>
<td>Cyclin-dependent kinase inhibitor. Involved in the cellular stress response</td>
<td>Upregulated</td>
<td>[86]</td>
</tr>
<tr>
<td>p53</td>
<td>Apoptosis. Induces Bax-mediated apoptosis</td>
<td>Interacts with</td>
<td>[72, 87]</td>
</tr>
<tr>
<td>p57/KIP2</td>
<td>Apoptosis. Inhibits cyclin-dependent kinases</td>
<td>Downregulated</td>
<td>[72]</td>
</tr>
<tr>
<td>p-JNK</td>
<td>Responsive to stress stimuli and play a role in the apoptosis pathway</td>
<td>Upregulated</td>
<td>[86]</td>
</tr>
<tr>
<td>WT-1</td>
<td>Transcription factor. Can modulate apoptosis by upregulating Bcl-2 [74]</td>
<td>Downregulated</td>
<td>[72]</td>
</tr>
<tr>
<td>ZPR1</td>
<td>Antiapoptosis. Prevents caspase-3 activation</td>
<td>Interacts, downregulated</td>
<td>[81, 88]</td>
</tr>
</tbody>
</table>

The Role of p53

The SMN protein also interacts with the proapoptotic p53 protein [6]. The p53 protein is a sequence-specific transcription factor that targets both mitochondrial and death receptor-induced apoptotic pathways, such as Bax, NOXA, and PUMA, resulting in cytochrome c release and Apaf-1/caspase-9 activation [83]. Although p53 is not subject to SMN-dependent regulation [80], it is thought that high levels of SMN are responsible for p53 localization to nuclear bodies [6]. Young et al. [6] determined that the SMN/p53 interaction prevented p53-mediated apoptosis. Not surprisingly, the truncated SMNΔ7 protein was unable to interact with p53 and prevent activation of p53 apoptotic pathways. The primary regulator of p53 is the MDM2 protein, which inhibits p53 transcriptional activity and targets p53 for proteasome degradation. MDM2 is a transcriptional target of p53 and, via an autoregulatory feedback loop, its expression is increased as p53 activity increases [84]. Interestingly, both MDM2 and its regulator, PSME3, are downregulated in SMA model mice [77], possibly secondary to reduced
SMN and p53 levels. The importance of MDM2 as a regulator of p53 has been demonstrated in MDM2 knockout mice whereby p53-driven apoptosis causes embryonic lethality [85].

What Role Does the Bcl-xL Protein Play?

The Bcl-x gene encodes several alternatively spliced mRNA’s, of which Bcl-xL is the predominant transcript [86, 87]. Unsequenced Bcl-xL targets the mitochondria, inhibiting cytochrome c release and Apaf-1-dependent caspase-9 activation, subsequently blocking apoptosis [88, 89]. Bcl-xL expression is high during embryogenesis [53] and is thought to play an essential role in CNS development, as Bcl-xL-deficient mice exhibit extensive apoptotic cortical and spinal neuronal loss [90]. In SMA, downregulation and irregular expression of Bcl-xL have been documented in human SMA fetal tissue [8] and SMA model mice [91]. However, since the identification of Bcl-2 as an interacting partner of SMN [5], Bcl-xL has been largely overlooked as playing a role in SMA pathogenesis. We recently reported that expression of Bcl-xL and SMN is coregulated, suggesting a common regulatory mechanism [9]. In addition, we reported that a regulator of both Bcl-x and SMN mRNA alternative splicing, the RNA-binding protein Sam-68, is reduced in SH-SY5Y cells when Bcl-xL or SMN is overexpressed [9]. Reduced levels of Sam-68 cause accumulation of both antiapoptotic Bcl-xL and full-length SMN2 transcripts [92, 93]. In SMA, Sam 68 regulation may be altered, resulting in abnormally high protein levels and potentially leading to the downregulation of Bcl-xL and increased exon 7 skipping in SMN2 transcripts.

The Role of the Neuronal Apoptosis Inhibitory Protein Gene

Lying immediately adjacent to the SMN gene, the neuronal apoptosis inhibitory protein (NAIP) gene is located within the 500-kb inverted duplication on chromosome 5. NAIP is the founding member of the human IAP protein family [94] that together inhibit many key caspases and procaspases. Interestingly, numerous studies have correlated NAIP deletions with SMA disease severity [95–100]. These studies show that up to 90 % of type I SMA patients have deletions in the NAIP gene. In contrast, deletions in NAIP are less frequent in types II and III SMA. Therefore, it is likely that NAIP gene expression is directly implicated as a modulator in SMA and absence of this gene exacerbates the SMA phenotype.

The NAIP protein directly inhibits caspase-3 and caspase-7 through the action of its baculovirus inhibitor of apoptosis protein repeat (BIR) [101]. Furthermore, NAIP suppresses apoptosis in neural tissues [102–104] and the loss of endogenous NAIP results in increased neuronal vulnerability [105]. In vivo, hippocampal pyramidal neurons from transgenic mice lacking the NAIP1 gene display an increased sensitivity to kainic acid-induced apoptosis [105]. However, these mice are morphologically normal and do not show any features or characteristics of an SMA phenotype. Attempts to determine the exact effects of NAIP deletions on SMA phenotype have, so far, not been successful, mainly due to the presence of six different NAIP genes [106], making in vivo modeling difficult.

Current Therapeutic Strategies Toward Treating Spinal Muscular Atrophy

Increasing Survival of Motor Neuron Protein Levels

Viral Delivery of Survival of Motor Neuron

The primary goal of current SMA therapeutics is threefold: firstly, to increase SMN protein levels; secondly, to promote the inclusion of exon 7 in SMN2 transcripts; and thirdly, to promote motor neuron survival. Considered to be the most promising of the current SMA therapeutic approaches, viral delivery of SMN presents an efficient method for systemic delivery of SMN protein. Demonstrating the feasibility of this approach for the first time, Azzouz et al. used a lentiviral vector to retrogradely increase SMN expression in motor neurons in an SMA mouse model [107]. Following this initial study, improvements in gene delivery efficiency have led to the rescue of a severe mouse model of SMA using an adeno-associated viral vector (AAV9), which can infect peripheral tissue as well as cross the blood–brain barrier [108]. However, it is still not clear what effects viral delivery will have on already diseased human motor neurons and if any immunological responses will occur.

Exon 7 Inclusion of Survival of Motor Neuron

An alternative approach to SMN1 gene replacement is the use of antisense oligonucleotides to alter SMN2 splicing. The antisense oligonucleotide approach targets pre-mRNA processing, reducing exon 7 exclusion in SMN2 mRNA and increasing full-length SMN transcripts and protein levels. Antisense oligonucleotide technology has previously been used to alter gene expression in different forms of cancer (reviewed in [109]) and has been used successfully to restore dystrophin expression in Duchenne muscular dystrophy patients [110, 111]. Applying this methodology to treating SMA has proven successful both in vitro [112] and in vivo using SMA mouse models [113]. However, oligonucleotide delivery requires a direct route of administration to the CNS, such as intrathecal or intracerebroventricular injections, and will require additional optimization for an efficient delivery to peripheral organs.
Neuroprotection and Antiapoptotic Approaches

**Survival of Motor Neuron-independent Therapies**

While current strategies have focused solely on the importance of increasing SMN levels, the use of neuroprotective therapeutics as either a separate or complementary treatment for SMA also needs to be considered. For example, neurotrophic factors have demonstrated therapeutic benefits clinically and in animal models of neurodegenerative diseases, such as ALS, Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease (reviewed in [114]).

**Cardiotrophin**

Cardiotrophin-1 (CT-1) regulates apoptosis by blocking the proapoptotic functions of p53 and Bax, activating the Akt prosurvival signaling pathway and promoting Bcl-2 expression [115]. CT-1 delivery has proven to be neuroprotective in in vitro models of cerebral ischemia [116], while intramuscular delivery of an adeno-virus-expressing CT-1 is able to delay motor impairment and muscle atrophy in an ALS mouse model [117]. With respect to SMA, Lesbordes et al. were the first to demonstrate the effective use of CT-1 treatment in an SMA mouse model [118]. In this study, overexpression of CT-1 in SMA mice resulted in increased survival, reduced NMJ disorganization, and diminished axonal degeneration [118]. Importantly, CT-1 is capable of reducing neuronal apoptosis by blocking caspase-3 and caspase-8 activation [119]. These findings suggest that there is potential for CT-1 to be used to reduce motor neuron apoptosis and prolong survival in SMA patients.

**Insulin-like Growth Factor**

Similarly, insulin-like growth factor (IGF-1) activates several prosurvival signaling pathways including PI3-kinase/Akt and Erk1/2. In addition, IGF-1 enhances motor neuron axonal growth [120] and is thought to act in a retrograde manner to exert a neuroprotective effect on motor neurons. Expression of IGF-1 reduces muscle wasting in a mouse model of Duchenne muscular dystrophy (mdx) and causes significant improvements in life span and motor function in animals models of spinal bulbar muscular atrophy [121] and ALS [122].

In a severe mouse model of SMA, transgenic expression of IGF-1 in skeletal muscle tissue improved median survival and also resulted in improved overall motor function [123]. While significant improvements were observed in this study, they remain modest in comparison to recent studies using SMN gene replacement or correction of SMN2 splicing using antisense oligonucleotide delivery [124–126]. In contrast, in a SMA type III mouse model, CNS-mediated delivery of IGF-1 did not improve overall survival or motor function but did reduce motor neuron loss [127]. These results suggest the neurotrophic and antiapoptotic effects of IGF-1 therapy may be more beneficial in severe forms of the disease, where motor neuron loss is greater. Current therapeutic strategies are also using IGF-1 in combination with SMN-dependent approaches [128], demonstrating the importance of achieving neuroprotection and gene replacement for maximal therapeutic benefit in SMA.

**Bcl-xL and Bcl-2**

Both Bcl-2 and Bcl-xL expressions are reduced in SMA fetus spinal cords [8], potentially contributing to the irregular neuronal morphology and increased levels of apoptosis observed during SMA development [59, 129]. With this in mind, Tsai et al. [91] first postulated that Bcl-xL may compensate for a deficiency in SMN levels, showing that in transgenic SMA type III mice, increased Bcl-xL expression resulted in improved viability and reduced muscle atrophy. Recently, Bcl-xL has also been shown to phenotypically rescue mouse motor neurons exhibiting all the effects of reduced SMN levels [130]. Extending on these studies, we have demonstrated a relationship between Bcl-xL and SMN expressions, whereby coexpression of both proteins provided additive protection from apoptosis [9]. From these studies, we postulate that Bcl-xL is an important regulator of SMN expression and may be involved in mediating the antiapoptotic effects of SMN.

In addition to Bcl-xL, there also exists a relationship between the Bcl-2 and SMN proteins [5, 76]. Coexpression of Bcl-2 and SMN causes a synergistic reduction in Bax-mediated apoptosis [5]. Hence, it is not surprising that levels of proapoptotic Bax protein are elevated in the spinal cords of SMA mice and that inhibiting Bax in SMA mice results in improved motor neuron numbers and median survival [60]. Therefore, targeting apoptosis in SMA appears to be a viable treatment approach and the use of Bcl-xL and/or Bcl-2 in future SMA therapies should be considered.

**Platelet-derived Growth Factor Prolactin and Erythropoietin**

Recently, both platelet-derived growth factor (PDGF) and prolactin have been shown to elevate SMN protein levels through different pathways [131, 132]. PDGF was found to inhibit GSK-3, an important regulator of p53 activity, via activation of the Akt signaling pathway. In comparison, prolactin receptor activation stimulates the Janus kinase 2 pathway (JAK2), resulting in STAT5-mediated transcriptional activation of SMN and other antiapoptotic proteins [131, 133]. In this study, prolactin treatment of a severe mouse model of SMA resulted in elevated full-length SMN protein levels in the CNS, improved motor function,
and enhanced survival [131]. Signaling via the Jak2/Stat5 pathway, erythropoietin (EPO) is another growth factor that could potentially show therapeutic potential in SMA. EPO is able to cross the blood–brain barrier [134] and several studies have demonstrated its effectiveness to ameliorate or reduce neuronal injury in cell culture and/or animal models of stroke, epilepsy, spinal cord injury, and ALS [135–138]. Interestingly, both PDGF- and prolactin-stimulated pathways result in SMN upregulation and the regulation of key survival proteins. This suggests that molecular regulation of SMN can be tightly controlled, independent of a splicing function, via different cell signaling pathways.

Stem Cells to Treat and Model Spinal Muscular Atrophy

Embryonic Stem Cells

Human embryonic stem cells (hESCs) were first isolated in 1998 and have generated much interest in their use to develop in vitro disease models and for stem cell transplantation [139]. Pluripotent hESCs are derived from the “inner cell mass” of blastocyst stage embryos. While embryonic stem cells are relatively easy to obtain and differentiate, ethical challenges relating to their source need to be overcome if they are to be considered as a mainstream therapy. Recently, embryonic stem cell-derived neural stem cells were successfully transplanted into SMA model mice [140]. In this study, intrathecal transplantation of ESC-derived neural stem cells demonstrated appropriate migration patterns, differentiated into motor neurons, and increased overall survival [140]. In SMA, stem cell transplantation of donor-derived hESCs may replenish motor neuron numbers but will not target reduced SMN protein levels in peripheral tissues. Therefore, for this approach to be feasible, it will need to be used in conjunction with other SMA therapies.

Induced Pluripotent Stem Cells

Reprogramming of human fibroblasts presents another potential source of pluripotent stem cells and bypasses the ethical issues surrounding obtaining hESC’s. While at present this method does not offer a therapeutic avenue, reprogramming of SMA fibroblasts into human SMA motor neurons has potentially opened the door to targeted drug screening and improved SMA therapeutics [141]. SMA-derived motor neurons show axonal deficits and increased levels of apoptosis but importantly respond to SMN-dependent therapies. In addition, SMA-derived motor neurons display increased caspase-3 activation and selectively blocking caspase-3 rescues these neurons from an apoptotic death [71]. Utilization of iPSCs to model SMA can potentially provide a reliable and accurate platform to identify and assess therapies aimed at increasing SMN levels and targeting apoptosis in SMA.

Clinical Trials

Completed and Potential Clinical Trials in Spinal Muscular Atrophy

Currently, there are no approved treatments to prolong the survival of SMA patients. Clinically, numerous compounds have been trialed to increase SMN transcript and protein levels, but to date, no therapeutic candidate has been adopted as a treatment for SMA. The first candidates identified for treating SMA were the histone deacetylase (HDAC) inhibitors, which were found to increase SMN2 expression [142]. Preclinical studies using HDAC inhibitors result in increased full-length SMN2 transcripts in vitro [143–145] and increased median survival in vivo [146]. However, the increased survival observed in HDAC-treated SMA mice could be due to an increased expression of several antiapoptotic genes as a result of the treatment [147]. Clinical trials of two HDAC inhibitors, phenylbutyrate and valproic acid, failed to show any significant clinical benefits in SMA patients [148–150]. Similarly, hydroxyurea enhances SMN2 expression in SMA patient cells [151] but has little or no clinical effects on SMA patients [152]. Currently, direct replacement of SMN using a scAAV9-expressing SMN appears the most promising therapeutic approach. Preclinical studies demonstrate dramatic improvements in systemic SMN expression and median survival in SMA mouse models [124, 125]. Furthermore, this approach results in sustained and systemic expression in nonhuman primates [153]. As a result of these studies and recent approval from the NIH Recombinant DNA Advisory Committee, phase I clinical trials using AAV9 to treat SMA are anticipated to commence in 2013.

Neuroprotective and antiapoptotic therapeutics could potentially target and reduce the early motor neuron loss that occurs in SMA patients. To date, clinical trials in SMA patients using the neuroprotective gabapentin have demonstrated negative or minimal results [154]. Neurtrophic factors have also been used in preclinical studies in numerous neurodegenerative diseases with success. However, in clinical trials of ALS, IGF-1, BDNF, and CNTF have failed to show any clinical benefit [155–158]. From these results, it appears that solely targeting apoptosis in SMA is not the answer. However, to combat the motor neuron loss due to elevated apoptosis occurring early in the disease, the addition of neurotrophic and/or antiapoptotic factors alongside SMN replacement may provide additional clinical benefits.

Conclusions

SMN is a multifunctional protein that interacts with and mediates the expression of numerous other proteins
involved in DNA repair, calcium handling, cell cycle, and apoptosis and which major function is the maintenance of motor neuron survival. Importantly, in SMA, the expression of critical modulators of cell viability is significantly affected (Table 1) that is likely to affect the disease phenotype. Expression of these genes in SMA may be affected through direct or indirect regulatory roles of the SMN protein and/or dysfunction of the snRNP complex, thereby causing altered splicing of these important genes [44]. The diverse range of proteins affected in SMA strongly supports a multifunctional role for the SMN protein. Further elucidation of SMN functions and downstream effects of SMN deficiency is likely to lead to a better understanding of the disease mechanisms that underlie impaired motor neuron survival in SMA.

