A DIFFERENTIAL RESPONSE TO NEWT REGENERATION EXTRACT BY C2C12 AND PRIMARY MAMMALIAN MUSCLE CELLS

Sarah Kawesa

This thesis is submitted as a partial fulfillment of the Ph.D program in

Cellular, Molecular and Medicine

Submitted April 31, 2013

Department of Cellular and Molecular medicine

Faculty of Medicine

University of Ottawa

AUTHORIZATION

Figure 4. Authorization has been granted from Dr. Michael Rudnicki for the use of the figure describing myogenesis in mammals (Bentzinger et al., 2012).

ABSTRACT

Tissue regeneration in mammals does not occur via the process of dedifferentiation, a process whereby differentiated cells lose their specialized characteristics and revert to a less differentiated state. McGann et al. (2001) showed that mouse C2C12 myotubes treated with newt extract derived from regenerating limbs can re-enter the cell cycle, fragment and proliferate (a characteristic of muscle dedifferentiation). However, the validity of these studies has been called into question since others have been unable to repeat them. My research attempts to replicate the results of McGann et al, and to carry them further. I examined several strategies for tracking the extract-treated cells and I also repeated the studies in a primary muscle culture system. Furthermore, I examined the effect of the extract on myoblast differentiation.

The most effective dedifferentiation assay that I developed involved the microinjection of myotubes with extract and with a GFP plasmid that allowed tracking of the injected cells. Cells were then examined for cell cycle re-entry using BrdU incorporation or Ki-67 immunostaining. In addition, immunocytochemistry and RT-PCR analysis were used to examine the expression or down-regulation of muscle-specific markers. Finally, a preliminary GeneChip analysis was conducted to examine which genes were up or down regulated following extract treatment.

The results show that newt extract is able to block the differentiation of confluent myoblasts, resulting in fewer multinucleated, myosin heavy chain expressing myotubes. However, when myoblasts were differentiated into myotubes and subsequently treated with newt extract, the results suggest that cell cycle re-entry and down-regulation of differentiation markers can occur in C2C12 myotubes, but not in primary myotubes.

Fragmentation though, was seen in both C2C12 and primary myotubes following treatment or injection with newt extract. Moreover, the fragmented cells appeared to be viable. Transcriptional profiling indicated that newt extract affects genes implicated in cell cycle, transcription, stress, chromatin modification, growth, cell adhesion, extracellular matrix, wound healing and microtubule binding. These findings confirm that mammalian myotubes can be induced to dedifferentiate following treatment with newt extract; however, a differential response was observed between C2C12 and primary muscle cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A1 newt	Newt myoblast cell line
AdCre	Adenovirus Cre recombinase
ARF	Alternate reading frame
AP	Alkaline phosphatase
AEC	Apical epidermal cap
ANOVA	Analysis of Variance
Ara-C	Arabinosylcytosine
bFGF	Basic fibroblast growth factor
BHLH	Basic helix-loop helix
BCA	Bicinchoninic acid
BrdU	Bromodeoxyuridine
C2C12	Mouse myoblast cell line
CDC2	Cell division cycle 2
cDNA	Complementary DNA
CDK4/6	Cyclin dependent kinase 4 and 6
CMV	Cytomegalovirus
DAPI	4', 6-diamidino-2-phenylindole
dpc	Days postcoitum
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleoside triphosphate
DM	Differentiation medium
DMEM	Dulbecco's Modified Eagle Medium

EDU	Ethynyl-2′-deoxyuridine
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
eGFP	Enhanced green fluorescence protein
ECM	Extracellular matrix
FBS	Fetal bovine serum
G0 phase	Gap 0 phase
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
GO	Gene ontology
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green fluorescence protein
GM	Growth medium
HS	Horse serum
HDGF	Hepatoma-derived growth factor
HCI	Hydrochloride
INK4	Inhibitor of cyclin-dependent kinase 4
Loxp	Locus of Crossover in P1
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MCM2	Mini-chromosome maintenance 2
M phase	Mitosis phase
MWCO	Molecular weight cut off
MC	Mononucleated cells

Msx-1	Msh homebox 1
MNC	Multinucleated cells
MCK	Muscle creatine kinase
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
MHC	Myosin heavy chain
MRF4	Myogenic regulatory factor 4
Nrad	Newt ras associated diabetes
OHRI	Ottawa Hospital Research Institute
Pax-3	Paired box 3
Pax-7	Paired box 7
PFA	Paraformaldehyde
Pen/Step	Penicillin-Streptomycin
PECs	Pigmented epithelial cells
PCR	Polymerase chain reaction
PES	Polyethersulphone
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PIC	Protease inhibitors cocktail
p34	Protein kinase
RT-PCR	Real-time polymerase chain reaction
RFP	Red fluorescence protein
RB	Retinoblastoma

pRB	Retinoblastoma protein
RB-/-	Retinoblastoma null-mutant
RNA	Ribonucleic acid
Six 1/4	Sine oculis-related homebox ¼
SV40	Simian vacuolating virus 40
NaOH	Sodium hydroxide
Shh	Sonic hedgehog
S phase	Synthesis phase
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
MS222	Tricaine methanesulphonate
TRIS-HCI	Trisaminomethane hydrochloride
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
Wnt	Wingless protein
YFP	Yellow fluorescence protein

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Catherine Tsilfidis for giving me the opportunity to work in her lab and on this project. I would also like to thank my thesis advisory committee members for their expertise and input on the project. I would like to thank the Tsilfidis lab, in particular Margaret Beddaoui, and Sarah Wassmer for all their encouragement, laughter and support. My friends Monika Antanya and Roshan Sriram, you guys rock.

I would like to thank my family for all their support and help. My mother, I love to death. I would like to thank you for the love, support and taking care of my little angel. Thank you to my niece, nephew, sisters and brother; especially Mildred and Leann for taking care of my daughter.

To the love of my life, my husband Paul, thank you so much for all your support. Your love and encouragement has kept me going through the tough times and good times. Thank you for driving me to work and waiting in the car until my experiments were done. Thank you for taking care of our daughter when I am at work. You are the best thing that ever happened to me. The words in this thesis are not even enough to express how appreciative I am to have you in my life. To my daughter, Malaika, I am so grateful to have a wonderful little person like you in my life.

Chapter 1. Background

Tissue regeneration is a complex process of replacing cells lost due to disease or injury (Brockes and Kumar, 2002). All eukaryotes have basic regenerative abilities such as wound closure and tissue repair. The most complex form of regeneration is known as epimorphic regeneration. This is mastered by the urodele amphibians where epimorphic limb regeneration involves 4 major phases: wound healing, dedifferentiation, blastema formation, and redifferentiation (Brockes and Kumar, 2002; Call and Tsonis, 2005). It is believed that the dedifferentiation process is the hallmark of regeneration (Tanaka, 2003), whereby differentiated cells re-enter the cell cycle and revert back to a progenitor phenotype (Kumar et al., 2000). Regeneration is limited in "higher" vertebrates. In mammals, once cells have completed the differentiation pathway, they are committed for life. Urodeles provide exceptions to that rule. Understanding the dedifferentiation process in newt regeneration is key to improving regenerative potential in humans.

Although mammalian muscle cells do not dedifferentiate during regeneration there is indication of dedifferentiation in *vitro*. C2C12, a mouse muscle cell line, is commonly used to study the dedifferentiation process. In order to further characterize dedifferentiation in mammals, dedifferentiation needs to be assessed in muscle derived from primary cells. This thesis examines dedifferentiation in both C2C12 cells and in primary mature multinucleated muscle cells.

1.1. Introduction to Regeneration in Vertebrates

All organisms have developed strategies to repair tissues and organs damaged by injury or disease. In mammals, regenerative ability is limited to a few tissues including the liver, pancreas, epithelium, muscle, bone and blood. In comparison to mammals, amphibian

regenerative ability is much greater. Regeneration in vertebrates is achieved by three different pathways as illustrated in Figure 1.

1.1.i. Compensatory hyperplasia regeneration

Humans are able to regenerate the liver via compensatory hyperplasia. This form of regeneration involves formation of new cells from pre-existing differentiated cells. An injury to the liver leads to an inflammatory response, whereby tissue debris is removed (Markiewski et al., 2004). This is followed by the regeneration response resulting from proliferation and differentiation of quiescent cells (eg. hepatocytes) leading to restoration of the missing portion of the liver (Michalopoulos, 2007). In humans, regeneration of the liver is completed within 8 to 15 days following partial hepatectomy (Michalopoulos, 2007).

1.1.ii. Stem cells activation

Injury to human skeletal muscle tissue leads to disruption of the muscle cell membrane within minutes. Subsequent to membrane disruption is the removal of the tissue debris. Skeletal muscle repair is achieved via stem cell activation. The cells that are involved in repairing muscle tissue are referred to as satellite cells. Satellite cells are located underneath the basal lamina of the myofiber. These cells are normally in a quiescent state, and upon injury they become activated, and they divide. One daughter cell remains a stem cell (self-renewal of the satellite cell population; Collins, 2006) and the other gives rise to a muscle progenitor cell. The progenitor cell goes on to divide and differentiate into a mature muscle myofibre or to fuse with existing myofibres (Carlson, 2005).

1.1.iii. Epimorphic regeneration

Amphibians, which are the champions in regeneration, amongst vertebrates repair their organs or tissues such as the limbs or lens via epimorphic regeneration. Immediately

after removal of the urodele lens, the pigmented epithelial cells (PECs) of the dorsal iris dedifferentiate, losing the characteristics that define their cell type such as their pigmentation (Ito et al., 1999). Proliferation of the depigmented cells ensues. Ten days after removal of the lens, a new lens vesicle is formed from the depigmented dorsal PECs, and synthesis of crystallins (the major lens proteins) is observed after 12 to 16 days (Tsonis et al., 2004). Lens regeneration as determined by protein expression and structure, is complete within 25 to 30 days, although the optical properties of the lens take considerably longer to regenerate (Sarah Wassmer, Ph.D student, unpublished work).

The urodele limb, which is described below in greater detail, has also been shown to undergo dedifferentiation following amputation. Following limb amputation in newts, wound healing occurs. Differentiated cells proximal to the amputated site dedifferentiate and proliferate to form a blastema composed of a heterogeneous mixture of progenitor cells (Yokoyama, 2008). The cells in the blastema will eventually re-differentiate and form a new limb.

1.1.iv. Limb regeneration in urodele amphibians

The limb has been the most intensely studied organ to understand epimorphic regeneration. In 1766, Spallanzani discovered that salamanders could regenerate their limbs (Dinsmore, 1996). In subsequent years, other urodele species were identified that are able to regenerate their limbs after amputation or injury. Studies on limb regeneration have been mostly obtained from species such as the European crested newt, *Triturus cristatus*, the Northern American eastern newt, *Notophthalmus viridescens*, the spotted salamander, *Ambystoma maculatum*, the ribbed newt, *Pleurodeles waltl*, found in the Iberian Peninsula and Morocco, and the axolotl, *Ambystoma mexicanum,* found in and around Mexico City (Maden, 1999).

Figure 2 illustrates the steps involved in limb regeneration in urodeles. The timing of the stages can be variable between animals depending on the health of the animal, and the conditions under which regeneration occurs (eg. temperature and nutrition). The following stages are based on the study by Iten and Bryant (1973) conducted in adult *N viridescens* at 25ºC.

1.1.iv.a. Wound healing

When a limb is amputated, epithelial cells that are near the site will migrate and cover the wound. Studies obtained from following a regenerating newt limb of *Notophthalmus viridescens* revealed the wound stump is covered by the migration of epithelial cells within 9 to 13 hours (Repesh and Oberpriller, 1980). After the wound is closed, the wound epidermis thickens to form the apical epidermal cap (AEC), which is visible by day 5 (Hay and Fischman, 1961). There is no detectable basement membrane underneath the AEC. The epithelial cells in the AEC are seen projecting into the underlying stump layer. In addition, noticeable dead cells or debris underneath the AEC are observed. Histolysis, which is caused by enzymatic degradation of the extracellular matrix (ECM), becomes evident by day 4 to 5 post amputation in adult urodeles (Hay and Fischman 1961). This could lead to a release of cells such as muscle, dermal or Schwann cells from their matrix.

1.1.vi.b. Dedifferentiation

Evidence of dedifferentiation of tissues beneath the AEC can be seen between days 5 and 6 post-amputation. The dedifferentiated cells are produced from differentiated cells including muscle, cartilage, dermis and bone that revert back to an undifferentiated state. Dedifferentiated cells have rounded nuclei with prominent nucleoli, increased nuclear and cytoplasmic ratios and increased numbers of free ribosomes (Hay and Fischman, 1961). In

muscle dedifferentiation, small mononucleated muscle fragments are visible underneath the AEC. These small fragments might result from fragmentation of multinucleated myotubes. Evidence of fragmentation of myotubes into mononucleated cells will be described further in section 1.2. By days 8 to 11 post-amputation there are fewer dead cells and debris underneath the AEC. Degeneration of nerves is visible in the stump tissues. Increases of mesenchymal cells, which are the products of dedifferentiation, are observed underneath the AEC (Boilly et al., 1991). The dedifferentiation process continues until approximately day 18 post-amputation.

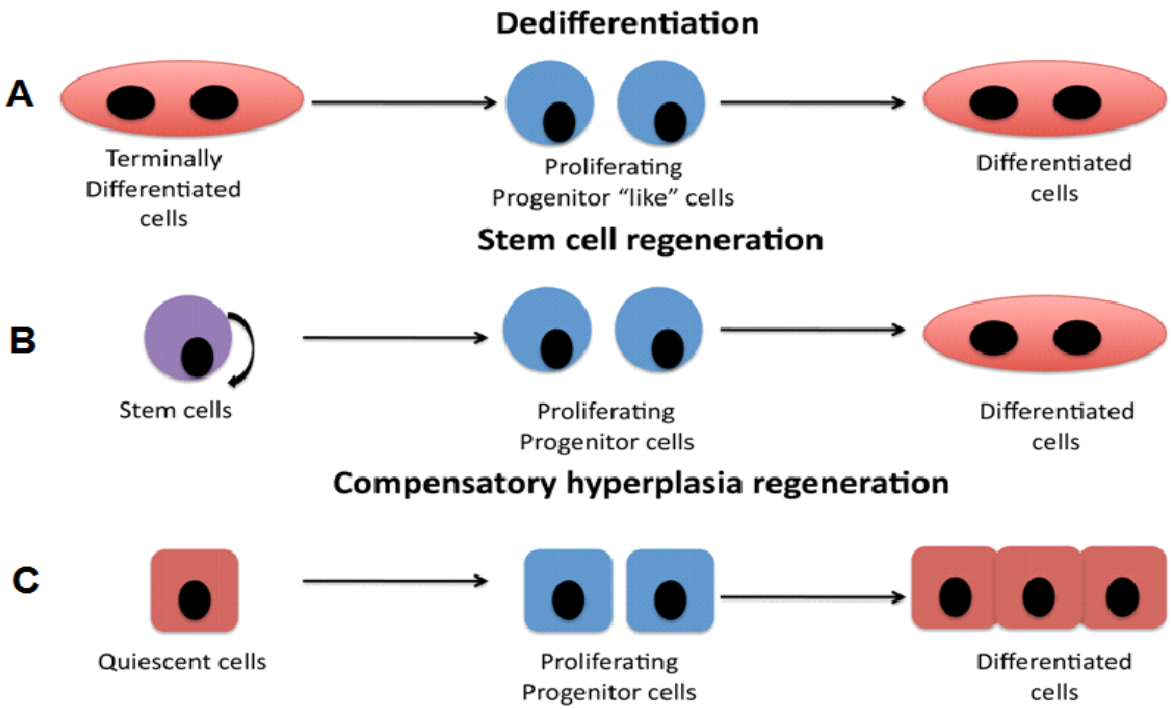


Figure 1. Mechanisms of tissue regeneration in vertebrates. (A) Dedifferentiation entails terminally differentiated cells reverting back to an undifferentiated progenitor "like", state which are capable of proliferating and redifferentiating into mature cells. This type of regeneration is observed in urodeles such as newts and axolotls. (B) Stem cell regeneration involves activation and proliferation of a stem cell, whereby one daughter cell converts into a progenitor cell that proliferates and differentiates into a mature cell type, while the other daughter cell remains a stem cell (eg. muscle regeneration in humans). (C) Compensatory hyperplasia observed in liver regeneration involves proliferation of quiescent hepatocyte cells into new hepatocyte cells.

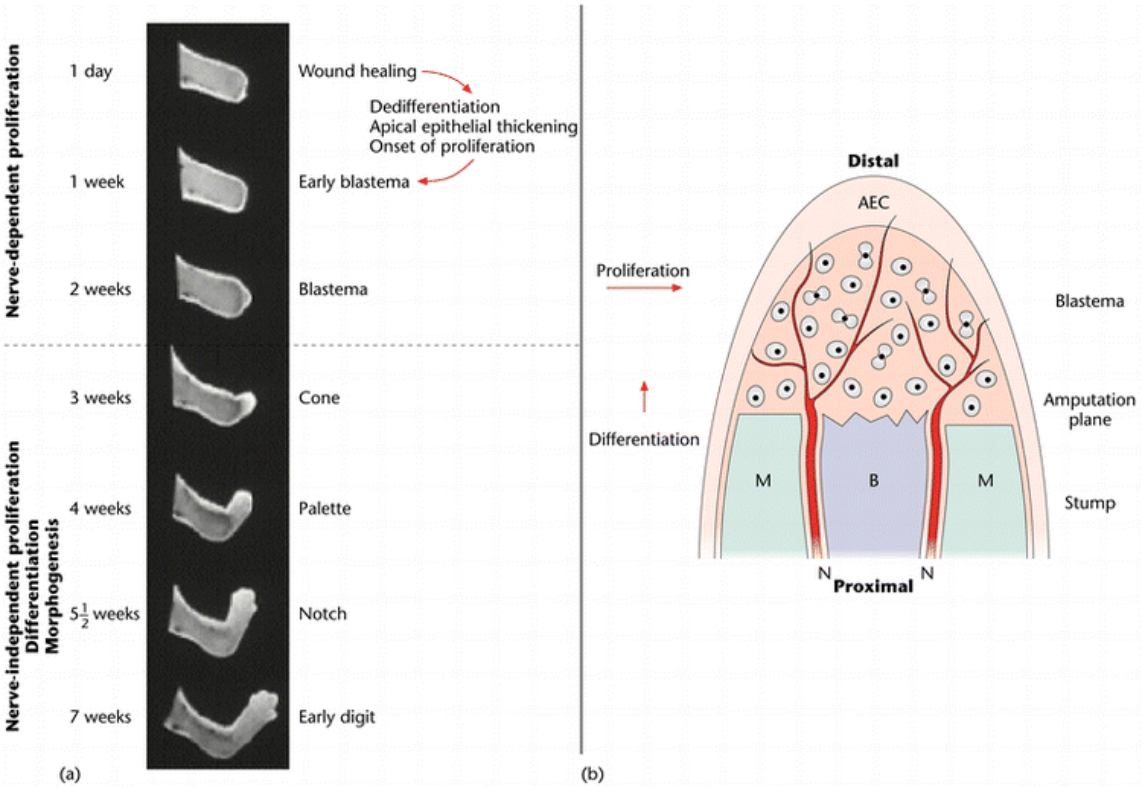


Figure 2. Phases involved in repairing an amputated limb of adult newt, *Notophthalmus viridescens*. (a) Amputation of the limb results in wound healing, dedifferentiation, apical epithelial cap (AEC) formation, blastema formation and proliferation, and differentiation. Within 1 week after amputation, the blastema is noticeable. Proliferation of the blastema cells is dependent on the presence of nerves. Differentiation and morphogenesis in the blastema is nerve independent after 3 weeks post-amputation. (b) Diagram representation of differentiated cells of the limb stump in the proximal region including muscle (M), bone (B), and nerve (N). The blastema, and the AEC are found in the distal regenerate. (Figure was obtained from (Ferretti, 2001).

