MQP-BIO-DSA-9388
MQP-BIO-DSA-0101

UPREGULATION OF NEURONAL GROWTH GENES DURING A THERAPY FOR RAT SPINAL CORD INJURY

A Major Qualifying Project Report
Submitted to the Faculty of the
WORCESTER POLYTECHNIC INSTITUTE
In Partial Fulfillment of the Requirements for the
Degree of Bachelor of Science
in
Biology and Biotechnology
by

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April 27, 2006

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ABSTRACT

Three dimensional cell-derived scaffolds charged with neuronal growth factors are currently being evaluated as therapeutics for healing spinal cord injuries in rats (Total ReCord, Inc). Our working hypothesis is that the growth factors stimulate neuronal re-growth, while the scaffold provides structural support for the elongating neuron. To determine if specific neuronal growth factor genes are activated by the therapy, an *in vitro* model was developed in which cultured human SHSY neuronal cells were treated with the growth factors used to charge the scaffold, and the upregulation of specific genes was monitored by RT-PCR. The data indicate the therapy causes the upregulation of a number of genes known to promote neuronal growth including, but not limited to Neuregulin-1, Fibronectin, GAP-43, and NGFR.

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ACKNOWLEDGEMENTS

We would like to take this opportunity to express our most sincere thanks to Professor Dave Adams. He has been an amazing professor, advisor and mentor. His passion for teaching and science is clearly evident through his courses and his interaction with students. His dedication to students and academics has shaped our extremely positive experiences at WPI. He welcomed us into his lab, and was kind and patient with us throughout the entire project. Without his guidance and support we would not be where we are today. We would also like to thank Total Record Inc. (Worcester, MA) for supplying us with both funding and their experimental therapy. We wish them success with this product and hope that it will be able to help many people in the future. Our thanks go out to JoAnn Whitefleet-Smith who allowed us to use the cell culture research facility. Without this experience our project would not have been possible. Next we would like to thank Kirk Stovall who provided us with a flask of SHSY cells with which we initiated our cell culture. He also provided us with guidance regarding cell culture techniques throughout our project that was extremely helpful. Lastly we would like to thank Turkan Arca. Her thesis was an extremely helpful guide for writing this paper.

BACKGROUND

The Need for Tissue Substitutes

Human organ failure and tissue loss result in approximately eight million surgical procedures performed annually in the U.S. at a cost of over $800 billion (Langer and Vacanti, 1993). Three main approaches have been utilized as therapy: 1. tissue transfer (the transfer of tissue from a healthy site in the same individual, or from another individual), 2. medical devices (the use of an artificial device to support the function of the lost tissue), and 3. drugs (the use of pharmacological agents to compensate for the lost tissue. Unfortunately, several problems often accompany these approaches, including: for the first approach, the limited number of tissue and organ donors, imperfect donor matches, and dependence on immunosuppressants; for the second approach, the limited durability and tissue compatibility of mechanical devices, for the third approach, the complexity of prolonged pharmacologic supplementation. To address these problems, new approaches are being developed, including the use of cells grown in scaffolds or matrices (living tissue equivalents, LTEs), and cell-derived matrices (CDMs) (for reviews see: Fuchs *et al*., 2001; Vacanti, 2003).

Living Tissue Equivalents (LTEs)

Living tissue equivalents (LTEs) are made by mixing viable cells with a soluble biopolymer solution (collagen, fibrin, and/or proteoglycans). The biopolymer solution serves as a scaffold, a physical support for cell attachment and tissue development. The scaffolds are designed to resemble the structure and composition of the extracellular

matrix (ECM) the cells normally interact with *in vivo*. Over the next few days of culture, the cells rearrange and partially degrade the biopolymer scaffold while synthesizing new proteins. LTEs have extensively been used as *in vitro* models for wound healing (for a review see: Grinnell, 2003), and are already in use as replacements for lost or damaged connective tissue (for example in burn patients, Apligraf™ from Organogenesis, Inc.).

Apligraf™ is the first FDA-approved tissue engineered product. It is a "living skin equivalent" containing a fibroblast populated matrix that serves as the "dermis", which provides structural and nutritional support to the growing epithelial cells of the epidermis (Eaglstein and Falanga, 1998; Parenteau, 1999). Apligraf™ produces no immunorejection since it does not contain antigen presenting cells. It has been hypothesized that the functionally flexible fibroblast cells adapt to the environment into which they have been placed, and take corrective action by producing the appropriate soluble factors (Lee, 2000).

The best characterized LTEs include cell-seeded collagen and fibrin gels. Collagen was one of the earliest and most widely used natural scaffold (Bell *et al*., 1979). More recently, stronger scaffolds made of poly-glycolic acid (PGA) and poly-lactic acid (PLA) have been used, but these are potentially immunogenic (Ye *et al*., 2000). LTEs have several advantages over the earlier versions that used totally synthetic scaffolds since the substrate is natural, and is conducive to cell spreading and extracellular matrix formation (Neidert *et al.*, 2002).

The major drawback of these gel-based LTEs is they are weak relative to normal connective tissues; it is important to create artificial tissues that can withstand strong *in vivo* mechanical forces (Gildner and Hocking, 2003). Other drawbacks include low

enzymatic and thermal stability when placed in *in vivo* conditions, and the presence of animal components (collagen, fibrin, serum) that can result in graft rejection.

