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RICE UNIVERSITY

First evidence of skelemin, a myosin-associated protein, in smooth muscle and its involvement in cell adhesion; and the role of the cell cycle in cell type choice during mammalian development

by

Jeanette M. Schwartz

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF ARTS

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ABSTRACT

First evidence of skelemin, a myosin-associated protein, in smooth muscle and its involvement in cell adhesion; and the role of the cell cycle in cell type choice during mammalian development

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Indirect immunofluorescence in conjunction with Northern and Western blot analysis were used to identify the presence of skelemin, a myosin-associated protein, in smooth muscle. Feline uterus was cryosectioned and triple-stained for skelemin, smooth muscle myosin, and desmin. I observed that skelemin antibodies colocalized with the myosin-II filaments as well as the desmin intermediate filament cytoskeleton. This is the first evidence of a myosin-associated protein within smooth muscle, and it raises the possibility that smooth muscle myosin may be more organized than has been assumed. Antisense oligonucleotide treatment was used to analyze the underexpression of skelemin protein in developing embryoid bodies. Skelemin underexpression was seen to cause loss of cell-cell as well as cell-substrate adhesion. Time lapse video microscopy was used to analyze the role of the cell cycle in cell type choice during mammalian development. Discussed are methods employed to improve resolution for accurate analysis.
ACKNOWLEDGMENTS

I would like to thank Maureen Price and Richard Gomer whose advice and expertise were instrumental in my scientific development. I would also like to thank Dr. Julian Allen and Dr. Rhonda Albright, for donating the samples of canine and feline muscle tissues, as well as Alan Bradley for the gift of the embryonic stem cells. I would like to thank the following people for their technical support: Kathleen Matthews and Larry McIntyre for the use of their cell culture rooms, Bernie Andruss for his assistance with the confocal microscopy, Spike Longoria for compiling videotape segments, Eric Hnath for his guidance with protein interaction analysis, Tod Romo for his assistance with preparing the figures, Derek Milner for his advice in culturing embryonic stem cells, and the members of the Gomer lab for their invaluable advice. Special thanks are extended to Ed Frank, Derrick Brazill and Debra Brock for their help in editing and preparing the manuscript. Finally, I would like to thank my loving husband and family for their support and words of encouragement.
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Chapter 3 Examination of the role of the cell cycle in mediating cell-type choice during mammalian development

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Chapter 1
Skelemin, an M-disc associated protein, is present in smooth muscle

Introduction

There are three different muscle types found in vertebrates: skeletal, cardiac, and smooth muscle. Although each of these muscle types contains the same contractile components, myosin, actin, as well as an intermediate filament cytoskeleton composed of desmin, the arrangement of these components in each specific muscle type is unique to the function it performs. Much of what is known about contraction and the cytoskeleton was obtained from the highly organized structure of skeletal muscle. Skeletal muscle is comprised of large, fusiform, multinucleate cells, each of which is made up primarily of myofibrils. The myofibrils are divided into contractile units called sarcomeres in which the strict architecture of the contractile apparatus is laid. Each sarcomeric unit is contained within a pair of thick bands, or Z-discs. Here the thin actin filaments are bound in a strict parallel conformation and extend inward toward the center of the sarcomere where they overlap the thick myosin filaments in a 2:1 ratio. Thus allowing for rapid contraction and tension production for load bearing. Adjacent myosin filaments are bound at a central region, or M-disc, by several different myosin-associated proteins including skelemin, a myosin-associated protein which cross-links the myosin filaments to the desmin intermediate
filament cytoskeleton which surrounds and links the myofibrils (Price, 1983 for review). Skelemin also wraps around each M-disc and links adjacent M-discs, forming an extramyofibrillar cage around each sarcomere (Price, 1987). This striated organization is also found within the mononucleate cells of cardiac muscle tissue which also require rapid contraction.

The least understood and most disputed arrangement of contractile and cytoskeletal domains is within smooth muscle. Compared to the semi-crystalline structure of the molecular components in striated muscle cells, smooth muscle cells appear to lack organization. The filaments in the elongated, spindle-shaped cells of smooth muscle are almost homogeneously distributed throughout the cytoplasm giving rise to a smooth appearance. The molecular components of smooth muscle cells are commonly characterized as members of either the cytoskeletal or contractile domains. This organization is believed to enable the smooth muscle cells to contract to one-fifth their original size, thus producing a larger contractile force over a greater period of time (Alberts et al., 1994, Wolfe, 1993). Immuno-colocalization and ultrastructural studies suggest that these two domains are linked at electron-dense bodies that are uniformly distributed within the cytoplasm and are also associated with the plasma membrane (Draeger et al., 1990).

The cytoskeleton of smooth muscle consists of bundles of intermediate filaments that run the length of the cell, forming a branched network (See Figure 1). These intermediate filaments consist primarily of desmin, within visceral smooth muscle, and of
Figure 1. Schematic illustration of the structural organization of the cytoplasmic and contractile components in the smooth muscle cell. The cytoplasmic domain comprises the cytoplasmic (CDB) and membrane-associated dense bodies (MADB) that are both penetrated and linked longitudinally by non-muscle actin. The desmin intermediate filaments (IF) branch throughout the cell and interact along the periphery of the CDBs. Plectin contained within the MADBs couples the IF to the plasma membrane. The contractile domain contains a loosely arranged network of actin and myosin-II filaments. The filaments are arranged diagonal to the long axis of the cell and may be linked from CDB-CDB or CDB-MADB as shown. Smooth muscle actin filaments may be coupled to the dense body periphery via actin cross-linking proteins, such as filamin. Caldesmon associates with the smooth muscle actin in the contractile domain where its C-terminus competes with binding of the myosin heads to the same N-terminal site of the actin. The N-terminus of caldesmon may wrap around the actin filament to bind the myosin neck.
vimentin and desmin or their copolymers, within vascular smooth muscle. They have a uniform diameter of 10 nm (Bagby, 1986). Transverse tissue sections repeatedly show the intermediate filaments interacting laterally along the perimeter of the cytoplasmic dense bodies (Small, 1995 for review). Immunofluorescence and electron microscopy have revealed that the intermediate filaments do not impinge upon the sarcolemma directly (Draeger et al., 1990). Instead, this association may be mediated by plectin within the membrane-associated dense bodies (Wiche et al., 1983) Plectin is an intermediate filament binding protein found in the intercellular adhesion structures, desmosomes and hemi-desmosomes (Wiche et al., 1983).

Immunocytochemical analysis has shown that the intermediate filaments do not comprise the entire cytoskeletal domain. In 1992, Kabsch and Vandekerckhove discovered six isoforms of actin that differed in their amino-terminal sequences. These isoforms include α-skeletal muscle, α-cardiac muscle, α- and γ-smooth muscle, and β- and γ-non-muscle or cytoplasmic actins. Vandekerckhove and Weber (1981) found that the β-cytoplasmic actin is distributed among the intermediate filaments of the cytoskeletal domain and constitutes 30% of the total actin within smooth muscle. β-cytoplasmic actin colocalizes with α-actinin, an actin cross-linking protein found within the cytoplasmic dense bodies and the membrane-associated dense bodies. Immunofluorescence staining of extended cells which were then allowed to contract revealed that the arrangement of the cytoplasmic dense bodies was highly geometric.
(Draeger et al., 1990). In extended cells, the cytoplasmic dense bodies align and appear to be strung together longitudinally by the β-cytoplasmic actin (North et al., 1994).