1.1.vi.c. Blastema formation

Histological studies have found that the blastema becomes evident within 1 week post-amputation of the newt limb of *Notophthalmus viridescens,* and continues to grow up to day 20 (Iten and Bryant, 1973). The cells that accumulate in the blastema are thought to originate from dedifferentiation of stump tissues including Schwann cells, muscle, dermis, and cartilage**,** as well as from resident stem cells (Kragl et al., 2009; Morrison et al., 2006). immunofluorescence staining of regenerating tissues of adult newts with 22/18 (an antigen found in regenerating tissues of urodeles) was found to be specific to blastema cells of regenerating limbs. The expression was not present in non-regenerating limbs. A subpopulation of muscle mononucleated cells in the blastema was positive for 22/18 and 12/101, a marker that stained myofibers. Schwann cells were also found to stain for 22/18 (Kintner and Brockes, 1985). Given that 22/18 positive cells are only present in the regenerating limbs, it might indicate that muscle or Schwann cells positive for 22/18 in the blastema are products of dedifferentiation.

The evidence indicating that blastema cells result from dedifferentiation of mature cells also comes from studies using cell tracking. Dextran-labeled newt multinucleated myotubes were implanted into regenerating limbs. One week later, labeled mononucleated cells were found in the blastema. In addition, the cells increased over time in the blastema implying that the implanted myotubes had broken down and become proliferating cells (Lo et al., 1993). In addition, multinucleated myotubes labeled with pseudotyped retroviruses expressing human placental alkaline phosphatase (AP), when implanted into the regenerating blastema of the newt, gave rise to mononucleated cells which incorporated

bromodeoxyuridine (BrdU) in their nuclei, suggesting that they were capable of re-entering the cell cycle (Kumar et al., 2000). BrdU is a marker used to label cycling cells in S-phase.

A recent study gave additional understanding to the origin of the blastema cells. This study discovered that cells in the axolotl blastema that were derived from tissues such as muscle, Schwann cells or dermis gave rise to tissues of similar origin during regeneration (Kragl et al., 2009). In this study, embryonic green fluorescent protein (GFP) positive dermis, muscle, and Schwann cell tissues derived from a transgenic salamander were grafted to a GFP negative animal. GFP positive cells were found in the blastema a week after amputation. (Kragl et al., 2009). The cells in the blastema generally maintained their identity. For example, muscle cells only contributed to muscle during limb regeneration (Kragl et al., 2009).

Although, it is suggested that blastema cells are products of dedifferentiation, there is also evidence of satellite cell contributions in the formation of the blastema. As mentioned above, satellite cells are located in the myofibers, and upon regeneration the cells become activated, proliferating, and fusing into myofibers (Peault et al., 2007). Satellite cells are characterized by the expression of markers such as Pax-7 and M-cadherins (Kuang and Rudnicki, 2008). Following amputation of the limb, Pax-7 expressing cells were discovered in the blastema of the regenerating newt limb (Morrison et al., 2006). The study also found that myofibers that were isolated from the newt forelimb did not fragment to give rise to mononucleated cells *in vitro* (Morrison et al., 2006). The type of cells used may account for the discrepancies between this study and other studies that support muscle regeneration in urodeles via dedifferentiation. Studies that examine urodele muscle dedifferentiation *in vitro* use myotubes (multinucleated cells), while Morrison, (2006) used myofibers to investigate

cell cycle re-entry. Evidence that supports either muscle dedifferentiation or satellite cell involvement in blastema formation during limb regeneration seems to be genuine. It will be interesting to see if blocking either mechanism will hinder muscle regeneration in urodeles.

1.1.vi.d. Redifferentiation and growth

This stage initiates differentiation of cells within the blastema. At around 22 days post-amputation in adult newts, differentiation of cartilage begins within the stump tissue, followed by differentiation of muscle and connective tissues (Iten and Bryant, 1973). The pallette structure becomes visible with appearance of the first digit at around day 33 post-amputation (Iten and Bryant, 1973). Around this time, tissues including cartilage, muscle, connective tissue, and nerves are well differentiated. By day 40, the patterning of the new limb becomes complete. The new limb will continue to grow larger and eventually will not be distinguishable from the intact limb.

1.2. Introduction to Dedifferentiation

The phenomenon referred to as dedifferentiation, where cells loose their differentiated characteristics, can be detected in cell types including muscle, cartilage, dermis, and Schwann cells during regeneration in urodeles (Hay and Fischman, 1961; Kragl et al., 2009). Dedifferentiated cells can be detected at the gene or protein expression level and by their alteration in function. At the gene and protein level, the cell undergoing dedifferentiation is found to down-regulate differentiation-specific markers and up-regulate progenitor markers. At the functional level, the cells regain the ability to proliferate. Post-mitotic cells are able to re-enter the cell cycle and eventually re-differentiate into their original cell type or trans-differentiate into other cell types. Most cell types in higher vertebrates lack the ability to dedifferentiate, with the exception of Schwann cells of

peripheral nerves (Chen et al., 2007). Injury to the nerve results in post-mitotic Schwann cells losing contact with axons leading to their dedifferentiation, followed by proliferation. In differentiating Schwann cells, the expression of transcription factors such as c-Jun is down regulated. However, an increase in c-Jun expression is found in Schwann cells undergoing dedifferentiation after injury (Parkinson et al., 2008). This implies that c-Jun is needed for Schwann cell dedifferentiation.

1.2.i. Histological evidence of dedifferentiation in amphibians

The work of Hay in 1958 and 1959 demonstrated that tissues close to the injured site begin to undergo dedifferentiation after amputation of the forelimb of larval *Ambystoma* (Hay, 1958; Hay, 1959). Observations obtained by electron microscopy revealed that the cells that accumulate in the stump tissue underneath the wound epidermis by day 3 to 6 post-amputation do not resemble the cells in their tissues of origin. For example, the endoplasmic reticulum of cartilage cells was found to be highly organized in differentiated cells. However, when these cells were undergoing dedifferentiation, the endoplasmic reticulum became disorganized and appeared rounder. The muscle cells undergoing dedifferentiation were also found to look different from the differentiated muscle cells. The changes observed between differentiated and dedifferentiated cells included disappearance of myofibers, as well as changes in cell organelles (Hay and Fischman, 1961). Moreover, deoxyribonucleic acid (DNA) synthesis occurred in cells undergoing dedifferentiation. Thymidine injected into the limbs of the newt *Triturus viridescens* was found in muscle and connective tissues located in the limb stump by day 4 post-amputation (Hay and Fischman, 1961). Although thymidine incorporation was mostly seen in muscle mononucleated cells, a few muscle multinucleated cells also incorporated thymidine in their

nuclei. Cell types including Schwann cells, fibroblasts, and loose connective tissue that were close to the amputation sites were also found to incorporate thymidine into their nuclei. The cells that were undergoing dedifferentiation were found to have larger nuclei and were "mesenchyma-like" in appearance (Hay and Fischman, 1961).

The changes observed in cells undergoing dedifferentiation occur because genes involved in differentiation are suppressed, while genes characteristic of the undifferentiated state are up regulated. The signals that trigger this shift are unknown. Nevertheless, remodelling of the extracellular matrix (ECM) is observed during the dedifferentiation process. The ECM proteins such as collagen and laminin, which are normally elevated during the time when cells are undergoing differentiation, are decreased in dedifferentiation (Gulati et al., 1983; Mailman and Dresden, 1976). During this initial phase of dedifferentiation, an increase in the expression of members of the matrix metalloproteinase (MMP) family is observed. MMP are proteases which function in degrading different types of extracellular matrix proteins (Birkedal-Hansen et al., 1993). The activity of MMP-9 can be detected in the early stages of limb regeneration in *Notophthalmus viridescens* (Vinarsky et al., 2005). Inhibition of protease activity in the regenerating newt limb by GM6001 (matrix-metalloproteinase inhibitor) causes scarring in the limb stump (Vinarsky et al., 2005). Another ECM protein that decreases during dedifferentiation is the Stump 1 antigen, an extracellular protein. This protein is abundant in un-amputated limbs of salamanders (Geraudie and Ferretti, 1998).

At the same time, the expression of ECM proteins such as tenascin, fibronectin, hyaluronate and type XII collagen are up-regulated during dedifferentiation (Tassava et al., 1996). Modification of the ECM could lead to changes in cell shape and reorganization of

cytoskeleton (Juliano and Haskill, 1993), and lead, in turn, to up regulation of signals that govern the un-differentiated state.

Some of the genes up regulated during dedifferentiation include newt *msx-1*, and *Nrad*. *Msx-1* is a member of the homebox gene family. During newt limb regeneration, Msx-1 expression is found in the blastema (Simon et al., 1995). Newt ras associated diabetes (*Nrad*), a subfamily of Ras-GTPases is also implicated in muscle dedifferentiation. *Nrad* expression was detected at high levels in skeletal muscle cells that were near the amputated site, and in the blastema at low levels following amputation of the newt limb in the Japanese newt *cynops pyrrhogaster* (Shimizu-Nishikawa et al., 2001).

1.2.ii. Muscle dedifferentiation in urodeles

Much of the evidence for the dedifferentiation process has been demonstrated using muscle cells. The structure of skeletal muscle cells is highly organized. Skeletal muscles are composed of multinucleated fibers arranged in bundles. Each fiber is made up of numerous myofibrils surrounded by the sarcoplasmic reticulum (Greising et al., 2012). After amputation of the urodele limb, skeletal myofibers lose their differentiated state, and re-enter the cell cycle. In addition, these cells fragment to produce proliferating mononucleated cells. The earliest indication that skeletal myofibers can undergo dedifferentiation during urodele limb regeneration came from observation studies using electron microscopy. These studies found that when myofibers were undergoing dedifferentiation, the sarcoplasmic reticulum broke up into vesicles. Boundaries between myofibers indicated fragmentation of myofibers. The generated fragments of mononucleate cells were seen to move away from the intact muscle tissue to develop into blastema cells (Hay, 1959). Furthermore, it was determined by using the autoradiographic technique that the fragment cells were able to synthesize proteins.

Incorporation of methionine-S^{35} was found in fragments of myofibers of adult newt *Triturus cristatus* limbs 5 days post-amputation (Bodemer and Everett, 1959).

Subsequent studies provided more evidence showing muscle cell involvement in the process of dedifferentiation. As previously stated, when Lo et al. (1993) microinjected multinucleated cells with the lineage tracer rhodamine-dextran and implanted them below the wound epithelium of the amputated hindlimb of the adult newt, they found labelled mononucleated cells were located in the blastema (Lo et al., 1993). Newt multinucleated cells that were labeled with pseudotyped retrovirus expressing human placental alkaline phosphatase (AP), when implanted into the regenerating blastema of the newt, *Notophthalmus viridescens,* also resulted in fragmentation (Kumar et al., 2000). A study by Velloso et al (2000) also found that generation of mononucleated cells from post-mitotic multinucleated cells proceeded in the absence of cell cycle progression. Cell cycle progression was blocked either by X-irradiation or by transfection of cyclin dependent kinase 4 and 6 (CDK4/6) inhibitor p16. When multinucleated cells were implanted into the newt regenerating limb, they gave rise to mononucleated cells in the limb blastema (Velloso et al., 2000). This suggests that cell cycle re-entry and fragmentation are independent events.

1.2.iii. Muscle fragmentation in urodeles in vitro

The newt A1 muscle cell line originally isolated from cultures of the newt limb is commonly used to examine dedifferentiation *in vitro*. In culture, newt A1 myoblasts can divide in the presence of rich nutrient culture and when the serum is reduced, the cells exit the cell cycle, fusing to form multinucleated myotubes (Ferretti and Brockes, 1988). Addition of newt extracts prepared from regenerating newt limbs were found to induce fragmentation of newt A1 multinucleated myotubes into mononucleated cells which were

able to proliferate (McGann et al., 2001). A recent study also discovered that multinucleated cells derived from primary myoblasts, which were generated from the myofibers of newts were able to undergo fragmentation when cultured on tenascin-C, an extracellular matrix protein (Calve and Simon, 2012). This study demonstrated that the extracellular matrix is essential for muscle dedifferentiation.

1.2.iv. Cell cycle

Muscle dedifferentiation does not only involve fragmentation but also involves cell cycle re-entry in multinucleated myotubes. The cell cycle is a series of events which takes place in a cell resulting in its division, differentiation or quiescence. Figure 3 shows the events involved in cell cycle progression in mammalian cells. When cells are in the G1-phase of the cell cycle, they prepare for DNA synthesis. In order for cells to progress to the next phase, which is the S-phase, they require the presence of growth factors. Once the cells pass through the *restriction point* in late G1-phase they will progress into the S-phase where DNA is duplicated (Johnson and Walker, 1999). The cells will continue through the G2-phase where DNA is prepared for division. Cell division occurs in the M-phase of the cell cycle (Johnson and Walker, 1999). Cells that are not dividing are considered quiescent or terminally differentiated cells. These cells will arrest in the G0-phase of the cell cycle. During G0-G1 transition, which is mediated by mitogens, cyclin D becomes synthesized and will bind and activate cyclin dependent kinase (CDK4/6) (Sanchez and Dynlacht, 2005). The cyclin D/CDK4/6 complex will phosphorylate and inactivate the retinoblastoma protein (pRb) (Connell-Crowley et al., 1997). As a result, pRb releases E2F, a transcriptional factor, which mediates activation of transcription genes like cyclin E and A (Sherr and Roberts, 2004). While cells are transitioning from G1 to S-phase, cyclin E activates CDK2 leading to

hyperphosphorylation of pRb (Johnson and Walker, 1999). The S phase of the cell cycle is under the control of cyclin A/CDK2, and the beginning of M phase is controlled by cyclinA/CDK1, then by cyclin B/CDK1 (Arellano and Moreno, 1997). Following mitosis, cells either proceed to the G1-phase, or differentiate and arrest in the G0-phase of the cell cycle.

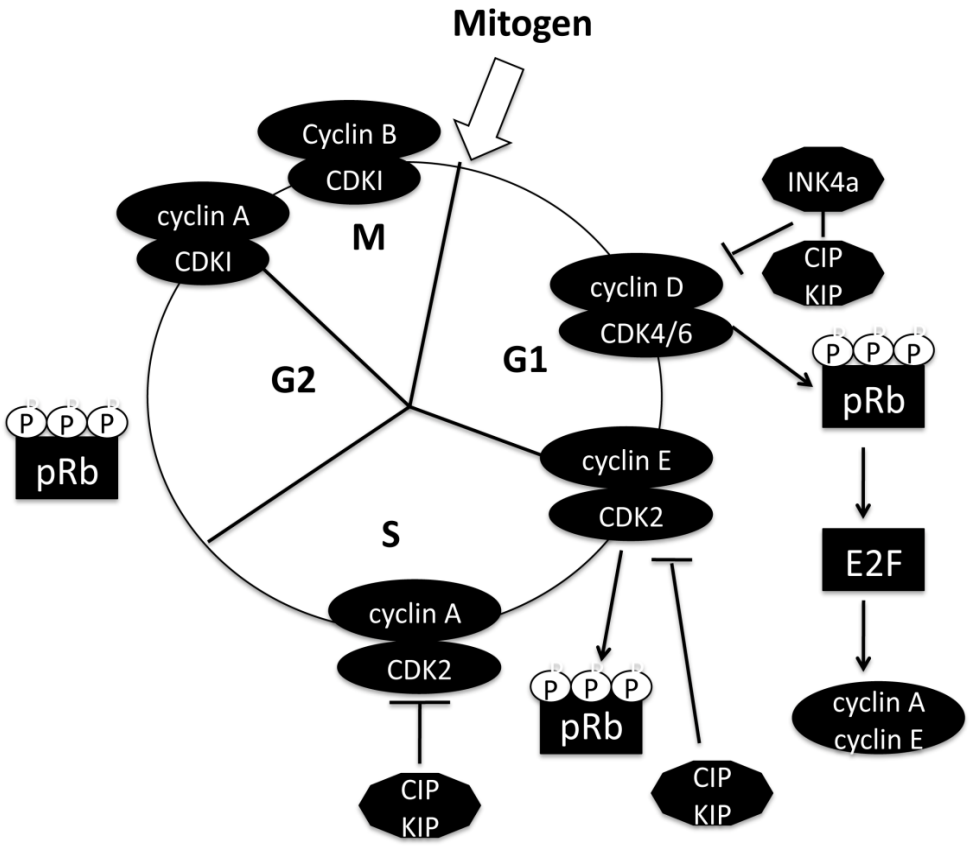


Figure 3. Cell cycle events in mammalian cells. In response to a mitogen, during G0-G1 transition, cyclinD/CDK4/6 complex is activated leading to phosphorylation and inactivation pRb. Phosphorylation of pRb by cyclinD/CDK4/6 causes release of E2F, which mediates activation of transcriptional genes like cyclin A and E. Activation of cyclin E will interact with CDK2 to allow G1-S phase transitioning. Cell cycle progression is inhibited when CDK inhibitors (INK4a and CIP/KIP) bind to CDK4/6, preventing the interaction with cyclin D. CIP/KIP family members also inhibit cyclinE/CDK2 interaction preventing cells from progressing to S-phase. The S-phase is under the control of cyclinA/CDK2, and is opposed by the CIP/KIP family. The beginning of the M-phase is under the control of cyclinA/CDKI, and subsequently by cyclinB/CDKI.