Cell-Derived Matrices (CDMs)

Cell-derived matrices (CDMs) are a type of LTE in which the scaffold for the seeded cells is derived directly from cells. Fibroblasts provide the best studied CDM system. Several research groups have demonstrated that under certain conditions fibroblasts can be induced to produce a thin three-dimensional sheet of extracellular matrix material (ECM) *in vitro* (Grinnell *et al.*, 1989; Clark *et al.*, 1997; Ishikawa *et al.*, 1997; L'Heureux *et al.*, 1998; Ohgoda *et al.*, 1998). The fibroblast CDM is predominantly composed of supermolecularly organized collagen (Grinnell *et al.*, 1989; Ishikawa *et al.*, 1997), and more closely approximates native tissue than reconstituted collagen gels (Clark *et al.*, 1997; L'Heureux *et al.*, 1998). Fibroblast CDMs support significantly faster rates of cell adhesion, migration, proliferation, and acquisition of *in vivo*-like morphology than reconstituted ECM (Cukierman *et al.*, 2001). During the culture period, the multilayered fibroblasts assume a bio-synthetic phenotype characterized by low cell proliferation, high collagen accumulation, fibrillar fibronectin organization, and the formation of actin stress fibers and focal adhesions (Kessler *et al.*, 2001).

The drawback of CDMs is they are very thin and take a long time to grow, generally several months (Auger *et al*, 2000), whereas collagen gels and fibrin gels can be developed in only a few days (Grinnell, 1994). However, recent advancements using chemically defined media for promoting high ECM synthesis shorten the culture time to

just a few weeks, while producing thicker stronger matrices (Billiar *et al*., 2000; 2005). Instead of using undefined serum supplementation, the new culture medium includes growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), dexamethasone, and L-3,3',5-Triiodothyronine, along with a few basic components necessary for cell growth (insulin, selenious acid, and a lipid precursor). Many growth factors, including bFGF, can accumulate and retain their activity for relatively long periods of time within the ECM (Aktas and Kayton, 2000; Bottaro *et al.*, 2002).

Total ReCord, Inc.

Recently, a new CDM system has been developed using human blood instead of cultured fibroblasts (Total ReCord, Inc., Worcester, MA; formerly New World Labs, Inc.). The blood-derived Regeneration Matrix™ system (RMx) has shown the ability to heal spinal cord injuries in a rat model. RMx in the future may show similar restoration of functionality in other models of CNS damage such as stroke, Multiple Sclerosis, Alzheimer's disease, Parkinson's disease, and ALS. This therapy is a powerful alternative to embryonic stem cells, and is the first acellular, non-drug based regenerative medicine. The RMx promotes the upregulation of neurtrophic factors, neurite outgrowth and the formation of new blood vessels to support new CNS tissue growth.

Neurotrophic Factors

A newly discovered family of proteins called neurotrophic factors (NTFs) has been found to be responsible for the maintenance of adult neurons, and the growth and

survival of neurons during development. NTFs also have the ability to control neuronal stem cell differentiation, which makes them an excellent target for a potential therapy. These proteins, which are present in the central and peripheral nervous system, also appear to have the ability to repair neuronal processes in damaged neurons. It has been shown that NTFs are synthesized when neuronal cells are under a stress such as ischemia (Abe, 2000). Additionally, exogenous administration of NTFs was also shown to protect brain tissue from oxygen deficiency. These factors are very likely involved in the mechanism of action of the RMx on the spinal cord neurons.

Genes of Interest in this MQP

Fibronectin-1

The Fibronectin-1 gene encodes a high molecular weight rod-like glycoprotein called Fibronectin. Fibronectin can be found in two forms: a soluble dimeric form found in the plasma, or as an insoluble glycoprotein dimer or multimer that serves as a linker to the extracellular matrix (Baron *et al*, 2000). Fibronectin is known to be involved in many processes including tissue repair, embryogenesis, wound healing, blood coagulation, host defense, and other cell adhesion and migration processes. Current literature shows that fibronectin stimulates axonal regrowth when implanted into damaged spinal cord tissue (King *et al*, 2005). These data suggests that fibronectin could be a key player in the mechanism of action of Total ReCord's RMx.

Neuregulin-1

The Neuregulin-1 gene encodes a 44-kD glycoprotein called Neuregulin. The gene produces a large number of isoforms of the protein, which have varying activities, by alternative splicing. These isoforms include glial growth factors, heregulins, and sensory and motor neuron-derived factor (Ho *et al.*, 1995). The varying isoforms of the protein are expressed in a tissue-specific manner, and differ significantly in their structure. Some forms of the protein contain immunoglobulin domains, whereas others contain kringle-like domains; however, all forms of the protein contain epidermal growth factor-like domains. All isoforms of the Neuregulin-1 gene act as ligands to the ERBB family of tyrosine kinase transmembrane receptors. The interaction of the protein with these receptors induces the growth and differentiation of epithelial, neuronal, glial, and other types of cells. These proteins have also been known to upregulate acetylcholine receptor genes.

GAP-43

The Growth-Associated Protein-43 (Gap-43) gene encodes a membrane and cytoskeletal associated phospho-protein that is expressed at high levels in neuronal growth cones during development and axonal regeneration (Chong *et al.*, 1994). Although GAP-43 is not necessarily essential for neuronal outgrowth, it plays an important role in axonal guidance and "decision points". Literature has shown increased GAP-43 expression in injured neurons which further suggests its regenerative tendency (Bradbury *et al*, 1995). The protein is considered a vital component of an effective regenerative response in the nervous system.

NGF Receptor

The NGFR gene encodes a 75-kD surface receptor glycoprotein called Nerve Growth Factor Receptor. This receptor is found on non-myelin forming Schwann cells, nerve fibers, and perineural cells (Fetsch *et al,* 2005). The protein contains an extracellular domain which contains four 40-amino acid repeats, with 6 cysteine residues found at conserved positions. This cysteine-rich area contains the Nerve Growth Factor binding domain. Literature suggests that NGFR mediates an axonal guidance function for restoring and developing spinal cord neurons. This was shown by increased levels of NGFR mRNA transcripts after a lesion was induced on rat spinal cords.

Trk-1

The Trk-1 gene encodes an auto-phosphorylating membrance-bound receptor called Neurotrophic Tyrosine Kinase Receptor. Upon neurotrophin binding, this kinase, phosphorylates itself and also members of the Mitogen-Activated Protein Kinase (MAPK) pathway. The Trk receptors play an important role in the regulation of development for both the central and peripheral nervous system (Bothwell, 1996). For example, the Trk protein acts as the main receptor for NGF, and participates in its primary signal transduction.