The contractile domain consists of the thick and thin filaments of myosin and actin, respectively, along with their associated proteins. Thick filaments are myosin polymers measuring 15-20 nm in diameter and 2.2 μm in length (Bagby, 1986; Cooke, 1983). Thick filaments are associated with thin filaments via cross-bridging of the myosin heads that occur at regular intervals of 14.3 nm (Cooke, 1983). In vitro analysis strongly suggests that the smooth muscle myosin filaments are side-polar (Craig and Megerman, 1977; Trybus, 1996). This means that myosin heads have one polarity on each side of an essentially rod-like filament with opposing polarity on opposite faces (See Figure 1). Compared to the bipolar polymerization of striated muscle myosin filaments which result in a central bare zone, the antiparallel polymerization of smooth muscle myosin filaments results in contiguous cross-bridging of the globular heads with bare zones only at the ends of the filaments (Cooke, 1983). This allows myosin filaments in smooth muscle cells to pull farther down the actin filament, thus increasing their degree of contraction compared to that of striated muscle.

Thin filaments consist primarily of α- or γ-actin and tropomyosin and are 4-8 nm in diameter (Bagby, 1986). Expression of these isoforms is dependent upon the type of contraction the smooth muscle will be performing. Large percentages of α-smooth muscle actin is found in vascular smooth muscle tissues which utilize
tonic contraction. Alternatively, large percentages of γ-smooth muscle actin is found in visceral smooth muscle tissue which requires phasic contraction, such as that within the organs of the gut and the esophagus (Adelstein And Sellers, 1996). Accordingly, the thin filaments are fairly evenly distributed throughout the cell and are arranged as rosettes around the thick filaments (Small, 1995 for review). Contractile thin filaments interact along the periphery of the cytoplasmic dense bodies and are bound to the membrane-associated dense bodies via the actin cross-linking proteins vinculin and talin (Draeger et al., 1990). (See Figure 1.) Filamin is colocalized within the membrane-associated dense bodies as well as within the cytoplasmic actin polymers (Small et al., 1986). These data suggest that filamin may cross-link the contractile and cytoskeletal actins.

The least understood structural aspect of smooth muscle is the association of the myosin filaments to the cytoskeletal and contractile domains. Within mature striated muscle tissue, a major factor of myosin organization is the M-disc, in which myosin filaments are linked together by myomesin, M-protein, and MM-creatine kinase (Thornell et al., 1990 for review). Because myosin filaments are homogeneously dispersed in smooth muscle cells, they appear to lack an equivalent of an M-disc. This raises the questions of how myosin filaments are maintained in position within the contractile domain and how they achieve leverage for contraction. Two elements are believed to maintain myosin in position within the smooth muscle cell (Small, 1995 for review). One is the association of caldesmon with both smooth muscle actin and myosin which has structural and
functional implications. Caldesmon protein is believed to wrap around the actin filament where it is able to bind the myosin neck region with its amino-terminus (Ikebe and Reardon, 1988; Haeberle, 1994). The caldesmon carboxyl-terminus inhibits the binding of myosin cross bridges through competition with the myosin heads for the same amino-terminal site on the smooth muscle actin (Hemric and Chalovich, 1990).

The other element believed to maintain position is the existence of a stabilizing factor of myosin filaments in vivo. Myosin filaments isolated from smooth muscle have been shown to be readily depolymerized by ATP in vitro (Onishi et al., 1978). Dephosphorylation of the myosin light chain results in depolymerization of the filaments. This results from the folding of the myosin tail into a stable, looped configuration which can be induced by phosphorylation to revert to an elongated monomer capable of polymerizing (Trybus, 1991). However, immunostaining using antibodies specific for myosin in the depolymerized state revealed that in both contracted and relaxed samples of smooth muscle, myosin monomers were found to encompass only 15% of the total myosin (Horowitz et al., 1994). This suggests that a stabilizing factor exists in vivo which binds the myosin tail preventing massive depolymerization.

As stated earlier, a major factor of myosin organization within mature striated muscle is the M-disc, in which myosin filaments are linked together by myomesin, M-protein, and MM-creatine kinase (Thornell et al., 1990). The periphery of the M-disc appears also to
be linked to the surrounding intermediate filament cytoskeleton (Price, 1987). Immunofluorescence has shown that skelemin, a 195 kDa protein, is associated with the M-disc, forming a ring around its periphery and linking neighboring M-discs (Price, 1987). Transfection of COS cells with whole skelemin and defined fragmented constructs showed that skelemin expression resulted in colocalization of myosin with the intermediate filaments (Price et al., in preparation). Colocalization by skelemin appeared to be dictated by the 60 kD carboxyl one-third portion.

Skelemin belongs to a family of intracellular proteins possessing motifs associated with both myosin-binding and cell adhesion. There are seven immunoglobulin superfamily C2 type motifs which flank five fibronectin type III-like motifs; five of the former are at the carboxyl-terminus. Skelemin is unique to the myosin-associated family in that it also contains two short intermediate filament helical core-like motifs, one at the amino-terminus and one at the carboxyl-terminus (Price and Gomer, 1993). At the amino-terminus of skelemin lies a 190 residue unique sequence (Price and Gomer, 1993). The predicted secondary structure of skelemin is largely β-sheet interrupted by short helices, turns and random coils (Price and Gomer, 1993).

We have identified the presence of skelemin protein in smooth muscle tissue. This is exciting because it was the first evidence of an M-disc associated protein in smooth muscle. This raises the possibility that skelemin may cross-link the myosin filaments to each other as well as to the desmin intermediate filament cytoskeleton.
This interaction could therefore provide the leverage through which myosin filaments initiate contraction. In this chapter, I provide evidence that a skelemin gene is transcribed and skelemin is produced in smooth muscle, that it is localized to smooth muscle cells, and that it colocalizes with both myosin-II filaments and desmin intermediate filaments within smooth muscle cells.
Table 1. Summary of the filaments of the contractile and cytoplasmic domains of smooth muscle cells and their associated proteins

<table>
<thead>
<tr>
<th>Filament</th>
<th>Protein</th>
<th>Cellular Location</th>
<th>Associated Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin</td>
<td>α- and γ- smooth muscle actin</td>
<td>Contractile domain Associated with the CDB and MADB</td>
<td>In MADB - filamin, α-actinin</td>
</tr>
<tr>
<td></td>
<td>β- cytoplasmic actin</td>
<td>Cytoplasmic domain Linking dense bodies longitudinally</td>
<td>In MADB - vinculin, talin, filamin, α-actinin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>In CBD - α-actinin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>in Cytoplasm - filamin</td>
</tr>
<tr>
<td>Thick</td>
<td>Myosin-II</td>
<td>Contractile domain</td>
<td>Caldesmon N-terminus, Actin via cross-bridges, Skelemin</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Desmin</td>
<td>Cytoplasmic domain Associated with the MADB and periphery of CDB</td>
<td>In MADB - plectin, in cytoplasm - Skelemin</td>
</tr>
</tbody>
</table>
Materials and Methods

Northern Blot

A Multiple Tissue Northern Blot (Clontech Laboratories, Inc., Palo Alto, CA) containing approximately 2 μg of poly A++ RNA per lane from each of eight different human muscle containing tissues was hybridized using a 0.63-kb (bases 4084-4717) Eco RI fragment of skelemin cDNA. This fragment encodes from the linker between the immunoglobulin C2 motifs 4 and 5, through 6, and 10 bases into the intermediate filament like core linker between motifs 6 and 7 (See Figure 2). The probe was prepared by maxiprep (Qiagen, Chatsworth, CA) of the cloned fragment in pBluescript followed by excision by EcoRI digestion. The fragment was separated by gel electrophoresis (1% Agarose, 1X TAE), identified by UV illumination and extracted from the agarose using Geneclean III (Bio 101, Vista, CA). Approximately 50 ng of probe was labeled with [α-32P]dCTP by the random-hexamer method (Boehringer Mannheim, Indianapolis, IN). Unincorporated nucleotides were removed using a NucTrap Probe Purification Column (Stratagene). The membrane was prehybridized in a 10X Denhardt's Solution (Maniatis), 50% formamide solution (Maniatis) for 3 h at 42°C in a Hybaid mini-hybridization oven (Woodbridge, NJ) The prehybridization buffer was then replaced with fresh buffer plus approximately 2 x 10^6 cpm/ml of labeled probe, and allowed to hybridize for 20 h at 42°C. The blot was washed 3 times for 10 minutes each in 2 X SSC/0.05% SDS solution at room temperature while agitating Autoradiography was done using
Figure 2. Domains of deduced amino acid sequence of skelemin cDNA, illustrating the motifs of the 60 kDa expression protein from which the anti-skelemin carboxyl one-third antibodies were raised. Also shown is region encoded by the 0.63 kb EcoRI fragment. Adapted from Price and Gomer, 1993.
preflashed X-Omat AR5 film (Kodak) with a Cronex Lightning Plus intensifying screen (Du Pont), at -70 °C for 8-36 hours.