1.2.v. Cell cycle control in differentiating myoblasts

Cultured myoblasts, deprived of growth factors will exit the cell cycle and fuse together to form multinucleated myotubes. Multinucleated cells are maintained in the G0-phase of the cell cycle through up-regulation of cell cycle regulators as well as basic-helix-loop-helix transcription factors (Andres and Walsh, 1996), which are discussed in section 1.3.i. During myoblast differentiation, cyclin D1, and cyclin dependent kinases (CDK) becomes down regulated (Jahn et al., 1994), while cyclin dependent kinase inhibitors including p18, p27, and p21 become up regulated (Halevy et al., 1995; Myers et al., 2004; Zabludoff et al., 1998). High levels of the hypophosphorylated pRb are observed during differentiation of myogenic cells (Kitzmann and Fernandez, 2001).

1.2.vi. Cell cycle re-entry in multinucleated cells in urodeles

It has been thought that once cells become terminally differentiated, they are incapable of synthesizing DNA again (Andres and Walsh, 1996). The newt regeneration process shows that this is not the case. Intraperitoneal injection of 5-Ethynyl-2′-deoxyuridine (EDU), a proliferation marker, resulted in EDU presence in the myonuclei of the myofibers of regenerating limbs (Calve and Simon, 2011). Thrombin, a blood clotting protein, also was found to induce cell cycle re-entry in newt A1 multinucleated cells (Tanaka et al., 1997), as measured by bromodeoxyridine (BrdU) incorporation. Although, thrombin protein induced cell cycle re-entry in newt cells, it was not adequate to push the cells to progress through the cell cycle. The cells were found to arrest in the G2-phase of the cell cycle. Addition of thrombin protein to C2C12 multinucleated cells (a mouse cell line), however, did not cause cell cycle re-entry (Tanaka et al., 1997). This shows that differentiated mammalian cells have lost the ability to respond to thrombin. The

mechanisms of cell cycle re-entry in newt multinucleated cells mediated by thrombin are uncertain. Nevertheless, thrombin has been shown to promote cyclin D1 expression in retinal pigment epithelium cells (Parrales et al., 2011).

Phosphorylation of pRb in newt multinucleated cells in response to serum is associated with cell cycle re-entry (Tanaka et al., 1997). This response can become inhibited by the expression of p16, a cyclin-dependent kinase inhibitor. p16 inhibits the cyclin/CDK complex, which initiates the phosphorylation of the retinoblastoma protein. Interestingly, cultured multinucleated myotubes from Rb-/- mice are able to re-enter the cell cycle following serum stimulation (Schneider et al., 1994). Furthermore, newt A1 myotubes that were treated with extract from early newt forelimb regenerates were able to re-enter the cell cycle as well as proliferate (McGann et al., 2001). The newt extract seems to contain factors that might push terminally differentiated muscle cells to re-enter and progress through the cell cycle. Identifying the mechanisms involved in inducing cell cycle re-entry in multinucleated cells by the treatment with regeneration-derived newt extract will be essential.

1.3. Regeneration properties of mammals

1.3.i. Skeletal muscle development and regeneration

Skeletal muscle in mammals is a stable tissue made up of multinucleated postmitotic muscle fibers (Greising et al., 2012). Skeletal muscle tissue develops from the mesodermal precursor cells derived from the somite. Figure 4 shows the formation of skeletal muscle cells during development and regeneration. Activation of the myogenic pathway results from the secretion of signals from the notochord, neural tube and ectoderm, including sonic

hedgehog (Shh*),* and Wingless protein (Wnt) (Cossu et al., 1996). Mice lacking Shh show defects in the limb muscles (Kruger et al., 2001).

During early muscle development, genes including *sine oculis-related homebox 1/4 (Six1/4)* and *paired box 3 (Pax3)* function to control myogenic specification. *Six 1 and 4* belong to a six genes family that encodes a six-type homeodomain (Bentzinger et al., 2012). Double knockouts of *Six 1* and *Six 4* show severe defects in hypaxial and epaxial muscles during development (Francetic and Li, 2011). *Splotch* mice embryos that are mutant in Pax3 also lack body muscles during development (Tajbakhsh et al., 1997).

Determination of mesodermal precursor cells to the myogenic lineage is regulated by the expression of myogenic regulatory factors (MRFs) Myf5 and MyoD (Kassar-Duchossoy et al., 2004). Myogenic regulatory factors are members of the basic helix-loop helix (BHLH) family. The MRFs regulate myogenic differentiation through heterodimerization of the BHLH domain with E-proteins and by binding to the E-box (CANNTG) sequence present in the promoters of muscle specific genes (Parker et al., 2006). Expression of *MyoD,* considered a master regulator gene for myogenesis, was found to activate muscle genes in cell types including fibroblasts, adipocytes, and neuroblastoma cells (Weintraub et al., 1989). The expression of Myf5 is first detected in the earliest somites of mice 8 days postcoitum (dpc), and MyoD at 8.5 dpc (Ott et al., 1991; Sassoon et al., 1989). In cell culture, Myf5 and MyoD are expressed in proliferating myoblasts (Braun et al., 1989; Tapscott et al., 1988).

Terminal differentiation of skeletal muscle cells involves activation of additional MRFs such as myogenin, and myosin heavy chain (MHC) (Miller, 1990). The expression of myogenin is first detected in the rostral somites of mouse at 8.5 days post coitum (dpc), and

MHC at 10.5 dpc p.c. (Sassoon et al., 1989). During differentiation, MHC is expressed in differentiated multinucleated myotubes in cell culture (Miller, 1990).

Injury to the muscle tissue leads to inflammatory response and degeneration of the muscle fibers. The activity of the matrix metalloproteases (MMP-9 and 2) is up regulated, and it is associated with breaking down of the ECM proteins of degenerating myofibers (Kherif et al., 1999). Degeneration of the myofiber leads to the activation of quiescent mononucleate precursor cells which reside along the myofiber, referred to as satellite cells, to repair the damaged tissue (Charge and Rudnicki, 2004). These satellite cells are located between the basal lamina and the sarcolemma of the muscle fibers. The transcriptional factor Pax7 is expressed in satellite cells, and in myogenic progenitor cells (Peault et al., 2007). These myogenic progenitor cells then proliferate, differentiate, and fuse into the damaged muscle fibers.

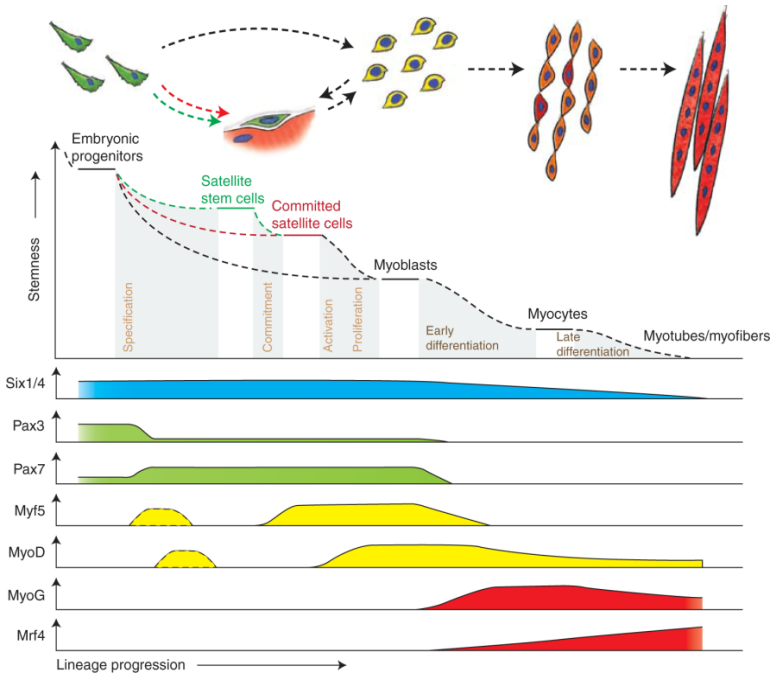


Figure 4. Myogenesis. During development, Six1/4 and Pax-3 expression is found in progenitor cells specified to become myogenic. Cells committed to the myogenic lineage express the early myogenic regulatory factors (MRFs) Myf5 and MyoD. Expression of Myf5 and MyoD is found in proliferating myoblasts. Once myoblasts are differentiated, the expression of Myf5 and MyoD is down regulated, while, myogenin (myoG) and Mrf4 is up regulated. Myocytes will fuse together to form myotubes (multinucleated cells) that express markers including MyoG and Mrf4. The expression of Pax-7 and Myf5 is found in satellite cells, which are committed to repairing injury of the muscle. The expression of Pax-7 is also found in satellite stem cells, which undergo self-renewal (Figure was obtained from (Bentzinger et al., 2012).

1.4. Mammalian muscle dedifferentiation

Mammalian muscle cells do not dedifferentiate during regeneration. This may be due to the absence of factors that initiate the process, or the cells' inability to respond to dedifferentiation stimuli. Nevertheless, there is indication of dedifferentiation *in vitro*. C2C12, a mouse muscle cell line, has been used to study the dedifferentiation process. C2C12 cells, when they are differentiated, exit the cell cycle and remain in the G0-phase of the cell cycle. These cells express mature markers such as myosin heavy chain (MHC), and myogenin (Andres and Walsh, 1996). Evidence for dedifferentiation in C2C12 is provided by a number of studies.

1.4.i. A1 newt/C2C12 hybrid

Velloso et al. (2001) found that hybrid multinucleated cells generated from the fusing of C2C12 and newt A1 myoblasts are able to re-enter the cell cycle in the presence of serum. These authors found that 6.8 % of C2C12 nuclei within the hybrid cells were able to incorporate BrdU. This suggests that factors produced by the newt cells allowed cell cycle re-entry in the mouse nuclei. It is possible that mammalian multinucleated cells are not able to divide in response to extracellular signals because the factors that allow newt multinucleated cells to re-enter the cell cycle could have been lost in mammalian multinucleated cells. On the other hand, C2C12 multinucleated cells are not totally unresponsive to proliferative signals. Up-regulation of immediate early genes such as c-fos, c-jun, c-myc and id-1 were observed in serum stimulated C2C12 myotubes suggesting that these cells that were confined to G0 were able to traverse to G1 (Tiainen et al., 1996).

1.4.ii. Msx-1

Msx genes are a family genes coding for homeobox-containing transcription factors that are normally expressed during vertebrate development and regeneration. In vertebrate development, *msx-1* has been shown to be expressed in the growing limb bud (Lallemand et al., 2005). Msx-1 has also been implicated in the inhibition of myogenesis (Bendall et al., 1999). The *msx-1* gene is involved in blastema formation during limb regeneration in newts (Simon et al., 1995). Expression is detected in the blastema of the adult urodele amphibian during the initial stages of regeneration but not detected in fully regenerated limbs (Simon et al 1995), suggesting that Msx-1 expression might be associated with dedifferentiation, or inducing dedifferentiation of fully differentiated cells. Evidence of involvement of Msx1 in dedifferentiation came from a study of ectopic over-expression of the gene in mouse C2C12 multinucleated cells. Over-expression of *msx1* in mouse multinucleated myotubes in culture induced fragmentation of multinucleated cells into proliferative mononucleated cells (Odelberg et al., 2000). This study also found that over-expression of *msx1* led to the reduction of muscle markers such as MyoD, myogenin and MRF4 and to transdifferentiation of C2C12 cells into cells expressing chondrogenic, adipogenic or osteogenic markers. These results suggest that Msx1 is an important factor in dedifferentiation.

1.4.iii. TWIST

Twist is a nuclear basic helix-loop-helix transcription factor. This gene has been shown to be involved in the formation of mesoderm in *Drosophila*. In mammals, the expression is found in cranial neural crest derivatives, and mesenchymal structures. The gene has been implicated in the inhibition of mesodermal cell lineages including muscles (Spicer et al., 1996). A recent study by Hjiantoniou et al. (2007) found that over-expression

of *TWIST* in C2C12 multinucleated cells resulted in induction of dedifferentiation. *TWIST* caused a reduction of myogenic markers such as MyoD, myogenin, and myosin heavy chain and an increase in cyclin D1 (Hjiantoniou et al., 2008). Fragmentation was observed in C2C12 myotubes treated with *TWIST*, which indicates that this gene can induce muscle dedifferentiation in mammalian cells.

1.4.iv. SV40 large T antigen

SV40 (simian vacuolating virus 40), which is an oncogene derived from the polyomavirus has also been implicated in causing dedifferentiation in muscle cells. A study by Endo et al (1998) found that adding large T antigen to C2SVT (a mouse skeletal muscle cell line that harbours the *T antigen* gene linked to an inducible promoter) caused cell cycle re-entry that proceeded to M phase, cytokinesis, cleavage and apoptosis. The large T antigen acts by inactivating *retinoblastoma (Rb)* and inducing *c-Jun* (Endo et al., 2004). These factors are essential in inducing cell cycle re-entry in differentiated cells.

1.4.v. Myoseverin, a microtubule destabilizing agent

The ability of C2C12 multinucleated to undergo fragmentation was also confirmed with a microtubule destabilizing drug called myoseverin (Rosania et al., 2000). Myoseverin is a trisubstituted purine molecule that binds to and disassembles microtubules. Addition of myoseverin to C2C12 myotubes caused cells to break down into mononucleated cells and to proliferate as analyzed by flow cytometry. In addition, myoseverin caused an increase in *myf5* mRNA level and CDK2, suggesting a reversal of the differentiated state into a proliferative state. Myoseverin also affects genes involved in extracellular matrix remodelling (Rosania et al., 2000). These results suggest that depolymerization of

microtubules may be a key event in the pathway leading to fragmentation of multinucleated cells.

1.4.vi. Inactivation of pRb and p19ARF

As mentioned above, cell cycle re-entry occurs in newt multinucleated cells when pRb is inactivated by phosphorylation (Tanaka et al., 1997). In mammals, suppression of pRb by siRNA induces cell cycle re-entry in C2C12 myotubes. In normal primary myotubes, inactivation of pRb did not result in cell cycle re-entry (Camarda et al., 2004; Huh et al., 2004). This might imply that mammalian cells have other mechanisms that hinder cell cycle re-entry in terminally differentiated cells. A study by Pajcini et al. (2010) found that inactivation of Rb and $p19^{ARF}$ in primary myotubes led to cell cycle re-entry. $P19^{ARF}$ is a tumor suppressor, and functions to cause cell growth arrest through p53 (Kamijo et al., 1997). In addition, inactivation of Rb and $p19^{ARF}$ led to down-regulation of differentiation markers including muscle creatine kinase (M-CK), MHC, and MRF4 and up-regulation of cyclin D1 and cyclin E. This study also found that in terminally differentiated myocytes, suppresion of Rb and $p19^{ARF}$ causes cell cycle re-entry and proliferation. The proliferating cells were found to be able to re-fuse again into multinucleated cells in culture. Furthermore, when the dedifferentiated myocytes were injected into the *tibialis anterior* muscle of mice, they were found to fuse with the existing muscle (Pajcini et al., 2010). This study demonstrates that normal primary muscle cells can also undergo dedifferentiation.

1.4.vii. Newt extract

The extracts prepared from regenerating newt limbs of *Notophthalmus viridescens* can induce newt and mouse multinucleated cells to re-enter the cell cycle, fragment, as well as proliferate (McGann et al. 2001). The authors found 18% of C2C12 multinucleated cells

treated with the blastema extract undergo cell cycle re-entry. The treatment also affected the expression of muscle proteins. There was a reduction in MyoD, myogenin and troponin T in C2C12 myotubes treated with the newt blastema extract. In addition, 10% of the mouse myotubes were seen to undergo fragmentation and proliferation. It appears likely that the newt blastema extract might contain more than one factor necessary for fragmentation, and cell cycle re-entry.

The dedifferentiation inducing factors in the newt extract are thought to be proteins. When McGann et al. (2001) subjected the newt extract to freeze-thaw cycles, its activity was reduced. Boiling the extract for 5 minutes at 95^0C also inhibited its activity. Furthermore, when the blastema extract was subjected to 1% trypsin for 30 minutes at 37^0C, the dedifferentiation signal was eliminated. Proteolytic analyses of the newt limb extract using the casein and gelatin zymography found that the newt extract contains MMP proteases (Vinarsky et al., 2005). Zymography is a technique that detects enzyme activity. Zymography detected MMPs with sizes ranging from 25 to 85 kDa. As mentioned above in section 1.2.ii, the expression of MMPs is detected during the initial stages of dedifferentiation during newt limb regeneration. MMPs may be essential in initiating dedifferentiation in multinucleated cells.

1.5. Characteristics of dedifferentiation in mammalian muscle cells

Dedifferentiation is not a phenomenon that naturally occurs in mammalian muscle cells during regeneration. This is an event observed in newt muscle cells during regeneration, which implies that urodele cells are different from mammalian cells. For example, serum treatment of urodele myotubes causes phosphorylation of the pRb protein and results in cell cycle re-entry (Tanaka et al., 1997). This effect is not seen in mammalian

myotubes. However, myotubes derived from a mouse myoblast cell line deficient in Rb are able to re-enter the cell cycle following serum stimulation (Schneider et al., 1994).

While cell lines such as C2C12 are ideal models to study reversal of differentiation, as the cells are able to maintain their ability to terminally differentiate *in vitro* (Andres and Walsh, 1996), they can become mutated with time. Recently, it was discovered that the C2C12 cell line has a deletion in the INK4a/ARF locus (Pajcini et al., 2010). The *INK4a/ARF* gene encodes p16, a cyclin-dependent kinase inhibitor, and p19, alternate reading frame (ARF) that controls p53 function (Chin et al., 1998). Since established cell lines can become altered with time, in order to further characterize dedifferentiation in mammalian cells, primary muscle cell lines should be used.

A number of studies that have attempted to induce dedifferentiation in primary myotubes have found that in normal cells, cell cycle re-entry does not occur (Camarda et al., 2004; Huh et al., 2004). In contrast to the studies by Schneider et al. (1994) that showed cell cycle entry in myotubes derived from an immortalized Rb-/- cell line, two studies have shown that Rb removal alone cannot cause differentiated myotubes to re-enter the cell cycle in a primary cell line. Differentiated myotubes that were infected with a Cre recombinase-carrying adenovirus (AdCre) to remove Rb, and treated with growth medium containing serum did not incorporate BrdU in their nuclei. Yet, the expressions of proliferating cell nuclear antigen (PCNA), cyclin E, cyclin A, DNA replication licensing factor (mini-chromosome maintenance, MCM2), and DNA ligase I was up regulated (Camarda et al., 2004). Huh et al. (2004) also found that myotubes lacking pRb that were differentiated from myoblasts derived from transgenic mice expressing MCK-Cre and Rb flanked by loxP sites did not undergo cell cycle re-entry when treated with serum. Furthermore, re-stimulation of

myotubes did not result in up-regulation of cyclin E. These studies indicate that ablation of pRb is not sufficient to cause primary multinucleated cells to progress into the S phase of the cell cycle. As mentioned above, Pajcini et al. (2010) found that inactivation of Rb and $p19^{ARF}$ in primary multinucleated cells resulted in cell cycle re-entry. In addition, inactivation of Rb and $p19^{ARF}$ led to down-regulation of differentiation markers including M-CK, MHC, and MRF4 and up-regulation of cyclin D1 and cyclin E. This study indicated that $p19^{ARF}$ might be the factor that impedes cell cycle re-entry in terminally differentiated muscle cells.