It is important that SMN-independent approaches to promote neuronal survival should not be ruled out as they may complement therapies aimed at increasing SMN protein levels. In particular, neuroprotective and antiapoptotic proteins have shown promising results in promoting neuronal survival, rescuing axonal defects and increasing endogenous SMN protein levels. As summarized in this review, a variety of proteins involved in survival/apoptosis are affected in SMA mouse models and patient tissues. A more complete understanding of the signaling pathways involving SMN is required to substantiate the key cellular mechanisms involved in SMA pathogenesis. However, the role of apoptosis in SMA pathogenesis is well-established and targeting the early-stage apoptosis that occurs in SMA may be paramount to attempting to completely alleviate the disease.

Acknowledgments These studies were supported by the Neuromuscular Foundation and Muscular Dystrophy Association of Western Australia.

Conflict of interest None.

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Chapter 3

General Methods and Materials
GENERAL METHODS

Methods not listed in this section are described in detail in the relevant chapters.

DNA and RNA Preparative Procedures

Plasmid extractions

Isolation of purified plasmid DNA from *E. coli* was performed using the Wizard® Plus SV Mini-preps DNA purification system kit (Promega). This procedure involved inoculating single colonies into 1.8 ml of 2 x Yeast extract and Tryptone (2 x YT) medium containing a suitable antibiotic. Cultures were grown in microfuge tubes fitted with PTFE membrane lids (Eppendorf LidBac), and incubated for 18 hours at 37°C with vigorous shaking (Eppendorf; 1400rpm). Cells were pelleted by centrifugation and plasmid DNA was prepared according to the manufacturer’s instructions. Plasmid DNA was eluted in 80 µl of sterile water for irrigation (WFI) and stored at -20°C.

Restriction fragment purification

DNA fragments generated from restriction enzyme digestions were separated by agarose gel electrophoresis and purified using the Wizard® SV Gel and PCR Clean-up System (Promega). The purified DNA generated was eluted from mini-columns in sterile WFI, and stored at -20°C for use in subsequent experiments.
RNA extraction

Total RNA was extracted from cells in culture or animal tissue samples using TRIZOL® Reagent, a mono-phasic solution of phenol and isothiocyanate, and used according to the manufacturer’s instructions. This method results in the isolation of RNA at a sufficient purity for use as a substrate for isolating full length gene products by PCR. Total RNA was resuspended in sterile WFI at 0.2-1.0 μg/ml and stored at -80°C.

First strand synthesis of complimentary DNA (cDNA)

In the annealing step, total RNA (typically 1.0 μg) was combined with the appropriate primer in WFI, incubated at 80°C for 5 minutes and cooled rapidly on ice for 5 minutes to prevent secondary structures reforming. Primers for isolating full length cDNA sequences consisted of either 100-500 ng of Oligo (dT)₁₅ (Promega) or 100 ng of gene specific primer. For analysing RNA transcript levels, 100 ng of random hexamers (Promega) were used.

For first strand synthesis, annealed primer-templates were gently mixed with 1 x M-MLV Reverse Transcriptase Reaction buffer (Promega), RNasin® Ribonuclease Inhibitor (1 unit; Promega), dNTP (0.5 mM), M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) 200 units. Reactions using; Oligo (dT)₁₅ were incubated at 40°C for 60 minutes, gene-specific primers were incubated at 40-55°C for 60 minutes and random hexamers were initially incubated at RT for 10 minutes, followed by 40-55°C for 50 minutes. Reactions were inactivated by heating to 70°C for 15 minutes. Complimentary
DNA was used as template for second strand synthesis and PCR amplification or otherwise stored at -20°C.

*Polymerase chain reaction (PCR)*

Polymerase chain reaction was used to generate fragments for vector construction, cloning and for assessing transgene transcript levels. Routinely, reaction components were combined with sterile WFI in a total volume of 50 µl, and comprised: 50-500 pg of template (genomic DNA, plasmid DNA or first strand cDNA), 1 x enzyme specific reaction buffer (as supplied by the enzyme manufacturer), 200 µM of dNTPs, 100 ng of each forward and reverse oligonucleotide primers and 1-2 units of the DNA synthesising enzyme. For reactions utilising Taq DNA polymerase, a Mg\(^{2+}\) concentration of 1.5 mM was used. Selection of a DNA polymerase was based on the final product and was chosen from either Taq DNA polymerase (Promega), high fidelity ThermalAce™ (Invitrogen), or high fidelity Pfu DNA polymerase (Promega). Cycling conditions for each reaction were dependent on the template source and GC ratio, primer to template annealing temperature, DNA polymerase and expected length and eventual use of the PCR product. Generally, after initial denaturation at 94-98°C of 2-3 minutes (1x), reactions were exposed to 20-35 cycles of; denaturation at 94-98°C for 30-45 seconds, primer template annealing at 50-60°C for 30-45 seconds, and extension at 68-74°C for a time calculation based on enzyme processivity and expected product length. A further 10 minute extension completed the cycling protocol.
PCR and restriction fragment purification

PCR products and DNA fragments generated by restriction enzyme digestion were purified from reactions or agarose gel slices using the Wizard® SV Gel and PCR Clean-Up System (Promega). Purified DNA was eluted from the mini-column in sterile WFI and was suitable for subsequent cloning.

DNA Modifying Procedures

Restriction enzyme digestion

Restriction enzyme reactions were performed on plasmid DNA for both preparative and analytical purposes. In general, restriction digests comprised DNA (0.2-3.0 µg) in sterile WFI and combined in a 20-80 µl reaction volume with; 1 x restriction enzyme buffer (supplied as a 10 x stock by the enzyme manufacturer), at least 4 units of enzyme per µg of DNA. Reactions were allowed to proceed for at least 2 hours at the temperature specified by the manufacturer. For double digests, reactions were either conducted separately followed by purification, or simultaneously if enzyme buffer conditions were compatible. Linearisation of plasmid vector DNA by restriction digestion was performed for 12-18 hours, using excess restriction enzyme. For directional cloning into expression vectors, whereby the use of two different restriction enzymes was necessary, digests were always performed sequentially.
**Ligations**

The ligation reaction components were combined in a total volume of 25 µl and comprised the following: plasmid vector DNA (25-50 ng); insert DNA (100-250 ng); 1 x T4 DNA ligase ligation buffer (as supplied as a 10 x stock by the enzyme manufacturer, Promega); T4 DNA ligase 4.5 units. Ligation mixtures were incubated at RT for 12-18 hours and stored at -20°C.

**Analytical Procedures**

*Gel electrophoresis*

Plasmid DNA, RNA, restriction enzyme fragments and PCR products were mixed with DNA loading dye (Promega) and electrophoresed in agarose gels (0.8-2.5% w/v) using a TAE buffer system. Gels were pre-stained with ethidium bromide (0.5 µg/ml) and bands were visualised by UV transillumination (Foto/UV21, Fotodyne transilluminator), sized against DNA molecular weight markers and imaged/digitised following capture with a Chemismart 3000 Gel documentation system (Vilber Lourmat).

*Quantification of DNA and RNA*

Where necessary, DNA and RNA samples were quantified spectrophotometrically using a Nano-drop (Thermo-Scientific). Briefly, absorbance measurements at two wavelengths, 260 nm and 280 nm, were used to calculate by the purity and concentration of starting
material. Pure preparations of RNA and DNA routinely gave $\text{OD}_{260}/\text{OD}_{280}$ ratios of approximately 1.8.

**DNA sequencing**

The ABI Prism Big Dye Terminator version 3.1 reaction-ready fluorescent labelling sequencing mix, employing Ampli Taq® DNA polymerase, was used to sequence double-stranded DNA. Seven microlitres of purified plasmid DNA, 2 µl of Big Dye Terminator version 3.1, and 1 µl of the appropriate primer were added together in a thin-walled microfuge tube. Cycling conditions were as follows; initial denaturation at 96°C for 1 minute, followed by 25 amplification cycles (30 seconds at 96°C, 30 seconds at 50°C and 4 minutes at 60°C) and a hold at 11°C. To precipitate the soluble DNA products, 20 µl WFI, 75 µl of 100% ethanol and 3 µl of sodium acetate were added to each of the samples. The samples were briefly vortexed, then incubated at room temperature for 20 minutes (protected from light), followed by centrifugation at 18,500 g for 30 minutes. The supernatant was carefully removed and the pellet was washed with 250 µl of 70% ethanol by inverting the tube several times. The samples were then centrifuged again at 18,500 g for 12 minutes, and the supernatant discarded. The pellet was dried for 2 minutes at 65°C in a PCR machine. Samples were processed by the Lottery West Biomedical facility: Genomics, Royal Perth Hospital.
SDS-PAGE electrophoresis

Proteins were routinely analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using pre-cast gels (Invitrogen system). Protein samples and Novex® pre-stained protein standards (Invitrogen) were combined with 2.4 µl of 10 x sample reducing agent (Invitrogen), 6 µl of 4 x LDS sample buffer (Invitrogen), the volume made to 25 µl with WFI and heated at 70°C for 10 minutes. A MOPS buffer system (Invitrogen) was used and NuPAGE antioxidant (Invitrogen) was included in the upper chamber, as directed by the manufacturer. Samples were loaded onto pre-cast NuPAGE® SDS gels (Invitrogen) and run at a constant 200 volts for 50 minutes.

Western blotting

For western blot analysis, proteins separated by SDS-PAGE were transferred to PVDF membranes (1 hour at 30 volts), using transfer buffer (Invitrogen). Membranes were first washed in PBS, blocked in PBS-T (0.1%) containing ovalbumin for 1 hour. Membranes were exposed to primary antibodies diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml) overnight at 4°C with gentle rocking (Table 3.3). Membranes were washed in PBS-T (0.1%) three times for five minutes, and then incubated with a secondary antibody coupled to horse radish peroxidase (HRP) for 1 hour at room temperature (Table 3.3). The membranes were washed three times for five minutes in PBS, and protein bands were visualized using the ECL detection reagent (Amersham).
Bacterial Procedures

Maintenance of bacterial cells

For long-term storage, both recombinant and non-recombinant bacterial cells were resuspended in 2 x YT medium containing 15% glycerol, aliquoted out into sterile 2 ml cryotubes (Nunc), snap frozen in liquid nitrogen and stored at -70°C. Cells were recovered from slightly thawed frozen aliquots using sterile loops or micropipette tips and used to inoculate agar or liquid culture media.

Preparation of chemically competent JM109 and KRX cells

A stock of frozen KRX competent cells (Promega) was thawed slowly on ice. Five microlitres was inoculated into 1 ml of 2 x YT media and incubated at 37°C for 1 hour with shaking (300 rpm). A 100 µl aliquot of the broth mixture was plated onto a pre-dried 2 x YT agar plate, and incubated at 37°C overnight. Two colonies were inoculated into two separate 2 ml tubes containing 2 x YT media; fitted with Eppendorf LidBac membrane filters and incubated overnight at 37°C with shaking (1400 rpm).

Three millilitres of overnight KRX broth was inoculated into 500 ml of fresh SOB medium, and incubated at 28°C with shaking at 225 rpm. Once the OD_{600} reached 0.4, the culture was transferred to an ice slurry for 10 minutes, and then centrifuged at 3,000 g in a pre-cooled bucket rotor for 10 minutes. The cell pellet was gently resuspended in 100 ml of ice cold TB, incubated on ice a further 10 minutes, and re-pelleted by centrifugation at 3,000 g
for 10 minutes. The cell pellet was gently resuspended in 18.6 ml of TB, mixed with 1.4 ml of DMSO, and incubated on ice a further 10 minutes. Aliquots of the cell mixture (0.6 ml) were transferred to 1.5 ml microfuge tubes, flash frozen in liquid nitrogen and stored at -80°C.

**Preparation of electro-competent BJ5183 E. coli cells carrying pAdeasy**

A 3 ml aliquot from a fresh overnight culture of *E. coli BJ5183* (pAdEasy) grown in LB broth media was inoculated into 500 ml of fresh LB broth media containing Ampicillin (50 μg/ml). Cells were grown at 37°C, with vigorous shaking, to an OD<sub>600</sub> of between 0.5-0.8. The cells were chilled on wet-ice for 30 minutes and then pelleted by centrifugation for 15 minutes at 4°C at 4000 g. After re-suspension in 500 ml of ice cold WFI, the cells were re-pelleted by centrifugation. This step was repeated and the cells were resuspended in 10 ml of ice-cold 10% glycerol (v/v in WFI). As a final step, the bacteria were re-pelleted, resuspended in 1.5 ml of fresh ice-cold 10% glycerol, aliquoted into fresh 0.6 ml tubes and snap-frozen in liquid nitrogen for storage at -80°C.

**Transformation of DNA into chemically competent cells**

Five to twenty microlitre volumes of either unligated, undigested plasmid DNA (10-100 ng) or ligation mixtures containing cDNA inserts and linearized plasmid vector DNA were gently mixed with 50-200 μl of freshly thawed ice-cold chemically competent *E.coli* cells in sterile 1.5 ml eppendorf tubes. Cell/DNA mixtures were incubated on wet-ice for 10 minutes and then heat-shocked at 42°C for exactly 45 seconds in a bench-top incubator.
(Thermomixer Comfort, Eppendorf) and then returned to ice for a further 2 minutes. One millilitre of fresh 2 x YT broth kept at room temperature was added to the cells and the culture incubated at 37°C for 1 hour. Cells were pelleted by centrifugation at 14,000 rpm for 1 minute and resuspended in 100 μl of 2 x YT. Aliquots of the bacterial suspensions were plated onto 2 x YT agar selection plates containing the appropriate antibiotic (50-100 μg/ml) and allowed to grow for 16-20 hours at 37°C. Where the identification of DNA inserts was necessary, those cells transformed with derivatives of pGEM-Teasy were plated onto agar supplemented with 0.1M IPTG and 40 μg/ml of X-gal. Colonies appearing white generally harboured plasmids containing DNA inserts.

Transformation of DNA into electro-competent cells

A 40 μl aliquot of electro-competent BJ5183 (pAdeasy) cells was thawed on ice and then gently diluted with fresh ice cold 10% glycerol. Five microlitres of recombinant plasmid DNA was added to the cells and gently mixed. The cell/DNA suspension was carefully transferred to a fresh sterile 2 mm electroporation cuvette (Bio-rad). Using a Gene Pulser Xcell™ electroporation system (Bio-rad), the cells were electroporated at the following conditions: 2.5 KV, 200 Ohms and 25 μF. Immediately following electroporation, the cells were resuspended in 1 ml of fresh 2 x YT media, transferred to a fresh 1.5 ml tube and incubated with gentle shaking at 37°C for 1 hour. Cells were pelleted by centrifugation at 14,000 rpm for 1 minute and resuspend in 100 μl of 2 x YT. Aliquots of the bacterial suspensions were plated onto 2 x YT agar selection plates containing kanamycin (50 μg/ml) and allowed to grow for 20-24 hours at 37°C.
GENERAL MATERIALS

Materials not listed in this section can be found in the relevant chapters.

Mammalian Cell Lines, Bacterial Strains, Adenoviral and Plasmid DNA vectors

Descriptions, sources and uses for *E. coli* strains, mammalian cell lines adenoviral genomes and plasmids are listed in tables 3.1, 3.2, 3.3 and 3.4.