**Immunoblotting**

Protein samples of striated and smooth muscle tissues were prepared from canine leg, diaphragm, bladder, large intestine, prostate and uterus. The large intestine and uterus were scraped in 5X Complete protease inhibitor cocktail (Boehringer Mannheim) on ice to isolate the taenia coli and the myometrium respectively. Tissue samples weighing 0.2 g were homogenized in 5 ml of 5X Complete protease inhibitor cocktail (Boehringer Mannheim) with a tissueemiser (Tekmar, Cincinnati, OH). Homogenates were stored in liquid nitrogen and samples were diluted fresh for SDS-PAGE in a 5X dithiothreitol/SDS loading buffer (Laemmli) to a final concentration of 1.5X. Samples were electrophoresed through an 8% SDS-polyacrylamide separating gel. Protein samples were transferred overnight onto Immobilon-P PVDF membrane (Millipore, Bedford, MA) using a tank transfer system (BioRad) containing a 20% methanol transfer buffer (25 mM Tris base, 192 mM Glycine, pH 8.3). Membranes were blocked for 1 h in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 9 mM Na$_2$HPO$_4$, 1 mM KH$_2$PO$_4$, pH 7.45) containing 3% BSA (Sigma, St. Louis, MO) at room temperature, while agitating; then stained for 1 h with 2.4 μg/ml of rabbit anti-skelemin polyclonal antibody directed against whole skelemin protein isolated from bovine heart (See Figure 2). Membranes were washed 3 X 10 min in PBS containing 0.5% Tween-20. Following the washes,
membranes were incubated 1 h in a 3000 X dilution of horse-radish peroxidase conjugated goat anti-rabbit antibody (Amersham), then washed as above. The membrane was rinsed in PBS, then exposed to X-Omat AR5 film (Kodak) for 5 - 20 min.

**Indirect Immunofluorescence**

Samples of liver were taken from an adult mouse that had been sacrificed by cervical dislocation. Samples of uterus were received from spayings of adult cats. Tissues were placed in a layer of O.C.T. embedding compound (Miles, Elkhart, IN) in a mold set on dry ice and then immersed into liquid nitrogen for quick-freezing. The samples were stored at -70° C until time of cryosectioning. Cross-sections of 8-10 μm were cut with an Accu-Edge Blade (Miles, Elkhart, IN) in an IEC cryotome (Needham Heights, MA), thaw-mounted onto coverslips, and fixed with 95% ethanol at room temperature for 10 minutes. Sections were blocked with 1% BSA in PBS for 15 minutes, and double-stained for 1 h at room temperature in 50-70 μg/ml of ammonium-sulfate or E-Z Sep (Pharmacia Biotech, Uppsala, Sweden) purified polyclonal rabbit antibodies directed against whole bovine skelemin (Price, 1987) or against the bacterially expressed carboxyl one-third region of mouse skelemin cDNA (Price and Gomer, 1993), and a monoclonal antibody directed against desmin (Clone DE-U-10, Sigma). Following three 5 minute washes in PBS, 0.05% Nonidet P-40, the sections were stained for 1 h at room temperature with affinity-purified fluorescein-conjugated goat anti-rabbit IgG F(ab')2 (Cappel/Organon Technika, Durham, NC)
and Cascade Blue-conjugated goat anti-mouse IgG F(ab')\(_2\) (Molecular Probes, Eugene, OR) and 0.17 units/100 μl of rhodamine-labeled phalloidin (FLUKA Chemical Corp, Rovkonkoma, NY) or 0.1 μg/ml of DAPI nuclear stain (Sigma). Sections were washed as above and mounted in a p-phenylenediamine anti-fade (Johnson and Araujo, 1981) to retard photobleaching. Phase-contrast/epifluorescence microscopy was performed using a Nikon Microphot-FX microscope equipped with filters which allow for narrow excitation/emission ranges to view the green, red, and blue labels of the same section individually (Omega Optical, Brattleboro, VT). Similarly, a triple-band filter (Omega Optical) was used, which filtered the three separate excitation/emission wavelengths simultaneously. Images were viewed under a 40 X fluorescence objective (Nikon). Photographs were taken with a Nikon FX-35WA camera on Fuji color SuperHG 1600 film and developed commercially.

**Confocal microscopy**

Feline uterus cross-sections, 10 μm thick, were prepared as described above and thaw-mounted onto coverslips in 95% ethanol at room temperature for 10 minutes. Sections were blocked with 1% BSA in phosphate-buffered saline for 15 minutes, and double-stained for 1 h at room temperature in 100 μg/ml of ammonium-sulfate or E-Z Sep (Pharmacia) purified polyclonal rabbit antibodies directed against whole bovine skelemmin (Price, 1987) or against the bacterially expressed carboxyl one-third region of mouse skelemmin cDNA (Price and Gomer, 1993), and a monoclonal mouse antibody
directed against desmin (Sigma) or a monoclonal mouse antibody directed against smooth muscle myosin (Clone hSM-V/SMNS-1, Sigma). Sections were washed as described above and were stained for 1 h at room temperature with affinity-purified fluorescein-conjugated goat anti-rabbit IgG F(ab')2 (Cappel) and Cascade Blue-conjugated goat anti-mouse IgG F(ab')2 (Molecular Probes). Sections were washed again and mounted in anti-fade as described above. Coverslips were sealed to the slides and viewed with a Zeiss Laser Scan Microscope 410 inverted microscope (Germany). Images were viewed under a 100 X oil immersion objective. Scanned images were assayed for colocalization. Images were magnified and processed for publication using Adobe Photoshop and Canvas and were printed on a Phaser 440 color printer.

Results

Skelemin is transcribed in smooth muscle cells

Most of what is known about the contractile and cytoskeletal proteins of muscle tissue was first developed from striated muscle. Skelemin, a myosin-associated protein localized at the M-disc of striated muscle tissue, was shown to cause association of the myosin containing structures and the desmin intermediate filament cytoskeleton in COS cell transfection assays (Price et al., in preparation). To investigate the presence of skelemin in smooth muscle, Northern blot analysis was used to determine whether skelemin is transcribed in tissues rich in smooth muscle. A multi-
tissue Northern blot containing a panel of poly-A+ selected mRNA prepared from human muscle was purchased from Clontech. The blot was probed with a 0.63 kb EcoRI cDNA fragment encoding part of the carboxyl one-third region of skelemin (Price et al., in preparation) (Refer to Figure 2 for skelemin fragments) revealing intense labeling of a 5.8 kb message in skeletal and cardiac striated muscle tissues corresponding to the size of the skelemin cDNA isolated from a mouse skeletal muscle cDNA library (Price and Gomer, 1993) (Figure 3). Longer exposures demonstrated relatively small amounts of 5.8 kb skelemin message in uterus, colon, small intestine, bladder, stomach, and prostate smooth muscle tissue samples. These results can be a ramification of the fact that the smooth muscle tissue is infiltrated and surrounded by copious amounts of connective tissue. Consequently, the percentage of muscle specific mRNA per sample of visceral tissue is much lower than that in striated muscle tissue mRNA. To measure the amount of mRNA loaded per lane, the blot was probed with β-non muscle actin. The two hybridization bands at 2.0 kb and 1.6 kb of the β-actin transcript is reflective of the β-actin isoforms found in muscle tissues and are not the result of degradation (Giovanna et al., 1991). It is obvious from Figure 3 that the lanes were not equally loaded. Therefore, the intensity of the bands is not representative of the rate of transcription of skelemin within smooth muscle.
Figure 3. Human Muscle Multiple Tissue Northern Blot from Clontech probed with the 0.63 kb cDNA fragment encoding the carboxy one-third of skelem. The 24 h exposure reveals hybridization in the smooth muscle tissues of the uterus, colon, small intestine, bladder, stomach, and prostate. The 8 h exposure reveals distinct bands in the skeletal and cardiac striated muscle tissues, and the smooth muscle of the prostate. A β-actin (non-muscle actin) probe was used to assess the amount of total RNA per lane.
Skelemim protein is expressed in smooth muscle tissue