1.6. Purpose of this thesis

McGann et al. (2001) found that the newt extract contains factors which are involved in promoting fragmentation of C2C12 myotubes. The fragmented mononucleated cells were able to proliferate when placed in growth medium. This study was able to demonstrate that mammalian multinucleated cells can undergo full dedifferentiation. However, the results of this study have been called into question by regeneration biologists. There have been numerous attempts to replicate this data without success. Moreover, the authors cannot definitively eliminate the possibility that the fragmentation of myotubes observed was the result of apoptosis and that the observed "proliferation" of these fragmented mononucleated cells did result from contaminating myoblasts in the culture. Since death assays were not conducted following extract treatment, and differentiated myotubes cultures are never free of contaminating myoblasts, the results obtained by McGann et al. (2001) are not conclusive. Moreover, the studies were conducted in the C2C12 cell line, which others have shown has accumulated mutations. The question still remains whether dedifferentiation can occur in normal primary muscle cells, and in particular, in the presence of newt extract. The question

about the validity of the results by McGann et al. (2001) as well as the lack of information on primary myotubes has led to the following objectives:

1.6.i. Objectives

Aim 1: To examine cell cycle re-entry in C2C12 and primary myotubes. The cells were treated or micro-injected with newt extract. Cell cycle re-entry in myotubes was assessed by staining the cells with BrdU or Ki-67 (markers for proliferating cells).

Aim 2: To examine fragmentation of multinucleated cells into mononucleated cells. Newt extract-treated myotube cultures or myotubes injected with newt extract and GFP to tag only myotubes were followed with live-imaging microscopy.

Aim 3: To examine the effect of newt extract on muscle proteins. This was analyzed by injecting multinucleate cells with GFP and treating the cells with newt extract followed by fixing and staining the cells with GFP and MHC. We expected to detect GFP positive cells which were negative for MHC and this would indicate that the newt extracts causes down-regulation of mature muscle markers.

Aim 4: To identify candidate genes involved in the dedifferentiation process in C2C12 and primary cells. Myotubes cultures were treated with newt extract followed by Affymetrix GeneChip analysis, and real-time PCR.

Chapter 2. Material and Methods

2.1. Animal handling and surgeries

Animals used in these studies were housed at the University of Ottawa Animal care Facility. All experimental procedures were approved by the University of Ottawa Animal Care Committee. The newt extract that was used in these experiments was collected from adult newts, *Notophthalmus viridescens* that were purchased from Charles D. Sullivan (Nashville, TN). The animals were anesthetized by immersion in 0.1% tricaine methanesulphonate (MS222, Sigma) in dechlorinated water and the initial amputation was done above the wrist. Forelimbs were reamputated at 5 days after the initial amputation and the regenerated tissues were collected. The forelimbs were then re-amputated 3 days later, and again after 1 day, as shown in figure 5. Non-regenerating tissues (referred to as intact extract in the text) were collected from the hand extremities of the adult newts, *Notophthalmus viridescens*. The tissues were immediately frozen down in liquid nitrogen and stored at -80^{0}C.

2.2. Preparation of Newt Limb Extract

Newt extract was prepared, as per the protocol of McGann et al. (2001). The primary newt extract (1^0) was prepared from first-time amputated tissues. The secondary extract (2^0) was prepared from animals that were previously amputated allowed to regenerate for 1 or 3 months before re-amputating. The frozen tissues were thawed and placed in 10 ml Dulbecco's Modified Eagle Medium (DMEM)/High glucose (Hyclone) supplemented with protease inhibitors cocktail (PIC) from Roche. One tablet of PIC was dissolved in 1.5 ml of distilled water. About 1 ml of the dissolved solution was added to 100 ml of DMEM. The tissues were homogenized using a VWR VDI 12 hand homogenizer (5-10min), and

Misonix XL2000 sonicator for 1-3 min. The homogenate was spun using a centrifuge for 25 min at 2000 x *g* @ 4^0C. The supernatant was then placed in a fresh tube and spun for 60 min at 100,000 x *g* @ 4^0C using an ultracentrifuge. The supernatant was filtered through a 0.40 μm filter (Fisher). The extract concentration was determined with a BCA (bicinchoninic acid) protein assay kit (Bio-Rad) and stored in 0.5 ml aliquots at -80^0C.

2.3. Preparation of Mouse Embryonic Fibroblast (MEF) Extract

The mouse embryonic fibroblast cells used in this experiment were obtained from Dr. Luc Sabourin's lab. MEF cells were grown to 90% confluency on 200 mm plates (Corning Inc.) in 10% fetal bovine serum (FBS, Hyclone) supplemented with 1X pen/strep (Penicillin-Streptomycin, Gibco), 1X L-glutamine (Gibco), and 1X sodium pyruvate (Sigma). The cells were then scraped off the plates and placed in 10 ml Dulbecco's Modified Eagle Medium (DMEM)/High glucose (Hyclone) supplemented with protease inhibitors cocktail (PIC) from Roche. One tablet of PIC was dissolved in 1.5 ml of distilled water. About 1 ml of the dissolved solution was added to 100 ml of DMEM. The cells were sonicated for 1-3 mins using the Misonix XL2000. The homogenate was centrifuged for 25 min at 2000 x *g* at 4^0C. The supernatant was then placed in a fresh tube and spun for 60 min at 10,000 x *g* at 4^0C using an ultracentrifuge. The supernatant was filtered through a 0.40 μm filter (Fisher). The cell extract concentration was determined with a BCA (bicinchoninic acid) protein assay kit (Bio-Rad) and stored in 0.5 ml aliquots at -80^0C.

2.4. Differentiation of C2C12 cells

The C2C12 cells used in this experiment were obtained from Dr. Luc Sabourin's lab (Ottawa Hospital Research Institute, OHRI). Myoblasts were grown to confluency on 100 mm plates (Corning Inc.) in 10% fetal bovine serum (FBS, Hyclone) supplemented with 1X

pen/strep (Penicillin-Streptomycin, Gibco), 1X L-glutamine (Gibco), and 1X sodium pyruvate (Sigma). To induce differentiation into multinucleated myotubes, the medium was switched from 10% FBS to 2% horse serum (HS, Sigma) differentiation medium (DM).

2.5. Isolation and differentiation of primary myoblasts

Primary myoblasts were isolated from either wild-type mice or transgenic mice expressing yellow fluorescent protein (YFP) in their mature differentiated muscle. The transgenic lines were provided by Dr. Michael Rudnicki (OHRI). Mice carrying loxp- stop-loxp sequence followed by the yellow fluorescent protein (YFP) inserted into the Rosa locus were crossed with mice expressing muscle creatine kinase (MCK)-Cre recombinase. Genotyping was conducted on deoxyribonucleic acid (DNA) extracted from tails of 21 day old pups. 1M NaOH / 0.5 M EDTA in water was added to the tissues at 95° C for 1 hr to lyse the tissues followed by adding the neutralizing buffer of 1 M Tris HCl (pH 8) in H_20. For the PCR reaction, 50 mM of $MgCl_2$ (Invitrogen) , 20 mM dNTP (Invitrogen), 0.5 µl of Taq polymerase, 5 µl of 10 X PCR Buffer (Invitrogen), 50 ng/ml of DNA, 100 µM of Cre-primer sequence (forward – 5' - ATGCTTCTGTGCGTTTGCCG and reverse – 5' CCTGTTTTGCACGTTCACCG) (Sigma) were combined in a 50 µl reaction. PCR was performed with an initial denaturation step of 94°C for 30 min, followed by 35 cycles of 96°C for 20 sec, 60°C for 20 sec, 72°C for 30 sec, and an extension step at 72°C for 2 min. Primary myoblasts used in this experiment were isolated from the hindlimb muscle (according to the protocol of Springer et al. (2002) in 4-6 week old mice. Isolated primary myoblasts were grown in Ham's F-10 medium (Sigma), supplemented with 20% fetal bovine serum (FBS, Hyclone), 4X pen/strep (Gibco), 1X fungizone (Gibco), 50 µg/ml gentamicin (Gibco) and 10 ng/ml of basic fibroblast growth factor (bFGF, Sigma). Cells were grown on

collagen-coated plates. Once the primary myoblasts reached 50-70% confluency the medium was changed to a DM of 2% horse serum to induce myotube formation.

2.6. Purification of myotubes

2.6.i. Ara-C Treatment

In order to remove contaminating myoblasts from the myotube cultures, cells were transferred from DM to growth medium (10% FBS) with 10 μM arabinosylcytosine (Ara-C, Sigma) for 48 hrs. After treatment with Ara-C, the cells were washed in phosphate buffered saline (1XPBS) and transferred back into DM for 24 hrs.

2.6.ii. Cell filtration

A second method for removing contaminating myoblasts was to pass the myotube cultures through a series of filters. Day 4 differentiated myotubes were passed through a 100-μm mesh (Fisher). Cell debris was retained on the filter. The eluate was passed through a 40-μm sieve to get rid of most of the contaminating myoblasts, and myotubes that were retained on the filter were plated on 60 mm plates (Corning Inc).

2.7. Tracking differentiated cells.

2.7.i Transfection of C2C12 cells with DNA Plasmids

Proliferating C2C12 myoblasts cultured in 10 % FBS were co-transfected with both 5-μg pCMV-EGFP/RFP (addgene) and 5-μg MCK-Cre (addgene) plasmids using Exgen500 reagent (Fermentas). When the cells reached confluency, the medium was switched to 2% HS to induce differentiation.

2.8. Newt extract treatment

Myotubes cultures were treated with newt extract using 2 methods. In the first method, extract was added to the culture medium. In the second method, myotubes were microinjected directly with newt extract.

2.8.i. Treatment of differentiated muscle in cultures with newt extract

Myotubes were treated with 0.3 mg/ml or 0.1 mg/ml of primary (1°) or secondary (2°) newt extract in 2 % horse serum or 10 % fetal bovine serum for 3 days. 1 mM of bromodeoxyuridine (BrdU) (Invitrogen) was added to the cells in the presence of newt extract. Control cells were originally treated with heat-inactivated (95°C for 5 mins) extract. Since this treatment produced no effect, subsequent controls were untreated in order to conserve extract and to reduce the number of newts required for generating extract.

2.8.ii. Microinjection

2.8.ii.a Preparation of extract and GFP

The newt extract fraction was prepared by adding 100 µl of crude extract of 2.5 mg/ml to a vivaspin 500 column of 30,000 molecular weight cut off (MWCO) polyethersulphone (PES) (Sartorius). The samples were spun down for 30 min at 16,200 x *g*. The >30 kDa fraction that was retained on the filter was resuspended in 25 µl. The concentration of the separated fractions (≤ than 30 kDa and ≥ than 30 kDa) were examined with a BCA protein assay kit (Bio-Rad) and stored at -80^{0}C. The procedures for preparation of extract were carried out at 4ºC. Myotubes cultured in 2 % horse serum were microinjected with 1:1 ratio of 4 mg/ml-newt extract and 100 ng/µl of pEGFP-C1 (addgene) GFP plasmid.

2.8.ii.b Preparation of extract from mouse embryonic fibroblasts (MEFs)

The MEF extract fraction was prepared by adding 100 µl of crude extract of 1.7 mg/ml to a vivaspin 500 column of 30,000 molecular weight cut off (MWCO)

polyethersulphone (PES) (Sartorius). The samples were spun down for 30 min at 16,200 x *g*. The >30 kDa fraction that was retained on the filter was resuspended in 25 µl. The concentration of the separated fractions (greater than 30 kDa) was examined with a BCA protein assay kit (Bio-Rad) and stored at -80^{0}C. The procedures for preparation of extract were carried out at 4ºC. Myotubes cultured in 2 % horse serum were microinjected with 1:1 ratio of 4.25 mg/ml-MEF extract and 100 ng/µl of pEGFP-Cl (addgene) GFP plasmid.

2.8.ii.c. Preparation of cells

Day 3 myotubes were purified using filtration (as per section 2.5.ii). Filtered C2C12 cells were plated on 35 mm non-coated plates, 0.8% sodium hyaluronate coated plates or 0.4% collagen coated plates, and primary cells were plated on either 0.8% sodium hyaluronate coated plates or 0.4% collagen coated plates. Microinjection was performed using a micromanipulator and a Zeiss inverted microscope. The DNA plasmid was loaded into a 'FemtoTip' needle (Eppendorf) using a microloader. For the injection, an injection pressure of 150 hPa, a maintenance pressure of 50 hPa, and an injection time of 0.1 s was used. About 25 to 60 myotubes were microinjected with the extract/plasmid or MEF/plasmid cocktail in either the cytoplasm or the nucleus. Right after the injection, the cells were transferred to 10 % FBS for 3 or 4 days. The control cells were microinjected with DMEM and GFP plasmid.

2.9. Immunocytochemistry

Control or extract-treated cultures were washed with 1 X PBS before fixation. Cells were fixed in 4% paraformaldehyde (PFA) for 5 minutes at room temperature, followed by 3 washes 1 X PBS for 5 minutes each. For the BrdU assay, several additional steps were required prior to the addition of the antibody. The cells were blocked in 5% goat serum

(Jackson Immuno Research), 0.3 % Triton X-100 (Sigma) in PBS for 30 min at room temperature prior to addition of the primary antibody. The cells were incubated in 1N HCl (Sigma) for 10 min on ice, followed by 2N HCl for 10 min at room temperature and for 20 min at 37^0C and washed in PBS (3X, 5 min each). To buffer the cells 0.1M borate buffer (ACP Chemical Inc) was added for 12 min at room temperature.

The primary antibodies incubated with the cells included the following markers: monoclonal antibody BrdU, 1:500 dilution (Molecular Probes); monoclonal antibody Ki-67, 1:200 dilution (Sigma); polyclonal antibody MF20, 1:50 (Santa Cruz Biotechnology); monoclonal antibody p21, 1:50 (BD (Pharmingen); monoclonal antibody p130, 1:50 (Santa Cruz Biotechnology); monoclonal antibody p27, 1:50 (Santa Cruz Biotechnology) and monoclonal antibody GFP, 1:200 (Invitrogen). The secondary antibodies included goat anti-mouse Alexa Fluor 488 (Invitrogen) and anti-rabbit Cy3 (Jackson Immuno Research, 1:400 dilutions for both). Examination of fluorescent signals was done using a Zeiss Axiovert or Nikon inverted microscope.

Percentage of cell cycle re-entry in myosin heavy chain (MHC) positive cells detected with the MF20 antibody was calculated by dividing the number of MHC +ve cells also positive for BrdU or Ki-67 with the total number of MHC cells X 100. Percentage of nuclei exhibiting cell cycle re-entry in microinjected cells (with both newt extract and GFP) was calculated by dividing the number of nuclei in GFP + ve myotubes that were also positive for Ki-67 with the total number of nuclei in GFP +ve myotubes X 100.

2.10. Assessing down-regulation of differentiation-specific muscle markers

Day 3 differentiated myotubes were filtered to remove myoblasts, as previously described. On day 4, about 25 to 60 myotubes (of greater than 3 myonuclei) cultured in 2 %

horse serum were microinjected with the pEGFP-C1 plasmid, as previously described. Right after the injection, myotubes were placed in either 2 % horse serum or 10 % fetal bovine serum with newt blastema extract for 3 or 6 days. The control myotubes were put back in either DM or GM without newt blastema extract. This was followed with fixing and immunostaining the cells with GFP and MHC.

2.11. Assessing the effect of newt extract on muscle cell cycle regulators

C2C12 myoblasts were allowed to differentiate for 5 days. Newt extract at the concentration of 0.3 mg/ml (1°) in growth medium (GM) was added to the differentiated cells for 3 days followed by immunostaining for p21 antibody.

2.12. Assaying fragmentation and proliferation

2.12.i. Fragmentation of multinucleated in the presence of newt extract

Fragmentation was assessed by following Ara-C and with newt-extract treated myotube cultures with a Nikon inverted or Zeiss live imaging microscope. C2C12 and primary cultures were differentiated for 2 days followed by Ara-C treatment as described above for 2 days. The cells were then placed back into DM for 1 day followed by newt extract for 3 days at a concentration of 0.3 mg/ml (1°) in DM. C2C12 cells were followed with the Zeiss live imaging microscope and photographed every 15 minutes, and primary myotubes were followed with the Nikon microscope each day for 3 days. Fragmentation and proliferation were also assessed in both primary and C2C12 GFP myotubes (of > 3 myonuclei) that were microinjected with a 1:1 ratio of GFP plasmid (100 ng/µl) and newt extract fraction of greater than 30 kDa (4 mg/ml) with the Zeiss live imaging microscope. Pictures were taken at 15 min intervals for 5 days.

2.13. Myoseverin treatment

Day 4 multinucleated cells in DM were treated with 10 μM myoseverin (Sigma) for 24 hrs. After 24 h, myoseverin was removed, and the cells were placed back in DM for 48 h to assess refusion.

2.14. Detection of apoptotic cells

Apoptotic cells were identified using terminal deoxynucleotidyl transferase (TdT)-mediated DUTP nick end labeling (TUNEL) using the ApopTaq Fluorescein or ApopTaq Red *In situ* Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. C2C12 differentiated cells, both untreated and treated with newt regeneration extract, were pre-fixed in 1% PFA in PBS (pH 7.4) for 10 min at RT. The cells were post-fixed at -20^{0}C for 5 minutes in 2:1 ethanol:acetic acid. After the washes, the fixed cells were incubated with the kit reagents, followed by MF20 antibody (1:50) staining and examined by fluorescence microscopy.

2.15. Assessing whether newt blastema extract inhibits muscle differentiation

Myoblasts were kept in growth medium (GM) until they became 70% confluent for primary cells and 100% for C2C12 cells. The myoblast cultures were then treated with 0.3 mg/ml of newt blastema extract in DM or GM for 4 days followed by immunostaining with MF20 (myosin heavy chain, MHC). The control cells were not treated with newt blastema extract.