Table 3.1  *Escherichia coli* strains used in this thesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ5183</td>
<td><em>endA sbcBC rec BC galK met thi- 1 bioT hsdR</em> (Strr) (host for pAdEasy Adenoviral vector)</td>
<td>QBiogene</td>
<td>Recombination permissive host to produce recombinant viral plasmid DNA</td>
</tr>
<tr>
<td>KRX</td>
<td><em>F’, traD36, ΔompP, proA+B+, lacIq, Δ(lacZ)M15</em> ΔompT, <em>endA1, recA1, gyrA96 (Nav), thi-1, hsdR17 (rλ, mλ+), e14- (McrA-), relA1, supE44, Δ(lac-proAB), Δ(rhaBAD):T7 RNA polymerase</em></td>
<td>Promega</td>
<td>General host for plasmid preparation, plasmid transformation and protein expression</td>
</tr>
<tr>
<td>JM109</td>
<td><em>endA1, recA1, gyrA96, thi, hsdR17 (rλ, mλ+), relA1, supE44, Δ(lac-proAB), [F’, traD36, proAB, lacIqZΔM15</em></td>
<td>Promega</td>
<td>General host for plasmid preparation, plasmid transformation, cDNA cloning and sequencing</td>
</tr>
</tbody>
</table>
Table 3.2  Cell lines used in this thesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>Compliments adenoviral replication</td>
<td>QBiogene</td>
<td>Cell line for viral preparation and amplification. Used for plasmid DNA transfections.</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Human neuroblastoma cell line.</td>
<td>Sigma</td>
<td>Cell line for differentiation into neuronal-like cells and most experiments.</td>
</tr>
<tr>
<td>U251</td>
<td>Human malignant glioblastoma cell line</td>
<td>Sigma</td>
<td>Used for characterisation of the secretory constructs</td>
</tr>
</tbody>
</table>

Table 3.3  SMA patient fibroblasts used in this thesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 SMA</td>
<td>Dermal fibroblasts derived from a type I SMA patient</td>
<td>Coriell Institute</td>
<td>Patient cells were used for experimentation and assessment of gene expression</td>
</tr>
</tbody>
</table>
Table 3.4  Plasmids used in this thesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAdEasy-1</td>
<td>A virulent viral genome containing site specific recombination sequences matching those on pCMV-Shuttle vector, ΔE1 gene and ΔE2 gene. AmR</td>
<td>QBiogene</td>
<td>Plasmid/genome for generating adenoviral vectors</td>
</tr>
<tr>
<td>pCMV-Shuttle</td>
<td>Contains CMV promoter, multiple cloning sites and SV40 polyadenylation signal sequence plus sites for directed recombination with pAd Easy and Kanamycin resistance (Km&lt;sup&gt;R&lt;/sup&gt;) selection marker.</td>
<td>QBiogene</td>
<td>Original adenoviral vector cloning plasmid</td>
</tr>
<tr>
<td>pCR4Blunt-TOPO®</td>
<td>The plasmid vector is supplied linearized with Vaccinia virus topoisomerase I covalently bound to the 3´ ends</td>
<td>Invitrogen</td>
<td>Direct insertion of blunt-end PCR products for sequencing</td>
</tr>
<tr>
<td>pEGFP-N1</td>
<td>CMV promoter, multiple cloning site with downstream EGFP cDNA sequence and SV40 polyadenylation sequence. Km&lt;sup&gt;R&lt;/sup&gt; selection marker.</td>
<td>Clontech</td>
<td>Source of EGFP cDNA, CMV promoter, and SV40 polyadenylation sequence.</td>
</tr>
<tr>
<td>pGEM-Teasy</td>
<td>Allows blue/white selection of recombinant clones, contains T7 and Sp6 RNA polymerase sequences for sequencing, and Ampicillin resistance (Amp&lt;sup&gt;R&lt;/sup&gt;) selection marker</td>
<td>Promega</td>
<td>T/A cloning and selective blue/white colony screening</td>
</tr>
</tbody>
</table>
Chemicals, Reagents and Enzymes

Chemicals and enzymes were obtained from the sources listed in Table 3.5. Unless otherwise indicated, all reagents were of analytical or biochemical grade.

Table 3.5  A list of chemicals and enzymes used in this thesis.

<table>
<thead>
<tr>
<th>Item</th>
<th>Details</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose (Biotechnology grade)</td>
<td></td>
<td>Ameresco</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Ampicillin (sodium salt)</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>Kanamycin sulphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bacterial culture media</td>
<td>Agar</td>
<td>Amresco</td>
</tr>
<tr>
<td></td>
<td>Bacto-trypotone</td>
<td>Difco</td>
</tr>
<tr>
<td></td>
<td>Bacto-Yeast extract</td>
<td>Difco</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Powdered</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bradford Reagent</td>
<td>Bio-Rad protein assay reagent</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Choroform</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Deoxynucleotide triphosphates (dNTPs)</td>
<td>dATP, dGTP, dCTP, dTTP</td>
<td>Promega</td>
</tr>
<tr>
<td>Dimethylsulphoxide (DMSO)</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Di-sodium hydrogen orthophosphate</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>DNA molecular weight marker standard (1.0 kb ladder)</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td></td>
<td>BDH</td>
</tr>
</tbody>
</table>
Table 3.5  A list of chemicals and enzymes used in this thesis (continued).

<table>
<thead>
<tr>
<th>Item</th>
<th>Details</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Isopropylthio-β-D-galactoside (IPTG)</td>
<td></td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>LY294002 (PI3-kinase/Akt inhibitor)</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>M-MLV Reverse transcriptase</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>Novex pre-stained protein molecular weight marker</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>Complete cocktail</td>
<td>Roche</td>
</tr>
<tr>
<td>Restriction enzymes</td>
<td>Bgl II, Cla I, Eco RI, Hind III, Kpn I, Mlu I, Pme I, Sal I, Sca I, Xho I</td>
<td>Promega, New England (NEB) Biolabs</td>
</tr>
<tr>
<td>RNasin Ribonuclease Inhibitor</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Trizol Reagent</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Trizma base</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween-20</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td></td>
<td>NEB</td>
</tr>
</tbody>
</table>
Antibodies

The antibodies used in this thesis and their sources are listed in table 3.6.

Table 3.6 A list of antibodies used in this thesis.

<table>
<thead>
<tr>
<th>Antibody (anti-)</th>
<th>Source</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-fodrin</td>
<td>Mouse (monoclonal)</td>
<td>MP Biochemicals</td>
</tr>
<tr>
<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Rabbit</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>Mouse (monoclonal)</td>
<td>Pharmagen</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Extracellular signal regulated kinase (ERK 1/2)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)</td>
<td>Rabbit</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Mouse (monoclonal)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat (secondary) conjugated with horse radish peroxidase (HRP)</td>
<td>Rabbit</td>
<td>Zymed</td>
</tr>
<tr>
<td>Histidine tag</td>
<td>Mouse (monoclonal)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mouse (secondary) conjugated with horse radish peroxidase (HRP)</td>
<td>Sheep</td>
<td>Amersham</td>
</tr>
<tr>
<td>Mouse Alexa 488 (secondary)</td>
<td>Goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Rabbit (secondary) conjugated with horse radish peroxidase (HRP)</td>
<td>Donkey</td>
<td>Amersham</td>
</tr>
<tr>
<td>Sam68</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Survival of Motor Neuron (SMN) protein</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>
Solutions and Culture Media

Unless otherwise stated, all general purpose solutions were prepared using Milli-Q water, autoclaved for stability and stored at room temperature. All media and solutions used in the preparation of chemically competent and electrocompetent bacterial cells were prepared in sterile water for irrigation (Baxter). Antibiotic stock solutions were prepared in WFI and sterilised by microfiltration using a 0.2 µm filter.

Table 3.7 Solutions and culture media used in this thesis.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x YT media</td>
<td>Bacto-tryptone (15 g), Bacto-yeast (10 g), NaCl (5 g) dissolved in Milli-Q water to a final volume of one litre.</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50 mg/ml stock. Dissoled in WFI, sterilised by microfiltration (0.2 µm) and stored at -20°C.</td>
</tr>
<tr>
<td>DNA loading dye</td>
<td>6 x stock prepared in 1 x TAE buffer and containing Bromophenol Blue (0.25%), Xylene Cyanol FF (0.25%) and 30% glycerol</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM stock solution was prepared by mixing 100 µl of each constituent (dATP, dTTP, dCTP and dGTP each at 100 mM) with 600 µl of WFI, dispensing 100 µl aliquots and stored at -20°C.</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Prepared as 10 mg/ml stock with WFI. Stored in the dark, at 4°C.</td>
</tr>
<tr>
<td>MgCl₂ (1M)</td>
<td>Dissolved in WFI and sterilised by microfiltration/0.2 µm.</td>
</tr>
<tr>
<td>MgSO₄ (1M)</td>
<td>Dissolved in WFI and sterilised by microfiltration/0.2 µm.</td>
</tr>
</tbody>
</table>
Table 3.7 Solutions and culture media used in this thesis (continued)

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS SDS running buffer</td>
<td>Supplied as 20 x stock (Invitrogen) and composed of MOPS (1 M); Tris Base (1 M); SDS (69.3 mM); EDTA (20.5 mM) made up in ultrapure water.</td>
</tr>
<tr>
<td>NuPAGE lauryl dodecyl sulphate</td>
<td>Supplied as 4x stock (Invitrogen) and composed of Glycerol (4 g); Tris-Base (0.682 g); Tris HCL (0.666 g); LDS (0.8 g); EDTA (0.006 g); Serva Blue G250 (0.75 ml of 1% solution); Phenol Red (0.25 ml of 1% solution) made up in 10ml of ultrapure water. Unadjusted 1x buffer is pH 8.5.</td>
</tr>
<tr>
<td>(LDS) sample buffer</td>
<td></td>
</tr>
<tr>
<td>NuPAGE transfer buffer</td>
<td>Supplied as 20 x stock (Invitrogen) and composed of Bicine (500 mM); Bis-Tris (500 mM); EDTA (20.5 mM); Chlorobutanol (1 mM) made up in ultrapure water. Unadjusted 1x buffer is pH 7.2.</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>10 x stock was prepared by dissolving 11.5 g of Di-sodium hydrogen orthophosphate anhydrous (80 mM), 2.96 g sodium dihydrogen orthophosphate (20 mM), 5.84 g sodium chloride (100 mM) in Milli-Q water to 1000 ml.</td>
</tr>
<tr>
<td>pH7.5</td>
<td></td>
</tr>
<tr>
<td>SOB++</td>
<td>Bacto-tryptone (20 g), Bacto-yeast (5 g), NaCl (0.5 g) and KCl (0.186 g) dissolved in WFI to a final one litre volume and sterilised. Prior to use, 20 ml of MgCl₂ (1M stock) and MgSO₄ (1M stock) was added to a final concentration of 10mM.</td>
</tr>
</tbody>
</table>
Table 3.7 Solutions and culture media used in this thesis (continued)

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAE</td>
<td>A one litre, 50 x stock solution was prepared by combining Trizma base (242 g), 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA (pH8.0) with Milli-Q water. Stored at RT.</td>
</tr>
<tr>
<td>TB Buffer</td>
<td>HEPES (10mM), CaCl2 (15mM), KCl (250mM) were dissolved in WFI and the pH 6.7 adjusted to with KOH, prior to adding MnCl2 (55mM). The solution was sterilised by microfiltration/0.2 μm and stored at -4°C.</td>
</tr>
</tbody>
</table>
Chapter 4

Survival of Motor Neuron (SMN) Protein Over-expression Prevents Calpain Mediated Cleavage and Activation of Procaspase-3 in Differentiated Human SH-SY5Y Cells

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Survival of Motor Neuron (SMN) Protein Over-expression Prevents Calpain Mediated Cleavage and Activation of Pro-caspase-3 in Differentiated Human SH-SY5Y Cells

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SURVIVAL OF MOTOR NEURON PROTEIN OVER-EXPRESSION PREVENTS CALPAIN-MEDIATED CLEAVAGE AND ACTIVATION OF PROCASPASE-3 IN DIFFERENTIATED HUMAN SH-SY5Y CELLS

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Abstract—Spinal muscular atrophy (SMA), a neurodegenerative disorder primarily affecting motor neurons, is the most common genetic cause of infant death. This incurable disease is caused by the absence of a functional SMN1 gene and a reduction in full length survival of motor neuron (SMN) protein. In this study, a neuroprotective function of SMN was investigated in differentiated human SH-SY5Y cells using an adenoviral vector to over-express SMN protein. The pro-survival capacity of SMN was assessed using an Akt/PI3-kinase inhibition (LY294002) model, as well as an oxidative stress (hydrogen peroxide) and excitotoxic (glutamate) model. SMN over-expression in SH-SY5Y cells protected against Akt/ phosphatidylinositol-3-kinase (PI3-kinase) inhibition, but not oxidative stress, nor against excitotoxicity in rat cortical neurons. Western analysis of cell homogenates from SH-SY5Y cultures over-expressing SMN harvested pre- and post-Akt/ PI3-kinase inhibition indicated that SMN protein inhibited caspase-3 activation via blockade of calpain-mediated proteolysis. This study has revealed a novel anti-apoptotic function for the SMN protein in differentiated SH-SY5Y cells. Finally, the cell death model described herein will allow the assessment of future therapeutic agents or strategies aimed at increasing SMN protein levels. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: SMN, anti-apoptotic, adenoviral, SMA, caspase-3.

With an incidence of 1 in 6,000 live births, spinal muscular atrophy (SMA), an autosomal recessive disorder characterized by progressive motor neuron loss and muscle weakness, is the leading genetic cause of infant mortality.

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Abbreviations: BDNF, brain-derived neurotrophic factor; DIV, on day in vitro; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; GFP, green fluorescent protein; H2O2, hydrogen peroxide; MOI, multiplicity of infection; PBS, phosphate buffered saline; PI3, phosphatidylinositol-3-kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMA, spinal muscular atrophy; SMN, survival of motor neuron protein; SMN1, survival of motor neuron 1; SMN2, survival of motor neuron 2

EXPERIMENTAL PROCEDURES

Cloning of human SMN cDNA and construction of adenoviral vectors

Total human RNA was isolated from HEK293 cells, reverse transcribed and amplified by PCR using a gene specific primer pair containing unique restriction sites (bold) and a Kozak sequence

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Dia was removed and replaced with fresh NB2° DIV 9, and the inhibitor cytosine arabinofuranoside. One third of the culture plates were coated with poly-D-lysine (50 μg/ml) and washed in cold DMEM/10% horse serum. Culture 96-well plates were transfected with 3 MOI of the adenovirus (Boulos et al., 2006). Briefly, pShuttle plasmid DNA (pRSV-SMN1/CMV/GFP) was linearized by Pmel digestion and introduced, by electroporation (Gene Pulser II, Bio-Rad, Hercules, CA, USA) into the Escherichia coli strain BJ5183 harboring the pAdeasy plasmid (Zeng et al., 2001). Recombinants were selected on media containing 50 μg/ml kanamycin, and their plasmid DNA analysed by Pael digestion. HEK293 cells grown to 90% confluency in 25 cm² flasks were transfected with 3 μg of Pael linearized recombinant plasmid DNA using Lipofectamine2000 (Invitrogen, Grand Island, NY, USA) and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA). Following the appearance of viral plaques (5–10 days), culture lysates were used for viral amplification in HEK293 cells. The SMN1 expressing adenoviral vector was designated AdRSV:SMN:CMV:GFP (AdRSV:SMN). Adenoviral particles were purified and concentrated from HEK293 cell lysates using the Adeno-X kit (BD Biosciences, San Jose, CA, USA). Viral titres were determined by end-point dilution assay as indicated by enhanced GFP reporter expression. Vectors consisting of an adenovirus expressing only green fluorescent protein (GFP; AdRSV:Empty:CMV:GFP; AdRSV:Empty) and an adenovirus over-expressing Bcl-xL (AdRSV:Bcl-xL:CMV:GFP; AdRSV:Bcl-xL) have been described previously (Boulos et al., 2006).

### Rat primary cortical neuronal cell cultures

All animal procedures were approved by the University of Western Australia Animal Ethics Committee. Establishment of cortical cultures was as previously described (Meloni et al., 2001). Briefly, cortical tissue from E18 to E19 Sprague–Dawley rats were dissociated in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 1.3 mM l-cysteine, 0.9 mM NaHC03, 10 μM papain (Sigma, St. Louis, MO, USA) and 50 U/ml DNaseI (Sigma) and washed in cold DMEM/10% horse serum. Culture 96-well plates were coated with poly-D-lysine (50 μg/ml; 70–150 μg/ml) and incubated overnight at room temperature. The poly-D-lysine and reverse (HindIII) was replaced with Neurobasal Media (containing 2% B27; 4% FCS; 1% horse serum; 62.5 μM 2-mercaptoethanol and 30 μg/ml penicillin). Neurons were plated at a density of 40,000 cells per well and cultures were maintained in a CO2 incubator (5% CO2; 95% air balance and 98% humidity) at 37 °C. On day in vitro (DIV) 4 half the media was removed and replaced with fresh NB2° DIV 9, and the cultures were maintained at 37 °C in 5% CO2.

### Control fibroblasts and SMA patient fibroblasts

Control human fibroblasts (AGO8814; Coriell Institute, Camden, NJ, USA) and SMA type I patient fibroblasts (GM03813; Coriell Institute) were maintained in DMEM containing penicillin (20 U/ml), streptomycin (20 mg/ml) and foetal calf serum (FCS; 5%; heat-inactivated) and incubated at 37 °C (5% CO2). SH-SY5Y cell propagation and differentiation

SH-SY5Y neuroblastoma cells were maintained in DMEM containing penicillin (20 U/ml), streptomycin (20 mg/ml) and FCS (5%; heat-inactivated) and incubated at 37 °C (5% CO2). To obtain differentiated SH-SY5Y cultures, cells were seeded into a 96 well plate (~30,000 cells/well) in 100 μl of DMEM (5% FCS) containing all-trans retinoic acid (15 μM; Sigma). After 3 days, half the media was replaced with serum free DMEM and retinoic acid (15 μM). Five days after plating, half the media was replaced with serum free DMEM containing brain-derived neurotrophic factor (BDNF; 2 μM; Sigma), and maintained for a further 3 days before use. Undifferentiated SH-SY5Y cells showed a rounded appearance with short processes, compared to retinoic acid treated SH-SY5Y cells which displayed a more neuronal-like morphology, with cells appearing triangular and showing longer processes (Encinas et al., 2000, James et al., 2004).

### Adenoviral transduction of SH-SY5Y cells in 96 well plates

Adenovirus was diluted in serum free DMEM media containing BDNF (2 μM) and added to differentiating SH-SY5Y cultures 5 days after plating. To ensure uniform transduction of each virus in SH-SY5Y cultures (multiplicity of infection; MOI: 35–50), GFP reporter expression was initially quantitatively measured with a fluorescence plate reader (SPECTROstar; Omega, NC, USA) and subsequently routinely assayed by fluorescent imaging (Olympus IX70; Olympus DP70 digital camera; Olympus, Melville, NY, USA). Cultures were used for experiments 3 days after viral transduction.

