Protein expression changes in spinal muscular atrophy revealed with a novel antibody array technology

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Summary
Autosomal recessive proximal spinal muscular atrophy (SMA) is a severe neurodegenerative disease of childhood causing weakness and wasting secondary to motor neuron dysfunction. Over 97% of cases are caused by deletions or mutations within the survival motor neuron (SMN) gene. The SMN protein is highly expressed within brain, spinal cord and muscle, and is decreased in SMA patients. It has been shown to have an important role in RNA metabolism, but the reason for the specific motor neuron loss is still unclear. We have used a novel antibody array technology to look for differences in the expression patterns of primary muscle cultures from a type II SMA patient and a normal control. A relatively small number of differences were found within a group of proteins that function as both RNA binding proteins and transcription factors. Interactions between a number of these proteins are well established, and three of them bind in turn to p53 which interacts with SMN. A number of the changes were confirmed with western blot analysis both in the primary muscle cultures and in skeletal muscle samples from SMA patients and controls. Changes at the mRNA level were also confirmed with oligonucleotide arrays. These results suggest that a common transcription pathway may be altered in the disease state, and suggests that down-regulation of transcription factors contributes to SMA pathogenesis.

Keywords: spinal muscular atrophy; proteomics; microarrays; transcription

Abbreviations: EGF-R = epidermal growth factor receptor; ERK = extracellular signal related kinase; gems = gemini of coiled bodies; INR = internally normalised ratio; JNK = c-jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MDM2 = mouse double minute 2; SMA = spinal muscular atrophy; SMN = survival motor neuron; VASP = vasodilator-stimulated phosphoprotein; WT-1 = Wilm’s tumour-1

Introduction
Autosomal recessive spinal muscular atrophy (SMA) is a severe neurodegenerative disease of childhood causing highly specific motor neuron loss. It has an estimated incidence of 1 in 6000–10 000 and a carrier frequency of 1 in 40, and is the second commonest genetic cause of death in childhood after cystic fibrosis (Pearn, 1978). The clinical phenotype of weakness and wasting is variable, but divided into types I to III based on age of onset and survival. All three types are caused by deletions or mutations within the telomeric copy of the survival motor neuron (SMN) gene, SMN1 (Lefebvre et al., 1995). The SMN gene is highly conserved throughout evolution and has been found in every organism studied, but in all other species exists as a single copy (Viollet et al., 1997; Miguel-Aliaga et al., 1999). Primates are unique in having two copies of the SMN gene, with an almost identical copy gene SMN2. Mutations within SMN2 have no phenotypic effect and are present in 5% of the normal population, but there is a single nucleotide difference within exon 7 that causes alternative splicing and production of an isoform lacking exon 7 (Lorson et al., 1999). This produces truncated protein, which is thought to be unstable and rapidly degraded.

The SMN protein is comprised of 294 amino acid residues and has a molecular weight of 38 kDa with no significant homology to any other known protein. It is a ubiquitously expressed protein found in both the cytoplasm and the
nucleus, where it is concentrated in punctate structures called ‘gemini of coiled bodies’ (gems) (Liu and Dreyfuss, 1996). Gems have a dynamic relationship with coiled bodies within the nucleus and the two structures often co-localise. Coiled bodies are known to have a role in RNA metabolism. Not all cells have coiled bodies and gems, and cardiac muscle and smooth muscle cells have neither (Young et al., 2000). SMN protein levels vary amongst different tissues with the highest expression in brain, spinal cord and muscle (Coovert et al., 1997). The levels are particularly high during development, with a decrease post-natally (Burlet et al., 1998).

Decreased levels of the SMN protein cause degeneration of motor neurons and a subsequent weakness and wasting that is progressive and often fatal. There is an inverse relationship with amount of total SMN protein and disease severity, and Coovert and colleagues have shown a decreased level of SMN that is most striking within the spinal cord and muscle of type I SMA patients (Coovert et al., 1997).

The SMN protein has been shown to self oligomerize (Lorson et al., 1998) and to interact with a number of proteins involved in RNA metabolism including fibrillarin, GAR1, small nuclear ribonucleoproteins, Sm core proteins and RNA polymerase II, as well as RNA itself (Fischer et al., 1997; Jones et al., 2001; Pellizzoni et al., 2001a, b). Dreyfuss and colleagues have identified a number of novel proteins, named Gemin 2 to 5, which form a multiprotein complex with SMN that shuttles back and forth between the nucleus and the cytoplasm and is involved with the formation, nucleus import and regeneration of spliceosomal small nuclear ribonucleoproteins (Paushkin et al., 2002). These splicing functions have been shown to be impaired both in the SMN2 product lacking exon 7 and in SMA patients.