2.16. Assessing whether newt blastema extract causes proliferation of myoblasts

2.16.i. Flow cytometry analysis

Semi-confluent myoblasts that were grown in GM for 48 h with or without extract were trypsinized and washed once in 10% FBS-DMEM. Myoblasts were then washed twice in PBS supplemented with 1mM EDTA (PBSE) and then fixed in 1 mL of PBSE by the drop

wise addition of 2 mL of 80% ethanol pre-chilled to –20 °C. The samples were then stored at –20 °C for a minimum of 2 hours, washed once in PBSE, resuspended in DNA content staining buffer (1.1% citrate buffer, 10 µg/µL propidium iodide (Sigma), 0.1 mg/ml RNase) and incubated for 30 minutes at 37 °C before being analyzed on a Beckman-Coulter flow cytometer using the Expo 32 software package.

2.16.ii. Hemocytometer analysis

Myoblasts were plated on 100 mm plates (Corning Inc.) in growth medium until they became semi-confluent. Cells were then trypsinized and resuspended in GM. The same number of cells (69.25 X 10^4 in 1 ml of GM or DM) was plated per plate either in the absence or presence of 1º and 2º newt extract at a concentration of 0.1 or 0.3 mg/ml. After 48 h in culture, the cells were re-counted using the hemocytometer.

2.17. Affymetrix GeneChip analysis

Ara-C treated myotube cultures in DM were treated with 0.3 mg/ml of newt blastema extract for 1 day. Ribonucleic acid (RNA) was isolated from cells (both untreated and treated with newt blastema extract) at 24 h using the Rneasy minikit from Qiagen. The quality of RNA was checked using a BioAnalyzer followed by Affymetrix GeneChip analysis. Analysis of gene expression was performed on two replicates of treated and non-treated dishes. Affymetrix analysis was done by the OHRI GENE Expression Facility using the Mouse Genome 430-v20 GeneChip. Data analysis was performed by Dr. Alan Mears (OHRI). Further analysis on the differentially expressed gene list was performed using DAVID Bioinformatics Resources 6.7. The genes were arranged into molecular function using Gene Ontology (GO) function.

2.18. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from extract-treated and control myotubes at 4, 8, 12, 24, 48 and 96 h after treatment. RNA was isolated using the Rneasy minikit from Qiagen. RNA concentration was determined on the spectrophotometer and the quality of RNA was verified by electrophoresis on a 1% agarose gel. Equal amounts of RNA were reverse transcribed with oligo dT-primers to give complementary DNA (cDNA) using the first strand cDNA kit from Bio-Rad. RT-PCR was conducted using the SYBR green kit from Bio-Rad. Gene-specific primers (Table 1) were designed using Primer Software Version 3. PCR was performed for *msx-1, pax-3,* and *myogenin* using Bio-RAD icycler at 95°C for 5 min followed by 35 cycles each of denaturing at 95°C for 30 sec, annealing at 58°C for 30 sec, followed by a 30 sec extension step at 72°C. PCR was performed for *PCNA* at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, followed by 72ºC for 30 sec. PCR was performed for *myf5* at 94°C for 2 min followed by 35 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec. Analysis of gene expression was calculated using the $2^{\Delta\Delta ct}$ model. Expression profiles were normalized against GAPDH. Controls (untreated cells) were assigned a value of 1 and all comparison was made relative to this. The quality of the PCR products was examined by electrophoresis on a 1% agarose gel.

2.19. Statistics

The data are presented as means +/- standard deviation. The statistical calculations were done using GraphPad Prism 5. Differences between control and treated samples were analysed using 2-way ANOVA or the student t-test. P-values less than 0.05 were considered to be significant.

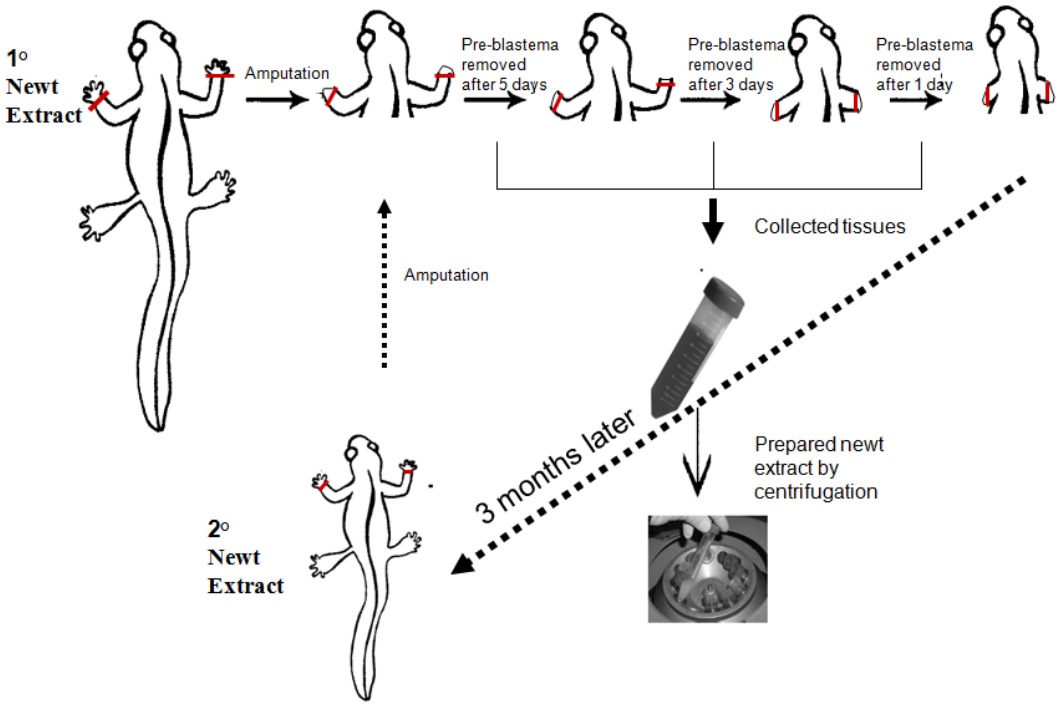


Figure 5. Isolation of newt blastema extract from the adult newt, *Notophthalmus viridescens:* The primary (1°) newt extract was collected by first amputating above the wrist of the newt forelimb to remove the intact tissue. After 5 days, the growing regenerating pre-blastema was removed and frozen in liquid nitrogen. After 3 days the limb was re-amputated, and then re-amputated again the following day. The 1, 3 and 5-day regenerates were pooled. The secondary (2^0) extract was prepared from animals that had been previously amputated, and allowed to regenerate for 1 or 3 months. Pooled tissues were subjected to homogenization and centrifugation as described in section *2.2*

Table 1 – Primers used in RT-PCR analysis

Gene		Sequence
MYOGENIN	FWD	5'-GCAATGCACTGGAGTTCG-3'
	REV	5'-ACGATGGACGTAAGGGAGTG-3'
MYF-5	FWD	5'-CTGTCTGGTCCCGAAAGAAC-3'
	REV	5'-AAGCAATCCAAGCTGGACAC-3'
MSX-1	FWD	5'-AGCTCTGCTGCCCTATACCA-3'
	REV	5'-CTTGGCCTCTGCATCCTTAG-3'
PAX-3	FWD	5'-CTGCACTCAAGGGACTCCTC-3'
	REV	5'-GTGAAGGCGAGACGAAAAAG-3'
PCNA	FWD	5'-GCTTGGCAATGGAACATTA-3'
	REV	5'-CAGTGGAGTGGCTTTTGTGA-3'
GAPDH	FWD	5'-TCGGTGTGAACGGATTTG-3'
	REV	5'-GGTCTCGCTCCTGGAAGA-3'

Chapter 3. Results

McGann et al., (2001) showed that mammalian myotubes treated with newt extract could re-enter the cell cycle and fragment into mononucleated cells. Subsequent to this study, numerous laboratories tried unsuccessfully to repeat these studies. The reason for this may be due to the variability of the extract obtained from one isolation to the next, even if the extraction protocol remained identical. In fact, the positive results described in this thesis were obtained from six extracts out of a total number of 20 extract preparation. The reasons for this variability are not entirely clear, but the age and health of the animals and the rate of regeneration probably has a large contributing factor.

3.1. Assessing cell cycle re-entry in mammalian myotubes

3.1.i. Cell cycle re-entry in ara-C treated C2C12 myotubes that were treated with newt extract

The first aim of my research was to replicate the results of McGann et al. In order to do this, C2C12 myoblasts were grown to confluency and induced to differentiate into multinucleated myotubes. Cultures were then treated with ara-C in order to remove contaminating myoblasts and to yield a purer myotube population. Ara-C is a cytosine analogue that incorporates into replicating DNA but is toxic to the cells because it interferes with chain elongation and DNA synthesis (Kufe and Major, 1982). After ara-C treatment, the cells were put back into differentiation medium (DM) for recovery for 1 day. Subsequently, the differentiated cells in GM or DM were treated with newt extract for 3 days in the presence of BrdU. Control cells were placed in GM or DM. This was followed by fixation and staining the cells with anti-BrdU antibody to assess cell cycle re-entry and MF20 to label MHC-positive myotubes. Figure 6A and Figure 7A show that C2C12

mononucleated cells, which are also positive for MHC (ie. myocytes) are capable of re-entering the cell cycle following treatment with the secondary newt extract at a concentration of 0.3mg/ml. 23% of MHC +ve mononucleated cells were induced to re-enter the cell cycle following treatment with newt extract in GM (figure 6B). Furthermore, 4% of the cells that re-entered the cell cycle are seen in mitosis (cytokinesis phase) of the cell cycle. It should be noted, however, that in a subsequent experiment involving a different batch of extract, fewer MHC+ve mononucleated cells were seen to enter the cell cycle (Figure 7B). This reiterates the fact that there are notable differences between batches of extract; some extracts showed significant cell cycle re-entry in myotubes and others showed minimal responses.

Cell cycle re-entry in MHC positive cells was not observed in ara-C differentiated cultures treated with the newt extract at a concentration of 0.3 mg/ml or 0.1 mg/ml in GM (figure 6B) or in cells treated with any extract in DM (Figure 6C). In addition, cell cycle re-entry was not observed in control cultures that were not treated with newt extract or cultures treated with the extract from intact limbs (figure 6 and figure 7). There was a significant decrease in multinucleated cells in cultures treated with newt extract in both GM and DM (figure 6B, C and figure 7B). It is possible that multinucleated cells in cultures treated with newt extract were fragmenting into mononucleated cells or dying and lifting off the plates. A significant increase in MHC +ve mononucleated cells was observed in cultures treated with newt extract in DM, indirectly suggesting that newt extract causes fragmentation of myotubes into mononucleated cells. This increase in the number of MHC +ve mononucleated cells could also occur from fragmented multinucleated cells in the process of undergoing apoptosis.

3.1.ii. Cell cycle re-entry in ara-C treated primary myotubes that were treated with newt extract

Cell cycle re-entry was assessed in extract-treated primary myotubes that were differentiated from myoblasts derived from transgenic mice expressing MCK-Cre/lox-Rosa-YFP. Upon differentiation, muscle creatine kinase (MCK), a marker for differentiated cells, became activated in these cells and this led to cre-lox recombination resulting in expression of yellow fluorescence protein (YFP) in differentiated muscle cells. First, myoblasts were differentiated for 2 days followed by ara-C treatment on day 3 and 4 in GM to eliminate proliferating myoblasts. After ara-C treatment, the cells were placed back in differentiation medium for 1 day to recover, followed by treatment with newt extract. Subsequently, the cells were fixed and immunostained with anti-BrdU and GFP antibodies. This approach was used because it allowed tracking of differentiated cells. However, the YFP expression could only be detected after myotubes culture differentiated for almost 7 days as shown in figure 8. Also, the expression of YFP was found to be very weak in myotubes, necessitating staining with GFP antibody.

Cell cycle re-entry was almost never observed in GFP +ve multinucleated or mononucleated cells treated with 1°newt extract at 0.3 mg/ml. In all the cultures treated with newt extract, only a single myotube showed BrdU incorporation in one of the nuclei (figure 9A). It is possible that a dividing cell incorporated BrdU before fusing with an existing myotube. Furthermore, a significant decrease in GFP +ve multinucleated cells was observed in cultures treated with newt extract (figure 9B). An increase in GFP +ve mononucleated cells was not observed, which might indicate that the decrease in multinucleated cells may have resulted from cells dying and lifting off the plates.

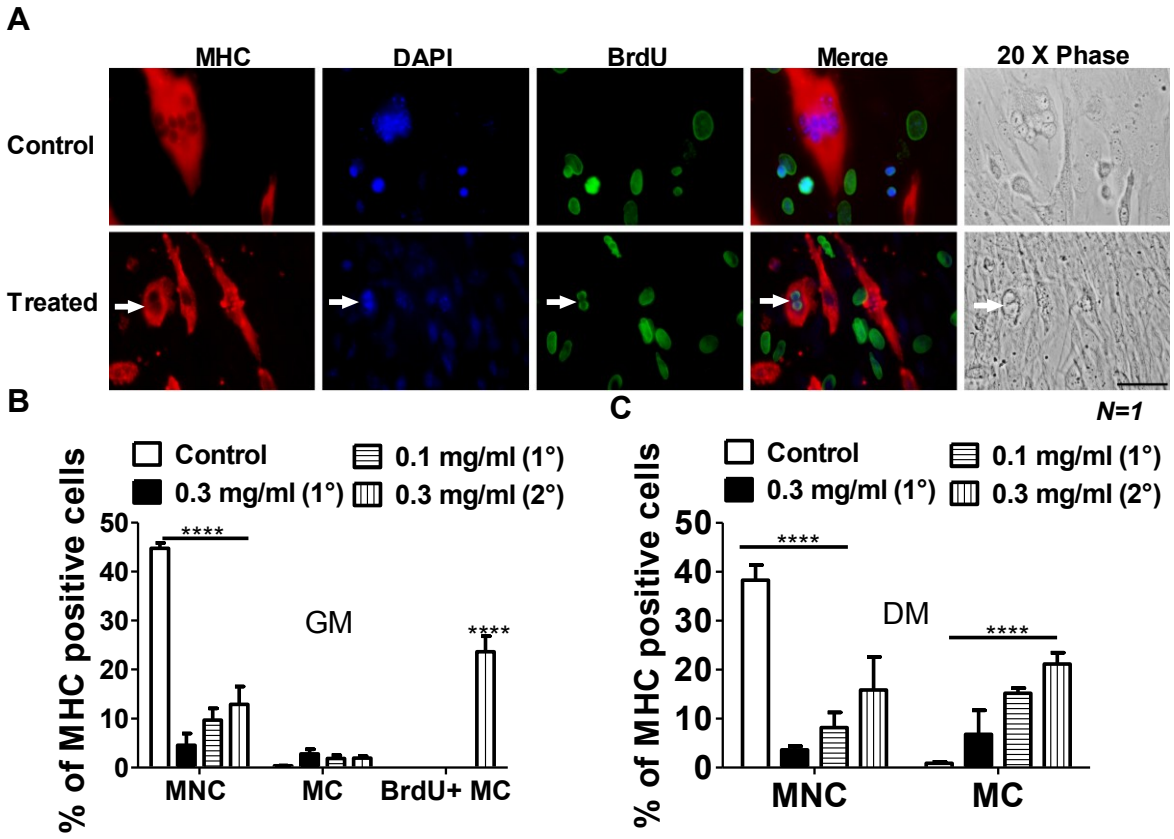


Figure 6. Cell cycle re-entry in C2C12 MHC +ve mononucleate cells treated with 0.3 mg/ml of 2^0 newt extract in GM. (A) Immunostaining shows MHC (red), BrdU (green), DAPI (blue), a merged image and 20 X phase in both control and treated cells. In newt-extract treated cells, the arrows are pointing to a MHC expressing cell that is also positive for BrdU. No cell cycle re-entry was found in MHC positive cells in the control cells. (B) Myotubes were treated with newt extract of various concentrations in the presence of growth medium (GM). 1º refers to primary extract and 2º refers to secondary extract (see Fig 5). The graph shows the percentage of myosin heavy chain (MHC) positive cells in multinucleated cells (MNC), mononucleated cells (MC) and MC positive for BrdU. The control represents cells that were not treated with newt extract. **(C)** Differentiated C2C12 cells were treated with 2^0 or 1^0 newt extract at various concentrations in differentiation medium (DM). Asterisk indicates significant differences (****$P < 0.0001$) between the control and treated groups. The experiment was performed in triplicate. Scale bar = 100 µm. *N=1* represents one biological sample with 3 replicates.