### Adenoviral transduction of rat primary cortical neurons and fibroblasts in 96 well plates

Adenovirus was diluted in pre-conditioned media and added to cells in culture. To ensure uniform transduction of each virus in both cortical neurons and fibroblasts (MOI: 70–90), GFP reporter expression was routinely assayed by fluorescent imaging (Olympus IX70; Olympus DP70 digital camera; Olympus). Cultures were used for experiments 3 days after viral transduction.

### Immunohistochemistry of SMA patient fibroblasts

SMA type I patient fibroblasts (GM03813; Coriell Institute) grown on cover slips were fixed in ice cold 4% formalin in phosphate buffered saline (PBS) for 1 h. Cover slips were rinsed in PBS-tween (PBS-T), blocked in 10% goat serum and exposed to an anti-SMN (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody, diluted in PBS-T, and exposed to a secondary antibody (Alexa Fluor 488 goat anti-mouse, 1:400; Invitrogen) diluted in PBS-T at 4 °C for 2 h. Cover slips were rinsed with PBS, mounted onto slides, and viewed using fluorescence microscopy.

### Cell injury models

**Hydrogen peroxide oxidative stress model.** To determine if the SMN protein could protect against oxidative stress, a hydrogen peroxide (H2O2) model was used. Bissonnette et al. (2004) has previously shown that in differentiated SH-SY5Y cultures exposed to 270 μM H2O2, cell viability was decreased by ~50%. Differentiated SH-SY5Y cultures were treated with 300 μM H2O2 in serum-free DMEM media, and cell death was assessed 16–18 h (37 °C; 5% CO2) after H2O2 addition.

**Inhibition of Akt/P13-kinase cell signalling apoptosis model.** To assess the potential anti-apoptotic function of the SMN protein, differentiated SH-SY5Y cultures were treated with a selective phosphatidylinositol-3 kinase (PI3) inhibitor, LY294002 (Sigma), which inhibits BDNF-mediated pro-survival signalling resulting in a predominantly apoptotic cell death (Fujiwara et al., 2006). This model involved replacing media in wells with serum-free DMEM...
media containing LY294002 (12.5–100 μM in DMSO), and incubating cultures for 16–18 h (37 °C; 5% CO2) prior to cell death assessment.

Glutamate excitotoxicity model. Cultures were treated on DIV12 with 100 μM glutamate for 5 min. Media was removed and replaced with a 50% NB2°/balanced salt solution and incubated for 1 h at 37 °C in 5% CO2. Cell death was assessed using the LDH assay.

CytoTox 96® non-radioactive cytotoxic assay (LDH assay)

Cell death assessment was performed using the CytoTox 96® non-radioactive cytotoxic assay (LDH assay; Invitrogen). This assay which measures LDH released from dead cells, following the enzymatic (LDH) mediated conversion of the tetrazolium salt substrate into its red formazan product (detected spectrophotometrically at 490 nm), was performed according to the manufacturers instructions. The amount of formazan product is proportional to the number of lysed/dead cells in culture.

Protein extraction from cells and western blotting

Proteins were extracted from SH-SY5Y cells using RIPA lysis buffer. Protein concentrations were determined using the Bradford assay (Bio-Rad), and equivalent amounts of protein (10 μg per lane) were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using pre-cast Bis–Tris gels (Invitrogen). For western blot analysis, proteins separated by SDS-PAGE were transferred to PVDF membranes. Membranes were briefly washed in PBS, blocked in PBS-Tween 20 (0.1%) containing ovalbumin (1 mg/ml) for 1 h and incubated in primary antibodies (β-catenin: 1:2000, BD Biosciences; SMN: 1:2500, Santa Cruz Biotechnology; Caspase-3: 1:2000, Santa Cruz Biotechnology; α-fodrin: 1:600, MP Biomedicals, Solon, OH, USA) diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml) overnight at 4 °C with gentle rocking. For protein detection, membranes were washed in PBS-T (0.1%), incubated with an HRP complexed secondary antibody (1:10000–1:25000 donkey anti-rabbit/sheep anti-mouse; GE Healthcare, Piscataway, NJ, USA) for 1 h at room temperature, washed in PBS, and visualized using ECL plus detection reagent (GE Healthcare). Quantification and protein band densitometry of western blots was undertaken using ImageJ (NIH) software.

Statistics

Statistical differences between experimental groups were determined by ANOVA, followed by post-hoc Fischer’s PLSD test. P<0.05 was considered statistically significant (* P<0.05; ** P<0.005; *** P<0.0005). Unless otherwise stated, all experiments were conducted at least three times.

RESULTS

Adenoviral mediated protein over-expression in differentiated SH-SY5Y cells and SMA patient fibroblasts

Transduction of differentiated SH-SY5Y cells was confirmed by fluorescence microscopy of GFP reporter expression (Fig. 1A). Western blot analysis of lysates from differentiated SH-SY5Y cells transduced with AdRSV:SMN and AdRSV:Bcl-xL confirmed the over-expression of SMN and Bcl-xL proteins respectively (Fig. 1B). In addition, immunohistochemical analysis confirmed SMN over-expression by AdRSV:SMN in SMA patient fibroblasts (Fig. 2). Over-expressed SMN protein appeared as cytoplasmic punctate structures consistent with previous studies (Gangwani et al., 2001).

![Fig. 1. Confirmation of adenoviral-mediated transduction and expression in differentiated SH-SY5Y cells. (A) GFP reporter expression in SH-SY5Y cells transduced with AdRSV:SMN, AdRSV:Empty and AdRSV:Bcl-xL. (B) Western blot analysis of protein extracted from differentiated SH-SY5Y cells transduced with either AdRSV:Empty, AdRSV:SMN or AdRSV:Bcl-xL. Expression of β-tubulin served as the loading control. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.](image-url)
SMN over-expression does not protect against hydrogen peroxide (oxidative stress)

AdRSV:SMN mediated over-expression of SMN in differentiated SH-SY5Y cultures did not protect cells from hydrogen peroxide exposure. By contrast, cultures transduced with the AdRSV:Bcl-xL vector exhibited reduced cell death following hydrogen peroxide exposure (Fig. 3).

SMN over-expression does not protect against glutamate (excitotoxicity)

AdRSV:SMN mediated over-expression of SMN in rat primary cortical neurons did not protect cultures from excitotoxic injury. However, cultures over-expressing the Bcl-xL protein showed reduced cell death following glutamate exposure (Fig. 4).

SMN over-expression protects against PI3-kinase/Akt inhibition (apoptosis)

A LY294002 dose response experiment using AdRSV:Empty vector transduced differentiated SH-SY5Y cultures revealed that a 25 μM dose resulted in ≈70% cell death (Fig. 5), and thus, this concentration was used for subsequent experiments.

AdRSV:SMN transduction of differentiated SH-SY5Y cultures resulted in significantly reduced cell death following LY294002 exposure (P<0.005; Fig. 6). Similarly, AdRSV:Bcl-xL transduced SH-SY5Y cultures also exhibited significantly reduced cell death following LY294002 exposure (P<0.0005; Fig. 6).

SMN protein reduces caspase-3 activity

The cytoskeletal protein, α-fodrin, is susceptible to cleavage by both caspase-3 and calpain. Therefore, a time course of caspase-3 and calpain activation based on α-fodrin cleavage following exposure to LY294002 was undertaken in differentiated SH-SY5Y cells. The time course revealed that the 120 and 145/150 kDa α-fodrin degradation products significantly increased 2 h after LY294002 exposure (Fig. 7). Therefore, the 2 h post LY294002 exposure time point was chosen to assess the effects of SMN over-expression on α-fodrin degradation in SH-SY5Y cells.

Differentiated SH-SY5Y cultures transduced with the AdRSV:SMN vector and exposed to LY294002 showed a significant reduction in α-fodrin 120 and 145/150 kDa degradation products (Fig. 8). Again, the effect was similar to that observed following transduction of SH-SY5Y cells with the vector, AdRSV:Bcl-xL (Fig. 8). The cleavage products are representative of caspase activity (120, 145/150 kDa products) and/or calpain activity (145/150 kDa product), demonstrating that both SMN and Bcl-xL reduce caspase activity, and potentially calpain activity, following LY294002 exposure.

![Fig. 2. Immunofluorescence analysis of AdRSV:SMN transduced SMA I patient fibroblasts. (A) Non-transduced SMA I patient fibroblasts probed with an anti-SMN antibody. (B) SMN protein detected in AdRSV:SMN transduced SMA patient fibroblasts. (C) GFP reporter expression in AdRSV:SMN transduced SMA I patient fibroblasts. (D) Merged image overlapping GFP reporter expression and SMN localization. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.](image1)

![Fig. 3. LDH assay comparing cell death between transduced differentiated SH-SY5Y cultures. Differentiated SH-SY5Y cultures transduced with AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty followed by hydrogen peroxide (300 μM) treatment for 18 h.](image2)

![Fig. 4. LDH assay comparing cell death between transduced primary rat cortical neurons. Primary cultures were transduced with AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty followed by glutamate (100 μM) treatment for 2 h.](image3)
SMN protein prevents calpain-mediated cleavage of the caspase-3 prodomain

Activation of procaspase-3 (32 kDa) requires cleavage of the 3 kDa amino-terminal to form a pro-apoptotic p29 subunit (29 kDa; McGinnis et al., 1999; Wolf et al., 1999). Western analysis of caspase-3 protein from differentiated SH-SY5Y cells transduced with AdRSV:SMN showed no detectable caspase-3 p29 subunit, unlike AdRSV:Bcl-xL and AdRSV:Empty transduced SH-SY5Y cells (Fig. 9). Western analysis of SH-SY5Y cells 2 h post LY294002 exposure, revealed that AdRSV:SMN transduced cells harbour reduced levels of the p29 subunit compared to AdRSV:Bcl-xL and AdRSV:Empty transduced cells (Fig. 9). In contrast, the caspase-3 20 kDa intermediate subunit, which is generated after caspase-9 cleavage of procaspase-3, showed no difference in levels between SH-SY5Y cells transduced with the different viral vectors (Fig. 9).

DISCUSSION

In SMA, a significant number of motor neurons in the spinal cord die by a process characteristic of apoptosis (Simic et al., 2000, 2008; Tsai et al., 2006). Furthermore, over-expression of SMN protein reduces cell death following apoptotic insults in rat primary cortical neurons, rat PC-12 cells and mouse NSC34 cells (Kerr et al., 2000; Vyas et al., 2002; Parker et al., 2008); however, to date, clarification of SMN’s specific pro-survival mechanism(s) is lacking. In characterizing a PI3-kinase/Akt inhibition apoptotic cell death model using differentiated SH-SY5Y human neuronal-like cells, we demonstrated that SMN protein over-expression reduces caspase-3 activation by preventing calpain-mediated cleavage of the caspase-3 prodomain (Wolf et al., 1999). Our data thus supports and extends previous findings that SMN plays a role in modulating apoptosis and reducing caspase-3 activity (Kerr et al., 2000; Vyas et al., 2002; Wang et al., 2005; Parker et al., 2008).

The activation of caspase-3 (32 kDa) involves a two stage processing event; the removal of its prodomain (3

Fig. 5. Dose-dependent induction of SH-SY5Y cell death by the PI3 kinase/Akt inhibitor, LY294002. LDH assay comparing cell death in AdRSV:Empty transduced differentiated SH-SY5Y cultures in response to increasing concentrations of LY294002 (12.5–100 μM).

Fig. 6. LDH assay comparing cell death in transduced differentiated SH-SY5Y cells. Measured LDH levels in AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty transduced SH-SY5Y cells treated with LY294002 (25 μM).
kDa) resulting in a 29 kDa subunit (p29) and subsequent spontaneous proteolytic cleavage of p29 into large and small subunits (17/12 kDa; Han et al., 1997; Deveraux et al., 1998; Meergans et al., 2000). SMN over-expression in untreated and dying SH-SY5Y cells resulted in the absence or reduction of the caspase-3 p29 subunit, indicating that prodomain removal was inhibited. Cleavage of the caspase-3 prodomain occurs via the action of calpain (Wolf et al., 1999). How SMN is able to block calpain cleavage of caspase-3 remains unclear. Interestingly, calpain also cleaves SMN protein, generating a 10 kDa and 28 kDa fragment, which may impart a pro-survival function (van Bergeijk et al., 2007; Walker et al., 2008). Alternatively, it is possible that SMN can block access to pro-caspase-3 cleavage in a manner similar to heat shock protein 27 (Hsp27), which is mutated in an axonal form of Charcot–Marie–Tooth disease (CMT2F), and like SMN, reduces caspase-3 activation and apoptosis (Pandey et al., 2000; Benn et al., 2002; Concannon et al., 2003; Reilly and Shy, 2009).

Bcl-xL is a well characterized potent anti-apoptotic member of the Bcl-2 family, which can protect against excitotoxic and oxidative injury (Xu et al., 1999; Dietz et al., 2007). Thus, as demonstrated in this study, Bcl-xL can also protect against non-apoptotic related cell death such as necrosis (Tsujimoto et al., 1997; Panickar et al., 2005). Hydrogen peroxide-induced oxidative stress triggers apoptosis at lower concentrations and necrosis at higher concentrations (Skulachev, 2006). Therefore, the H2O2 concentration used in this study may cause predominantly necrotic cell death which explains why SMN over-expression was not neuroprotective. This study also supports findings by others that SMN protein over-expression does not protect neuronal cultures against glutamate-induced excitotoxicity (Cisterni et al., 2001). In our model, glutamate exposure causes calpain activation without cas-

**Fig. 8.** Comparison of α-fodrin degradation patterns following over-expression of SMN and Bcl-xL proteins. (A) Western blot analysis of α-fodrin in differentiated SH-SY5Y cells transduced with AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty and treated with LY294002 (2 h, 25 μM). (B) Densitometric analysis of the 145/150 kDa α-fodrin degradation product; (C) Densitometric analysis of the 120 kDa α-fodrin degradation product.

**Fig. 9.** Comparison of the caspase-3 p29 subunit status in transduced differentiated SH-SY5Y cells. (A) Western blot analysis of caspase-3 after SMN and Bcl-xL over-expression and exposure to LY294002 (2 h; 25 μM); (B) Densitometric analysis of the caspase-3 p29 subunit.
pase-3 activation (Meade et al., 2010). The lack of protection seen in the excitotoxic model is consistent with the notion that SMN can only protect against caspase-3 dependent apoptosis (Vyas et al., 2002; Wang et al., 2005; Parker et al., 2008).

Expression of Bcl-xL protein is reduced during fetal development in SMA patients (Soler-Botija et al., 2003). However, in our study, Bcl-xL over-expression did not reduce calpain-mediated cleavage of caspase-3 p29 subunit levels in untreated and dying SH-SY5Y cells, suggesting that it exerts its anti-apoptotic function through a different mechanism to SMN. Curiously, Bcl-xL over-expression can increase lifespan, improve motor function and reduce motor neuron degeneration in SMA mice (Tsai et al., 2008). Thus, our results suggest that treatments aimed at halting neuronal degeneration in SMA may benefit from the manipulation of both SMN and Bcl-xL survival pathways.

In summary, this study has shown that SMN over-expression reduces caspase-3 cleavage activation by specifically blocking calpain-mediated cleavage of the caspase-3 prodomain. However, the data presented in this study is preliminary and requires further verification in vivo using a relevant animal model. In addition, we have established a novel apoptotic model of SMN functionality in a human neuronal cell line, which can be used to further elucidate the pro-survival mechanisms of the SMN protein, as well as a basis to select and test potential drug targets.

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Chapter 5

Co-regulation of SMN and Bcl-xL Expression: Implications for Neuroprotection in Spinal Muscular Atrophy

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Co-regulation of SMN and Bcl-xL Expression: Implications for Neuroprotection in Spinal Muscular Atrophy

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CO-REGULATION OF SURVIVAL OF MOTOR NEURON AND BCL-XL EXPRESSION: IMPLICATIONS FOR NEUROPROTECTION IN SPINAL MUSCULAR ATROPHY

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Abstract—Spinal muscular atrophy (SMA), a fatal genetic motor disorder of infants, is caused by diminished full-length survival of motor neuron (SMN) protein levels. Normally involved in small nuclear ribonucleoprotein (snRNP) assembly and pre-mRNA splicing, recent studies suggest that SMN plays a critical role in regulating apoptosis. Interestingly, the anti-apoptotic Bcl-x isoform, Bcl-xL, is reduced in SMA. In a related finding, Sam68, an RNA-binding protein, was found to modulate splicing of SMN and Bcl-xL transcripts, promoting SMN7 and pro-apoptotic Bcl-xS transcripts. Here we demonstrate that Bcl-xL expression increases SMN protein by ~2-fold in SH-SY5Y cells. Conversely, SMN expression increases Bcl-xL protein levels by ~6-fold in SH-SY5Y cells, and ~2.5-fold in the brains of transgenic mice over-expressing SMN (PrP-SMN). Moreover, Sam68 protein levels were markedly reduced following SMN and Bcl-xL expression in SH-SY5Y cells, suggesting a feedback mechanism co-regulating levels of both proteins. We also found that exogenous SMN expression increased full-length SMN transcripts, possibly by promoting exon 7 inclusion. Finally, co-expression of SMN and Bcl-xL produced an additive anti-apoptotic effect following PI3-kinase inhibition in SH-SY5Y cells. Our findings implicate Bcl-xL as another potential target in SMA therapeutics, and indicate that therapeutic increases in SMN may arise from modest increases in total SMN. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: SMN, Bcl-xL, Sam68, PrP-SMN, additive, co-expression.