We have observed from Northern blot analysis strong evidence of skelemim in smooth muscle. Next, we wanted to assess the size of skelemim protein in smooth muscle muscle. We extracted protein from striated muscle samples of canine skeletal muscle and diaphragm, and from smooth muscle samples of canine bladder, taenia coli, uterine myometrium, and prostate gland. Samples were standardized for myosin heavy chain representation onto an 8% SDS-PAGE and Western blotted onto PVDF membrane. Skelemim protein was detected using a polyclonal rabbit antibody raised against whole skelemim isolated from bovine heart (Price, 1987). Specificity of polyclonal anti-skelemim antibodies was determined previously (Price, 1987) and have been proven to react with mouse skelemim (Price and Gomer, 199). As expected, a band corresponding to approximately 195 kDa was seen in the striated muscle samples, diaphragm and leg muscle (See Figure 4). A slightly lower molecular weight skelemim band at approximately 190 kDa was detected in the smooth muscle samples. This result is exciting because the transcript size of skelemim in all muscle types appeared to be the same length (see Figure 5); yet the protein translated was ~ 5 kDa smaller. Our RNA gels, however, can not resolve the difference between the striated and smooth muscle skelemim messages that gives rise to this 4-5 kDa change.
Figure 4. (A) Coomassie Brilliant Blue staining of canine skeletal and smooth muscle.

-195 KDa

97.4 KDa

116 KDa

205 KDa

Molecular Weight Markers

Leg muscle

Diaphragm

Uterine myometrium

Prostate

Bladder coli

- Demonstrate the difference between smooth skeletal and smooth muscle samples. Skelemin was detected using a rabbit polyclonal serum illustrating the amount of sample loaded per lane. (B) Western blot of canine skeletal and smooth muscle.

Molecular Weight Markers

Leg muscle

Diaphragm

Uterine myometrium

Prostate

Bladder coli
Skelemin localizes to smooth muscle cells as determined by indirect immunofluorescence staining

Northern and Western blot analysis revealed that skelemin is both transcribed and translated in organs rich in smooth muscle. However, these organs are comprised of many different cell types. Therefore, to localize skelemin to smooth muscle cells specifically, indirect immunofluorescence labeling was performed on cryosections of adult cat uterus, as well as sections of mouse liver as a negative control. Sections were triple-stained using polyclonal anti-skelemin antibodies, a monoclonal anti-desmin antibody, and phalloidin for filamentous actin. Two separate anti-skelemin antibodies were used: antibody raised against whole skelemin isolated from bovine heart and antibody raised against the bacterially expressed carboxyl one-third cDNA fragment isolated from mouse skeletal muscle (See Figure 2 for motifs). Immunostained sections of liver were negative for skelemin protein (data not shown).

Smooth muscle cells are dispersed in both transverse and longitudinal bundles among the connective tissue of visceral organs such as the uterus. The connective tissue permeates the smooth muscle cell bundles and attaches the cells to each other as well as to other bundles. Phase contrast microscopy of the immunostained adult feline uterus sections (Figures 5 and 6) demonstrate this arrangement. The phase contrast images show the distinct spindle-shaped morphology of the elongated smooth muscle cells which are smooth in appearance and stand out against the myriad of rounded connective tissue. Desmin antibodies and phalloidin were used to distinguish the elongated, tapered cells of smooth muscle from the
**Figure 5.** Indirect immunofluorescence staining of an 8 μm thick cryosection of adult feline uterus. The section was triple-stained with anti-desmin, and anti-skelemin antibodies, and with phalloidin to identify filamentous actin. Colocalization of all three labels is visualized through a triple-cube filter. Phase-contrast shows the distinct bundles of smooth muscle fibers among the vast connective tissues. Magnification 400X. Bar indicates 50 μm.
intervening connective tissue as seen in Figure 5. These longitudinal sections demonstrate the interlacing architecture of the smooth muscle fibers within the uterus. The centrally located nuclei are stretched and twisted in a manner corresponding to the orientation of the cells. Immunostaining using the polyclonal antibodies raised against whole skelemin showed a similar fibrous staining pattern similar to desmin and actin (Figure 5). The triple-stain image illustrates the overlap of the skelemin antibody staining with that of desmin and actin, and confirms the specificity of the skelemin antibody to muscle tissue. The carboxyl one-third of skelemin has been shown to associate myosin filaments and desmin intermediate filaments in striated muscle. Therefore, to determine if smooth muscle skelemin contains the region which mediates this association, indirect immunofluorescence staining of uterus cryosections was done using polyclonal antibodies raised against the carboxyl one-third of skelemin. These antibodies react with whole skelemin and with the 60 kDa carboxy terminus of skelemin, not with either the amino 66 kDa or the middle 57 kDa of skelemin (Price and Gomer, 1993). Antibodies against desmin, and phalloidin to stain filamentous actin were also used. Once again, desmin staining indicated the smooth muscle tissue within the section (Figure 6). The carboxyl one-third anti-skelemin antibody positively stained with a pattern similar to the desmin and actin labels. Thus, the 60 kD portion of skelemin implicated in associating the myosin filaments and desmin intermediate filaments is present in smooth muscle skelemin.
Figure 6. Indirect immunofluorescence staining of an 8 μm thick cryosection of adult feline uterus. The section was triple-stained with anti-desmin, and with a polyclonal antibody directed against the carboxyl 60 kDa of skelemin, and with phalloidin to identify filamentous actin. Colocalization of all three proteins is visualized through a triple-cube filter. Phase-contrast shows the bundles of smooth muscle fibers among the vast connective tissues. Magnification 400X. Bar indicates 50 μm.
Skelemin colocalizes with both myosin-II and desmin in smooth muscle cells as determined by confocal microscopy

Higher resolution microscopy was needed in order to elucidate the distribution of skelemin within smooth muscle cells with respect to desmin and myosin-II to determine if skelemin associates with these filaments as it does in striated muscle. I double-stained cryosections of uterus using polyclonal anti-skelemin antibodies with either anti-desmin or anti-smooth muscle myosin. Confocal microscopy using a Zeiss LSM 410 inverted microscope system and a 100 X lens was used to obtain 0.2 μm thick optical sections at higher resolution than from previous immunofluorescence studies.

Figure 7 illustrates two separate panels of longitudinal sections. Panel A compares the arrangement of the desmin intermediate filaments with respect to skelemin. Colocalization can be seen as yellow in the double label image. This is the result of the green and red fluorescence overlap. Skelemin appears to be distributed more widely throughout the smooth muscle cells than is desmin. White arrows identify an area where the desmin filaments lie inside a gap that is bordered on either side by skelemin. Skelemin appears to be colocalized at the periphery of the desmin filaments in these gaps. This arrangement was seen throughout the section. White arrow heads identify regions of direct colocalization of the desmin and skelemin signals. It appears from these sections that skelemin envelops the desmin intermediate filament cytoskeleton. Panel B illustrates the arrangement of skelemin with respect to concentrations of myosin. Here, skelemin lies within the space created between two areas of myosin concentration (white arrows)
Figure 7. Longitudinal 0.2 μm optical sections of adult feline uterus. The sections were double-stained with anti-skelemin antibodies and either anti-desmin (Panel A) or anti-smooth muscle myosin (Panel B). White arrow heads demonstrate regions of colocalization. White arrows demonstrate regions where skelemin occupies the areas defined by the desmin or myosin staining and colocalizes along their periphery. Magnification 1000 X. Bars measure 25 μm.
where it colocalizes with the myosin at its periphery. Skelemin also appears to be colocalized along the entire length of the myosin (white arrow heads). These patterns of colocalization suggest that skelemin may associate myosin filaments to each other as well as to the desmin intermediate filament.