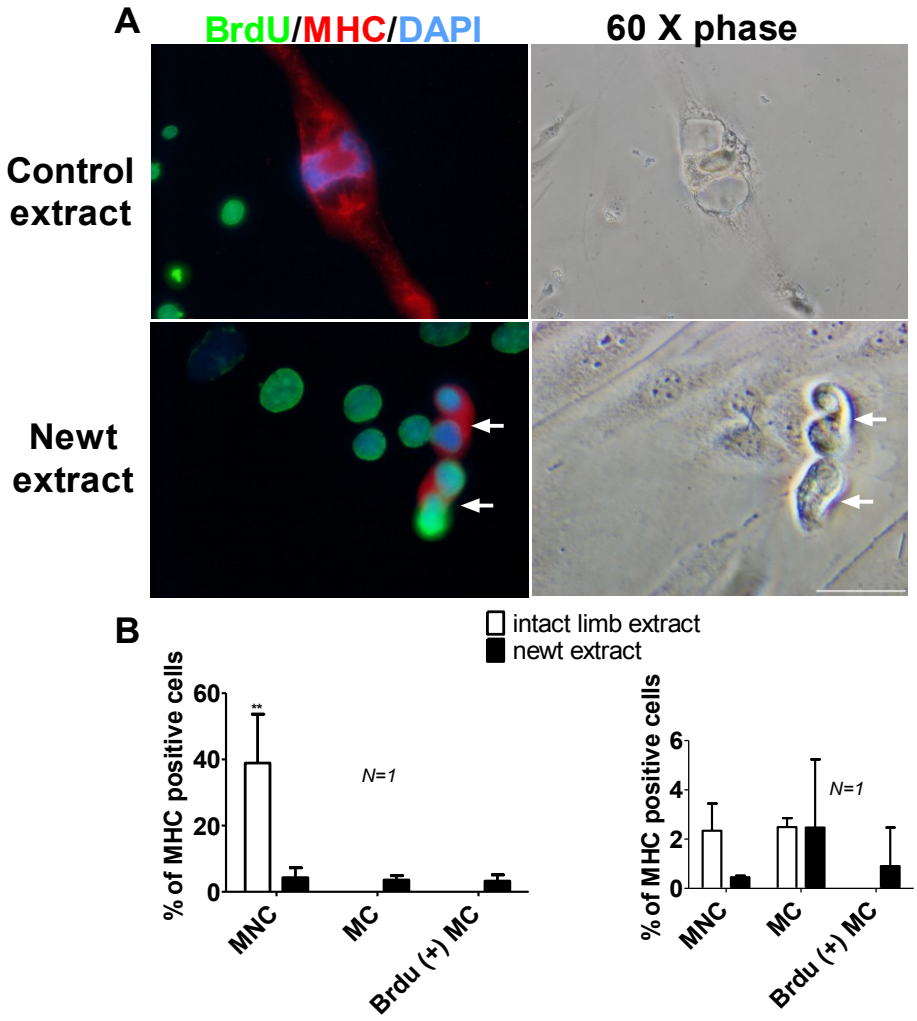


Figure 7. Regenerating limb extract induces cell cycle re-entry in filtered C2C12 cultures. (A) Immunostaining of a merged image showing MHC (red), BrdU (green), and DAPI and the corresponding phase image in both control (extract from intact limb) and treated cells (regenerating limb extract). In regenerating limb extract-treated cells, arrows are pointing to a MHC expressing cell that is also positive for BrdU. No cell cycle re-entry was found in MHC positive cells in the intact limb extract cultures. **(B)** Cell counts show percentage of MHC-positive cells in multinucleated cells (MNC), mononucleated cells (MC) and mononucleated cells that have incorporated BrdU (only seen in cultures treated with regenerating limb newt extract). Each experiment was performed in triplicate. Asterisks indicate significant differences (**$p < 0.01$) between the intact limb extract and the regenerating limb extract treated cultures. Scale bar = 200 μm

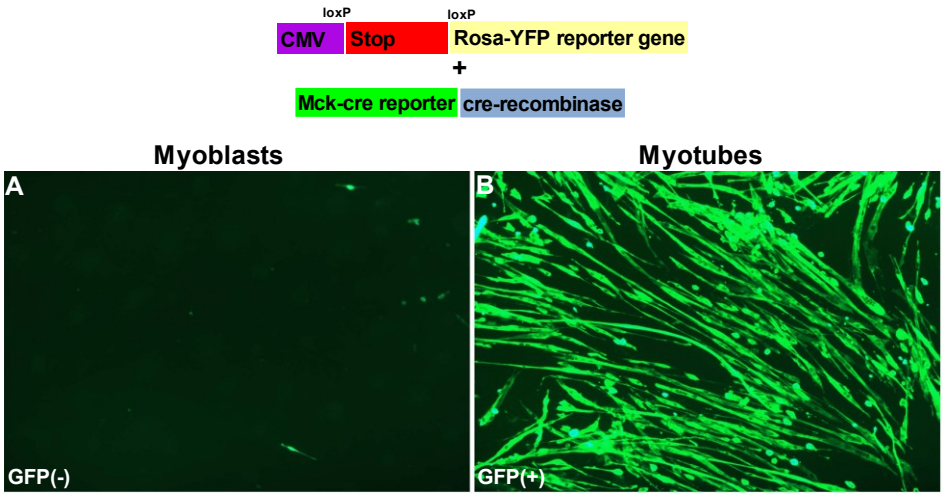


Figure 8. YFP expression in 7 day myotube cultures derived from MCK-cre/Rosa-YFP mice. (A) Shows immunostaining of myoblast isolated from MCK-cre/Rosa-YFP transgenic mice negative for GFP and (B) myotube culture positive for anti-GFP (green).

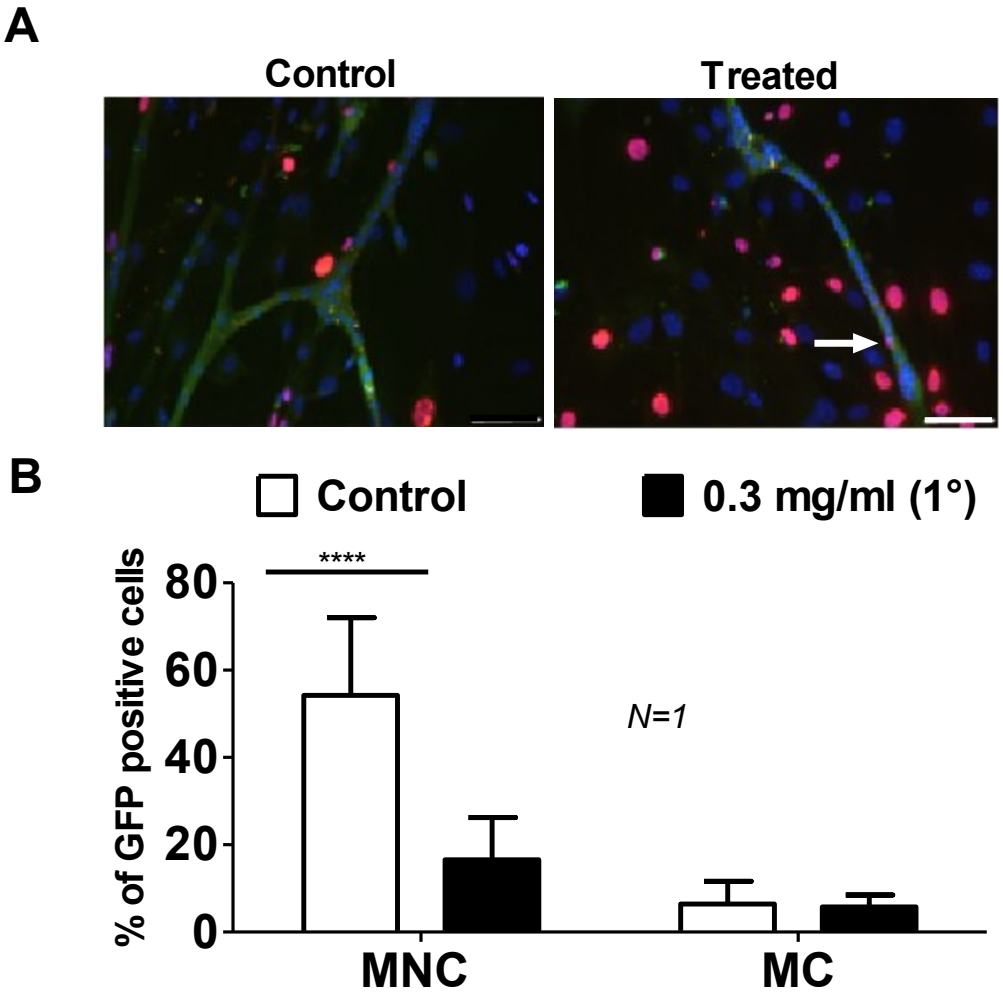


Figure 9. Primary GFP+ve myotubes from MCK-cre/lox-Rosa-YFP transgenic mice do not undergo cell cycle re-entry following treatment with the 1^0 newt extract. (A) Immunostaining shows control cells (not treated) and multinucleated cells treated with 0.3 mg/ml 1° newt extract. Cells are stained for BrdU (red), DAPI (blue), which stains the nuclei, and GFP (Green). Arrow in the treated sample shows a BrdU+ve nucleus within a GFP+ve myotube. (B) Graph shows percentage of GFP positive cells multinucleated (MNC) and mononucleated (MC) cells. Asterisks indicate significant difference (****$P < 0.0001$) between the control and extract treated group. The experiment was performed in septuplicate. Scale bar = 100 μm.

3.1.iii. Assessing apoptosis in ara-C and extract-treated myotubes

The decrease in multinucleated cells treated with Ara-C and newt extract might result from cells dying and lifting off the plate. It has been shown that myoblasts die in response to low mitogen concentrations, though differentiated myotubes are resistant to this condition (Wang and Walsh, 1996). C2C12 differentiated cultures treated with ara-C and subsequently treated with newt extract for 3 days seem to have fewer, as well as distorted myotubes, suggesting that these cells might be undergoing apoptosis. In order to detect apoptosis, ara-C treated C2C12 differentiated cells were treated with newt extract at different concentrations and stained using terminal dUTP nick end labelling (TUNEL). Cells in differentiation medium treated with 1°and 2° newt extract showed a significant increase in TUNEL positive nuclei in MHC positive myotubes in comparison to the control (figure 11). A reduction in MHC positive multinucleated cells and no increase in mononucleate cells in differentiation medium cultures treated with newt extract might suggest that growth factors are needed for dedifferentiation. Surprisingly, apoptosis was also observed in the ara-C control cultures in differentiation and in growth medium (figure 10 and 11). In one experiment, almost 45% of the control myotubes had TUNEL positive nuclei following transfer to growth medium (figure 11). Moreover, in some cases, control cells had even more TUNEL positive nuclei than cells treated with 2º extract (figure 12). This suggests that ara-C treatment might have contributed to cell death in myotubes. However, a combination of ara-C treatment and newt extract, particularly 1° extract, seems to cause more cell death than ara-C alone.

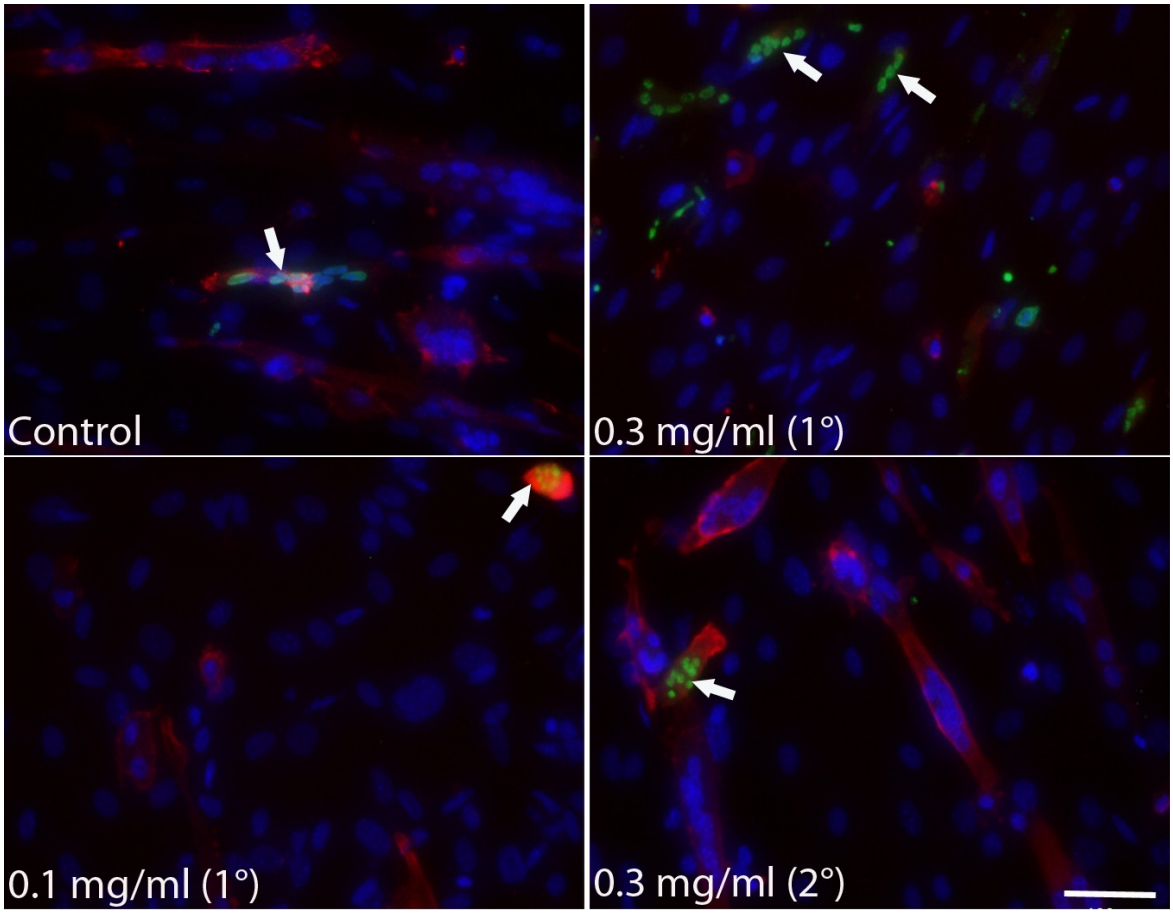


Figure 10. TUNEL staining of ara-C and extract treated C2C12 differentiated muscle cells. Immunostaining identifies TUNEL positive nuclei (green) in multinucleated cells positive for MHC (red). DAPI (blue) stains the nucleus. Arrows are pointing to TUNEL positive nuclei in controls (not treated) and cultures treated with 0.1 mg/ml (1°), 0.3 mg/ml (1°) and 0.3 mg/ml (2°) newt extract. Scale bar =100 μm.

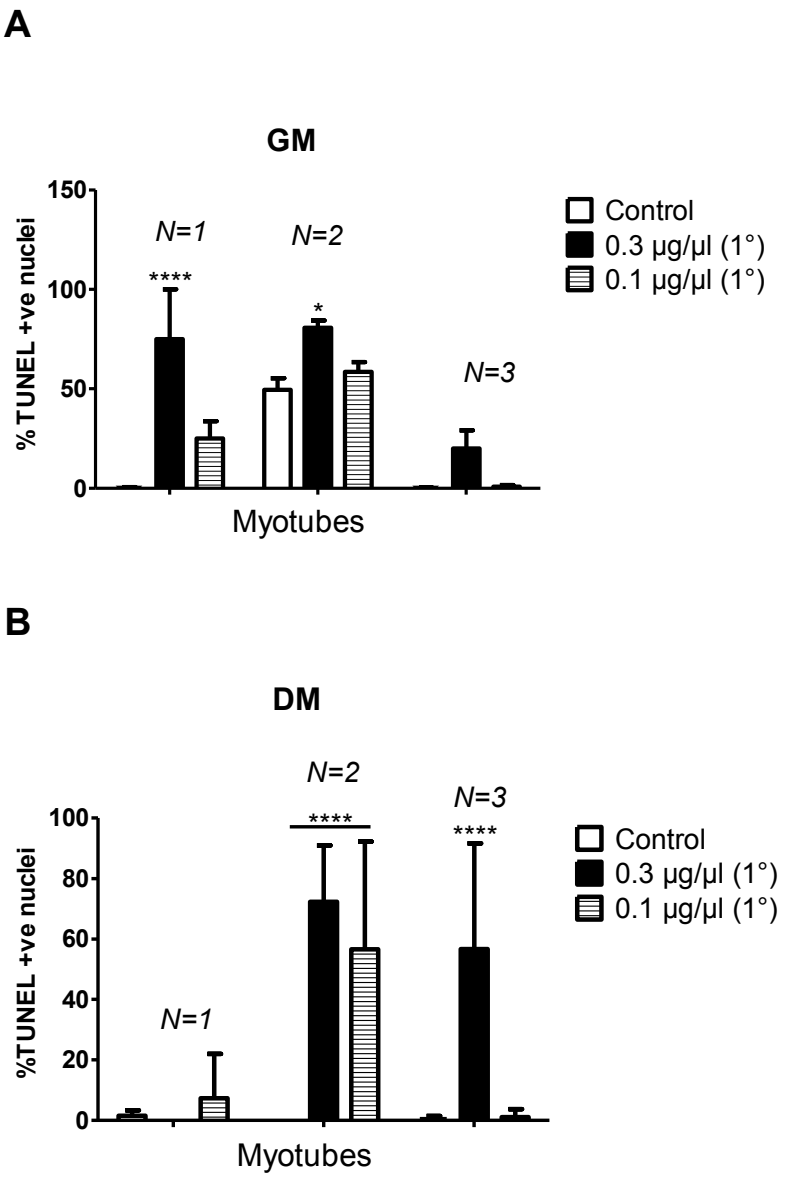


Figure 11. TUNEL assay indentifies apoptotic cells in ara-C treated C2C12 differentiated cells. (A) Percent TUNEL positive nuclei in multinucleated cells not treated (control) or treated with the primary newt extract at different concentrations in growth medium (GM). (B) Percent TUNEL positive nuclei in multinucleated cells not treated (control) or treated with the primary newt extract at different concentrations in differentiation medium (DM). N=1, N=2 and N=3 represent experiments done at different days with different batches of newt extract. Each experiment was performed in triplicate. Asterisk indicates significant difference (****$P < 0.0001$) between the control and 0.3 mg/ml (1°) and 0.1 mg/ml (1°) group.

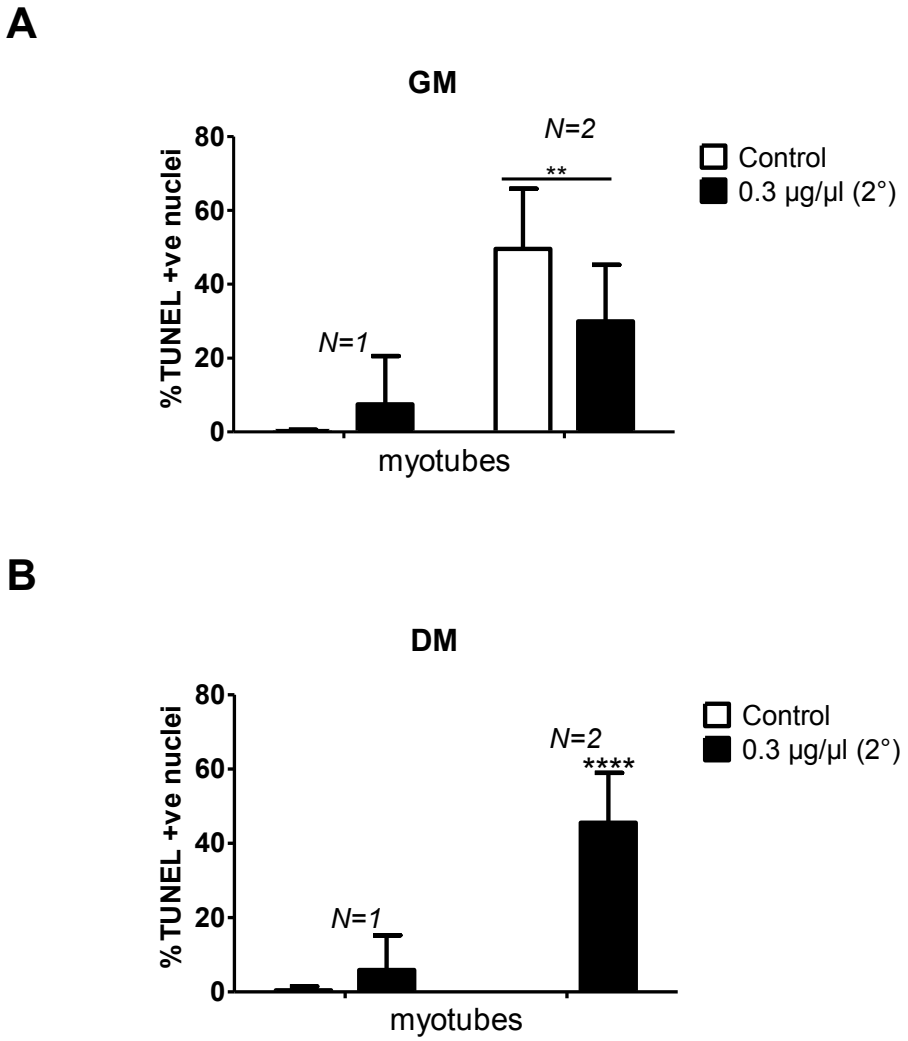


Figure 12. TUNEL assay identifies apoptotic cells in ara-C treated C2C12 differentiated cells. (A) Percent TUNEL +ve nuclei in multinucleated cells not treated (control) or treated with the secondary newt extract at a concentration of 0.3 mg/ml in growth medium (GM). (B) Percent TUNEL positive nuclei in multinucleated cells not treated or treated with 0.3 mg/ml of newt 2º extract in differentiation medium (DM). N=1, and N=2 represent experiments done at different days with different batches of newt extract. Each experiment was performed in triplicate. Asterisk indicates significant difference (**P < 0.01, and ****p< 0.0001) between the control and 0.3 mg/ml (2°).