SMN has been shown to interact directly or indirectly with other proteins including Bcl2 and p53, which are both involved in apoptosis (Iwahashi et al., 1997; Young et al., 2002). The interaction with Bcl2 is somewhat controversial, as other groups have not been able to replicate it (Coovert et al., 2000). Other binding partners include profilin II, which has a specific neuronal expression pattern, and several transcription factors e.g. ZR1 (Giesemann et al., 1999; Gangwani et al., 2001). Despite this bewildering array of binding partners, the precise role of SMN in the motor neuron degeneration of SMA is still unclear. SMN clearly has a housekeeping role within the cell, as no patients have been reported with absence of both gene copies and knockout of the single gene present in other organisms is universally lethal (Schrank et al., 1997; Miguel-Aliaga et al., 1999, 2000; Owen et al., 2000).

Motor neurons are cells that have a unique combination of high metabolic activity, long axonal processes and termination at a neuromuscular junction. The deficiency of the SMN protein could lead to a specific neuromuscular deficit in several ways. First, SMN could have a specific role related to RNA metabolism or transcription within the motor neuron or the axonal process. Secondly, there could be faulty muscle signalling from the skeletal muscle causing motor neuron death. There is evidence for this view with nerve–muscle cell culture models established from SMA patient muscle biopsies, where the spinal cord explants degenerate far more rapidly than expected and the muscle cells do not fuse as rapidly (Braun et al., 1997). Melki and colleagues have used Cre/loxP recombination within mice to delete exon 7 of SMN within first the neurons alone and then the muscle alone (Cifuentes-Diaz et al., 2001). Both mouse models had marked wasting and shortened life span, but the muscle mutant displayed much more severe muscle necrosis. They concluded that the SMN gene defect led to a degenerative process of muscle fibres and postulated a muscle specific role for SMN. Finally, the defect in SMA patients could be caused by another, as yet unknown function, of SMN.

Microarray technology is now commonly used to produce expression profiles of cDNA populations. Numerous groups have published genes or groups of genes within known pathways that are differentially regulated in disease, but it is recognised that there is a high degree of ‘false positives’ within the results. This can be due to a number of factors including repetitive DNA sequences and cross-hybridization. Not all genes found can be confirmed using techniques such as real-time PCR and still fewer can be shown to be up- or down-regulated at the protein level. At best, the correlation between DNA and protein levels is 25–50% (Shoemaker and Linsley, 2002).

In this paper, we use a novel technology, the antibody array, to look for proteins that are up- and down-regulated in SMA muscle cultures compared with normal controls. The antibody array is a new technique enabling protein differences to be assayed directly by hybridising fluorescently labelled protein onto a glass slide spotted with 380 different antibodies. Although there are a number of mouse models available to study SMA (Monani et al., 2000), the antibody array was designed primarily for human protein and only 60% of the spotted antibodies cross react with mouse. It was therefore necessary to find an appropriate human sample. Both spinal cord and muscle have a clear disease phenotype, both tissues express SMN in large amounts during development and both show a clear decrease in the SMN levels in SMA. There are obvious difficulties in obtaining motor neurons or spinal cord tissue. It was therefore decided to use primary muscle cultures established from a type II SMA patient and an age- and sex-matched normal control on the array. Muscle culture rather than biopsy samples were used to ensure reproducibility of the samples and a homogeneous cell population. Primary muscle cultures have not previously been used to study SMA.

Reproducible protein differences were found hybridising the primary muscle cultures to the antibody array. We show that the primary muscle cultures show a clear difference in SMN protein levels in western blot analysis. The antibody array showed that a number of proteins involved in both transcription and RNA binding are down-regulated in the disease process, and that these proteins are binding partners within a putative transcription pathway.
Material and methods

Cell culture
A commercial cell line, the Clonetics Human Skeletal Muscle Cell Line (Clonetics SkMc 2845) was used as a normal control. This was established from the biceps muscle of a 15-year old female. The undifferentiated myoblasts were maintained in Clonetics Basal Medium and Bullet kit at 37°C and 5% CO₂, and differentiated by switching to Dulbecco’s Modified Essential Medium (DMEM) with 5% horse serum, 2 mM glutamine and 50 units/ml streptomycin and penicillin. The medium was changed every 3–4 days.