INTRODUCTION

Spinal muscular atrophy (SMA) is an often fatal autosomal recessive genetic disease typified by the degeneration of anterior horn cells of the spinal cord, muscle weakness and atrophy. With an incidence of one in 6,000 live births, SMA is the most common genetic cause of infant mortality. Clinically characterized by profound muscle weakness, hypotonia and trunk paralysis, SMA is classified into 3 main subtypes (I–III) based on disease severity and age of onset (Munsat and Davies, 1992).

SMA is a monogenic disorder whereby the survival of motor neuron (SMN1) gene is mutated or absent in more than 95% of cases (Lefebvre et al., 1995). During human evolution, a 500 kb inverted duplication of the SMN1 locus has given rise to a second copy of the gene, termed SMN2. The SMN2 gene is essentially identical to SMN1 except for a single translationally silent nucleotide change (C→T) at position 6 of exon 7, which causes exclusion of exon 7 in SMN2 transcripts (Lorson et al., 1999; Monani et al., 1999). Consequently, only about 10% of SMN2 protein is functional, while the other 90% is truncated and unstable. The occurrence of multiple SMN2 gene copies in patient groups correlates with increased full-length SMN transcripts, greater amounts of functional protein, and reduced disease severity (Lefebvre et al., 1997; Feldkotter et al., 2002).

The SMN protein is highly expressed during embryonic development and ubiquitously expressed within the cytoplasm and nucleus of cells (La Bella et al., 1998). SMN plays a role in the assembly of small nuclear ribonucleoproteins (snRNPs), a component essential for pre-mRNA splicing (Liu et al., 1997; Pellizzoni et al., 1999). Among other functions, SMN also interacts with key regulators of cell survival, such as Bcl-2, ZPR1 and p53 (Iwahashi et al., 1997; Gangwani et al., 2001; Young et al., 2002), and appears to possess an important anti-apoptotic function (Kerr et al., 2000; Vyas et al., 2002; Anderton et al., 2011).

Curiously, like SMN, expression of the anti-apoptotic Bcl-2 family member, Bcl-xL, is also reduced in SMA patients and SMA mouse models (Soler-Botija et al., 2003; Tsai et al., 2008). Bcl-xL is highly expressed in

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the CNS and regulates cell survival by inhibiting the pro-apoptotic Bax and Bak proteins (Merry and Korsemeyer, 1997). Importantly, Bcl-xL expression rescues SMA-like motor defects (Garcera et al., 2011) and increases the life-span of transgenic type III SMA mice (Tsai et al., 2008). The co-occurrence of reduced Bcl-xL and SMN in SMA suggests co-regulated expression of these two proteins. Interestingly, phosphorylation of the RNA-binding protein, Sam68, promotes exon 7 inclusion in SMN2 transcripts and splicing of the pro-survival Bcl-xL transcript (Paronetto et al., 2007; Pedrotti et al., 2010).

We used recombinant adeno-viral vectors and transgenic mice to investigate the potentially important relationship between SMN and Bcl-xL expression. Expression of SMN strongly increased Bcl-xL expression in SH-SY5Y cells, and in PrP-SMN mouse brain tissue. Remarkably, expression of both SMN and Bcl-xL reduced total Sam68 levels, supporting the notion that SMN positively regulates its own expression, possibly by modulating Sam68 levels. Finally, we demonstrate that co-expression of Bcl-xL and SMN has an additive neuroprotective effect against phosphatidylinositol-3 kinase (PI3-kinase) inhibition-induced apoptosis, thereby identifying Bcl-xL as a valid therapeutic target in SMA treatment.

EXPERIMENTAL PROCEDURE

SH-SY5Y and type 1 SMA patient fibroblast cell maintenance

SH-SY5Y neuroblastoma cells and type I patient fibroblasts (GMO3813; Coriell Institute, Camden, NJ, USA) were maintained in Dulbecco’s modified eagle medium (DMEM)-containing penicillin (20 units/ml), streptomycin (20 mg/ml) and fetal calf serum (FCS; 5–10%; heat-inactivated), and incubated at 37 °C in 5% CO2. The neuronal differentiation of SH-SY5Y cells by exposure to retinoic acid (15 μM; 5 days) has previously been described (Anderton et al., 2011).

Adenoviral vectors and transduction of cells in culture

Recombinant adenoviruses were prepared according to the method of He et al. (1998), with some modifications (Boulos et al., 2006). Adenoviral vectors expressing Bcl-xL (AdBcl-xL), SMN (AdSMN) and a control vector (AdEmpty) have been described previously (Boulos et al., 2006; Anderton et al., 2006). Recombinant adenoviruses and transduction of each adenoviral vector, cultures were routinely assessed for GFP reporter expression using epi-fluorescent imaging (Olympus IX70; Olympus DP70 digital software). Transgenic PrP-SMN mice (line 92; (Gavrilina et al., 2008)) were obtained from Prof. Kevin Talbot (University of Oxford) and maintained on an FVB/N background. Non-transgenic FVB/N littermates provided wild-type (WT) controls. Mice were killed at 2 months of age by lethal injection (sodium pentobarbital, 100 mg/kg, IP). Whole brain and lumbar spinal cord were dissected out and snap-frozen. These experiments were approved by the Howard Florey Institute Animal Ethics Committee (permit number 10-024).

Protein extraction and Western blotting

Protein was extracted from mouse tissue or cultured cells using RIPA lysis buffer and homogenization. Western blotting has been described previously (Anderton et al., 2011). Briefly, membranes were blocked in PBS-Tween 20 (0.1%)-containing ovalbumin (1 mg/ml) for 1 h and incubated in α-fodrin (1:1000; MP Biomedicals, Solon, OH, USA), Bcl-x (1:3000: BD Biosciences, San Jose, CA, USA), β-tubulin (1:10000; Santa Cruz, Santa Cruz, CA, USA), Sam68 (1:5000; Santa Cruz), or SMN (1:3000; Santa Cruz) primary antibodies diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml). Proteins were detected using a HRP-complexed secondary antibody (1:15000–1:35000 donkey anti-rabbit/sheep anti-mouse; GE Healthcare, Piscataway, NJ, USA), and visualized using ECL plus detection reagent (GE Healthcare). Quantification and band densitometry of Western blots was undertaken using ImageJ (NIH) software.

Quantitative real-time-PCR

Total RNA was harvested using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Five hundred nanograms of total RNA was DNase treated (TURBO DNA-free; Applied Biosystems, Foster City, CA, USA) prior to 10 μl being used for cDNA synthesis using SuperScript III RT (Invitrogen) primed with random hexamers (Invitrogen) at 100 ng/μl according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems). Amplification of specific PCR products was performed in triplicates in a total reaction volume of 15 μl- containing 2 μl cDNA template, forward and reverse primers (300 nM), and 2 x Fast SYBR® master mix reagents (Invitrogen). Primers used for SMN amplification (forward; 5’ GTC CAG ATT CTC TTG ATG 3’) and GAPDH amplification (forward; 5’ ACA GTC AGC CGC ATC TTC TT 3’) and reverse; 5’ ACG ACC AAA TCC GTT GAC TC 3’). Amplifications were performed at 95 °C for 6 min and for 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 30 s at 72 °C. For quantitative PCR analysis, differences in gene expression were assessed relative to the levels of an internal control (Pfaffl, 2001).

One step RT-PCR

One hundred nanograms of total RNA was used in reaction with SuperScript III One Step RT-PCR with Platinum Taq (Invitrogen) as per manufacturer’s instructions. The SMN transcript was amplified across exons 4–8 using PCR primers (Forward: 5’ AAG TCT CCT GGA AAT TCA G 3’ and Reverse: 5’ TGG TGT CAT TTA GTG CTG CTC T 3’). The amplification protocol followed 55 °C for 30 min, 94 °C for 2 min and 28 cycles of 94 °C for 40 s, 56 °C for 1 min and 68 °C for 1 min. PCR products were fractionated on a 2% agarose gel.

PI3-kinase inhibition apoptotic model

Assessment of the anti-apoptotic function of SMN using the PI3-kinase inhibitor, LY294002 (Sigma, St. Louis, MO, USA), has previously been described (Anderton et al., 2011). Briefly, LY294002 was diluted in serum-free DMEM to a final concentration of 25 μM prior to addition to differentiated SH-SY5Y cells.
Actinomycin D treatment of differentiated SH-SY5Y cells

Inhibition of mRNA transcription was achieved by adding actinomycin D (Sigma: 5 µM final concentration) to differentiated SH-SY5Y cells maintained in serum-free DMEM. Total RNA was harvested at 0, 4 and 8 h following actinomycin D addition and subjected to quantitative real-time PCR analysis.

Cell viability assessment

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS; Promega, Madison, WI, USA). The MTS assay measures the mitochondrial conversion of the tetrazolium salt to a water-soluble brown formazan salt, which is detected spectrophotometrically at 495 nm. MTS absorbance readings from controls and treatments were converted to percentage viability.

Statistics

Statistical differences between experimental groups were determined by ANOVA, followed by post-hoc Fischer’s protected least significance difference (PLSD) test. A \( p < 0.05 \) was considered statistically significant. Unless otherwise stated, all experiments were conducted at least three times.

RESULTS

SMN expression induces Bcl-xL expression in differentiated SH-SY5Y cells

To determine if SMN protein levels could affect Bcl-xL protein levels, protein lysates of AdSMN-transduced SH-SY5Y cultures were subjected to Western blot analysis. The results indicate that SMN expression in SH-SY5Y cells increased Bcl-xL protein levels (Fig. 1A). Densitometric analysis of western blots showed an approximate 6-fold increase in Bcl-xL protein levels compared to AdEmpty-transduced cultures (Fig. 1A). We were unable to detect the pro-apoptotic Bcl-xS protein in in vitro studies, most likely due to the very low expression of Bcl-xS in healthy neuronal cells (Roth and D’Sa, 2001).

Bcl-xL expression induces SMN expression in differentiated SH-SY5Y cells

Following these results, we expressed Bcl-xL to determine if it similarly affected SMN protein levels. Western blot and densitometric analysis revealed Bcl-xL expression increased SMN levels by 2-fold (Fig. 1B). Interestingly, the resulting increase in SMN protein levels was several fold lower, giving the first indication of potentially different mechanisms used to up-regulate each protein.

SMN and Bcl-xL additively block PI3-kinase inhibition induced apoptosis in SH-SY5Y cells

Previous work has identified a synergistic anti-apoptotic action of SMN and Bcl-2 (Iwahashi et al., 1997). Given the closely related functions of Bcl-2 and Bcl-xL, we postulated that co-expression of Bcl-xL and SMN could show a similar pro-survival anti-apoptotic effect. To test this hypothesis, we utilized a PI3-kinase inhibition cell death model described previously (Anderton et al., 2011). Similar to previous studies, expression of Bcl-xL or SMN significantly increased SH-SY5Y cell viability (Anderton et al., 2011) compared to controls (Fig. 2A). As postulated, following PI3-kinase inhibition, co-expression of Bcl-xL and SMN significantly increased cell viability.

Fig. 1. Co-regulation of SMN and Bcl-xL in differentiated SH-SY5Y cells. (A) Western blot and densitometric analysis of Bcl-xL levels following adenoviral expression of Bcl-xL, SMN and empty vectors. SMN expression significantly increased Bcl-xL protein levels approximately 6-fold \((**P < 0.01, n = 4 \text{ experiments})\), as indicated by densitometric analysis. (B) Western blot and densitometric analysis of SMN levels following adenoviral expression of Bcl-xL, SMN and empty vectors. Conversely, Bcl-xL expression induced a 2-fold increase in SMN protein levels \((^*P < 0.05, n = 4 \text{ experiments})\).
compared to either SMN or Bcl-xL expression (Fig. 2A). The differences in cell viability can be attributed to an additive anti-apoptotic effect when Bcl-xL and SMN are co-expressed. To investigate these findings further, caspase-3 and calpain cleavage patterns of α-fodrin, were determined. Consistent with the viability data, co-expression of SMN and Bcl-xL appeared to reduce α-fodrin cleavage, as evidenced by reduced levels of the 145/150 and 120 kDa cleavage product (Fig. 2B).

**Bcl-xL and SMN expression increases endogenous full-length SMN transcript levels in differentiated SH-SY5Y cells**

Real-time PCR was used to determine if exogenous Bcl-xL or SMN expression in SH-SY5Y cells could impact on endogenous SMN transcript levels. To distinguish between endogenous and adenoviral exogenous SMN, we used a reverse primer complimentary to the 3’ untranslated region of the SMN gene (Fig. 3B). Full-length SMN transcript levels were increased between ~1.4 and ~1.7-fold in Bcl-xL- and SMN-expressing SH-SY5Y cell cultures respectively, compared to control cultures (Fig. 3A).

To determine if exogenous Bcl-xL and SMN protein expression affect endogenous SMN mRNA transcript levels, we treated differentiated SH-SY5Y cells (transduced with either AdSMN, AdBcl-xL, or AdEmpty), with the transcriptional inhibitor, actinomycin D. Using quantitative real-time PCR, we measured endogenous SMN mRNA transcript levels at 4 and 8 h post actinomycin D addition relative to AdEmpty-transduced cells (Fig. 3C). We found that endogenous SMN mRNA transcript levels in AdSMN- and AdEmpty-transduced cells rapidly declined to below basal levels (set as fold induction 1; Fig. 3C) at 4 and 8 h post actinomycin D addition. By contrast, although SMN mRNA transcript levels in AdBcl-xL-expressing cells declined, they remained above basal levels at 4 h and just below basal levels at 8 h (Fig. 3C).

**Exogenous SMN increases full-length endogenous SMN transcripts in SMA patient fibroblasts**

To determine if exogenous increases in SMN can influence SMN2 splicing, we expressed SMN in type 1 SMA patient fibroblasts. We found that exogenous increases in SMN expression correlated with an increase in full-length endogenous SMN transcript levels, proportional to exon-7 excluded transcripts (Fig. 3D). We next assessed if Bcl-xL expression had a similar effect on SMN splicing in SMA fibroblasts. We found that Bcl-xL expression did not increase full-length SMN transcript levels proportional to exon-7 excluded transcripts (Data not shown).

**Bcl-xL and SMN expression reduce Sam68 levels in SH-SY5Y cells**

Our evidence is that splicing of endogenous SMN transcripts can be regulated by exogenous SMN expression.
To this end, we investigated the levels of Sam68, a protein that directly affects the splicing of both SMN and Bcl-xL transcripts. Depletion of Sam68 leads to increases in full-length SMN and Bcl-xL protein (Paronetto et al., 2007; Pedrotti et al., 2010). Therefore, Sam68 protein levels were assessed to determine if Bcl-xL and/or SMN expression could alter levels of this protein. Interestingly, Sam68 levels were reduced 2-fold in SH-SY5Y cultures expressing either Bcl-xL or SMN, compared to control cultures (Fig. 4).

**Fig. 3.** SMN and Bcl-xL expression increase endogenous SMN transcripts. (A) Quantitative real-time PCR of endogenous SMN transcript levels following adenoviral expression of SMN and Bcl-xL in differentiated SH-SY5Y cells (*P < 0.05; **P < 0.01). (B) Schematic showing the SMN primers used in quantitative real-time PCR. To detect only endogenous SMN transcripts, the reverse primer was positioned within the untranslated region. (C) Quantitative real-time PCR of endogenous SMN transcript following actinomycin D (5 μM) treatment. Differentiated SH-SY5Y cells were transduced with AdSMN, AdBcl-xL or AdEmpty prior to treatment with actinomycin D for 0, 4 and 8 h. (D) Gel RT-PCR and densitometric analysis of endogenous full-length and Δ7 SMN transcripts following a dose-like adenoviral expression of SMN in type I SMA fibroblasts.

**Bcl-xL levels are increased in PrP-SMN transgenic mouse brain but not spinal cord**

To determine whether co-regulation of SMN and Bcl-xL expression occurs *in vivo*, we used a transgenic mouse overexpressing SMN. Brain and spinal cord mouse tissue from PrP-SMN mice revealed that SMN levels were increased between 3 and 3.5-fold in brain and spinal cord tissue compared to levels in wild-type mice (Fig. 5). Levels of the pro-survival Bcl-xL protein were increased...
2.5-fold in PrP-SMN brain tissue. Conversely, the ratio of Bcl-xS/Bcl-xL was decreased 2-fold in PrP-SMN brain (Fig. 5A). Curiously, Bcl-xL protein levels and the ratio of Bcl-xS/Bcl-xL were unchanged in spinal cord tissue (Fig. 5B).