PROJECT PURPOSE

Total ReCord, Inc. (formerly New World Laboratories) has developed a human blood cell-derived regenerative matrix (RMx) that has been experimentally shown to promote the healing of spinal cord injuries in rats. RMx consists of a polymerized cell-derived scaffold loaded with a proprietary mix of growth factors. The mechanism of how RMx regenerates spinal neurons is unknown, as is the relative roles played by the scaffold versus the loaded growth factors.

Our lab is interested in investigating the mechanisms responsible for the ability of RMx to heal those injuries by developing an *in vitro* cell culture-based system for analyzing changes in gene expression induced by the interaction of neuronal cells with RMx. The purpose of this MQP was to use RT-PCR to analyze the expression levels of several candidate genes related to neuronal growth in control cells versus cells treated with the proprietary growth factor mixture to determine which genes are being induced in neuronal cells by the growth factor component of the RMx.

Although RMx has primarily been tested in rats for cord regeneration, RMx is constituted from human blood cells and human growth factors, so we chose the SH-SY5Y human neuroblastoma cell line as the model system. The cells were cultured in therapeutic (RMx polymerizing medium) and control media, and RNA was extracted for analysis by RT-PCR. Our working hypothesis is that the RMx polymerized scaffold serves to guide the axons, while the growth factors induce neuronal growth. Discovering the role of these genes in neuronal regeneration could lead to new advances in the treatment of spinal cord injuries in humans.

MATERIALS AND METHODS

SH-SY5Y Cell Culture

Cell Properties and ATCC Recommendations

The SH-SY5Y human neuronal cell line was originally established in 1970 from the bone marrow biopsy of a four year old girl with a metastatic neuroblastoma. The source of the tumor was identified as the brain tissue. The cells were purchased from American Type Culture Collection (ATCC) (Catalog No. CRL-2266). Although ATCC indicates SH-SY5Y cells (abbreviated SHSY) grow as a mixture of suspension and adherent cells with an epithelial morphology, in our hands this is predominately an attached cell line, with some floaters. They are reported to grow in clusters with multiple, short and fine neurites. These cells will aggregate, form clumps and float in culture. The ATCC recommended subculture ratio was 1:20 to 1:50, with a doubling time of around 48 hours, but in our hands a 1:2 split was optimal. Recommendations also included growth in a 5% CO_2 incubator at 37˚C, with medium renewal every 4-7 days.

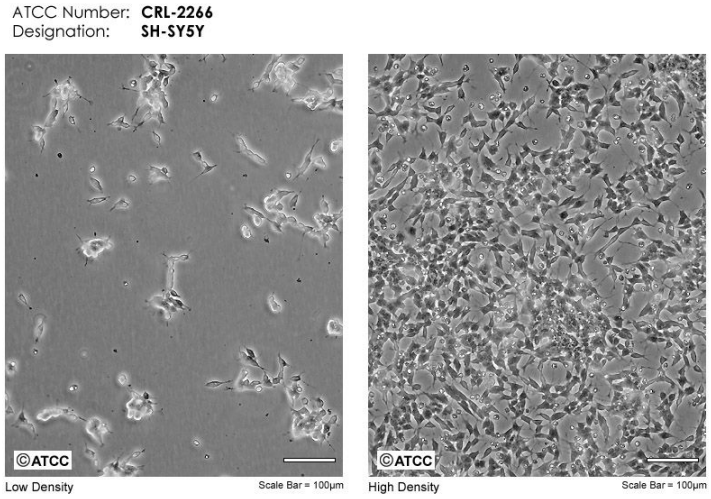

Figure 1. Micrographs of SHSY Cells in Culture (ATCC).

Preparation of Complete Culture Medium

The medium used for subculturing was prepared as recommended by ATCC. A 1:1 mixture of DMEM and F-12 (Dulbecco's Modified Eagle's Medium with non-essential amino acids and Ham's F-12 Nutrient Mixture) was used. DMEM/F-12 (ATCC) contained 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate and 1200 mg/L sodium bicarbonate. The medium was supplemented with Fetal Bovine Serum (FBS) (to 10%) (i.e. 50 ml of 100% FBS added to a "500 ml" DMEM/F-12 bottle) and Gentamycin (to 5 µg/ml) (i.e. add 250 µl of a 10 mg/ml Gentamycin stock to the 500 ml bottle). After adding the media components, the complete medium was sterilized using a 0.2 µm filtration unit with a vacuum pump. The medium was stored at 4˚C, and pre-warmed in a 37˚C water bath before use.

Cell Plating, Subculturing and Maintenance

A T-25 flask of SHSY cells containing about 6 ml of medium was provided by Kirk Stovall. Cells were allowed to grow to 70-80% confluency, then split 1:2 into a T-75 flask containing about 15 ml of medium (Table 1). Cell splitting used cell scrapers instead of trypsinization since breaking apart small clumps of cells was not critical for these experiments. Splitting was repeated until the cells reached 70-80% confluency in a T-150 flask containing about 25 ml of medium. When performing 1:2 splits, the cells were split every 3-4 days.

Table 1. Routine SHSY Culture Volumes (ml)

Flask	T-25	T-75	T-150
Volume of Medium	6 ml	15 ml	25 ml

Cell Treatment

Cells were grown to 70-80% confluency in T-150 flasks, then the medium was siphoned to waste and replaced with 25 ml of serum-free DMEM (control) or 25 ml of therapeutic growth medium provided by Total ReCord, Inc. (Worcester, MA). The cells were incubated at 37˚C in the CO_2 incubator for 48 hours, then RNA extraction was performed.