A primary muscle culture from a 14.5-year old female type II SMA patient was established by serial passaging of a trypsinised muscle biopsy. The biopsy was taken from the paraspinal muscle during surgery. Once established, the undifferentiated myoblasts were maintained in Ham’s F10 medium supplemented with 0.1% dexamethasone (v/v), 1% insulin (v/v), 15% fetal calf serum (FCS), 50 units/ml streptomycin and penicillin, 0.1% Epidermal Growth Factor (10 μg/ml v/v) and 0.1% fibroblast growth factor (10 μg/ml v/v). The myoblasts were induced to differentiate by growing them to confluence and then switching the culture medium to DMEM with 2% horse serum, 2 mM glutamine and 50 units/ml streptomycin and penicillin, and incubating them at 37°C at 10% CO₂. Half the medium was changed every 3–4 days. Both normal and SMA primary muscle cultures were grown for 7 days before harvesting them for either protein or RNA.

Skeletal muscle samples
The SMA muscle biopsies were taken from two type III SMA patients following local ethics committee approval (a kind gift of Professor K Bushby, University of Newcastle-upon-Tyne). A normal muscle sample was obtained from the paraspinal muscle of a young adult male undergoing surgery following trauma (a kind gift of Professor F Muntoni, Hammersmith Hospital, London). A non-SMA disease control was taken from the biceps muscle of a male patient with a genetically confirmed mitochondrial myopathy (a kind gift of Professor D Turnbull, University of Newcastle-upon-Tyne). The samples were snap-frozen, then homogenised in the Clontech lysis buffer.

Antibody array
The Clontech antibody array was used. This consisted of 380 individual antibodies spotted in duplicate upon a glass slide with two slides provided per experiment. The human primary muscle cultures were used for the experiment and the manufacturer’s protocol was followed. In brief, 150 mg of each of the primary cultures were pelleted, and the cell pellets were washed three times in phosphate-buffered saline (PBS) before being flash frozen in liquid nitrogen. The samples were thawed and homogenised in non-denaturing buffer (Clontech), then diluted after the protein concentration had been measured with the BCA kit (Pierce, Rockford, IL, USA) and each sample was labelled with either Cy 3 or Cy 5 dye (Amersham) before being passed through a PD desalting column (Amersham). Two slides were provided for reverse colour labelling to allow normalization of the samples. Hence samples Normal-Cy5 and SMA-Cy3 were mixed and added to slide 1, and Normal-Cy3 and SMA-Cy5 were mixed and added to slide 2. Total protein (10 μg) was added to each slide and hybridized at room temperature for 30 min before a series of washes. The slide was dried and scanned using a GRI 780 slide scanner and Imagene™ software. The subsequent text files were analysed with Clontech software (as recommended) and Genespring™ software to produce the scatter plots and correlation values.

Data analysis
The scanner file was analysed with Imagene™ software to produce a text file with signal intensities. The replicate values within each slide were averaged and an internally normalised ratio (INR) was calculated using Microsoft® Excel where:

\[
\text{INR} = \sqrt{\text{Ratio 1/Ratio 2}}
\]

and ratios 1 and 2 correspond to slides 1 and 2.

Ratio 1 = Normal-Cy5 relative fluorescent units/SMA-Cy3 relative fluorescent units and Ratio 2 = SMA-Cy5 relative fluorescent units/Normal-Cy3 relative fluorescent units.

The average INR is calculated for each antibody and duplicates that varied by >30% were discarded. An INR >1.3 or <0.7 was considered significant within the Excel software developed specifically for the antibody array by the manufacturers. Scatter plots and correlation coefficients were created within the Genespring™ software using a per slide normalization.

Western blot analysis
Total protein (10 μg) in the Clontech buffer was added to an equal volume of sodium dodecyl sulphate (SDS) buffer and boiled for 5 min before being separated on 8 or 10% SDS–polyacrylamide gels in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) at 100 V. Rainbow coloured molecular weight markers (10 μl) (Amersham Pharmacia) were used as protein standards. The gel was transferred onto nitrocellulose membrane (Schleicher and Schuell) at 80 V for 1 h in transfer buffer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS, pH 8.3).

To ensure even protein loading, the membranes were then stained with Sypro Ruby Protein Blot stain (Bio-Rad) as per the manufacturer’s instructions and imaged on a Typhoon 8600 phosphoimager (Molecular Dynamics).