**DISCUSSION**

The loss of motor neurons by apoptosis is an often overlooked contributor of SMA disease pathogenesis (Simic et al., 2000; Tsai et al., 2006; Simic, 2008). This is surprising given that SMN prevents apoptosis by reducing/blocking caspase-3 activation (Vyas et al., 2002; Parker et al., 2008; Anderton et al., 2011), and interacts with key regulators of survival such as p53, Bcl-2 and ZPR1 (Iwahashi et al., 1997; Gangwani et al., 2001; Young et al., 2002). Similarly, Bcl-xL, which prevents apoptosis by blocking Bax, Bak and cytochrome-C-mediated apoptotic pathways (Gonzalez-Garcia et al., 1995; Kim et al., 1997; Merry and Korsmeyer, 1997) is down-regulated in both SMA patients and animal models (Soler-Botija et al., 2003; Tsai et al., 2008). Moreover, Bcl-xL deficient mice show extensive cell death in differentiating and immature neurons (Motoyama et al., 1995), thereby establishing an essential role for Bcl-xL in neuronal survival and development.

In a previous study we demonstrated that SMN or Bcl-xL over-expression reduced SH-SY5Y cell death and caspase-3 activity following PI3-kinase inhibition-induced apoptosis (Anderton et al., 2011). In the present study, we show that co-expression of SMN and Bcl-xL in SH-SY5Y cells provides an additive anti-apoptotic protective effect after PI3-kinase inhibition. Our findings are in line with a previous study showing a synergistic anti-apoptotic effect between Bcl-2 and SMN following Bax-mediated apoptosis in HeLa cells (Iwahashi et al., 1997). The additive protective effect of SMN and Bcl-xL co-expression in the PI3-kinase inhibition apoptotic model, indicate that protection is occurring via different pathways/mechanisms. Analysis of α-fodrin cleavage patterns support this additive effect, determining that caspase-3 activity is further reduced following SMN and Bcl-xL co-expression. Thus, our results suggest that combining SMN and Bcl-xL replacement therapy may be more effective in reducing motor neuron cell death in SMA, than SMN replacement alone.

In this present study, we also investigated if the expression of SMN and Bcl-xL are related. By doing so, we demonstrated that SMN and Bcl-xL expression is positively co-regulated in vitro, and that this co-regulation may involve the RNA-binding protein, Sam68. We report for the first time that Sam68 is down-regulated by SMN or Bcl-xL expression in SH-SY5Y cells, which implicates reduced Sam68 protein levels as a potential mechanism contributing to increased SMN or Bcl-xL expression. However, SMN expression resulted in a much higher fold induction of Bcl-xL protein, suggesting the activation of other related pathways for this protein. For example, Stats activation induces expression of Bcl-xL, as well as SMN (Kochendoerfer et al., 2003; Farooq et al., 2011).

To determine if our findings were reflected in vivo, we investigated Bcl-xL expression in transgenic mice expressing SMN driven by the mouse prion protein promoter (PrP-SMN). Previous studies have used the mouse PrP promoter to deliver high transgene expression in neurons (Wang et al., 2005). Gavrila et al. (2008) showed that an increase in SMN expression in PrP-SMN mice was limited to brain and spinal cord tissue (Gavrila et al., 2008). We report similar findings, in that SMN is increased 3–3.5-fold in both tissues. In addition, we demonstrated for the first time that Bcl-xL is up-regulated in the brain of PrP-SMN transgenic mice, possibly due to alternate splicing of Bcl-x. This relationship in brain tissue

![Fig. 4. SMN and Bcl-xL expression markedly reduce Sam68 protein levels. A representative Western blot of Sam68 levels and densitometric analysis relative to β-tubulin levels following adenoviral expression of SMN, Bcl-xL, and an empty vector in differentiated SH-SY5Y cells (**P < 0.01).](image-url)
is potentially important, as both Bcl-xL and SMN are key regulators of apoptosis during embryogenesis (Motoyama et al., 1995; Schrank et al., 1997; Simic et al., 2000), and a deficiency in either protein causes defects in brain development (Roth and D’Sa, 2001; Wishart et al., 2010). However, unlike mouse brain, Bcl-xL levels were not increased in PrP-SMN mice spinal cord tissue, highlighting the occurrence of tissue specific regulatory mechanisms. Alternatively, it is possible that another mechanism is responsible for increased Bcl-xL levels in brain tissue, and that the specific down-regulation of Sam68 and its splicing function observed in human cells may not occur in mice due to a species specific regulatory mechanism (Paronetto et al., 2007; Pedrotti et al., 2010). Nevertheless, our study suggests combining Bcl-xL and SMN delivery may be of benefit in reducing apoptosis and further brain developmental abnormalities in SMA.

Current therapeutic approaches to treat SMA are aimed at increasing SMN protein levels, most commonly by correcting defective SMN2 splicing or viral expression of SMN1 (Lorson et al., 2010; Park et al., 2010). In this study we also report that exogenous SMN expression increases endogenous full-length SMN1 and/or SMN2 transcripts in both SH-SY5Y cells and SMA patient fibroblasts. Following actinomycin D treatment, SMN transcript levels in cells transduced with AdSMN compared with the AdEmpty control vector decreased at a similar rate. Our findings suggest that the increase in endogenous SMN transcript levels observed in AdSMN-transduced cultures is unrelated to stabilization of endogenous SMN mRNA transcript. While the exact mechanisms involved in SMN self auto-regulation requires further elucidation, it is known that the ability of SMN protein to interact with itself greatly affects SMN protein stability and cell survival (Morse et al., 2007). Our data are also supported by a previous finding, whereby reduced SMN protein levels exacerbate SMN exon 7 skipping, causing a further reduction in SMN protein (Jodelka et al., 2010). Furthermore, we

Fig. 5. Bcl-xL expression is elevated in PrP-SMN mouse brains. SMN and Bcl-xL protein levels were detected from either wild-type or PrP-SMN brain and spinal cord tissue. (A) SMN and Bcl-xL protein levels were significantly increased relative to b-tubulin levels in PrP-SMN brain tissue compared to controls (**P < 0.01; ***P < 0.01). A significant difference in in the Bcl-xS/Bcl-xL ratio was also observed in the brain tissue of PrP-SMN mice compared with controls (P < 0.05). (B) SMN protein levels were significantly increased in PrP-SMN spinal cord tissue (**P < 0.01). Densitometric analysis revealed no significant differences in Bcl-xL protein levels or the Bcl-xS/Bcl-xL ratio.
also demonstrated the effectiveness of increasing SMN protein and transcript levels following Bcl-xL adenoviral expression. Investigation of how Bcl-xL expression affected SMN transcript levels revealed no changes in splicing of SMN2 in SMA patient fibroblasts, but, interestingly, showed a trend toward increased stability of endogenous SMN mRNA transcripts. Taken together, our findings provide evidence that the exogenous delivery of SMN or Bcl-xL protein to patients may increase the amount and/or stability of endogenous full-length SMN2 transcripts, and thus potentially enhance the effectiveness of this treatment approach.

In summary, by correlating SMN and Bcl-xL protein levels, this study provides further evidence for the role of apoptosis in SMA pathogenesis. In addition, we confirm a positive feedback-loop regulating SMN expression, whereby Sam68 levels are decreased by exogenous SMN delivery, thereby leading to a concomitant increase in endogenous full-length SMN2 transcripts. Taken together, our findings provide further evidence of secondary pathways by which the cell can regulate and increase SMN expression.

AUTHORS’ CONTRIBUTIONS

RSA carried out most of the experiments and conducted the data analysis. LLP and CG carried out quantitative real-time PCR and RT-PCR. BJT supplied and dissected transgenic and control mice. BPM participated in conceptual design and helped to draft and revise the manuscript. CM and SDW participated in experimental design and interpretation of data. FLM was involved in drafting and revising the manuscript. RSA and SB conceived of the study and participated in its design and wrote the manuscript. All authors read and approved of the final manuscript.

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Chapter 6

Investigation of a Recombinant SMN Protein Delivery System to Treat Spinal Muscular Atrophy

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Investigation of a Recombinant SMN Protein Delivery System to Treat Spinal Muscular Atrophy

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Signed…………………………………… Signed………………………………………….
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INVESTIGATION OF A RECOMBINANT SMN PROTEIN DELIVERY SYSTEM TO TREAT SPINAL MUSCULAR ATROPHY

Abstract

Spinal muscular atrophy (SMA), the most common genetic cause of infant death, is a neurodegenerative disorder affecting motor neurons. SMA results from a loss in full-length survival of motor neuron (SMN) protein due to deletions/mutations in the SMN1 gene. In this study, we assessed the ability of cell-penetrating peptides (CPP) to deliver recombinant SMN protein to cultured neurons as a prelude for a potential therapeutic to treat SMA. Firstly, we confirmed that E. coli produced recombinant GFP protein fused to TAT (YGRKKRRQRRR; TAT-GFP) transduced rat cortical neurons in a concentration dependent manner. However, due to low yields of recombinant TAT-SMN protein obtainable from E. coli, we investigated the potential of a modified TAT (TATκ: YARKAARQARA) or R9 (RRRRRRRRRR) peptide downstream of the fibronectin (FIB) secretory signal peptide to generate recombinant CPP-fused SMN. While U251 cells transduced with an adenoviral vector expressing CMV-FIB-TATκ-SMN secreted recombinant TATκ-SMN protein, we did not detect TATκ-SMN protein transduction of cortical neurons. Further, purified TATκ-SMN was unable to transduce SH-SY5Y cells, nor block apoptosis following LY294002 treatment of these cells. Our findings indicate that TATκ is not a suitable CPP to deliver SMN protein to neurons. Nonetheless, we have developed a novel method to generate full-length recombinant SMN protein using a mammalian expression system, which can be used to explore the application of other CPPs to deliver SMN protein as a treatment for SMA.

Keywords

- Spinal muscular atrophy
- Recombinant SMN protein
- Cell penetrating peptides
- TAT
- Adenoviral vectors

List of abbreviations

BBB - Blood-brain barrier
CPP - Cell penetrating peptide
DIV - Day in vitro
DMEM - Dulbecco's Modified Eagle Medium
FCS - Foetal calf serum
FIB - Fibronectin
GFP - Enhanced Green Fluorescent Protein
MOI - Multiplicity of infection
PBS - Phosphate buffered saline
PI3 - Phosphatidylinositol-3 kinase
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA - Spinal muscular atrophy
SMN1 - Survival of Motor Neuron 1 gene
SMN - Survival of motor neuron protein

Introduction

Spinal muscular atrophy (SMA), an often fatal autosomal recessive genetic disease, is characterised by the selective degeneration of spinal cord motor neurons, which causes muscle weakness and atrophy. With an incidence of one in 6,000 live births, SMA is the most common genetic cause of infant mortality. Clinically, SMA is characterised by profound muscle weakness, hypotonia and trunk paralysis, and depending on disease severity and age of onset [1], is classified into 3 main phenotypic subtypes (I-III). To date, there is no cure for SMA, making the development of potential therapies a high priority.

SMA is a monogenic disorder in which the survival of motor neuron (SMN1) gene is mutated or absent in more than 95% of cases [2]. During human evolution a 500kb inverted duplication of the SMN1 locus has given rise to a second copy of the gene, termed SMN2. The SMN2 gene is essentially identical to SMN1 but a translationally silent nucleotide change (C→T) in exon 7 causes the exons’ exclusion from SMN2 transcripts [3,4]. Consequently, only about 10% of the survival motor neuron 2 (SMN2) protein is functional, while the other 90% is truncated and unstable [5]. The occurrence of multiple SMN2 gene copies in patient groups correlates with increased full-length SMN transcripts, greater amounts of functional protein, and reduced disease severity [6,7]. Therefore, increasing SMN protein levels in SMA patients presents a feasible treatment approach, and has previously improved the phenotype and survival rates in SMA animal models [8].

While several candidate therapeutic strategies targeting SMN levels have been developed to treat SMA, they have been of limited success. For example, histone deacetylase (HDAC) inhibitors, antisense oligonucleotides, direct viral delivery, neuroprotective factors and exercise have all showed promise in pre-clinical trials [8-13]. In addition, several HDAC inhibitors, phenylbutyrate and valproic acid have failed to show any significant clinical benefits in SMA patients (reviewed in [14]). Therefore, there is a need to develop and assess new approaches to treat SMA.
In an earlier study, recombinant SMN was fused to the neuronal targeting diphtheria toxin fragment (TTC-DTx) and evaluated for cell transduction in primary neuronal cultures [15]. Whilst this approach proved unsuccessful, and cells failed to take up exogenous SMN protein, it highlighted a concept whereby exogenous SMN protein could be delivered to neurons if a suitable cell targeting peptide was available. In this respect, the 11 amino acid TAT peptide, which is derived from the HIV-1 TAT protein can mediate the efficient cellular transduction of protein cargos in vitro [16]. Moreover, TAT-fused peptides (and related peptides; e.g. Arginine-9; R9) can cross the blood brain barrier (BBB) and impart therapeutic benefits in animal models of disease [17-19].

Therefore, in this study, we investigated the feasibility of related TAT peptides (TATx and R9) to deliver recombinant SMN protein to neurons. In doing so, we developed a mammalian adenoviral vector secretory expression system to generate SMN protein and then assessed the delivery potential of SMN fused to TATx to enter neurons.

Experimental procedures

Rat primary cortical neuronal cell cultures

All animal procedures were approved by the University of Western Australia Animal Ethics Committee. Establishment of cortical cultures was as previously described [20]. Briefly, cortical tissue from E18 to E19 Sprague-Dawley rats were dissociated in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Melbourne, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM NaHCO3, 10 U/ml papain (Sigma, St. Louis, MO, USA) and 50 U/ml DNaseI (Sigma) and washed in cold DMEM/10% horse serum. Before seeding, wells (96 well microtitre plate; Nunc) were coated with poly-D-lysine overnight (50 ml/well: 50 µg/ml; 70 - 150K, Sigma). Excess poly-D-lysine solution was then removed and replaced with Neurobasal (containing 2% B27 supplement; 4% foetal bovine serum; 1% horse serum; 62.5 mM glutamate; 25 mM 2-mercaptoethanol; 10 µg/ml streptomycin and 10 mg/ml penicillin). Neurons were plated to obtain approximately 10,000 viable neurons in each well on day in vitro 12. Neuronal cultures were maintained in a CO2 incubator (5% CO2, 95% air balance, 98% humidity) at 37°C. On day in vitro 4, one third of the culture medium was removed and replaced with fresh Neurobasal/2% B27 containing the mitotic inhibitor cytosine arabinofuranoside (1 µM final concentration; Sigma). On day in vitro 8, one half of the culture medium was replaced with NB/2% B27.

Generation of fibronectin fused constructs

Relevant DNA sequences for each of the construct components are shown in Table 1. Briefly, the rat fibronectin sequence was fused to either a TATx-HA or R9-HA sequence. Constructs containing an SMN or GFP sequence were generated with a 6xHis sequence for protein purification and as an epitope for detection by Western blot analysis.

Cell maintenance, differentiation and transfection

HEK293, U251 and SH-SYSY cells were maintained in DMEM containing penicillin (10 µg/ml), streptomycin (10 µg/ml) and foetal calf serum (FCS; 5-10%; heat-inactivated), and incubated at 37°C in a 5% CO2 incubator. The differentiation of SH-SYSY cells using retinoic acid (15 µM; 5 days) has previously been described [21]. HEK293 cells (150,000) seeded in 24 well plates were transfected using Lipofectamine 2000® in OPTI-MEM serum-free media (Invitrogen, Melbourne, Australia) according to the manufacturer’s recommendations.

Adenoviral vectors and transduction of cells in culture

Recombinant adenoviruses were prepared according to the method of He et al. 1998 [22], with some modifications [23]. The FIB-TATx-SMN and FIB-TATx-GFP cDNA fragments were released by restriction enzyme digestion and ligated into a modified pShuttle plasmid (pShuttle-CMV/WPRE; [23]). Briefly, pShuttle plasmid DNA was linearized by Pmel digestion and electroporated (Gene Pulser II, Bio-Rad, CA, USA) into the Escherichia coli strain BJS183 carrying the pAdEasy plasmid [24]. HEK293 cells were transfected with 3 mg of PacI linearized recombinant plasmid DNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Following the appearance of viral plaques (5-10 days), culture lysates were used for viral amplification in HEK293 cells. Adenoviral particles (AdCMV-FIB-TATx-SMN or AdCMV-FIB-TATx-GFP) were purified and concentrated from HEK293 cell lysates using the Adeno-X kit (BD Biosciences, San Jose, CA). Adenoviral vectors expressing SMN (AdSMN) and a control vector (AdEmpty) have been described previously [21]. For adenoviral transduction of cells, adenovirus was diluted in serum free DMEM media for use at an appropriate MOI (50-300) prior to addition to cells in cultures.