Figure 8 illustrates two separate panels of transverse optical sections. Panel A compares the arrangement of the desmin filaments with skelemin. Both longitudinal and transverse desmin bundles are represented. Once again, skelemin appears to both colocalize (white arrow heads) as well as envelop (white arrows) desmin. Skelemin fluorescence emulates a diamond pattern when viewed in cross section. This same pattern is seen in Panel B where it is representative of both the myosin and skelemin staining. Intense colocalization, appearing as yellow fluorescence in the double label, is seen between myosin and skelemin (white arrow heads). The scaffold formed by the myosin and skelemin staining is consistent with the criss-cross arrangement believed to be manifested by the contractile components and the dense bodies. Thus, skelemin may be linking myosin filaments to each other as well as to the intermediate filament backbone.
Figure 8. Transverse 0.2 μm optical sections of adult feline uterus. The sections were double-stained using anti-skelemim antibodies and either anti-desmin (Panel A) or anti-smooth muscle myosin (Panel B). White arrow heads demonstrate regions of colocalization. White arrows demonstrate regions where skelemim occupies the areas defined by the desmin or myosin staining and colocalizes along their periphery. Magnification 1000 X. Bars measure 25 μm.
Discussion

The dual binding properties of skelemin make it a good candidate for stabilizing myosin-II filaments in smooth muscle regardless of the contractile state. Therefore, we have investigated the presence of skelemin, an M-disc associated protein, in smooth muscle tissues. Northern blot analysis comparing mRNA from a panel of human striated and smooth muscle tissues revealed that the ~5.8 kb skelemin mRNA is actively transcribed in both muscle types. This was the first discovery of a myosin-associated protein in smooth muscle tissue. It was not surprising that the hybridization signals were much less intense in the smooth muscle lanes. Within mature striated muscle, desmin comprises about 0.35%, myosin comprises about 50%, and skelemin comprises only about 0.1% of the total protein (Price, 1987). However, in smooth muscle, desmin constitutes 8-10%, and myosin compromises only 5-10% of the total protein (Cooke, 1976; Johnson and Yun, 1979). Therefore, this result could be a reflection of the amount of skelemin transcribed. However, these data can not be strictly interpreted as representing transcription rate since as the β-cytoplasmic actin probe illustrates, the lanes were not equally loaded. If the amount of skelemin is relative to the amount of myosin in striated and smooth muscle cell-type then skelemin transcript and protein in smooth muscle would be at barely detectable levels. This is what we found.
Western blot analysis of smooth muscle skelemin revealed an interesting result. Skelemin protein appears to have an ~5 kDa lower molecular weight in smooth muscle than in striated muscle. This difference could be the result of alternative splicing of the pre-mRNA transcript or differential protein modifications specific for each muscle type. As few as 50 residues could be responsible for this small difference between the two proteins. This equals an approximately 150 nucleotide splice in the smooth muscle skelemin transcript which would be undetectable by Northern blot analysis. This phenomenon has been previously observed in smooth muscle myosin heavy chain isoforms in which an alternative pre-mRNA splice site causes a 4 kDa difference between the isoforms. The difference was shown to result from alternative splicing at the 3' end of the transcript where insertion of an exon encoding 9 residues and a termination signal results in the shorter isoform (Barany, 1996). The existence of skelemin isoforms could have physiological relevance to the difference in the arrangement of the myosin-II filaments between the two muscle types as well as to the difference in their contractile forces. Further investigation into the amino acid sequence of smooth muscle skelemin, as well as using PCR to determine its mRNA sequence could determine the nature of these two possible isoforms.

Identification of skelemin by Western blotting was somewhat difficult to achieve. Skelemin appears to be highly susceptible to proteolysis. It was therefore necessary to homogenize all samples on ice in 5X protease inhibitor cocktail and prepare samples for SDS-
PAGE directly from the frozen homogenate. Samples in SDS could not be re-used. Furthermore, skelemin separates just below myosin-II on an SDS-PAGE. Thus, we had to load less total protein in order to detect skelemin. Additionally, it was necessary to use an 8% polyacrylamide SDS gel to enhance separation of the high molecular weight proteins. These technical refinements may be employed to detect other myosin-associated proteins in smooth muscle.

Low magnification analysis of skelemin immunofluorescence labeling has shown that skelemin localizes to the smooth muscle cells in visceral organs such as the uterus. Confocal microscopy of uterus smooth muscle revealed that skelemin associates with both the desmin intermediate filaments as well as the myosin-II filaments. Longitudinal sections showed that skelemin lies between the myosin-II filaments suggesting that skelemin may link myosin filaments to each other. Transverse sections showed that skelemin colocalizes with myosin filaments as well as extending outward in a diamond-like pattern. The scaffold formed by the myosin and skelemin labels is consistent with the criss-cross arrangement believed to be manifested by the contractile components and the dense bodies (Small, 1995 for review). Thus, skelemin may be associating myosin filaments of juxtaposed contractile units in smooth muscle in much the same way that it associates neighboring M-discs in striated muscle. Furthermore, skelemin was also seen to colocalize with the desmin intermediate filaments. This suggests that skelemin may function to associate the myosin-II filaments to the desmin cytoskeleton in smooth muscle much as it does in striated muscle tissue.
Figure 9 illustrates the updated model of the molecular network of the smooth muscle cell. Skelemin is shown cross-linking the myosin filaments to the desmin intermediate filament cytoskeleton as well as linking the contractile units. It appears that skelemin may serve as an extramyofibrillar cage encasing the contractile units in smooth muscle.

**Future Work**

This thesis has revealed many interesting properties of skelemin protein in smooth muscle. The possibility of skelemin isoform differences between smooth and striated muscle tissues is very exciting. PCR analysis of smooth muscle skelemin subclones can be performed to further elucidate the ~5 kDa difference between the two skelemens. Furthermore, *in situ* hybridization of sections of tissues rich in smooth muscle will be performed to confirm that skelemin mRNA transcripts are localized in the visceral rather than the vascular smooth muscle cells. The subject of whether skelemin is a component of the vascular smooth muscle cytoskeleton remains to be investigated. This topic is clinically relevant to the study of smooth muscle cytoskeleton remodeling in adapting to hypertension, or quick shape change in organs such as the bladder or large intestine. Furthermore, we studied only normal non-pregnant uterus. It would be interesting to study the role of the smooth muscle cytoskeleton in remodeling the post-partum uterus.
Figure 9. Modified schematic illustration of the structural organization of the cytoplasmic and contractile components in the smooth muscle cell. Skelemin protein may link the myosin-II filaments to each other as well as to the desmin intermediate filament cytoskeleton. Skelemin may also link the contractile units as an extramyofibrillar cage.
Chapter 2
Antisense oligonucleotide analysis of the role of skelemin in cell adhesion and myogenesis