After blocking the membranes for 1 h in Tris-buffered saline (TBS-T) (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with the primary
antibody. The SMN, vasodilator-stimulated phosphoprotein (VASP) and epidermal growth factor receptor (EGF-R) antibodies (Transduction Laboratories) were used at 1:1000 dilution. The c-jun N-terminal kinase (JNK), JNK-P, p38 and extracellular signal related kinase (ERK) antibodies (Cell Signalling) were used at 1:250 and the mouse double minute 2 (MDM2) and DP1 antibodies were used at 1:500 (BD Pharmingen). Membranes were washed three times in TBS-T prior to incubation for 1 h with either horse radish peroxide (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG) or HRP-conjugated donkey anti-mouse IgG (Jackson Laboratories) diluted 1:3000 in TBS-T. Western blots were developed with the ECL chemiluminescence (Amersham Pharmacia) and exposed to Kodak X-OMAT LS film.

**Oligonucleotide arrays**
The HuFocus genechip system (Affymetrix) was used as per the manufacturer’s protocol. Total RNA (5 μg) prepared with the RNaseasy kit (Qiagen) with DNase, was amplified with a single round of in vitro transcription and hybridized onto the genechip. Both the Normal and the SMA sample were hybridized in duplicate. The data was analysed using the Genespring™ software.

**Results**

**Validation of primary muscle cultures as a model to study SMA**
It was first necessary to establish primary muscle cultures and ascertain that the SMN protein was decreased within the SMA patient sample to confirm that this was a valid model for use on the array. The primary muscle cultures were grown as described and differentiated for 7 days before harvesting them for protein. At this stage, they were confluent and had formed multinucleated myotubes which exhibited spontaneous contractile activity. The morphological appearance was identical between the two cultures and a typical example of one of the primary muscle cultures is shown in Fig. 1A. Before use on the antibody array, the cells were harvested for protein and western blot analysis was carried out, probing with the SMN antibody. A clear difference was seen between the normal samples and the SMA samples, where the total level of protein was markedly decreased in the latter. Each western blot was performed three times on two separate sets of cultures and a typical example is shown in Fig. 1B.

**Antibody array**
The antibody array was carried out as per the manufacturer’s instructions (summarised above). Ten microgram of each of the final protein mixes was used on slides 1 and 2. The slides were scanned immediately on a range of laser intensities to ensure that none of the spots were saturated and so that all data remained within a linear range. The slides were scanned at the appropriate wavelength for first Cy3 and then Cy5, and the two images were then merged with Imagene™. Each slide had control spots of bovine serum albumin (BSA) at the four corners, which hybridized strongly with both labels and helped to determine the best laser power. There were also several blank spots, which acted as negative controls. None of these spots had signal above that of the local background. A typical section of slide 2 is shown in Fig. 2. There is a range of signal intensities, but most of the spots are yellow representing equal hybridization of the two protein samples. Arrows highlight the BSA control dots and an antibody (spotted in duplicate) that looks green, indicating stronger hybridization by the normal protein.

**Data analysis confirms a number of differentially expressed proteins**
The data was then analysed initially with Imagene™ and then within Excel and Genespring™ to produce INRs. Over 90% of the proteins had INRs of 0.9±1.1. Only proteins that had ratios >1.3 or <0.7 were analysed further, based on the dynamic range of the array.

The correlation between the two experiments was 0.98 and the scatter plot of normal signal ratio compared with that of the SMA sample showed the majority of data had an INR close to 1 (Fig. 3). Nine genes were up-regulated in the
normal sample and two genes were up-regulated in the SMA sample. These are shown in Table 1 with a description of function.

**Western blot analysis of primary muscle culture protein confirms the antibody array results**

Western blot analysis was then carried out on the samples using both the protein sample used on the array in the non-denaturing Clontech buffer and separate protein samples in denaturing buffer. Each western blot was carried out a minimum of three times on two separate sets of primary muscle cultures; typical results are shown in Fig. 4A and B.

A reproducible difference was shown probing with total JNK1, with specific bands seen at the predicted molecular weights of 53 and 46 kDa, respectively. JNK1 is a stress activated map kinase and its increase could be a non-specific stress response. Therefore, two other map kinases, p38 and ERK, were tested; no difference was shown, implying that the difference in JNK1 was disease-specific. p38 produces a single band with a molecular weight of 38 kDa and ERK produces a doublet at 50 kDa (Fig. 4). The phosphorylated JNK1 antibody was also tested and, interestingly, the reverse pattern was seen with an increase in protein in the SMA patient sample. This was also repeated on several occasions, but was seen consistently. Typical results are shown in Fig. 4.