To further confirm that ara-C treatment can induce apoptosis in myotubes, the assay was repeated in non-ara-C treated C2C12 differentiated myotube cultures. The experiment was divided into two; ara-C treatment and non-ara-C differentiated muscle cultures. The ara-C treated cells were differentiated for 2 days in DM followed by ara-C treatment on day 3 and 4 in GM, and the cells were placed back in DM for 1 day. The non-ara-C treated cultures were differentiated for 2 days in DM, switched to GM for 2 days in the absence of ara-C, and back again in DM on day 5. Both the ara-C and non-ara-C differentiated cultures were treated with the same batch of 1° newt extract for 3 days at 0.3 mg/ml in GM, followed by TUNEL staining. Figure 13A shows a reduction in the number of nuclei found within myotubes in ara-C cultures treated with newt extract. In other words it appears that ara-C treatment in combination with extract treatment is inducing the death of larger myotubes and leaving behind only small myotubes with reduced number of nuclei. This reduction of multinucleated cells was not observed in non-ara-C cultures treated with newt extract (figure 13A). Very few TUNEL positive cells were observed in non-ara-C treated multinucleated cells treated with newt extract (figure 13B). Furthermore, when just control cells were examined in the presence or absence of ara-C, a significant reduction in the number of nuclei within myotubes was observed in the presence of ara-C in comparison to non-ara-C control cultures (figure 14). The ara-C appears to have caused larger myotubes to lift off the plate or die, as 3 days post treatment, very few multinucleated cells were present.

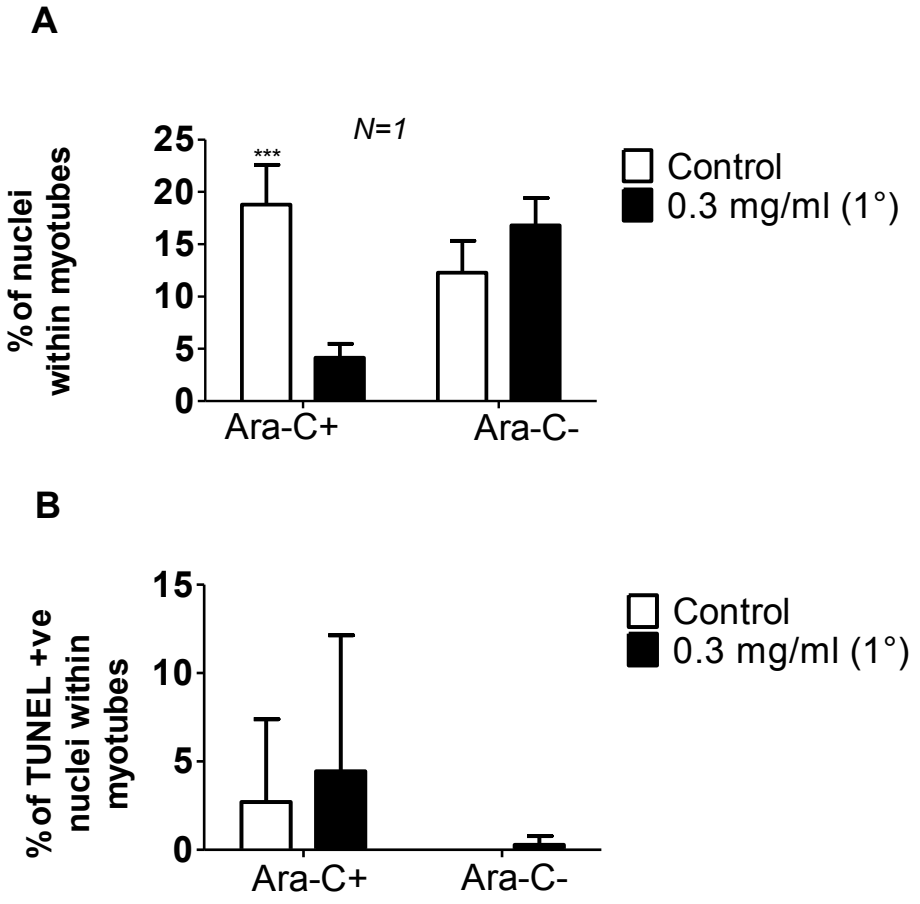


Figure 13. Effect of ara-C treatment on C2C12 differentiated cells. The graph shows (A) the percentage of nuclei within myotubes in controls or cultures treated with 1º newt extract in the presence of (+) or absence (-) of ara-C. (B) Percentage of TUNEL+ve nuclei within control or extract treated myotube cultures with (+) or without (-) ara-C treatment. The experiment was performed in triplicate. Asterisks indicate significant difference (***P < 0.001) between the control and 0.3 mg/ml of 1° extract.

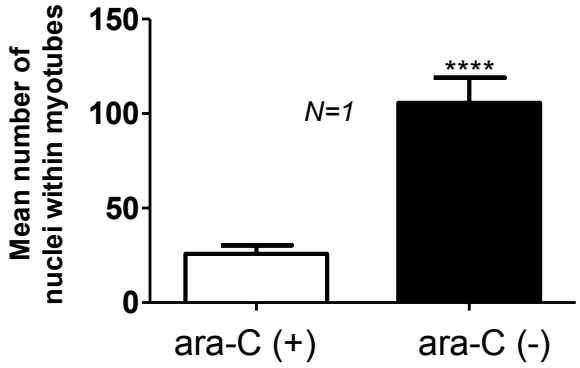


Figure 14. Effect of ara-C treatment on C2C12 differentiated cells. The graph shows ara-C treated (+) and ara-C non treated (-) control cells. Results are presented as mean $\pm$ SD. The experiment was performed in triplicate. Myotubes were treated with ara-C and then allowed to recover for 24 hrs. Three days later, cells were fixed, stained with MHC and the total number of nuclei within myotubes was counted.

3.1. iv. Assessing cell cycle re-entry in filtered myotube cultures

Ara-C treatment to remove contaminating myoblasts in the myotube cultures proved to be problematic, as shown by the increased cell death of myotubes. Therefore, filtration was used to decrease the myoblast contaminants in the differentiated myotube cultures.

Myotubes were separated from mononucleated myoblasts through sieving. Myoblasts were grown to confluency and differentiated for 3 days. On day 4, the differentiated cultures were passed through a 100-μm mesh to remove clumps of cells. The flow-through was passed through a 40 μm filter to allow the contaminating mononucleated cells to pass through. The myotubes retained on the filter were replated into 6-well plates. Following this procedure, a plate can contain about 70% multinucleated cells and myoblasts comprise less than 30% of the population. On day 5 the purified multinucleated cells were treated with the newt extract for 3 days in the presence of BrdU followed by staining for BrdU or MHC. The control cells were either untreated or were treated with heat-inactivated extract. BrdU incorporation was found in the multinucleated cells treated with 2^0 newt extract at 0.3 mg/ml (figure 15A). Five percent of MHC +ve multinucleated myotubes were also positive for BrdU after 72 hours of extract treatment (figure 15B). No cell cycle re-entry was seen in control cultures (figure 15B). Interestingly, when myotubes were not filtered prior to addition of the extract and treated for 48 hrs, 20% of the myotubes re-entered the cell cycle. This may be because filtration removes many of the small myotubes, which are the most likely to respond to the extract. Cell death was less common when ara-C was eliminated, as indicated by TUNEL staining (figure 16) and by comparison with figure 12.

Cell cycle re-entry was also assessed in non-ara-C treated primary myotubes derived from the MCk-cre/lox-Rosa-YFP mice. Cell cultures that were differentiated for 3 days were

filtered as described previously, to remove myoblasts. On day 5 the cells were treated with newt extract for 3 days in the presence of BrdU followed by staining for BrdU or MHC. The control cells were not treated with newt extract. The newt extract did not induce cell cycle re-entry in primary myotubes (figure 17A) treated with 2^0 newt extract at 0.3 mg/ml. DNA synthesis was mainly found in mononucleated cells. A significant decrease in multinucleated cells and increase in myocytes (1 or 2 nuclei positive for MHC) was observed in cultures treated with newt extract in comparison to the control (figure 17B). The decrease in multinucleated cells and the increase in myocytes might indirectly indicate that the newt extract causes fragmentation of multinucleated cells into myocytes.

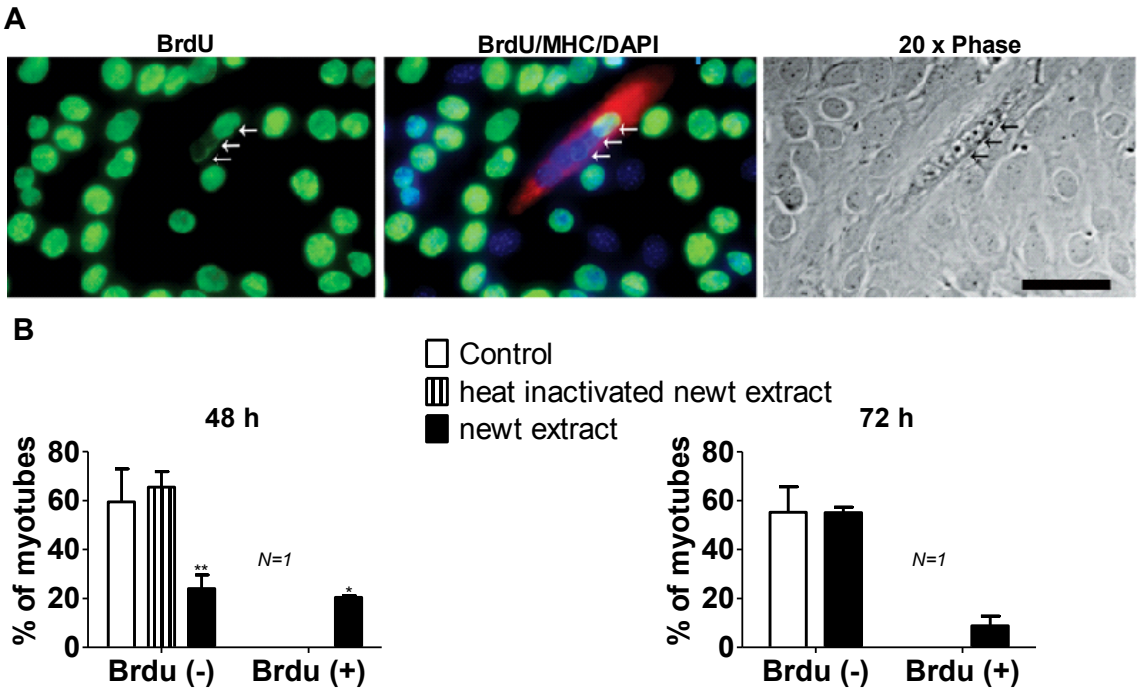


Figure 15. Cell cycle re-entry in filtered C2C12 myotubes treated with newt extract. **(A)** C2C12 myotubes treated with newt extract for 3 days and then stained for MHC (red), DAPI (blue), and BrdU (green) indicating cell cycle re-entry. **(B)** Cell counts show percentage of MHC-positive cells in myotube cultures that have incorporated BrdU in their nuclei in growth medium with 2º newt extract at a concentration of 0.3 mg/ml after 48 h (left panel) or 72 (right panel) of treatment with newt extract . Controls were cultures in growth medium and not treated with newt extract. Heat inactivated newt extract represent cultures in growth medium with newt extract inactivated by heat at 95° C for 5 min. Each experiment was performed in triplicate. Asterisks indicate significant differences (**$p < 0.01$ and *$p<0.05$) between the controls and the extract treated. Scale bar = 50 μm

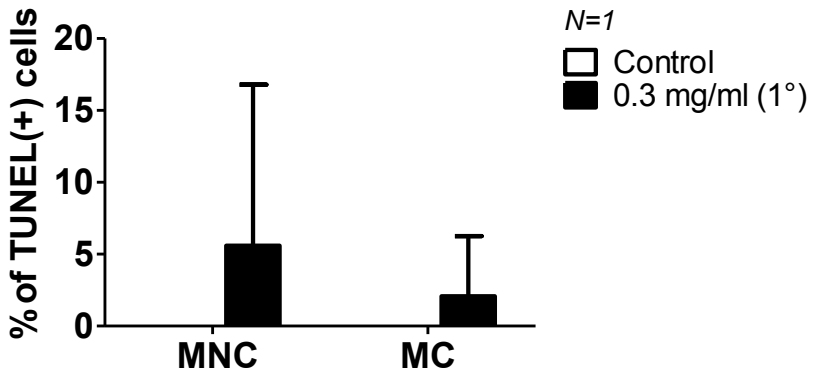


Figure 16. Effect on filtered C2C12 differentiated cells 48 hours after newt extract treatment. The graph shows percentage TUNEL positive multinucleated cells (MHC) and mononucleated cells (MC) that were not treated (control) or treated with newt extract at a concentration of 0.3 mg/ml (1°). The experiment was performed in triplicate.

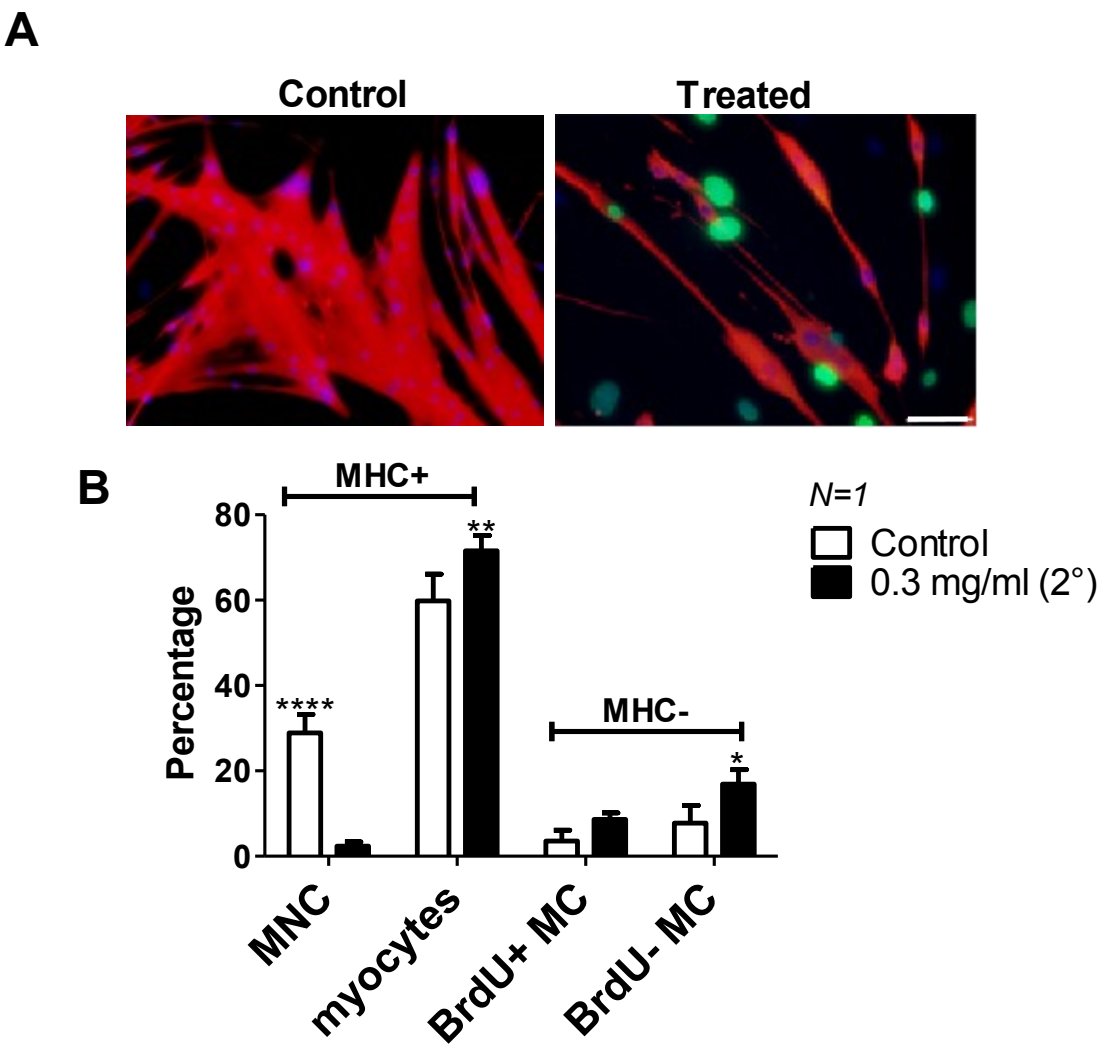
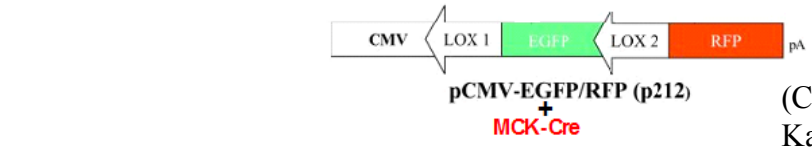


Figure 17. Primary myotubes from MCk-cre/lox-Rosa-YFP mice treated with the 2^0 newt extract. **(A)** Immunostaining shows control (not treated) and treated multinucleated cells with 0.3 mg/ml (2°) stained for BrdU (green), DAPI (blue) which stains the nuclei, and MHC (red). **(B)** Graph shows percentage of myosin heavy chain (MHC+) positive multinucleated (MNC) and myocytes and MHC- mononucleated cells (MC). Asterisks indicate significant difference (****$P < 0.0001$, **$P < 0.01$ and *$p < 0.05$) between the control and 0.3 mg/ml (2°) group. The experiment was performed in triplicate. Scale bar = 100 μm.