Cell Freezing

When SHSY cells were to be frozen, a T-150 flask was cultured to 70-80% confluency. The old medium was removed and a small volume of new medium was added. The cells were scraped into the new medium and transferred to a 15 mL conical tube. The cells were spun in the clinical centrifuge for five minutes on the medium setting and the supernatant removed. The cell pellet obtained from one T-150 flask was resuspended in 1 ml of freezing medium containing DMSO (Gibco) and placed into a cryovial. The vial was stored at –80˚C overnight insulated in styrofoam to ensure a slow cell freezing, and then transferred to a liquid nitrogen tank.

SHSY Morphology

Adherent SHSY cells either had a pyramidal morphology with short neurites or an elongated morphology with no neurites. Floating SHSY cells were observed to have a round swollen morphology.

Extraction of Total Cellular RNA

Total cellular RNA was extracted from SHSY cells using a chloroform / phenol extraction procedure (adapted from Clontech's Atlas Pure Total RNA Labeling System protocol). If two T-150 flasks were used for each experimental group, then these flasks were pooled together. The medium was removed and replaced with a small amount of 1X phosphate buffer saline solution (PBS). A cell scraper was used to dislodge the cells from the flask. The solution was then poured into a 15 ml conical tube. The cells were collected by centrifugation for 5 minutes in a clinical centrifuge (25˚C) at medium speed. The supernatant was discarded except for a small volume which was used to transfer the cells into a 1.5 ml eppendorf tube on ice (hereafter all steps were performed on ice, and centrifugations were at 4˚C). The tubes were microfuged for 30 sec at maximum speed to pellet the cells. The entire supernatant was discarded. The cell pellet from two T-150 flasks now exists in one eppendorf tube. In order to lyse the cells, 500 µl of denaturing solution (Clontech) was added to each pellet representing either the therapy-treated or non-treated (control) samples. The cells were thoroughly resuspended by pipeting up and down, and vortexing. They were then incubated on ice for 5-10 minutes. After microfuging for 5 minutes at 4˚C at maximum speed (13K) to pellet cell debris, 500 µl of the cleared supernatant was transferred into a 2 ml eppendorf tube. Phenol extraction was performed by adding 1000 µl (2 volumes) of buffer-saturated phenol (4˚C) into each tube and vortexing for 1 min for a thorough extraction. The tube was incubated on ice for 5 min, after which 300 µl chloroform (25˚C) was added. The tube was vortexed again for 1 min, and incubated on ice for 5 more minutes. The tubes were microfuged for 10

minutes at 4˚C at 13K, and the upper aqueous phase (about 500 µl) was aliquotted into a 1.5 ml eppendorf tube.

For isopropanol precipitation, a double volume of 4˚C isopropanol (usually 1 ml) was added to the aqueous phase in the eppendorf tube. The tubes were gently inverted to mix, but vortexing was avoided. After incubating on ice for 10 minutes, the tubes were microfuged for 15 minutes at 4˚C at 13K to pellet the RNA. After all the supernatant was discarded, the small white RNA pellet was visible. The RNA pellet was washed by adding 500 µl of –20˚C 70-100% ethanol, and vortexing briefly. The tubes were further microfuged for 5 minutes at 4˚C at 13K, and the supernatant was discarded, being very careful to not lose the loose RNA pellet. The pellets were air-dried overnight with a Kimwipe® over the open cap of the tube. The dry pellet was dissolved in 40 µl of RNase free distilled water (Clontech). If the pellets from more than one tube of the same sample were to be combined, the pellets were dissolved serially in a total of 40 µl. Usually the tube was briefly warmed in hand or in a 50˚C water bath to facilitate dissolution, and the tubes were briefly microfuged to ensure placement of the liquid in the tube bottom before OD analysis.

The RNA concentration was determined by absorbance at 260 nm. 1 µl of RNA solution was added into 1000 µl dH_2O, and the OD was taken at 260 nm zeroed against dH_2O. The RNA concentration was calculated using the following formula: RNA concentration in µg/µl = OD value x 42 µg/ml x 1000 dilution factor x 1 ml/1000 µl. The RNA solution was then diluted with RNase-free dH_2O to a 1 µg/µl final concentration in a 0.5 ml eppendorf tube, and stored at –80˚C. The RNA was thawed on ice before use in

RT-PCR experiments. Typical yields of SHSY RNA using this protocol are shown in Table-2 below.

Table 2. Routine SHSY RNA Yields

Culture Size	OD 260 on 1:1000	Concentration	Yield
Two T-150 Flasks	0.033	1.4 µg/µl	55 µg RNA

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the RETROscript Kit from Ambion, sometimes using SuperTaq Polymerase and MMLV (Ambion) to supplement the kit. A 20 µl RT reaction was set up in a 0.5 ml tube as follows: 2 µg of RNA (2 µl of 1 µg/µl RNA), 2 µl of 50 µM oligo(dT) primer, and 8 µl dH_2O. The tube contents were briefly microcentrifuged, and the RNA was denatured at 85˚C for 3 minutes in a thermocycler. Following denaturation, the tubes were immediately placed on ice to prevent RNA renaturation, briefly microfuged and put back on ice. 2 µl of 10X RT Buffer, 4 µl of 2.5 mM dNTPs and 1 µl 10U/µl RNase Inhibitor were added into each tube. Usually this was prepared as a master mix, and 7 µl was allocated into each tube. After adding 1 µl of 100 U/µl MMLV-RT, the tubes were flicked to mix, microcentrifuged briefly, and immediately placed into the thermocycler. Reverse transcription was allowed to take place for 60 min at 42˚C, followed by a 10 min denaturation step at 92˚C. cDNA samples were either used immediately for PCR, or stored at -20˚C.