Introduction

Skelemin protein is a member of a unique family of myosin-associated proteins that contains both fibronectin type III motifs as well as immunoglobulin C2 motifs (Price and Gomer, 1993). As a member of the immunoglobulin superfamily, skelemin resembles many adhesion molecules that appear to be responsible for cell-cell as well as cell-substrate association. These include neural cell adhesion molecules (N-CAM), fasciclin II, neuroglian, and β4-integrin (Price and Gomer, 1993). In addition, fibronectin type III motifs have been implicated in fibronectin's homophilic and heterophilic binding of cells to the extracellular components responsible for migration and adhesion and may function in this manner in other proteins containing these motifs. Recently, while investigating proteins that bind β3-integrin, Kumar Reddy of the Cleveland Institute, isolated skelemin protein from a two-hybrid assay. β3-integrins are involved in both cell-cell as well as cell-substrate adhesion. Thus, skelemin protein may be involved in mediating cell adhesion. To study the role of skelemin in cell adhesion and myogenesis, I have employed the use of embryonic stem cell technology. Embryonic stem (ES) cells are pluripotent tissue culture cell lines derived from the inner cell mass of a developing mouse
blastocyst (Robertson, 1987). These cells retain the phenotypic properties of the mammalian embryo when cultured under the proper conditions. When allowed to differentiate, ES cells can give rise to a diversity of cell types in vitro (Robertson, 1987). ES cells can aggregate to form colonies of pluripotent cells surrounded by an outer layer of endodermal cells which secrete α-foetal proteins to the inner cell mass (Robertson, 1987). These aggregates known as embryoid bodies (EBs) have been shown to follow as ordered a sequence of development in vitro as the embryo in vivo (Keller, 1993). The EBs are formed by suspending cell-containing droplets from the lid of a petri dish and incubating 4-5 days. When plated out onto gelatin coated tissue culture grade dishes, EBs will attach to the surface and spread outward, giving rise to differentiation of muscle cells as well as many other cell types (Robertson, 1987). Within 2 days of plating EBs, a rhythmic contraction indicative of cardiocytes can be seen within the central region of the EBs. Within 7 days, twitching myotubes will appear toward the periphery, and within 15 days, the wave-like contraction of smooth muscle can be seen toward the center of the EB.

Skelemin's binding properties may make it difficult to construct knock-out transgenic mice. Therefore, antisense and sense 24-mer oligonucleotide constructs were used to assay the phenotype of under-expression of skelemin during differentiation. Antisense oligonucleotides have been shown to be a valuable tool in blocking mRNA translation, providing a means by which to study the under-expression of skelemin during development (Shoemaker et al., 1990).
Materials and Methods

Cell Culture
Frozen AB 2.2 clone #011 passage 13 ES cells were obtained as a gift from the laboratory of Dr. Alan Bradley (Baylor College of Medicine, Houston, TX). ES cells were quick thawed in a 37 °C water bath. Cells were diluted by slowly adding 5 ml of M15 media [high glucose DMEM culture medium containing GlutaMAX (Gibco BRL) supplemented with 15% ES qualified fetal bovine serum (Gibco BRL), 50 units/ml penicillin, 50 μg/ml streptomycin (Gibco, BRL), 150 μM α-monothioglycerol] dropwise while agitating (Robertson, 1987). ES cells were pelleted and resuspended in 5 ml M15 media and plated onto gelatin type A (Sigma) coated tissue culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) and set in a 37° C, 5% CO₂, water jacketed incubator (Forma Scientific, Marietta, OH) to proliferate. To maintain the pluripotent phenotype during proliferation, culture medium was supplemented with Leukemia Inhibitory Factor (LIF) (Gibco BRL) at a concentration of 1000 units/ml. Culture media was changed and supplemented with LIF daily. Once the ES cells reached 80% confluency, approximately 2 days following plating, the cells were passaged. Cells were washed with 5 ml Hanks’ Balanced Salt Solution (HBSS) Ca++, Mg ++ free (Gibco BRL) for 10 min at 37° C. The wash was removed and replaced with 2.5 ml of 0.125% trypsin-EDTA in HBSS and incubated 3 min at 37° C. Cells were pipetted vigorously using a sterile transfer pipette to dissociate cell colonies. This suspension was transferred to a 15 ml Falcon tube (Becton
Dickinson) and centrifuged. Cell pellets were resuspended in 3-5 ml of M15 and counted as a 1:10 dilution on a hemocytometer. ES cells were seeded at 5 x 10^6 cells per 100 mm tissue culture dish in 10 ml of M15 plus LIF for propagation and incubated as above.

**Antisense/Sense oligonucleotide treatment**

Sense and antisense sulfur modified-oligos were created to the start of the skelemin message including the last few bases of the 5' UTR (See Figure 10 below) (Oligos, etc, Wilsonville, OR).

<table>
<thead>
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<th>Skelemin message</th>
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<tr>
<td></td>
<td>GGATGTCTCTGCCCCTTTATCAGC</td>
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<td></td>
<td>GGATGTCTCTGCCCCTTTT</td>
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**Figure 10.** Sequence of skelemin antisense and sense S-oligonucleotides. Translational start site of skelemin is underlined.

The sulfur modification provides stability to the oligonucleotides. ES cells were diluted in 2 ml of M15 differentiation media (high glucose DMEM culture medium containing GlutaMAX (Gibco BRL) supplemented with 15% fetal bovine serum (Sigma), 50 units/ml penicillin, 50 μg/ml streptomycin, 150 μM α-monothioglycerol) such
that there were 600-800 cells per 20 μl drop. The M15 differentiation media contains the same components as proliferative M15 with the exception of the source of the fetal bovine serum. This serum has been shown to give rise to greater percentages of muscle cell differentiation (personal communications, Derek Milner). The dilutions were treated with 500 μM antisense or sense oligonucleotides to a final concentration of 1.25 μM. Cells were then plated as 20 μl drops onto petri dish lids. The petri dish bottoms were filled with 500 μl of sterile water and the lids were inverted on top such that the drops were hanging to force aggregation for embryoid body formation (Robertson, 1987). The hanging drops were placed in the 37°C, 5% CO₂ incubator and left undisturbed for 4.5 days (Robertson, 1987). Following the 4.5 days, the EBs were removed from the petri dish lid using wide bore pipette tips and plated into 12 multi-well type-A gelatin coated tissue culture dishes (Becton Dickinson). Antisense treated EBs were plated simultaneously into media treated with 1.25 μM antisense, and media lacking antisense oligonucleotide treatment for recovery assays. Sense treated oligonucleotides were plated into media that was continuously treated with 1.25 μM sense oligonucleotide. A series of EBs that never received oligonucleotide treatment were used as a control.

Photography and videomicroscopy
Phase contrast microscopy was performed using a Nikon Diaphot microscope. Photographs were taken using a Nikon DR-3 camera on
TMAX black and white p3200 film (Kodak) and developed commercially. Videomicroscopy was performed using an Ikegami Tsushinki video camera (Japan) and recorded onto T120 professional VHS videocassette (3M, St, Paul, MN) using an RCA VHS 4 head videosystem (Thomson Consumer Electronics, Indianapolis, IN).

**Results**

I have performed a series of experiments to determine when antisense oligonucleotide treatment is most effective during development. EBs were formed in media containing antisense or sense oligonucleotides at 5 μM, 2.5 μM, 1.25 μM, and 0.6 μM, and in media lacking oligonucleotides. The EBs were plated out into tissue culture dishes such that one series was treated with oligonucleotides during the formation of the EBs. The other series was not treated. Following EB formation, EBs from the pretreated series were exposed to the following conditions. One group received continued oligonucleotide treatment at the initial concentration. Another group was allowed to recover from oligonucleotide pretreatment in media lacking oligonucleotides. EBs from the untreated series were exposed to both sense and antisense oligonucleotides at the above concentrations. In both series, EBs were formed and allowed to differentiate without oligonucleotide treatment as a control for normal development.