The VASP antibody produces two bands ~55 kDa, which are known to be produced by varying phosphorylation state. Two bands were seen with a clear decrease in the SMA samples compared with the normal samples. Probing with the DP1 antibody also produced the predicted molecular weight band of 55 kDa, with a clear and reproducible difference between the normal and SMA sample.

**Western blot analysis in patient skeletal muscle biopsies demonstrates a disease-specific effect**

Western blot analysis was then carried out on in vivo samples by using skeletal muscle biopsies from patients. Skeletal
muscle from two type III SMA patients was compared with both a control skeletal muscle sample and a patient with a mitochondrial myopathy. The patient with mitochondrial myopathy was used to demonstrate that the effect seen was specific to SMA, rather than a non-specific effect secondary to muscle wasting. Each western blot was carried out three times. This also confirmed the differences seen in the JNK1, VASP, MDM2 and DP-1 antibodies. These results are shown in Fig. 4C.

**mRNA changes within the differentially expressed proteins**

Differences were shown in the protein levels of 11 proteins in total and five were confirmed independently with western blot analysis. To examine these proteins further, the corresponding mRNA levels were analysed using oligonucleotide microarrays. Five micrograms of total RNA from normal and SMA primary muscle cultures was hybridized onto the HuFocus genechip (~8700 genes represented) and each sample was hybridized in duplicate. The correlation values of each of the duplicates was 0.99. mRNA levels were decreased significantly in the SMA sample in p53, DP1, Ku70, VASP and Wilm's tumour protein-1 (WT-1). p53 was investigated as it binds to both SMN and a number of the other proteins found to be up-regulated in the normal sample.

There was no significant change in MDM2, JNK1, CDC25C and CD28. EGF-R was not represented on the array. There was a clear trend seen when looking at all the transcription factors on the array, with down-regulation of transcription factors within the SMA samples. Out of 78 genes listed as transcription factors within the Simplified Gene Ontology system, 59 were more highly expressed in the normal sample. These results are summarised in Table 2 and Fig. 5.

**Discussion**

Understanding the pathway of action of the SMN protein is central to understanding the pathogenesis of childhood SMA. The protein has an essential housekeeping role in all cells, but a decrease rather than a complete absence of the protein only leads to abnormalities of motor neuron and muscle. Whether the abnormalities in muscle are primary or secondary to the motor neuron deficit are still unclear.

The levels of the SMN protein in muscle have been shown to decrease in SMA patients, but muscle cultures have not been studied before. The comparison of primary muscle cultures between SMA patient and normal controls is a novel approach to try and understand molecular pathogenesis. It was therefore important to confirm a difference at the protein level between the total level of SMN in the patient sample and...
that of the normal control. There was a definite, reproducible
difference on western blot analysis, confirming a decrease in
SMN protein within this model. There is clearly a muscle
phenotype; however, this study may not reflect changes
within the motor neurons themselves. The advantage of a
tissue culture model rather than a muscle biopsy is the
possibility of producing repeat samples from the same
homogeneous cell population. This allows a variety of studies
to be repeated with a similar sample without introducing
variability due to different disease stages or different muscle
composition. This is particularly important in array studies to
minimize variation due to factors other than the disease
process.

Microarrays were designed to try to uncover pathways that
are differentially regulated between different DNA popula-
tions, and notable advances have been made within oncogene
pathways and with the understanding of cell cycle control
(Primig et al., 2000; Ross et al., 2000). These systems have
had the advantage of well-defined cell populations and a large
amount of existing knowledge about genes within the
pathways. However, somewhat less progress has been made
within other diseases and systems. One problem has been the
deluge of information provided by a large mouse or human
chip with several thousand genes being screened and a false
positive level that is often very similar to the number of genes
within the filtered gene lists. The large quantity of false
positive results can mask the genes that are truly differentially
expressed and are difficult to validate in terms of time and
cost. A significant number of the cDNAs spotted onto
microarrays comes from expressed sequence tags rather than
characterised genes, which also hinders functional analysis.
An alternative approach is to study protein levels directly, but
here too, there are difficulties created by the sheer complexity
of the protein structure and the complex folding of proteins
that are necessary for their function.