PCR

Each 50 µl PCR reaction was set up in a 0.5 ml eppendorf tube containing the following (added in the order indicated): 2.5 µl 10 µM sense primer, 2.5 µl 10 µM antisense primer, 39.6 µl of a master mix (containing 5 µl of 10X PCR Buffer, 2.5 µl of 2.5 mM dNTP's and 32.1 µl dH2O), 5 µl of the cDNA template from the above RT reaction, and finally 0.4 µl of 5U/µl Taq polymerase (2 units) to reach a final volume of 50 µl. The tubes were flicked to mix, and microfuged briefly before placing them in the thermocycler. The PCR reaction conditions for our experiments were as follows: 2 min initial denaturation at 94˚C; 35 cycles of amplification, including a 30 sec denaturation at 94˚C, 30 sec annealing at 55˚C, and 40 sec elongation at 72˚C; and a final 5 min elongation at 72˚C.

Gel Electrophoresis of PCR Amplicons

PCR reactions were analyzed on 3.5% NuSieve gels in 1X TAE buffer, containing 1 µg/ml ethidium bromide. 10 µl of PCR sample was mixed with 1 µl of 10X DNA sample buffer (containing 0.1X dyes) in an eppendorf tube, microfuged briefly, and mixed by pipeting up and down before loading onto the gel. Gel electrophoresis was for 60 V for 90 minutes for small units, and 2 hours for larger units. Gels were visualized under a UV trans-illuminator, photographed using a digital camera, and the band intensities were quantified using Scion Image Software (NIH).

RESULTS

Regeneration Matrix™ (RMx) is a type of "smart scaffold" derived from the extracellular matrix (ECM) of human blood, and "polymerized" in the presence of a rich medium containing a proprietary combination of growth factors. RMx has successfully been used to restore walking in a rat model for spinal cord injury (Total ReCord, Inc.). Our lab is interested in the mechanism of how RMx works, and has a working hypothesis that the RMx scaffold serves to guide elongating neurons, while growth factors present in the ECM portion (and added to the scaffold from the medium during polymerization) serve to induce neuronal growth.

To test this hypothesis, our lab has developed an *in vitro* model in which cultured human SH-SY5Y (usually abbreviated SHSY) cells are treated with either complete RMx (smart scaffold), scaffold without growth factors (plain scaffold), or growth factor medium (smart medium) to try to distinguish the effect of each critical component. The purpose of this MQP was to use RT-PCR to determine whether genes known to function in neuronal growth are upregulated in the model using the smart medium by itself.

The first goal of this project was to perform a preliminary series of RT-PCR reactions with a collection of 23 human neurological primer sets in our lab to determine which primer sets appropriately amplify their gene targets in SHSY cells. Table III lists the 23 primer pairs tested in these preliminary experiments, and the expected amplicon size. SHSY cells were cultured to approximately 70% confluence in T-150 flasks prior to treatment with smart medium or DMEM (control medium) for 24 or 48 hours prior to RNA extraction.

Gene Primer	Amplicon Size
12S rRNA	122 bp
12S rRNA	430 bp
16S rRNA	251 bp
16S rRNA	325 bp
ATP Synthase	353 bp
CNTF	235 bp
FGF-9	351 bp
Fibronectin-1	196 bp
GAP-43	407 bp
GDNF	192 bp
GDNF Receptor-2	240 bp
L-11 Ribosomal Protein	184 bp
L-19 Ribosomal Protein	257 bp
NCAM-1	308 bp
Netrin G1	369 bp
Neuregulin-1	383 bp
Neurotropin-3	304 bp
Neurotropin-6 gamma	326 bp
NGF Receptor	401 bp
S-19 Ribosomal Protein	293 bp
Somatostatin Receptor-1	247 bp
trk-2 Receptor	378 bp
trk-3 Receptor	339 bp

Table III: RT-PCR Primer Sets Tested on Human SHSY RNA in Preliminary Experiments. The sizes of the expected amplicons (bp) are shown in the right column. Primers were previously designed by D. Adams for our laboratory. Highlited genes produced the expected amplicons, and were chosen for further analysis.

Total cellular RNA was extracted as described in the Materials and Methods section of this report. Reverse transcription of the RNA to yield cDNA was followed by PCR for 35 cycles (see Materials and Methods), and the amplicons were analyzed by gel electrophoresis. Twelve of the genes (highlighted in Table III) were chosen for further study since they produced amplicons of the expected sizes (data not shown).

Additional flasks of cells were grown to 70-80% confluency, and once again treated for 48 hrs with either smart medium or DMEM prior to RNA extraction. RT-PCR was performed using the twelve primers highlighted in Table III, and the products were

analyzed by gel electrophoresis. Φ-X174/*Hae III* was used as a DNA marker. Figure 2 shows the Φ-X174/*Hae III* (left lane) with fragment sizes (labeled within the range of studied amplicons), next to a 100 base-pair ladder (right lane) to confirm the Φ-X174/*Hae III* fragment sizes.

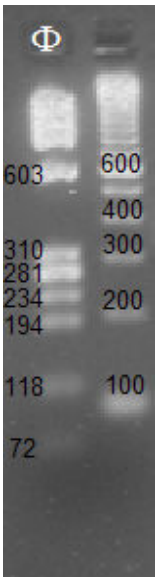

Figure 2: DNA Marker Size Designations. Comparison of Φ-X174/*Hae III* marker (left lane) with a 100 base-pair ladder (right lane). The right lane designations were used to help assign the lane 1 designations.

Figures 3-5 show the results of RT-PCR experiements with the chosen primers. Figure 3 shows the results of RT-PCR experiments using primers for GDNF, FGF-9, GDNF Receptor-2 (GDNFR), and Netrin G1 (Netrin). The expected amplicon size (in base-pairs) for each set of primers is given below each set of lanes. GDNF and Netrin-1 appear to be downregulated by smart medium, while FGF-9 and GDNF-R are upregulated.