Oligonucleotide treatment following EB formation did not result in abnormal phenotypes. The EBs differentiated as normal in all experimentals as well as controls. Oligonucleotide concentrations
above 2.5 μM resulted in aberrant differentiation not only within the antisense treated experimentals, but also within the sense treated controls. This may have been a result of high salt concentrations within the oligonucleotide mixture. However, oligonucleotide treatment at or below 2.5 μM added before EB formation resulted in abnormal phenotypes of the antisense treated EBs only. This abnormality resulted in either complete dissociation of the EB within 24 hours (Figure 10 A) or intact EBs that failed to attach to the petri dish (Figure 10 B). Within 2 days, those EBs that remained intact began to contract while floating in the medium. This is shown in Figure 10 B. The outer cells of the unattached, rounded EB are loosely associated. Video microscopy revealed that these outer cells were being broken free of the EB by the rhythmic contraction of the differentiated cardiocytes. The group of EBs allowed to recover from antisense oligonucleotides attached within 2 days and differentiated as normal (Figure 10 C). Interestingly, antisense recovery EBs developed myotubes that were more prominent than the sense and untreated controls (Figure 10 D, E, and F). EBs formed in sense oligonucleotides attached and differentiated at the same rate as the untreated controls (Figure 10 E and F respectively).

To determine if there is a turning point in development where cells cannot recover from antisense oligonucleotide treatment, I performed experiments in which EBs were formed in the presence of antisense oligonucleotide and treated with antisense oligonucleotide for 4 days after which they were allowed to recover. These cells did not form muscle cells even 2-3 weeks following oligonucleotide removal. Other cell types had differentiated.
Figure 11. Comparison of the phenotypes of embryoid bodies (EBs) treated with sense and antisense oligonucleotides. EBs treated with continued exposure to 1.25 μM skelemin antisense oligonucleotides, either dissociate after 24 hours (A) or remain intact but lose cell-substrate adhesion after 48 hours (B). EBs allowed to recover from antisense oligonucleotide treatment adhere to substrate after 48 hours (C) and display prominent myotubes after 15 days (D). EBs treated with continued exposure of 1.25 μM skelemin sense oligonucleotides (E) adhere and differentiate as would normal untreated EBs (F). Magnification 100 X for A, C, D, E, F. Magnification 200 X for B to illustrate the loose association of the outer layer.
**Discussion**

Antisense oligonucleotide treatment combined with ES cell technology has provided an essential tool for manipulating the expression of proteins and observing their effects during embryonic development (Shoemaker *et al.*, 1990; Van der Krol *et al.*, 1988). Addition of antisense oligonucleotides must be added prior to EB formation to observe abnormal development. The EBs are so densely populated that treatment afterwards is not sufficient to affect the majority of the cells. Furthermore, the oligonucleotides may not be able to penetrate to the inner cells of the EBs. A final concentration of 2.5 μm or below of skelemin antisense/sense oligonucleotide is adequate to obtain abnormal phenotypes in the antisense treatment only. Antisense skelemin oligonucleotides appear to inhibit early cell-cell as well as cell-substrate interactions. Thus, skelemin may be involved in cell adhesion.

Antisense recovery EBs were delayed in attachment and subsequent differentiation. This delay may be compensatory in nature reflecting the recovery rate for protein production subsequent to skelemin deficit resulting from antisense oligonucleotide treatment. Interestingly, recovery EBs formed prominent myotubes. This may be due to the cell population at the time of differentiation. After the pretreated antisense oligonucleotide EBs are plated, some dissociation occurs. Single cells that are incapable of attaching to the surface are no longer viable. As a result, the prominent myotubes may be visible due to a simple lack of crowding by connective tissue.
Skelemin antisense and sense experiments performed by Ed Frank with C2C12 cells, a predispositioned mouse muscle cell line support these results. Antisense skelemin treatment resulted in loss of cell-cell adhesion initially, followed by loss of cell-substrate adhesion with continued treatment. Indirect immunofluorescence of normal, sense treated C2C12 cells using anti-skelemin antibodies displayed small spikes of filamentous skelemin protein within the outer edge of the cell membrane where two myogenic cells were aggregating to fuse. Where cell-cell adhesion had occurred, skelemin protein was aligned at the cellular junctions. Immunofluorescence of the antisense treated C2C12 cells resulted in loss of skelemin staining. These data support the observations of skelemin's cell adhesion properties during EB formation and differentiation. Furthermore, they demonstrate a direct loss of skelemin protein resulting from skelemin antisense treatment.

Kumar Reddy has found in two-hybrid studies using portions of skelemin protein, that the cytoplasmic tail of β3-integrin binds to the immunoglobulin C2 motifs 4 and 5 of skelemin. This could be the molecular basis for the observed skelemin phenotype. Thus, skelemin has been shown to have three different and essential binding properties. It binds myosin of the contractile domain, desmin of the cytoskeletal domain, and now β3-integrin. Thus, skelemin may be involved in mediating the cytoskeletal response of a cell to external stimuli such as contact with another cell or the substrate.
Future Work

The use of skelemin antisense oligonucleotides suggests that skelemin may be involved in cell adhesion. Further investigation of this premise would include Western blot analysis comparing the presence of skelemin protein in adhered cells verses dissociated cells resulting from EBs formed in sense and antisense oligonucleotides. To determine that the oligonucleotides are specific for skelemin, Northern blot analysis can be performed using the end-labeled oligonucleotides as a probe of embryoid body RNA.
Chapter 3
Examination of the role of the cell cycle in mediating cell-type choice during mammalian development

Introduction

Cell type choice can be mediated by cell autonomous or extrinsic mechanisms. Cell autonomous response can occur through asymmetrical division or cell cycle stage recognition. Intercellular communication can occur through secreted factors or through direct cell contact. Cell cycle dependence of cell-type choice has been seen in development of *Dictyostelium discoideum*. In this organism pre-stalk cells arise from cells in S-phase or early G2 at the time of starvation, and pre-spore cells develop from cells in late G2 or M-phase at the time of starvation (Gomer and Firtel, 1987). To explore the role of the cell cycle in cell-type choice during mammalian development, I employed the use of mouse embryonic stem cells. As mentioned earlier, ES cells retain the phenotypic properties of the embryo. When allowed to differentiate, ES cells can give rise to a diversity of cell types *in vitro* (Robertson, 1987). This property provides a means through which early cell type differentiation can be traced and manipulated more easily than within the developing embryo.
Materials and Methods

ES cell culture
ES cell line AB 2.2 clone #011 was donated by Dr. Alan Bradley (Baylor College of Medicine, Houston, Texas). Cells were cultured as described in Chapter 2.

Immunostaining
ES cells were plated onto gelatin coated glass coverslips placed in 12 well plates (Becton Dickinson) with a seeding density of 5 X 10^4 cells per well. Cells were covered with 1.5 ml of M15 media plus LIF at a final concentration of 1000 units/ml and set at 37°C, 5% CO₂ for 24 h. Afterwards, LIF was removed by replacing the media with fresh M15 media. The cells were then allowed to differentiate. Coverslips were fixed at daily intervals in 100% ice cold anhydrous methanol for 15 minutes. ES cells were immunostained using the following antibodies: monoclonal rat anti-glial fibrillary acidic protein (Clone 2.2B10, Zymed, San Francisco, CA) at 50 μg/ml, mouse anti-neural filament 70 kDa + 200 kDa (Clone 2F11, Fisher) supplied in the working dilution, polyclonal rabbit anti-neural filament 200 (Sigma) diluted 1:80, monoclonal mouse anti-myosin light chain kinase IgG2b (Clone K36, Sigma) diluted 1:100, monoclonal mouse anti-smooth muscle myosin (Clone hSM-V, Sigma) diluted 1:500, monoclonal mouse anti-vimentin (Clone V9, Fisher) at 10 μg/ml, and monoclonal mouse anti-keratin protein (Clone AE1, Fisher) supplied in the working dilution. Staining procedures were followed as
described in Chapter 1. Secondary antibodies were used accordingly including: fluorescein conjugated goat (Fab')2 anti-rabbit IgG (Cappel), fluorescein conjugated goat (Fab')2 anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL), fluorescein conjugated goat (Fab')2 anti-mouse IgG2b (Southern Biotechnology Associates), Texas red conjugated goat F(ab')2 anti-mouse IgG (Jackson, West Grove, PA), and Cascade blue conjugated goat F(ab')2 anti-mouse IgG (Molecular Probes). Coverslips were mounted in anti-fade and analyzed for single positive, dual positive or negative immunofluorescence using a Nikon Microphot-FX microscope equipped for phase contrast and epifluorescence. Cells were counted and averaged for graphical analysis using Microsoft Excel.