The antibody array is a novel technology, which tries to
address some of these problems. It has 380 individual
antibodies spotted in duplicate with >96% compatibility to
human tissue. Many major cellular pathways such as signal
transduction, apoptosis, cell cycle control and transcription
are well represented. There are also a wide variety of
structural proteins. This antibody array is a sensitive method
determining differences in levels of protein between two
different samples.

Technically, the array worked well with a correlation
coefficient of 0.99. This implies a strong degree of similarity
between the two data sets and means that the differences
present between the two samples are more likely to be real.
The dynamic range of signal intensity on the array was over
10,000, showing that proteins expressed at a wide range of
levels can be assessed. The differences produced were shown
at a range of intensities not just the higher range. In total, nine
proteins were up-regulated in the normal sample compared
with the SMA patient sample. Only two proteins were up-
regulated in the SMA sample compared with the normal. The
reason for the bias towards the normal sample could be due to
a number of factors. First, this could be a real result reflecting
down-regulation of a number of proteins within a pathway to
produce disease. Secondly, it could reflect the composition of
antibodies on the array. Only a relatively small number of
proteins are represented and it could be that further proteins
up-regulated in the SMA patient are not present. Obviously,
no novel proteins are represented so information can only be
produced about existing, well-characterised antibodies.

Out of the proteins that were shown to be differentially
regulated, five were studied further with western blot analysis
and, in all cases, a clear difference was shown between
normal and SMA samples. This was confirmed with both the
protein from the array and protein from further primary
cultures used as biological replicates. This confirmed the
ability of the array to distinguish differences in protein levels
accurately.

This data was then strengthened with western blot analysis
of in vivo skeletal muscle biopsy samples using SMA patient

Table 1 Differentially expressed proteins within the antibody array

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMA sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC25C</td>
<td>Cell cycle control and transcription factor</td>
<td>0.8</td>
</tr>
<tr>
<td>CD28</td>
<td>Immune cell surface marker</td>
<td>0.8</td>
</tr>
<tr>
<td>Higher in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
<td>1.4</td>
</tr>
<tr>
<td>JNK1</td>
<td>Stress-activated MAPK</td>
<td>1.42</td>
</tr>
<tr>
<td>Psme3/PA28-γ</td>
<td>Proteosome degradation</td>
<td>1.47</td>
</tr>
<tr>
<td>p57/KIP2</td>
<td>Pro-apoptotic cyclin dependant kinase inhibitor</td>
<td>1.47</td>
</tr>
<tr>
<td>Ku70</td>
<td>DNA repair protein, also binds RNA</td>
<td>1.5</td>
</tr>
<tr>
<td>DP-1</td>
<td>Transcription factor, binds JNK1</td>
<td>1.6</td>
</tr>
<tr>
<td>WT-1</td>
<td>Transcription factor and binds RNA</td>
<td>1.7</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal growth factor receptor</td>
<td>1.8</td>
</tr>
<tr>
<td>MDM2</td>
<td>Nuclear oncoprotein and binds transcription factors</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>such as DP1, p53. Also binds RNA</td>
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Fig. 4 Western blot analysis of some of the proteins identified as differentially regulated on the antibody array. For all blots, even protein loading was first shown with Sypro Ruby red protein blot stain (data not shown). (A) Confirms a clear difference between normal (N) and SMA (S) with total JNK1 antibody. Unrelated map kinases p38 and ERK are also shown. There is no difference in the protein levels. Using the phosphorylated JNK1 antibody gives the reverse result with a clear increase in the SMA protein compared with normal. (B) Three other antibodies (VASP, DP1 and MDM2) are also shown and confirm the difference shown on the array. (C) Skeletal muscle samples are also shown with the antibodies JNK1, VASP and MDM2 with the same increase in protein levels in the normal sample (labelled N) and a non-SMA sample with a mitochondrial myopathy (labelled C) compared with two separate SMA type III patients (S).
skeletal muscle samples compared with a normal control. The same differences were confirmed; moreover, there was no similar change in the levels of these proteins when another non-SMA disease sample was used, which demonstrates that these changes are disease-specific.

The proteins identified have not been shown previously to bind directly to SMN, but a number of them do have clear links to each other and to the regulation of transcription and cellular proliferation. There are two proteins up-regulated in the SMA sample; CDC25C is involved in cell cycle control and CD38 is an immune surface marker. Neither of these currently have obvious links to the disease process, so they could represent proteins whose levels alter within individual cells over time or represent cross-hybridization within the array.