Generation of recombinant GFP and TAT-GFP proteins in E. coli

GFP or TAT-GFP cDNA flanked by KpnI (5’ end) and Xhol (3’ end) restriction enzyme sites was inserted into a variant of the E. coli pET28a expression vector (Novagen, Merck KGaA, Darmstadt, Germany) containing compatible sites. The growth of KRX cells carrying this plasmid and induction of CYP4 protein was essentially as per the manufacturer’s recommendations.

Table 1. Sequences used in the construction of the TAT and R9 secretory constructs.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIB</td>
<td>CTCAAGGGTCCGGACCCGGGGCCTGCTGCTGACAGTCCCGGTGGGACATCGGTGGTCCCGAACCAGAAGAGCAAGAGGGCCGCCGC</td>
</tr>
<tr>
<td>TATx</td>
<td>TACGCTGTAAGGACGTCTAGGCTTGTCGCCCTGGAACGCAAGAGCAAGAGGGCCGCCGC</td>
</tr>
<tr>
<td>R9</td>
<td>GGCGACGCTTGTCGCCCTGCTGACAGTCCCGGTGGGACATCGGTGGTCCCGAACCAGAAGAGCAAGAGGGCCGCCGC</td>
</tr>
<tr>
<td>HA</td>
<td>CTATCCTAGGGTCCAGATGACCA</td>
</tr>
</tbody>
</table>
Generation and purification of recombinant TAT-GFP and TAT-SMN proteins from mammalian cells

Human U251 glioma cells cultured in DMEM containing 5% FCS were transduced with AdCMV-FiB-TATx-SMN or AdCMV-FiB-TATx-GFP (MOI of 30) and incubated for 48 hours prior to cell lysis and protein extraction. Cell supernatants were harvested, combined with lysis buffer (20 mM Tris-HCl; pH 7; 200 mM NaCl; 10% w/v glycerol). Protein purity and concentrations were determined by SDS-PAGE electrophoresis followed by Coomassie staining and Bradford assays (Bio-Rad) respectively.

Transduction of recombinant proteins in culture

Recombinant proteins (0.5 μM to 5 μM) generated from bacterial or mammalian cells were tested for transduction activity in the following manner. Media was removed and proteins diluted in 100 μl of serum free DMEM media were added to cells in a 96 well plate for 1 hour. The cultures were washed 3 times with balanced salts solution (BSS; 116 mM NaCl mM, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 1 mM NaH2PO4 pH 7) prior to the analysis by fluorescent microscopy, cell viability assessment or protein lysis preparation.

PI3-kinase inhibition apoptotic model

Assessment of the anti-apoptotic function of SMN using the phosphatidylinositol-3 kinase (PI3-kinase) inhibitor, LY294002 (Sigma), has previously been described [21]. Briefly, LY294002 was diluted in serum free media to a final concentration of 25 μM prior to addition to differentiated SH-SYSY cells.

Cell viability assessment

Cell viability was assessed using the MTS assay (Promega, Madison, WI, USA). The MTS assay measures the cellular conversion of tetrazolium to the water-soluble formazan product, which is detected spectrophotometrically at 495 nm.

Protein extraction and western blotting

Proteins were extracted from cultured cells using RIPA lysis buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate and 1 mM EDTA). Western blotting has been described previously [21]. Briefly, membranes were blocked in PBS-Tween 20 (0.1%) containing ovalbumin (1 mg/ml) for 1 hour and incubated overnight with SMN primary antibodies (1:3000; Santa Cruz, Santa Cruz, CA, USA), HIS primary antibodies diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml). Proteins were detected using a HRP complexed secondary antibody (1:15000-1:35000 donkey anti-rabbit/sheep anti-mouse; GE Healthcare, Piscataway, NJ, USA), and visualised using ECL plus detection reagent (GE Healthcare). Quantification and band densitometry of Western blots was undertaken using ImageJ (NIH) software.

Immunohistochemistry

U251 glioma cells were grown in 24 well plates and transduced with adenoviral vectors for 48 hours. Cells were fixed in 10% formaldehyde, rinsed in PBS, blocked with 10% normal goat serum for 10 minutes, and exposed to anti-SMN antibody (1:200; Santa Cruz) diluted in PBS with 1% goat serum and incubated at 4°C overnight. Cells were washed in PBS prior to the addition of the secondary antibody (Alexa Fluor 594 goat anti-mouse, Invitrogen; 1:400) and incubated for 1 hour at 4°C. Fluorescence microscopy (Olympus IX70; Olympus DP70 digital camera) was used to analyse adenoviral vector protein expression.

Results

Recombinant TAT-GFP is capable of transducing rat cortical neurons

Fluorescence microscopy and western analysis confirmed that E. coli generated recombinant TAT-GFP, but not GFP, was capable of transducing cultured neurons in a concentration dependent manner (Figure 1A and 1B). Transduction of TAT-GFP was highly effective (60%-80% of cells GFP positive) at the two highest concentrations (2 and 5 μM) tested. By contrast, transduction of the native GFP was not detected at any concentration.

The expression of TAT-SMN and TAT-SMN-GFP in E. coli

Next, we set out to generate a recombinant TAT-SMN protein in E. coli. Whilst recombinant, TAT-SMN protein was not evident by SDS-PAGE (Figure 2A), presumably due to low yields,
Western analysis revealed a protein band, consistent with the predicted molecular weight of TAT-SMN protein (Figure 2B).

Given the high recombinant TAT-GFP protein recovery rates we obtained from E. coli, we postulated that GFP may enhance SMN protein recovery. Therefore, we generated and expressed the SMN protein as a larger fusion protein with GFP (TAT-SMN-GFP). However, although TAT-SMN-GFP fusion protein yields were significantly increased as revealed by SDS-PAGE analysis, two distinct protein bands were detected (Figure 2C). Western analysis using a specific anti-SMN antibody detected multiple bands similar to and larger than the molecular weight of the SMN protein (Figure 2D). These results suggest that the TAT-SMN-GFP fusion protein may be subject to cleavage, degradation or incomplete translation in E. coli.

**Generation of recombinant SMN fused to the fibronectin signal peptide**

The development of a plasmid vector capable of secreting TAT-fused GFP from HEK293 cells has been previously described [25]. The study determined that efficient secretion of intact TAT-fused proteins requires the furin proteolytic sites within TAT to be mutated. Hence, the original TAT sequence YGRKKRRQRRR was modified to YARKAARQARA (referred to as TATκ; [25]). Importantly, TATκ was shown to retain protein transduction efficiency. In our system, we used the adenoviral pShuttle plasmid expression vector, which incorporates the woodchuck post-transcriptional regulatory element (WPRE) for high-level expression [26]. GFP or SMN protein was fused to the rat fibronectin (FIB) signal peptide sequence and either the TATκ or R9 CPP sequence (Figure 3A).

**FIB-CPP-GFP transfected HEK293 cells show a secretory phenotype**

To determine if FIB-TATκ-GFP and FIB-R9-GFP secretory constructs induce the characteristic appearance of a secretory protein, HEK293 cells were transfected and assessed via fluorescence microscopy. Consistent with previous findings [25,27], HEK293 cells transfected with the pShuttle FIB-TATκ-GFP plasmid exhibited a weak intracellular GFP signal (Figure 3B), in which GFP fluorescence appeared to be
localised to the cell membrane. By contrast, HEK293 cells transfected with the FIB-R9-GFP plasmid showed strong punctate cytoplasmic fluorescence (Figure 3B).

Confirmation of adenovirual vector expression of FIB-TATκ-GFP and FIB-TATκ-SMN proteins in U251 cells

Using U251 cells, we assessed the efficacy of adenoviral (MOI 30) mediated expression of FIB-TATκ-GFP by fluorescence microscopy, and FIB-TATκ-SMN by immuno-fluorescence (Figure 4A). Cells transduced with AdCMV-FIB-TATκ-GFP exhibited membrane localised GFP fluorescence consistent with GFP secretion (Figure 4A). Immuno-fluorescent staining for SMN of AdCMV-FIB-TATκ-SMN transduced cells was cytoplasmic and diffuse (Figure 4A). By contrast, immuno-fluorescent staining for SMN in cells transduced with the AdCMV-SMN control vector was cytoplasmic, but punctate in appearance.

Using GFP, SMN and HIS antibodies, Western analysis of lysates from U251 cells transduced with the different adenoviral vectors confirmed the expression of TATκ-GFP and TATκ-SMN proteins (Figure 4B). To confirm that TATκ-GFP and TATκ-SMN were secreted, cell culture supernatants from transduced U251 cells were analysed by Western analysis. Both the TATκ-GFP and TATκ-SMN proteins were detected in culture supernatants using the HIS antibody and their respective protein specific antibodies (Figure 4C).

Recombinant TAT fused proteins can be purified from transduced U251 cell lysates and culture supernatants

Recombinant SMN protein derived from human cells, such as U251 cells, are likely to be correctly folded and post-translationally modified, which reportedly is required for full functional activity [28]. Recombinant TATκ-GFP and TATκ-SMN proteins were isolated and purified from culture medium, and from U251 cell lysates, using the 6xHIS tag and Ni²⁺ affinity chromatography (Figure 5A). Following SDS-PAGE analysis protein bands consistent with the predicted molecular weights for TATκ-GFP and TATκ-SMN proteins were only detected from U251 cell lysate preparations at very low yields (estimated at 0.1 mg/ml; Figure 5B). However, following Ni²⁺ affinity chromatography of adenovirally transduced U251 cell culture supernatants and lysates, Western analysis detected purified forms of the TATκ-GFP and TATκ-SMN proteins from both preparations (Figure 5C).

Purified TATκ-SMN and TATκ-GFP proteins do not transduce cortical neurons

To determine if recombinant TATκ proteins purified from supernatants and cell lysates could transduce cortical neurons, we added TATκ-GFP or TATκ-SMN proteins (0.5-1 μM) to cultures. However, Western blot analysis of lysates failed to detect any transduced TATκ-SMN or TATκ-GFP protein (Figure 6A). Similarly, recombinant TATκ-fused proteins purified from supernatants of U251 cultures infected with the FIB-TATκ-GFP and FIB-TATκ-SMN expressing adenoviral vectors was also unable to transduce neurons when compared to controls (Figure 6B).

TAT-SMN does not rescue SH-SY5Y cells from apoptosis

While protein transduction of TATκ-SMN was not detectable by Western analysis (Figure 6A and 6B), we hypothesised that even undetectable levels of TATκ-SMN was potentially functional and neuroprotective. Thus, to assess the functionality of transduced U251 cell supernatant and purified TATκ-SMN protein, we used an apoptotic cell death model that we had developed previously, in which exogenous SMN over-expression protects SH-SY5Y cells from LY294002 mediated PI3-kinase/Akt inhibition [21]. Treatment of differentiated SH-SY5Y cells with supernatant from transduced U251 cells was ineffective at reducing LY294002 induced cell death compared to controls (Figure 6C). In addition, purified TATκ-SMN protein (~0.5 μM) from U251 transduced cultures was ineffective at reducing LY294002 induced SH-SY5Y cell death (Figure 6D).

Discussion

The most promising therapies currently being developed to treat SMA are aimed at increasing SMN protein levels, indirectly, by redirecting SMN2 splicing or directly, by using viral delivery of an SMN transgene [29,30]. However, a number of SMA therapies, developed on the basis of promising animal and in vitro experimentation, have either failed to provide therapeutic benefits or remain in preclinical testing [31,32]. Thus, it is imperative to explore
and develop novel therapeutic approaches. One possibility involves the delivery of SMN protein directly to the central nervous system using CPPs, such as TAT, which are capable of transporting their cargo across the blood-brain barrier (BBB) and into cells of the central nervous system [33].

In the present study, we first confirmed that the TAT peptide can promote the transduction of the GFP protein across neuronal membranes. Consistent with previous studies [33-35] we observed a dose dependent increase in the transduction of a TAT fused GFP. To determine if TAT could facilitate the transduction of SMN into cells, we generated an E. coli expression vector to express a TAT-SMN recombinant protein. However, only small amounts of TAT-SMN protein were recovered, suggesting that protein was retained within inclusion bodies. To overcome this hurdle, we generated a fusion TAT-SMN-GFP protein to improve solubility and increase SMN protein yield. However, while total protein yield was increased, Western analysis showed that the SMN fusion protein was proteolytically cleaved and/or inefficiently translated in the E. coli expression system. Thus it would appear that the future success in generating an SMN CPP fusion protein will require an improved host expression system, redesign of the expression construct, including the elimination of any putative protease cleavage sites, and improvements to the protein purification methodology.

In lieu of resolving the problems outlined above, we opted for an alternative to the bacterial expression of TAT-SMN protein. In this instance we developed an adenoviral vector to secrete CPP-fused proteins from mammalian cells. This approach was also adopted because it broadened the applicability of CPPs for in situ therapeutic protein delivery. The secretion of proteins from mammalian cells is well characterised [36], and has shown potential in the treatment of neurodegenerative disorders [37-39]. Fusion of a therapeutic protein to the fibronectin signal peptide, an endoplasmic reticulum targeting motif, triggers its secretion via the constitutive pathway. Secretion is initiated following cleavage of the signal peptide and is completed by molecular trafficking events which deliver the protein to the plasma membrane [40]. In deciding a CPP to adopt for the secretory system, we chose to compare the action of a modified TAT peptide (TATx) and R9. While the TATx peptide is not cleaved by the convertase enzyme furin, R9 is cleaved by this enzyme [41]. Transient transfection of pShuttle plasmids expressing FIB-TATx-GFP in HEK293 cells appeared to confirm that TATx fused GFP was secreted, as only weak cytoplasm expression was observed. In contrast, HEK293 cells transfected with the pShuttle plasmid expressing FIB-R9-GFP revealed GFP to accumulate as aggregates within the cytoplasm, as would be expected if the fusion protein was being cleaved by furin.

To achieve protein expression in multiple cell lines, we constructed adenoviral vectors expressing FIB-TATx-SMN and the control protein FIB-TATx-GFP. A similar approach using an adeno-associated viral vector to express FIB-galanin demonstrated that galanin was efficiently secreted and capable of targeting NMDA receptors [37]. Immuno-detection/fluorescence and Western analysis of AdCMV-FIB-TATx-SMN and AdCMV-FIB-TATx-GFP transduced U251 cells confirmed secretion of the proteins. In addition, molecular weight estimates based on Western analysis confirmed
that the FIB signal peptide was removed, consistent with secretion via the constitutive pathway. Further, immuno-fluorescent staining of AdCMV-FIB-TATκ-SMN and AdCMV-FIB-TATκ-GFP transduced U251 cells confirmed the characteristic low level cytoplasmic localisation of the respective proteins. Together, these results provided confirmation of the methodological basis for using this system to produce secretable CPP-fused recombinant proteins.

To purify secreted recombinant proteins from our mammalian expression system, we incorporated a 6xHIS tag to the N-terminal of each protein. Next, we confirmed that TATκ-SMN and TATκ-GFP could be purified and concentrated using the HIS tag from both the cell culture supernatant and from cell lysate. In addition, Western analysis of the purified TATκ-SMN and TATκ-GFP using an anti-HIS tag antibody confirmed that the proteins retained their N-termini indicating the presence of an intact TATκ peptide sequence. While recombinant proteins were successfully purified, the yields were low. Due to limitations in the culturing conditions such as low overall cell numbers and reduced culture volumes compared to bacterial systems, low recombinant protein yields are commonly encountered when using small scale eukaryotic expression systems. Nonetheless, proteins purified from eukaryotic cell lysates are free of potential toxic bacterial cell products and are more likely to possess the necessary post-translational modifications. Finally, it should be noted that the SMN secretory system could easily be scaled-up to produce enough TATκ-SMN protein for assessment in SMA mouse models.

We observed that purified TATκ-GFP or TATκ-SMN protein from transduced U251 cells was unable to transduce cortical neurons. To exclude the possibility that the purification process may have affected TATκ transduction activity, culture medium from adenoviral transduced CMV-FIB-TATκ-GFP and CMV-FIB-TATκ-SMN cells was added directly to neuronal cultures. Similar to the purified TATκ-GFP or TATκ-SMN proteins, culture medium containing these proteins did not result in any detectable neuronal transduction. Our

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**Figure 5.** Purification and assessment of TATκ-GFP and TATκ-SMN recombinant proteins from mammalian U251 cells. (A) Schematic depicting recombinant protein purification methodology. (B) Coomassie staining of recombinant TATκ-fused proteins purified from U251 cellular extracts and supernatants by 6xHis tag affinity purification. (C) Western blot analysis of TATκ-fused proteins purified from U251 cellular extracts and supernatants by 6xHis tag affinity purification.
results contradicted those of a previous study using the TATκ peptide, which demonstrated TATκ-GFP secretion from a pSeTag2-TATκ-GFP transfected stable cell line, and subsequent transduction of the protein in H357 and Saos-2 cultures [25]. However, it should be noted that the contrasting results could be due to primary neuronal cultures being used in the present study, while established non-neuronal cell lines were used in the [25] study. We previously demonstrated that SMN or Bcl-xL protein over-expression can reduce SH-SY5Y cell death and caspase-3 activity following PI3-kinase inhibition induced apoptosis [21,42]. In the present study, we aimed to replicate the neuroprotective function of SMN by transducing cells with purified TATκ-SMN protein and culture medium containing TATκ-SMN protein. Treatment of SH-SY5Y cells with TATκ-SMN showed no improvement in cell viability compared to controls. The inability of TATκ-SMN protein to reduce PI3-kinase inhibition induced apoptosis could be related to the inability of the SMN fusion protein to transduce SH-SY5Y cells and reach therapeutic intracellular levels, or interference of the TATκ peptide with SMN activity. This study has developed an adenoviral expression vector capable of secreting and facilitating the purification of recombinant proteins expressed in mammalian cells. Although purified TATκ-SMN protein did not show functionality in transduction or viability assays, this may be due to low protein yields. Nonetheless, the methodology described in this study could be applied in other situations where secreted recombinant proteins with or without a fusion to a CPP may have a clinical benefit.