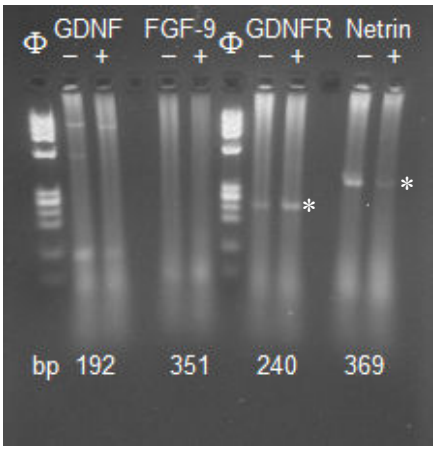

Figure 3: RT-PCR Results For GDNF, FGF-9, GDNFR, and Netrin-1. (-) denotes control cells, and (+) denotes cells treated with "smart medium" for 48 hrs. Gene designations are shown above each lane. Expected amplicon lengths are shown below each set

Figure 4 shows the results of PCR experiments using primers for growth-associated protein-43 (GAP-43) (GAP), Fibronectin-1 (Fibro), CNTF, and Neuregulin-1 (NG-1). GAP-43, Fibronectin, and Neuregulin-1 appear to be strongly upregulated, while CNTF (not easily seen in this figure) was downregulated.

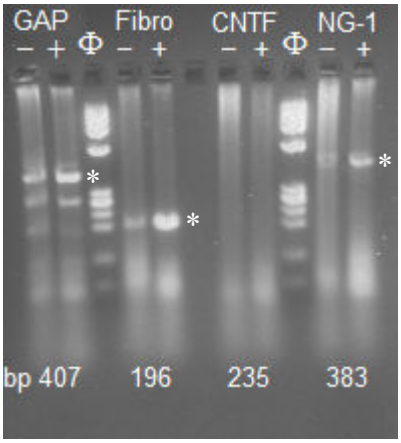

Figure 4: RT-PCR Results for Growth-Associated Protein-43 (GAP), Fibronectin (Fibro), Ciliary Neurotrophic Factor (CNTF), and Neuregulin-1 (NG-1). (-) denotes control cells, and (+) denotes cells treated with "smart medium" for 48 hrs. Gene designations are shown above each lane. Expected amplicon lengths are shown below each set.

Figure 5 shows the results of the RT-PCR experiments for ATP Synthase (ATP), NGF Receptor (NGFR), trk-3 Receptor (trk3), and trk-2 Receptor (trk2). NGFR, trk3, and trk2 appear to be upregulated, while ATP synthase is downregulated.

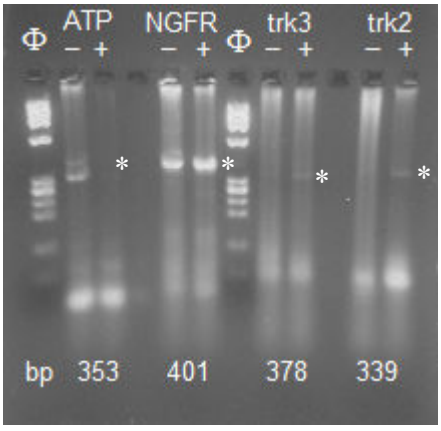

Figure 5: RT-PCR Results for ATP Synthase (ATP), Nerve Growth Factor Receptor (NGFR), trk3 Receptor (trk3), and trk2. (-) denotes control cells, and (+) denotes cells treated with "smart medium" for 48 hrs. Gene designations are shown above each lane. Expected amplicon lengths are shown below each set.

In order to determine whether any of the apparent changes in gene expression are statistically significant, repeated independent RT-PCR reactions (4-5 trials) were run on a

selected subset of four genes (Fibronectin, GAP-43, Neuregulin-1 and NGF Receptor) chosen for further analysis based on their relatively strong upregulations and excellent amplicon signals. The results of these experiments (and those of the previous gels) were quantitated using Scion Image software. The resulting band intensity data were used to calculate the fold-upregulation for each independent reaction. The results were normalized to GAPDH housekeeper. Microsoft Excel was used to calculate the mean (average) upregulation, standard deviation, and *p*-values for each gene. A one-tailed t-test was used to calculate *p*-values with a hypothesized mean difference of zero (Figures 6-11).

For fibronectin, the control cells exhibited a mean band intensity of 19.65 (N=4), versus a mean intensity of 57.22 in 48 hr smart medium-treated cells (Figure 6), while the mean fold-upregulation relative to GAPDH was 2.93 ($p = 0.005$) (Figure 7).

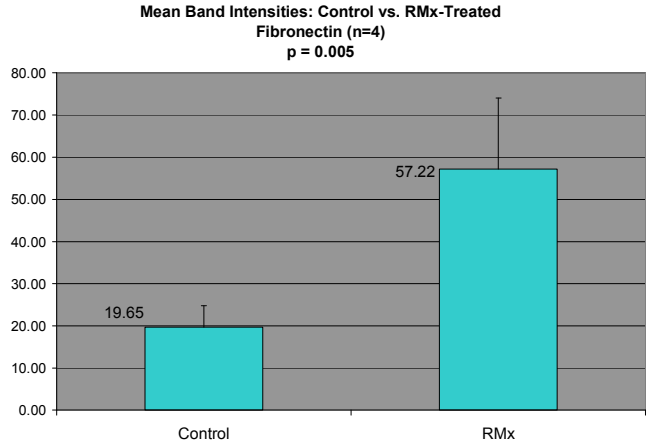

Figure 6: Mean Band Intensities (N=4) of Control versus Smart Medium for Fibronectin. N= 4. p = 0.005. Error bars denote one standard deviation.