The proteins that are down-regulated in the SMA sample can be examined in their functional groups. First, four out of the nine proteins are proteins that can act as both transcription factors and RNA binding proteins. Ku70, DP-1, WT-1 and MDM2 have all been shown to bind RNA and to act as transcription factors (Cassiday and Maher, 2002). This is of particular interest given the known functions of SMN. The SMN protein has been shown to bind RNA directly as well as acting as a putative transcription factor; it binds to the nuclear transcriptional activator E2 (Strasswimmer et al., 1999) and also to a DEAD box helicase Gemin 3 (previously known as DP103) which has known transcriptional activity (Charroux et al., 1999; Campbell et al., 2000). SMN also binds the multifunctional protein, p53 (Young et al., 2002), which is involved in cell cycle control, DNA repair, transcription activation and apoptosis (Cassiday and Maher, 2002). Its expression level is decreased in the SMA sample compared with the normal control in our oligonucleotide microarray. MDM2 is a transcriptional target of p53 and binds to a domain in the N-terminus of the p53 protein. The induction of p53 transcriptional activity leads to increases in MDM2 RNA and protein levels. Thus, it appears that an auto-regulatory feedback loop exists between these two proteins, which keeps the growth suppressive functions of p53 in check during normal cell cycling (Haines, 1997). In response to DNA damage, p53 protein transiently stabilizes and accumulates in the nucleus, where it performs its role as a transcription factor. Phosphorylation of p53 by MDM2 increases its sequence-specific DNA-binding activity (Kubbutat et al., 1998). p53 and MDM2 both bind DP-1 as part of their transcriptional activity (Sorensen, 1996; Loughran and La Thangue, 2000). It is likely, therefore, that MDM2 and DP1 are both down-regulated secondary to decreased levels of the SMN-p53 nuclear complex. It has been shown recently that JNK1 tethers DP1 and phosphorylates the transcription factor E2F1 in a fashion analogous to AP1, where it tethers to one component and phosphorylates other constituents (Wang et al., 1999). JNK1 is a stress-activated mitogen-activated protein kinase (MAPK) that is also down-regulated in the SMA sample on the antibody array. MAPKs have been implicated as regulators of differentiation. The biological effect of MAPK signalling in the nucleus is achieved by signal-responsive transcription factors and JNK1 is known to be up-regulated as a response to a variety of cellular stresses (Whitmarsh and Davies, 1998). If the array result was merely a non-specific effect of cellular stress due to some variation in tissue culture conditions, then other stress-related MAPKs might be expected to increase as well; however, when the protein expression levels of two other MAPKs, p38 and ERK1, were examined, no such change was found. If the change was a secondary change due to degeneration of the SMA myotubes, then the level of JNK1 would be more likely to be raised in the SMA sample rather than decreased relative to the normal sample. The increase in JNK1 therefore seems specific, so the other roles for this protein should be examined. The increased amount of phosphorylated protein in the SMA sample is interesting and may be caused by a number of factors such as a separate effect of the phosphorylated protein in the SMA process. Phosphorylation of JNK1 has been shown to be increased in skeletal muscle atrophy (Mizukami et al., 2001).

WT-1 is also a transcription factor and is known to bind both MDM2 and DP1 (Cassiday and Maher, 2002), so is likely to be altered in the SMA disease state. Ku70 is the regulatory subunit of the DNA-dependent protein kinase that phosphorylates many proteins, including p53, RNA-polymerase II and many transcription factors such as c-Jun, c-Fos and many more. It is a multifunctional protein implicated in many important cellular metabolic processes such as DNA double-strand break repair and transcriptional regulation. The mechanism underlying the regulation of all the diverse functions of Ku is still obscure, but it is decreased in the SMA sample possibly as a result of the down-regulation of p53 and the other affected proteins.

Another protein down regulated in the SMA sample is a member of the proteosome complex. The Psme/PA28-γ protein plays a role in proteosome degradation (Claverol et al., 2002) and Psme/PA28-γ is down-regulated in the SMA sample (Table 1). Activation of MDM2 is critical in the p53

Table 2 Fold changes within the transcription factors that alter on the antibody array