**Time Lapse Photography**

Coverslips were fitted with a 2 cm X 2 cm double well chamber sealed with paraffin wax to retain liquid. ES cells were seeded at 5 X 10⁴ cells per well in a total volume of 1.5 ml of M15 culture media treated with 1000 units/ml of LIF to prevent differentiation. The cells were filmed at 300X magnification using an ImagePoint scientific cooled CCD video camera (Photometrics Ltd., Tucson, AZ). This was achieved by setting a warmed Nikon TMS phase-contrast microscope into a 37° C, 5% CO₂ incubator. A constant 5 volt light source was directed through both a standard blue lamp filter and a green interference filter to remove heat (Nikon). One image was recorded every eight seconds on a Sony 8 mm Watchcorder (Sony). After 24 h, the cell positions were marked and the media was
changed to a LIF free equivalent. Images were recorded for another 24 h. Following video recording, cells were fixed for 2 minutes in 3% formaldehyde warmed to 37°C. Cells were then washed in 70% ethanol at room temperature for 10 minutes. Finally, cells were fixed again for 10 minutes in 95% ethanol. The filmed cells were immunostained using rat anti-glial fibrillary acidic protein and mouse anti-myosin light chain kinase, and assayed for positive immunofluorescence labeling. Acetate drawings of the cells contained within the filmed screen were made for identification during the immunofluorescence assays.

Results and Discussion

We wanted to study cell-type choice at the earliest time point in order to determine exactly when and what conditions resulted in the expression of certain gene products. Therefore, we used indirect immunofluorescence to determine which proteins were significantly expressed early in differentiation. Cells which were allowed to differentiate were fixed at 1 day intervals and immunostained for the expression of either keratin, the muscle specific proteins vimentin, smooth muscle myosin, and myosin light chain kinase, or the neuronal specific proteins neural filament 70 kDa and 200 kDa, and glial fibrillary acidic protein. Epifluorescence microscopy revealed that neural filament 200, glial fibrillary acidic protein, myosin light chain kinase, and smooth muscle myosin were the most represented cell types during the first 3 days of differentiation.

Next, we wanted to narrow the earliest timepoint at which the
cells start expressing these proteins. To determine the best time frame for filming the cells, I seeded at a density of 5X10^5 ES cells onto gelatin coated coverslips and allowed them to proliferate 24 hours in media containing LIF. After 24 hours, the media was replaced with media lacking LIF and the cells were allowed to differentiate. Samples were fixed at time points 0 LIF removal; 12 hours post LIF removal; 24 hours post LIF removal; and 48 hours post LIF removal. I double-stained the coverslips for each time point with antibodies against each of the following: glial fibrillary acidic protein and smooth muscle myosin, and neural filament 200 and myosin light chain kinase. Cells were recorded as single positive, dual positive or negative. Graphical analysis of these results (See Figure 12) reveals that less than 1% of the cells were positive for glial fibrillary acidic protein, neural filament 200, smooth muscle myosin and myosin light chain kinase as early as 1 day. This is the first evidence of specific cell type commitment at such an early time point.

To trace the lineage of the differentiated cell types, I seeded 5x10^4 ES cells onto gelatin coated glass coverslips and recorded images using a time lapse video recorder. I found that a maximum of 5 volts of light could be used safely if coupled with blue and green interference filters. The cells were filmed for 24 hours in media containing LIF. Afterwards, the media was replaced with media lacking LIF and the cells were allowed to differentiate another 24 hours. This allowed me to identify the stage of the cell cycle the ES cells were in when LIF was removed based on the time of division. An acetate drawing of the filmed ES colony was made and used to
Figure 12. Time course analysis of the percentage of ES cells expressing neural filament 200 kDa, myosin light chain kinase, smooth muscle myosin, and glial fibrillary protein.
record the location of positive cells. The coverslips were fixed and double-stained using antibodies against glial fibrillary acidic protein and myosin light chain kinase as selected from figure 11. I attempted to trace the lineage of the positive cells by watching the film in reverse order. I was able to successfully film several lineages; however, the cells were dividing and growing on top of each other forming a spherical colony. The microscope and camera are limited to one plane of focus during the filming; therefore, the cells I was tracing tended to get lost in the colony or move out of the focal plane.

Es cell survival is dependent upon cell density; therefore, high cell number is required for growth. As a result, cell colonies became large as cells divided and stacked upon each other. This made discerning individual cells difficult. I have tried several methods to overcome this problem and increase individual cell resolution. I have tried to focus on small colonies of 5-10 cells. However, small colonies were less suitable to provide for the 1% positives and had a tendency to be less viable and/or mutated.

Another possible solution was to lower seeding density while maintaining cell viability and the pluripotent phenotype. To accomplish this, I tried to isolate a conditioned media that would mimic a high seeded density environment. I had collected media at 1 day and 2 day intervals from ES cells growing at high density and performed parallel experiments using serial dilutions of conditioned medium on various densities of cells. Another parallel series of experiments was performed using dilutions of recombinant CMF, a density sensing factor isolated from \textit{D. discoideum}, and sterilized
bovine skim milk which contains many nutritive elements. None of the conditioned media improved viability at low seeding density. I noticed on the time lapse film that the cells aggregated and formed large colonies. Therefore, I explored methods to slow cell movement and intercellular adhesion. First, I tried to lower the temperature at which the cells were growing to 30° C to slow cell movement not interfering with the cells’ ability to divide. However, the heat emitted by the camera and microscope within the incubator maintained a constant temperature of 35° C which was not enough to slow the cells. Next, I tried parallel dilutions of EDTA ranging from 100 μM to 10 mM final concentration. However, low levels of EDTA had no effect and high levels prevented the cells from attaching to the gelatin surface of the culture dish. No concentration of EDTA slowed movement without disturbing cell attachment.

Initial time lapse analysis was recorded using a Panasonic PL200 CCD video camera. This camera did not project high quality images with the low light intensity required for cell viability. To improve the quality and resolution of the images projected, we obtained an ImagePoint scientific cooled CCD video camera. This camera had many programmable features which allowed for increased resolution by increasing the length of the exposure, digitizing the image, expansion of the gray scale, and control of the image amplifier gain. However, this camera is limited to one plane of focus. Further refinements of the image would be to film the cells using a digital imaging board that would record serial sections of the ES cells at defined time intervals. This would allow one to view the
differentiating colony in three dimensions. Thus one could follow the positive cells through the colony without disturbing too many differentiation factors or introducing stressful conditions for mutation selection.

**Future Work**

Several refinements must be made to increase resolution of recording individual cells. These refinements can be achieved in part by isolating a conditioned medium that would allow for increased survival rates at lower seeding densities thereby alleviating the problem of overcrowding and enabling one to discern individual cells. However, care must be taken to prevent introducing external signals that may influence differentiation of one particular cell type. Another technique to increase resolution could be the use of a digital imaging board. This system would make serial sections through the ES colony. Such optical sectioning could minimize the problem of the cells moving out of focus and thus permit them to be followed in their normal state without disrupting cell contact.
REFERENCES


# Appendix A

## Table of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDB</td>
<td>Cytoplasmic dense body</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>MADB</td>
<td>Membrane-associated Dense Bodies</td>
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<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>NF 200</td>
<td>Neural filament 200 kDa</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>O.C.T.</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SM Myo</td>
<td>Smooth muscle myosin</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium Chloride/Sodium Citrate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate/EDTA</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
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</table>