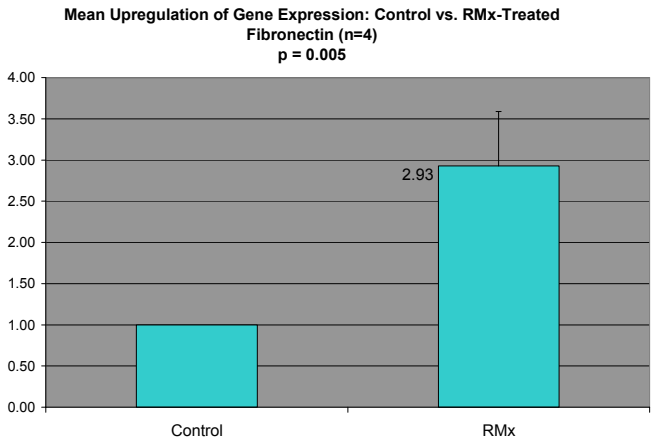

Figure 7: Mean Fold-Upregulation of Fibronectin (N=4) Relative to GAPDH. Error bars denote one standard deviation.

For Neuregulin-1, the control cells exhibited a mean band intensity of 21.28 (N=5), versus a mean intensity of 40.10 in 48 hr smart medium-treated cells (Figure 8), while the mean fold-upregulation relative to GAPDH was 1.97 (p = 0.022) (Figure 9).

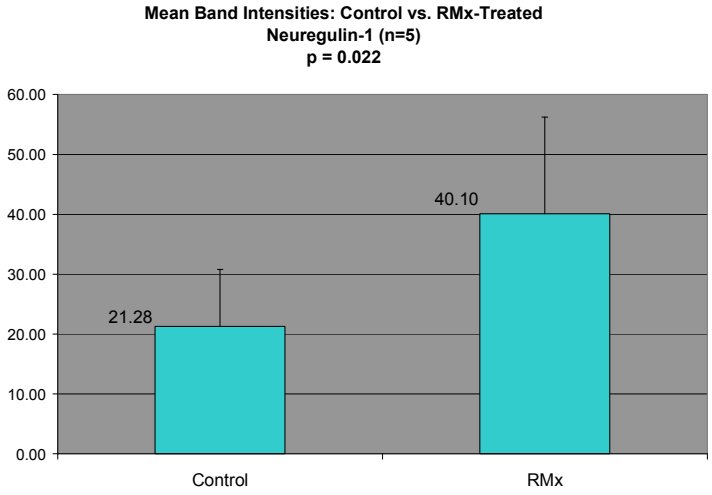

Figure 8: Mean Band Intensities of Control Versus Smart Medium for Neuregulin-1. N=5. p = 0.022. Error bars denote one standard deviation.

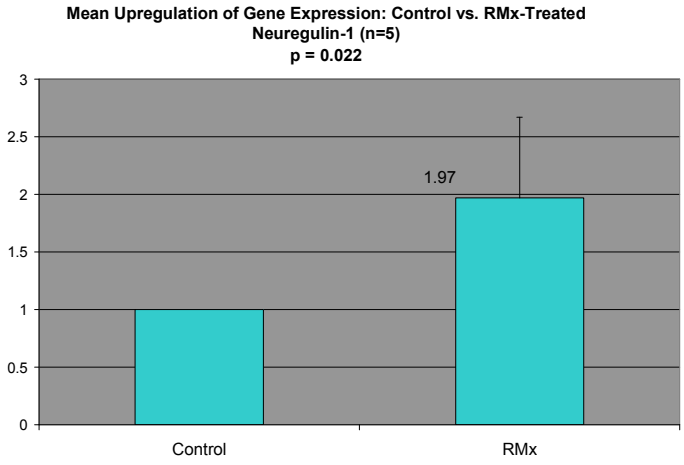

Figure 9: Mean Upregulation of Neuregulin Relative to GAPDH . N=5. p = 0.022. Error bars denote one standard deviation.

For NGF-Receptor, the control cells exhibited a mean band intensity of 31.11 (N=4), versus a mean intensity of 57.58 in 48 hr smart medium-treated cells (Figure 10), while the mean fold-upregulation relative to GAPDH was 1.91 (p = 0.007) (Figure 11).

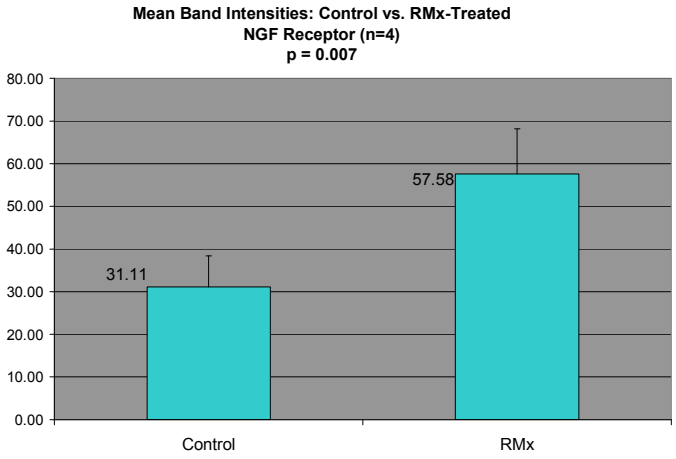

Figure 10: Mean Band Intensities of Control Versus Treated Samples for the NGF Receptor. N=4. p = 0.007. Error bars denote one standard deviation.

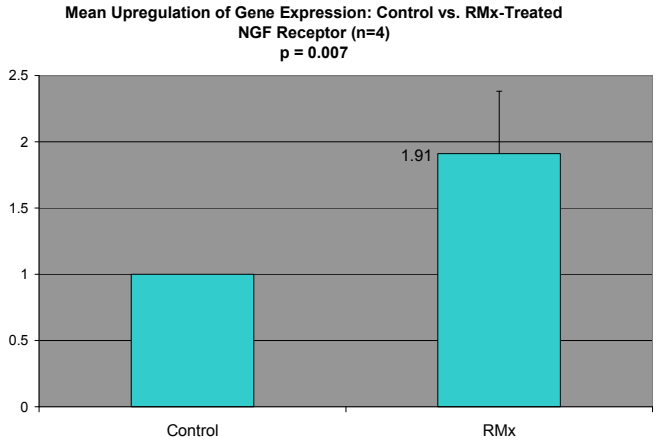

Figure 11: Mean Fold Upregulation of NGF-R Relative to GAPDH. N=4. p = 0.007. Error bars denote one standard deviation.