<table>
<thead>
<tr>
<th>Gene name</th>
<th>mRNA fold changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNK1</td>
<td>1.2 N&gt;S</td>
</tr>
<tr>
<td>VASP</td>
<td>1.9 N&gt;S</td>
</tr>
<tr>
<td>Ku70</td>
<td>4 N&gt;S</td>
</tr>
<tr>
<td>DP-1</td>
<td>1.2 N&gt;S</td>
</tr>
<tr>
<td>CDC25C</td>
<td>No change</td>
</tr>
<tr>
<td>CD38</td>
<td>No change</td>
</tr>
<tr>
<td>p57/KIP2</td>
<td>1.3 N&gt;S</td>
</tr>
<tr>
<td>Psme3/PA28-γ</td>
<td>No change</td>
</tr>
<tr>
<td>MDM2</td>
<td>Absent values on array</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Not on array</td>
</tr>
<tr>
<td>WT-1</td>
<td>3 N&gt;S</td>
</tr>
<tr>
<td>p53</td>
<td>1.9 N&gt;S</td>
</tr>
</tbody>
</table>

N = normal; S = SMA.
pathway because the MDM2 protein marks p53 for proteosome-mediated degradation. The effect of p53 within the cell is transitory and its degradation is necessary for its regulation. The 26S proteasome complex represents the major component of the ATP/ubiquitin-dependent protein degradation pathway. This pathway is responsible for the degradation of intracellular proteins tagged with multi-ubiquitin chains and thus regulates many processes in the cell such as progress through the cell cycle and gene transcription. If p53 and its associated transcription factors are down-regulated,

Fig. 5 Scatter plot showing the known transcription factors (functional description from the Simplified Gene Ontology system) with the normalised Normal patient expression values plotted against the SMA patient expression values. Lines showing the midpoint and 2 fold up and down are shown. Data produced with Genespring™.

N > SMA 1.5 fold

SMA > N 1.5 fold

58 genes

19 genes
then this affects the levels of the proteins required for their degradation, which could explain the decrease seen in this protein in the SMA sample.

If the SMN protein is down-regulated, this is likely to affect its binding partners such as p53 and other proteins within its pathway of action. These proteins have not previously been associated with SMN and, in this paper, we have shown that a number of proteins within pathways that regulate transcription are down-regulated in diseased cells. We can hypothesize that there is down-regulation of a transcription pathway which is triggered by the decreased levels of SMN and p53. MDM2 is decreased, which will cause a decrease in the phosphorylation of p53; DP1 and JNK1 are also decreased, with WT-1 and Ku70 both having modulatory effects. JNK1 has a number of important downstream effects on cellular proliferation, which are therefore likely to be altered. The interactions of the proteins discussed so far are summarised in Fig. 6.

Interactions of the proteins that are down-regulated in SMA cells

Interactions of the proteins that are down-regulated in SMA cells

p57/KIP2 is a cyclin-dependant kinase inhibitor (CKI) and thus has a negative regulatory effect on the cell cycle machinery. It acts as a critical terminal effector of signal transduction pathways that control cell differentiation. Cell-cycle arrest is thought to be required for differentiation of muscle cells. It has been shown that p57/KIP2 controls the differentiation of skeletal muscle. Mice lacking p57 fail to form myotubes and display increased proliferation and apoptotic rates of myoblasts (Yang et al., 1999). Braun and colleagues demonstrated abnormalities of muscle satellite cells from SMA patients in culture, and noted an increase in the time taken for the cells to fuse compared with normal controls (Braun et al., 1995). The increase in p57/KIP2 in normal cultures would be consistent with subtle abnormalities in differentiation in the SMA cultures, although this is likely to be a downstream effect.

EGF-R has a diverse range of cellular functions but acts as a growth factor and is known to be abnormally up-regulated in many different cancers. Its down-regulation has been shown to cause cell cycle arrest and apoptosis (Schlessinger, 2002) and, in this study, we have shown down regulation in the SMA sample. VASP is a protein which plays a role in membrane stabilization, filament formation and actin dynamics in muscle. Integral membrane proteins such as actin and tubulin are known to be altered in a wide range of disease processes (Loureiro et al., 2002). There may well be some impairment of these processes at a cellular level, but it is unclear at present whether the alteration of VASP levels is a primary or secondary effect.

In summary, we have described a useful model to study the gene and protein expression changes in SMA and have validated a novel antibody array technology. Changes on the array have been confirmed and a number of the proteins bind to each other within known transcription pathways raising the possibility that, when SMN protein levels decrease, a number of other transcription factors are also decreased and this has an adverse effect on cell development and function. Further work needs to be carried out to understand more about these proteins role in the disease process, but these results give novel insights into the pathways affected by the disease process and contribute to our understanding of the pathogenesis of SMA.

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References


Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci USA 1999; 96: 6307–11.