Acknowledgements

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Chapter 7

General Discussion
GENERAL DISCUSSION

Background

Spinal muscular atrophy is a debilitating and fatal disease in which there is degeneration of motor neurons and progressive muscular paralysis. Molecular studies have determined that the disease is linked to a critical reduction in SMN protein levels within neuronal populations of the central nervous system (CNS) (Coovert et al., 1997). However, while a strong correlation exists between SMN levels and disease, the complete function of the SMN protein has yet to be elucidated, leaving many questions unanswered. Arguably, a more complete understanding of the exact mechanisms used by the SMN protein to promote neuronal survival will also allow for improved understanding of SMA disease pathogenesis and progression. Moreover, there are currently no effective therapeutic approaches that can slow down or limit the disease severity and progression in SMA patients. Thus, there is an urgent need to explore the neuroprotective function(s) of the SMN protein, as it could lead to the development of novel targeted therapies for SMA.

Significance of Findings Generated From This Thesis

The role of apoptosis in SMA

Apoptosis involves the removal of unwanted cells that would normally impede the survival of an organism. This commonly occurring phenomenon is an essential requirement for normal development in the CNS, and functions to rapidly remove excess neurons, and neurons that fail to establish the appropriate synaptic connections
(Elmore, 2007; Oppenheim, 1991). In SMA, previous studies have reported significant dysregulation in apoptosis, anti- and pro-apoptotic gene expression, and neuronal migration (reviewed in Chapter 3). Studies from this thesis have pointed to an anti-apoptotic role for SMN in both neuronal and non-neuronal cells, findings which have revealed a novel role for SMN in regulating pro-survival cell signalling. Further, a large component of this thesis aimed to explore, review and better understand the anti-apoptotic properties of the SMN protein.

*Development of an in vitro apoptotic model to assess SMN activity*

While *in vitro* studies have assessed SMN expression and cell survival (Parker et al., 2008; Vyas et al., 2002; Wang et al., 2005), no previous study had used a human cell line or a specific pro-survival pathway inhibitor to induce apoptosis in order to investigate SMN protein function. The aim of this study was to establish a reliable and convenient apoptotic cell death model to assess the pro-survival function(s) of the SMN protein. The results of this study demonstrated that following differentiation, human SH-SY5Y cells appear neuron-like and become more sensitive to cellular insults. This hypersensitivity stress is not unexpected, as previous work has reported elevation in neuronal-specific markers (Korecka et al., 2013) and a neuronal-like phenotype (Agholme et al., 2010; Encinas et al., 2000) following cellular differentiation.

Following SMN over-expression in differentiated SH-SY5Y cells, this study demonstrated a lack of neuroprotection following hydrogen peroxide treatment, and confirmed that SMN expression does not protect cells from glutamate-induced excitotoxicity (Cisterni et al., 2001). These results indicate that the SMN protein cannot function to prevent necrotic-induced cell death, at least in the selected conditions.
(Chapter 4). To establish a selectively apoptotic cell death, a PI3-kinase/AKT inhibitor, LY294002, was used to induce apoptosis through a predominately caspase-dependent pathway. The apoptotic nature of this in vitro model was confirmed by studying alpha-fodrin cleavage patterns and confirming caspase-3 activation (Anderton et al., 2011) in differentiated SH-SY5Y cells.

Studies on the anti-apoptotic role of SMN

Using the newly developed apoptotic cell death model, SMN protein over-expression was shown to significantly improve cell viability and reduce caspase-3 activation following treatment with the PI3-kinase/AKT inhibitor (Chapter 4). Caspase-3 is commonly activated by the removal of its 3 kilodalton pro-domain, generating an activated 29 kilodalton subunit (Han et al., 1997; Meergans et al., 2000). Over-expression of the SMN protein blocked the cleavage of this 3 kilodalton pro-domain, resulting in a reduction in activated caspase-3 protein. While similar studies have identified a caspase-3 reduction following SMN expression (Parker et al., 2008; Vyas et al., 2002; Wang et al., 2005), this study is the first to demonstrate a specific mechanism by which the SMN protein promotes cell survival.

The relationship between the SMN and Bcl-xL proteins

To further understand the cellular pathways used by the SMN protein, this thesis examined one of the key regulators of cell survival, the B-cell lymphoma extra-large (Bcl-xL) protein. A member of the Bcl-2 family, Bcl-xL is important in neuronal development (Motoyama et al., 1995) and prevents apoptosis by blocking Bax, Bak and cytochrome-C-mediated apoptotic pathways (Gonzalez-Garcia et al., 1995; Kim et al.,
Previous studies have shown that both Bcl-2 and Bcl-xL are down-regulated in the CNS during development in SMA patients (Soler-Botija et al., 2003). Further, an interaction between Bcl-2 and SMN highlighted the importance of this family of anti-apoptotic proteins in SMA (Iwahashi et al., 1997). In exploring the effect of SMN over-expression, this study observed Bcl-xL up-regulation in differentiated SH-SY5Y cells \textit{in vitro}, and in the brains of transgenic mice over-expressing SMN (PrP-SMN; Chapter 5). However, while elevated Bcl-xL expression was observed in whole brain tissue, no changes were observed in spinal cord tissue. These results are intriguing, as clinically, SMA affects the anterior horn of the spinal cord. Thus, such findings suggest a relationship with Bcl-xL may be specific to populations of cells located within the brain, but require further experimentation to investigate this. In addition, this relationship extended both ways, and Bcl-xL over-expression elevated SMN protein levels in SH-SY5Y cells. Not surprisingly, studies aimed at treating SMA have used Bcl-xL expression to partially rescue SMA deficits \textit{in vitro} (Garcera et al., 2011) and \textit{in vivo} (Tsai et al., 2008). Findings from this study have established a clear correlation between SMN and Bcl-xL expression, strengthening the link between SMN and the regulation of survival.

\textit{Insight into pathways used to promote SMN expression}

Current research into SMA has focussed on therapies to promote full-length SMN protein expression. In this thesis, several mechanisms to promote SMN expression were identified. One such mechanism involved the Src substrate associated in mitosis of 68 kilodaltons (Sam68), an RNA binding protein, which negatively modulates the splicing of both the \textit{Bcl}-\textit{x} and \textit{SMN} genes (Paronetto et al., 2007; Pedrotti et al., 2010). While investigating Sam68, this study showed that over-expression of SMN or Bcl-xL proteins
reduced Sam68 protein expression (Chapter 5). The finding of reduced Sam68 expression suggests that a possible feedback loop exists which promotes the expression of both SMN and Bcl-xL proteins. While previous studies have shown that over-expression of Sam68 reduced Bcl-xL and full-length SMN protein levels (Paronetto et al., 2007; Pedrotti et al., 2010), this study is the first to show that levels of the SMN and Bcl-xL protein can feed backwards to affect Sam68 levels.

To confirm a feedback mechanism affecting SMN protein levels, this study explored what effect SMN over-expression had on endogenous full-length SMN transcript. Self-regulation of SMN has previously been shown in SMA patient cells, whereby reduced SMN protein levels result in a further reduction of SMN expression (Jodelka et al., 2010; Ruggiu et al., 2012). While confirming a self-regulatory mechanism, this study implicated a Sam68 mediated pathway in the promotion of exon-7 inclusion in SMN2 transcripts. Specifically, these results indicate that SMN and Bcl-xL over-expression may be affecting the phosphorylation status of Sam68. Therefore, this study has suggested that upstream promoters of SMN expression may also be affected following reduced SMN protein levels in SMA (Anderton et al., 2013), presenting further potential targets in SMA therapeutics.

Confirmation of TAT mediated protein transduction activity and the development of a novel TAT-SMN protein

While numerous therapies have shown promise in vitro and in SMA animal models, there is currently still no effective treatment for SMA. This study hypothesised that it may be possible to deliver a recombinant SMN protein fused to a CPP directly to cells. A similar approach has previously been attempted, whereby a recombinant SMN
protein was fused to the tetanus toxin C-Fragment. While the transduction of SMN appeared to be impeded (Francis et al., 2004), this approach has since been used with success for the delivery of numerous proteins to the CNS (Toivonen et al., 2010). To overcome problems facing SMN transduction, this project developed a novel SMN protein fused to the CPP, TAT. Cell penetrating peptides, such as the TAT domain, offer an established means of delivering therapeutic proteins across the blood brain barrier (BBB) and into cells (Fawell et al., 1994; Schwarze et al., 1999).

To confirm TAT functionality, this study first showed that a TAT-fused green fluorescent protein (GFP) readily transduced cortical neurons in a dose-dependent manner (Chapter 6). Subsequently, this study attempted to generate a recombinant TAT-SMN protein. In contrast to the production of TAT-GFP, initial results showed extremely low amounts of recoverable TAT-SMN protein, possibly because of extensive inclusion body formation within the host *E. coli* cells. To overcome this problem, a TAT-GFP-SMN fusion protein was generated to increase protein solubility and avoid inclusion body formation. However, the fusion protein appeared to be cleaved, most likely by endogenous *E. coli* proteases. For future work, a better understanding of the SMN protein chemistry and use of a superior purification technique would be required to enable successful generation of TAT-SMN from bacteria.

*Development of a novel fibronectin signal peptide fusion vector*

An additional aim of this project was to develop a novel vector capable of secreting recombinant TAT-fused SMN protein. This vector served two purposes, a mammalian system for the generation of recombinant proteins, and a novel therapeutic for use in
In designing the vector it was necessary to include an appropriate secretory signal, six histidine residues for protein purification and an efficient CPP for protein transduction. The rat fibronectin (FIB) sequence was utilised in this vector because of its efficient secretion of fused proteins via the constitutive secretory pathway. The FIB sequence has been used to secrete GFP and the neuroprotective protein, Galanin, in both in vitro and in vivo disease models (Haberman et al., 2003). Further, an adeno-associated virus (AAV) expressing FIB-Galanin successfully attenuated seizures and neuronal death in epileptic animal models (Haberman et al., 2003; McCown, 2006).

Incorporation of the TAT peptide ensured that the recombinant proteins contained an inherent transduction activity. While recent studies had demonstrated the feasibility of generating TAT-fused proteins from mammalian secretory vectors (Flinterman et al., 2009; Koutsokeras and Kabouridis, 2009; Ma et al., 2012), the newly designed vector included additional modifications to improve protein expression, secretion and recovery, such as a histidine tag and woodchuck post-transcriptional regulatory element (Boulos et al., 2006). These results confirmed that the TAT-fused proteins were expressed and secreted at the predicted molecular weights, indicating that cleavage of the FIB signal sequence occurred prior to secretion. In addition, TAT-GFP and TAT-SMN were generated efficiently using human cell lines, increasing the likelihood of proteins bearing the appropriate post-translational modifications.

Purification and assessment of a novel TAT-SMN protein from mammalian cells

Recombinant TAT-SMN and TAT-GFP protein secreted from adenovirally transduced cells were collected and purified using the six histidine residues engineered onto the carboxyl end of these proteins. While the purified protein was extremely pure, the total
yield was lower than expected. Low protein yields are a common problem faced when using eukaryotic expression systems. Nevertheless, the successful purification of secreted protein demonstrated the capacity of this vector for recombinant protein production.

The final aim of this thesis was to assess the recombinant TAT-SMN protein for functionality. As purification via the histidine tag confirmed the presence of the downstream TAT peptide, this study then assessed the transduction activity of the TAT-SMN and TAT-GFP proteins. Purified TAT-SMN or TAT-GFP did not appear to transduce cortical neurons in culture, nor did supernatants containing these proteins. These results indicated a lack of protein activity pre- and post-purification. In addition, despite a lack of detectable transduction in neuronal cultures, TAT-SMN recombinant protein was assessed in the previously described in vitro apoptotic model (Chapter 4). In contrast to adenoviral over-expression of SMN, the addition of TAT-SMN protein was not able to reduce apoptosis in differentiated SH-SY5Y cells (Chapter 6). This negative finding suggested that therapeutically sufficient levels of SMN protein were not entering cells. In comparison, similar studies have shown that secreted TAT-fused proteins can transduce cells when cultured in the presence of secreting cells, or incubated with the secreted protein (Flinterman et al., 2009; Koutsokeras and Kabouridis, 2009). The exact reason for the lack of detectable transduction of secreted TAT-SMN and TAT-GFP proteins in the present study has not been determined and requires further investigation.
Study Importance

The results from this study have led to the establishment and assessment of an in vitro apoptotic model for the evaluation of SMN protein function. Using this model, results have shown that over-expression of SMN protein protects human differentiated SH-SY5Y cells from an apoptotic cell death stimulus, re-affirming an important anti-apoptotic function of this protein. In addition, for the first time this work identified a specific cellular mechanism used by SMN to block caspase-3 activation and thereby reducing apoptosis. Using this model, this thesis also identified a novel relationship between the SMN and Bcl-xL proteins. This important relationship confirmed an essential role for the SMN protein in survival, and will allow future work to explore a previously neglected pathway in SMN expression. Finally, this project generated and assessed a novel recombinant TAT-SMN protein as a potential therapy for SMA. While this study succeeded in generating recombinant TAT-SMN, protein transduction was not detected, indicating that in its present format, a TAT-fused delivery system is not a viable therapeutic strategy for SMA.

Conclusions and Future Implications in SMA Research

The results presented in this thesis have explored and added to our understanding of the mechanisms of action of the SMN protein. This work established an important function for the SMN protein in normal cell survival and a novel function in blocking caspase-3 activation. Further, the identification of a potential auto-regulatory SMN pathway, and the identification of a reciprocal relationship with the Bcl-xL protein has greatly improved knowledge on SMN protein function. Since the discovery of these findings, expression of SMN has been linked to Notch signalling (Caraballo-Miralles et al., 2013)
and the activation of the Akt/CREB pathway (Branchu et al., 2013), processes involved in neurogenesis and neuronal pro-survival respectively (Datta et al., 1999; Xiao et al., 2009). However, recent evidence has suggested crosstalk between ERK and Akt pathways, whereby inhibition of ERK can promote Akt/CREB activation and SMN2 expression in SMA mice (Branchu et al., 2013). Encouragingly, combined with findings from this thesis, future research into pathways promoting SMN expression will provide insight into treating SMA.

In addition to mechanistic studies on the SMN protein, the newly developed apoptotic model presented in this thesis has the potential for use as a screening tool in the assessment of therapies aimed at increasing SMN protein levels. Further, previous in vitro models using non-human cell lines have lacked the smn2 gene, thereby limiting their future use in therapeutic drug screening in SMA research. However, since the development of this apoptotic model, induced pluripotent stem cell derived SMA motor neurons have shown promise in accurately modelling the disease in vitro (Ebert et al., 2009; Sareen et al., 2012). Confirming results from this thesis, deficits in SMA motor neurons could be alleviated by the addition of a caspase-3 inhibitor (Sareen et al., 2012). Taken together, these findings have confirmed the need for future investigation in the targeting of apoptotic cell death and pro-survival pathways for the treatment of SMA.

Over the past decade, several therapies have shown promise in elevating SMN levels in preclinical studies, but none have translated into an effective therapy (Anderton et al., 2013). This study decided to approach this conundrum by generating a vector to secrete recombinant TAT-fused SMN protein as a therapy for SMA. Despite limitations in transduction activity, future studies have the potential to modify this protein by altering the CPP or SMN protein itself. Interestingly, recent research has identified potent
neuroprotection in some CPP’s, such as polyarginine and TAT (Meloni et al., 2013), justifying the pursuit of this methodology in treating neurological disorders. However, during the completion of this thesis, several advances in SMN viral delivery have been made. For example, adeno-associated viral SMN delivery can increase SMN protein levels and improve survival in severe SMA mouse models (Foust et al., 2010). In addition, the SMN adeno-associated vector has been shown to increase SMN protein levels in the spinal cord of macaques (Dehay et al., 2012; Samaranch et al., 2012). Based on the promising results in mice and macaques the SMN expressing adeno-associated vector is on track to commence phase 1 SMA clinical trials. In conclusion, this study has made advances in understanding the functions of the SMN protein in vitro, and generated a novel secretory vector with potential future applications in recombinant protein generation.
References


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