For GAP-43, the control cells exhibited a mean band intensity of 31.75 (N=5), versus a mean intensity of 48.94 in 48 hr smart medium-treated cells (Figure 12), while the mean fold-upregulation relative to GAPDH was 1.66 (p = 0.010) (Figure 13).

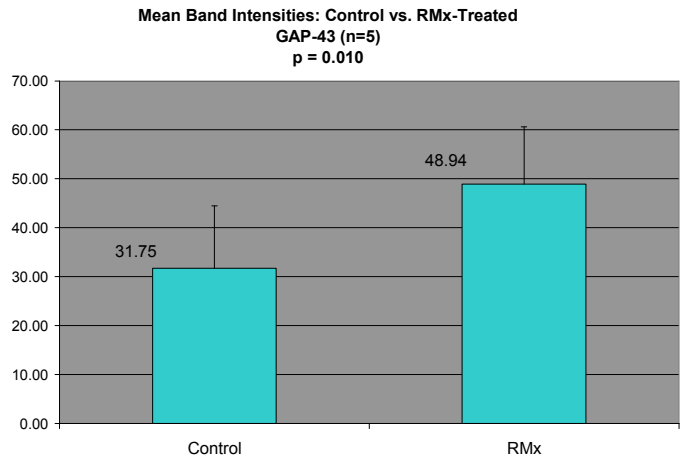

Figure 12: Mean Band Intensities (N=5) of Control Versus Treated for GAP-43. p = 0.01. Error bars denote one standard deviation.

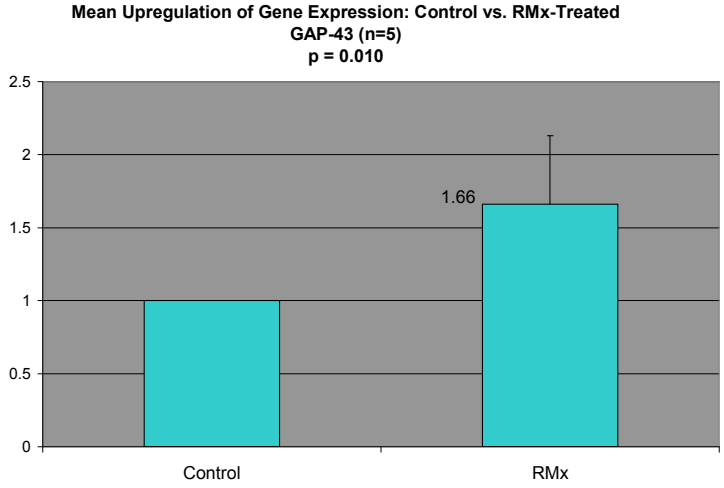

Figure 13: Mean Fold Upregulation of GAP-43 Relative to GAPDH. N=5. p=0.01. Error bars denote one standard deviation.

DISCUSSION

The purpose of this project was to determine if specific neuronal growth genes are upregulated in human SHSY cells by Total ReCord's growth factor-containing medium used to "charge" or "load" the scaffold portion of their Regeneration Matrix™ treatment. RNA extraction of control and therapy treated cells followed by reverse transcription polymerase chain reaction (RT-PCR) was chosen as a methodology to achieve this goal. Oligo-dT was used to create complementary DNA (cDNA) for the PCR reaction. Synthetic oligonucleotide primers complementary to 23 potential human genes of interest were tested against SHSY RNA in a preliminary series of experiments. cDNA was amplified by PCR and amplicons were separated by gel electrophoresis. Four genes that showed strong amplicons of the expected sizes, and appeared to be upregulated by the treatment (GAP-43, NGF-R, Neuregulin-1 and Fibronectin) were tested further in repeated trials to determine statistical significance. All four genes showed statistically significant upregulations by the growth factor treatment, indicating they might be involved in the recovery and regrowth of central nervous tissue in the rat model.

These genes each play a known important role in the regeneration and repair of spinal cord tissue. Fibronectin has been shown to act as a cellular "scaffold" that aids in tissue growth and elongation (Baron *et al*, 2000; King *et al*, 2003). Neuregulin, a neurotophic factor, plays a crucial role in the promotion of growth and differentiation of neuronal cells (Ho *et al*, 1995). GAP-43 is a key glycoprotein which plays an important role in the guidance of the neuronal growth (Chong *et al*, 1994; Bradbury *et al*, 1995). This protein helps "guide" the newly growing tissue in the correct direction. Lastly NGF-R has two important functions in this proposed mechanism. NGF-R, like GAP-43

acts as an axonal guidance factor, directing growing neurons to the correct destination. However, it also acts as a receptor for Nerve Growth Factor, another neurotrophic factor that promotes the growth and differentiation of neuronal cells (Fetsch *et al*, 2005).

To further elucidate the molecular mechanism of this RMx therapy, many other experiments could be performed. The regrowth of neuronal tissue requires the transport of blood to the new tissue. Therefore, angiogenesis, the development of new blood vessels should occur at a level higher than normal. For this reason we propose looking for the upregulation of angiogenic factors in the rat model. Monitoring the upregulation of these factors could be done by RT-PCR or ELISA.

For a more global view of the effects of RMx, expression profiling could be performed using a microarray on either SHSY RNA, or RNA isolated from regenerating rat neurons *in vivo*. This would allow one to relatively quickly determine the effects of the drug on every gene represented on the microarray (28,000). This could lead to the discovery of other proteins and signal transduction pathways that are playing a key role in this therapy. Kinase assays could also be performed on SHSY cell lysates to determine which signal transduction pathways are activated by the growth factors or RMx. In addition, if the cord regeneration model could be extended to mice, a large reservoir of pre-existing knock-out strains could be tested with the therapy to identify genes critical for its function.

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