3.1.v. Assessing cell cycle re-entry in labelled differentiated muscle cultures

The previous experiments have shown cell-cycle re-entry in C2C12 multinucleated myotubes treated with newt extract. In order to eliminate the possible explanation that BrdU incorporating myoblasts are fusing with existing myotubes to give these results, we used a 2-plasmid labeling system to identify differentiated cells. Labeling myotubes allows tracking of differentiation and the tracked cells can then be followed through dedifferentiation. To label cells, proliferating C2C12 myoblasts were co-transfected with a ratio of 1:1 (5 μg) of the plasmids pCMV-EGFP/RFP, and MCK-Cre (Bruning et al., 1998; Kaczmarczyk and Green, 2001). The first plasmid encodes a loxP-flanked green fluorescent protein (GFP) gene driven the by cytomegalovirus (CMV) promoter, and a red fluorescent protein (RFP, figure 18). With this system, myoblasts expressed GFP. During differentiation, MCK-Cre was activated leading to excision of the loxP flanked GFP gene, resulting in expression of RFP in myocytes or multinucleated myotubes (figure 18 and 19). The RFP expressing cells were then treated with 0.3 mg/ml (1°) of newt extract in DM, and BrdU was added to the cells on day 3. On day 4, the cells were fixed and stained for BrdU. Figure 20, shows cell cycle re-entry in RFP expressing myocytes following treatment with newt extract. Images of RFP cells were taken each day with a Nikon inverted microscope.

Although this particular system seems ideal to track differentiated cells *in vitro,* expression from the plasmids was not robust. After transfecting the cells with both plasmids, the expression of GFP was detected in almost 40 % of the cells within 24 h. However, over time the expression of GFP diminished. By the time RFP was detected, there are fewer cells in culture expressing the fluorescence tags. For this reason, an alternative method was devised, which is discussed in the next section.



(Construct modified from Kaczmarczyk and Green, 2001)

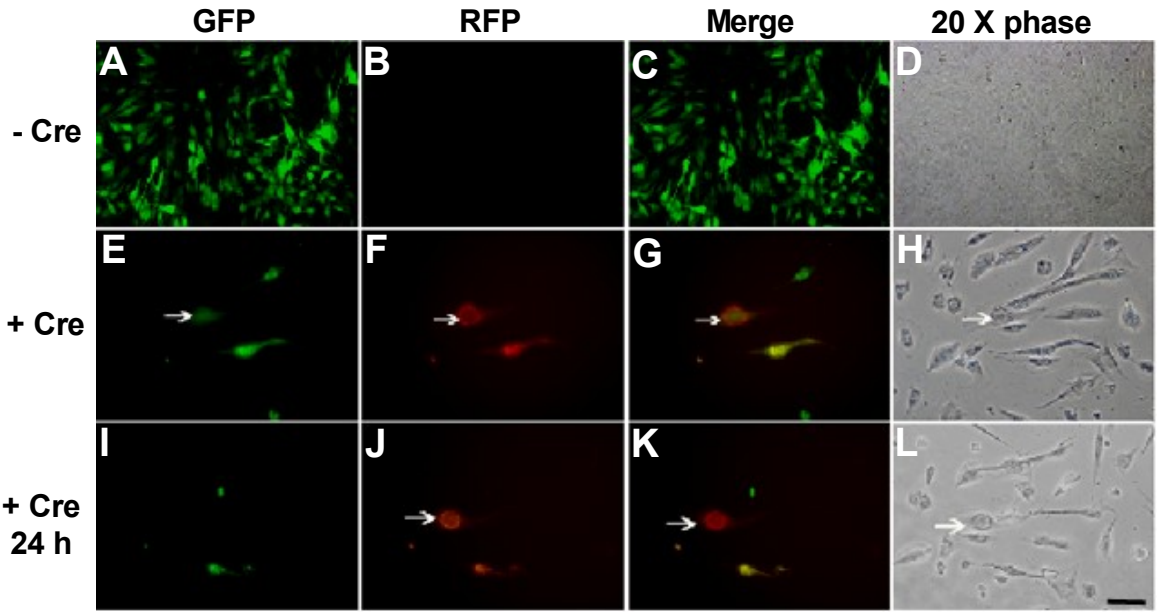


Figure 18. RFP expression in C2C12 differentiated muscle cells. C2C12 myoblasts transfected only with the pCMV-GFP/RFP plasmid express GFP (green) (A-D). In the presence of muscle creatine kinase (MCK)-Cre, GFP expressing cells switched to red fluorescence protein (RFP) expression (red) (E-H). The arrow points to a cell making a switch from GFP to RFP. After 24 h, the GFP expression could not be detected in a RFP expressing cell (I-L). Scale bar = 100 μm.

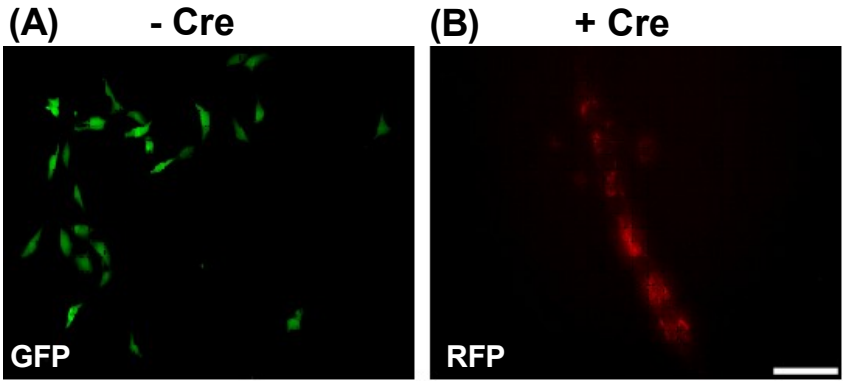


Figure 19. RFP expression in C2C12 differentiated myotubes. C2C12 myoblasts transfected with the pCMV-EGFP/RFP plasmid, and the MCK-Cre plasmid**. (A)** Myoblasts without cre express GFP, and in the presence of Cre recombinase (B) the expression of RFP becomes detected in multinucleated myotubes or myocytes. Scale bar = 100 µm.

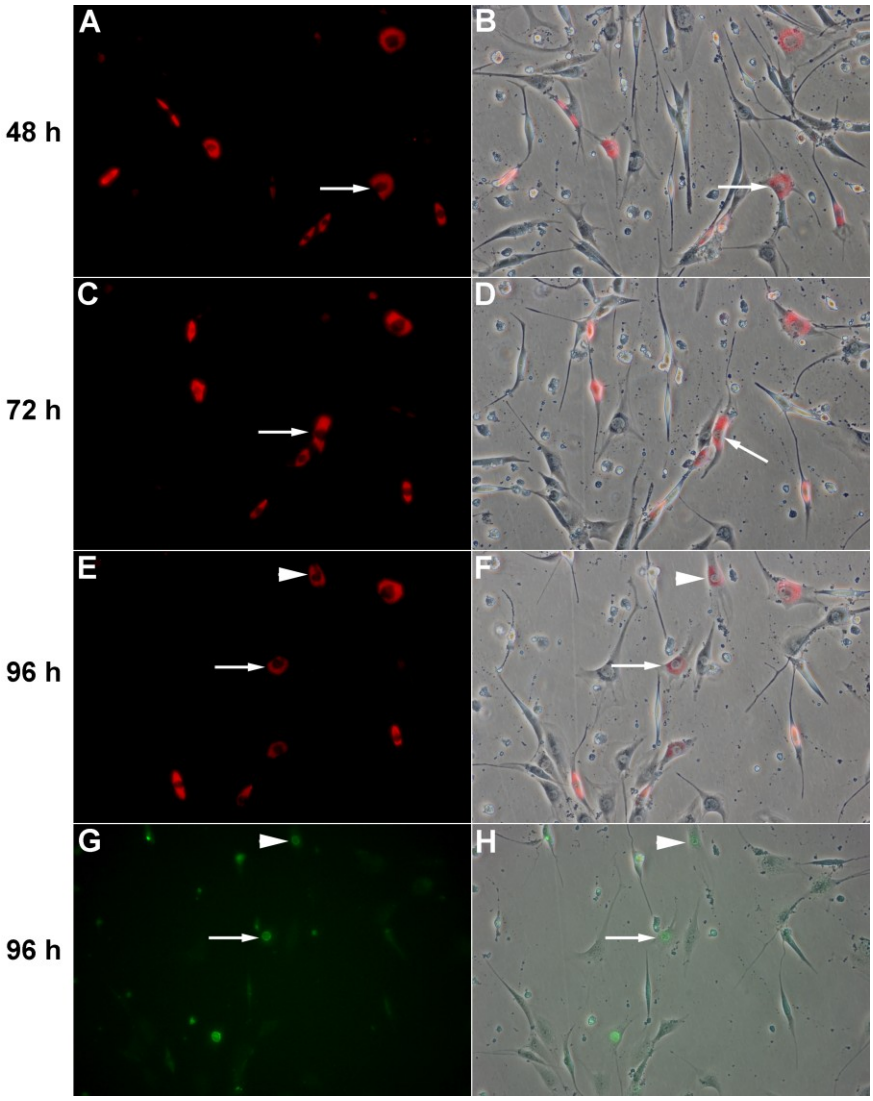


Figure 20. Cell cycle re-entry in RFP expressing C2C12 differentiated myotubes following treatment with newt extract. C2C12 myoblasts were transfected with both pCMV-EGFP/RFP plasmid, and the MCK-Cre plasmid in GM**.** Cells were grown to confluency and differentiated. When RFP was detected, newt extract at a concentration of 0.3 mg/ml (1°) was added to the cells in DM. BrdU was added to cultures that were treated with newt extract after 72 h. After 96 h, the cells were fixed and stained with BrdU (green). Images of RFP cells were taken at 48 h, 72 h and 96 h with a Nikon inverted microscope. The white arrow is pointing to the same RFP +ve cell at 48 h, 72 h (time when BrdU was added to culture), and 96 h. The differentiated cultures were fixed and stained for BrdU (green) after 96 h in culture in the presence of newt extract. The arrowhead points to another RFP +ve cell which is also positive for BrdU. Scale bar = 100 μm.

3.1.vi. Assessing cell cycle re-entry in microinjected C2C12 myotubes

Microinjection of multinucleated cells guarantees that only differentiated muscle cells are targeted. This ensures that GFP labelled cells positive for a proliferating marker were a product of differentiated myotubes. In preparation for microinjection, day 3 differentiated C2C12 or primary myotube cultures were passed through 100 and 40 μm sieves as described in the Materials and Methods section. The resulting C2C12 myotubes were plated on non-coated 35 mm plates and the primary cells were plated on 35 mm collagen coated plates in 2% HS. Multinucleated cells (of greater than 3 myonuclei) were microinjected with both newt extract and the GFP plasmid (figure 21). The control cells were microinjected with the GFP plasmid and Dulbecco's Modified Eagle Medium (DMEM). After microinjection, the cells were transferred to growth medium containing 10% fetal bovine serum (FBS) for 3 or 4 days. This was followed by staining the cells with Ki-67 or BrdU (a proliferation marker) and GFP or MHC (a differentiation marker).

C2C12 multinucleated myotubes that were injected with both the crude newt protein and GFP re-entered the cell cycle (figure 22A). The first time that this was done (N=1 in figure 22B), only 1.8% of the nuclei in MHC labeled multinucleated cells exhibited cell cycle re-entry as shown by BrdU staining (figure 22B) in comparison to myotubes injected with DMEM/GFP. This was repeated (N=2 in Figure 22B), and only 0.6% of the nuclei in MHC labeled myotube cells showed cell cycle re-entry. However, when the crude extract was fractionated, and C2C12 multinucleated myotubes were injected with the newt protein fraction greater than 30 kDa and GFP, a much greater cell cycle re-entry response was seen in comparison to myotubes injected with the crude extract. Approximately 45% of the nuclei in GFP labeled multinucleated cells exhibited cell cycle re-entry as shown by Ki-67 staining

(figure 23). In addition, mononucleated cells which were GFP and Ki-67 positive were only found in cultures injected with the greater than 30 kDa protein fraction and GFP (figure 23F-J). Cell cycle re-entry was not observed in multinucleated cells injected with DMEM and GFP (figure 23).

It is possible that the cell cycle re-entry effect seen on myotubes with the regenerating extract had nothing to do with the regeneration process, per se, but was related to the fact that the extract was derived from proliferating cells. We therefore tested an extract isolated from a different source of proliferating cells. Mouse embryonic fibroblast (MEF) cells, which are used as feeder cells to support stem cell growth (Garfield, 2010) were grown to 90 % confluency, and an extract was prepared, as described in section 2.3. Because the extract protein fraction of greater than 30 kDa was more effective in inducing cell cycle re-entry on myotubes, we therefore decided to test the same protein fraction of MEF cells on myotubes. Myotubes were microinjected with both MEF extract and the GFP plasmid. After microinjection, the cells were transferred to growth medium containing 10% fetal bovine serum (FBS) for 4 days. This was followed by staining the cells with Ki-67 and GFP. C2C12 myotubes that were injected with both the MEF protein fraction greater than 30 kDa and GFP did not undergo cell cycle re-entry (figure 24). This indicates that the regenerating extract contains factors which induce cell cycle re-entry in myotubes.

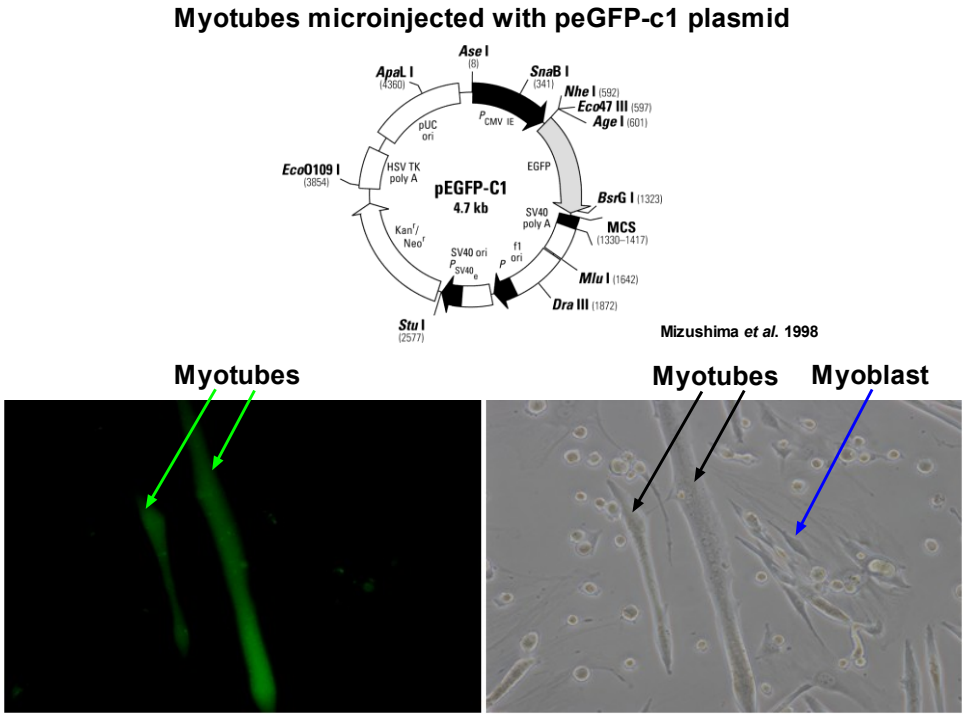


Figure 21. GFP expression in myotubes following injection with GFP plasmid. Live images of myotubes injected with GFP (green) that were captured by Nikon microscopy.

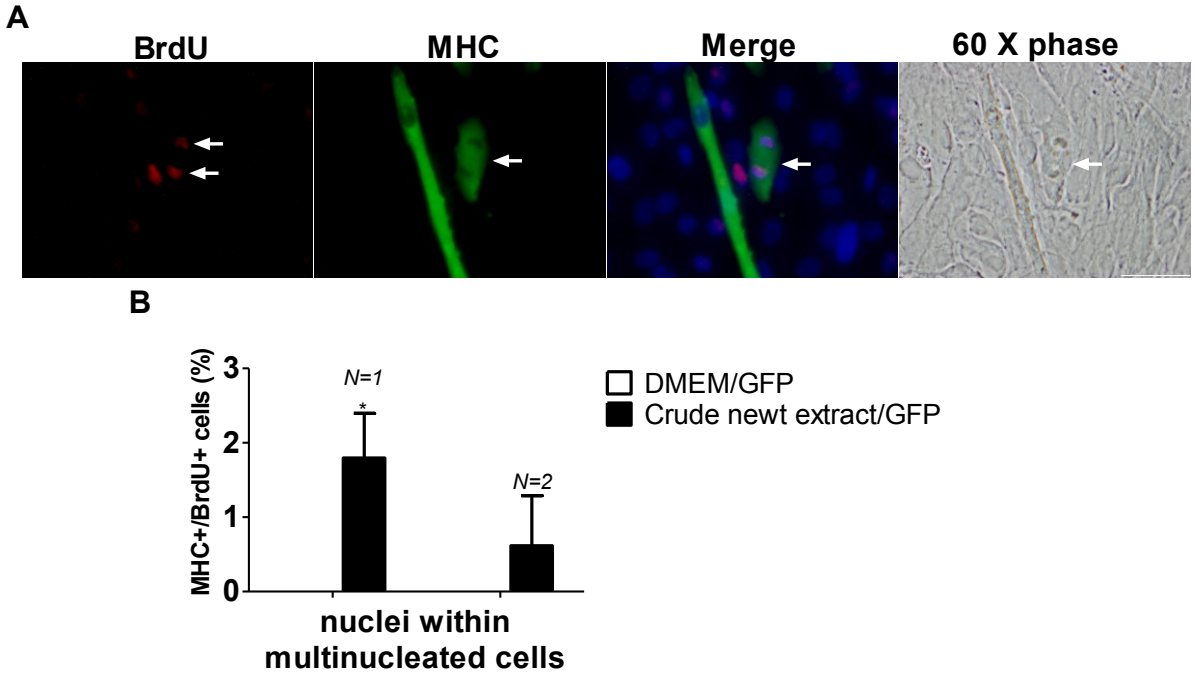


Figure 22. Cell cycle re-entry in C2C12 cells plated on non-coated dishes that were microinjected with both 2^0 newt extract (crude extract) and GFP. (A) C2C12 cells were stained with BrdU (red), DAPI (blue), and MHC (green). Arrows identify MHC positive myotubes that were injected with the newt extract undergoing cell cycle re-entry. (B) Cell counts show the percentage of nuclei within multinucleated myotubes positive for both MHC and BrdU in cultures that were injected with newt extract. Each experiment was performed in triplicate. Asterisks indicate significant difference (*$p < 0.5$) between the cells injected with DMEM/GFP and newt extract/GFP. Scale bar = 200 μm.

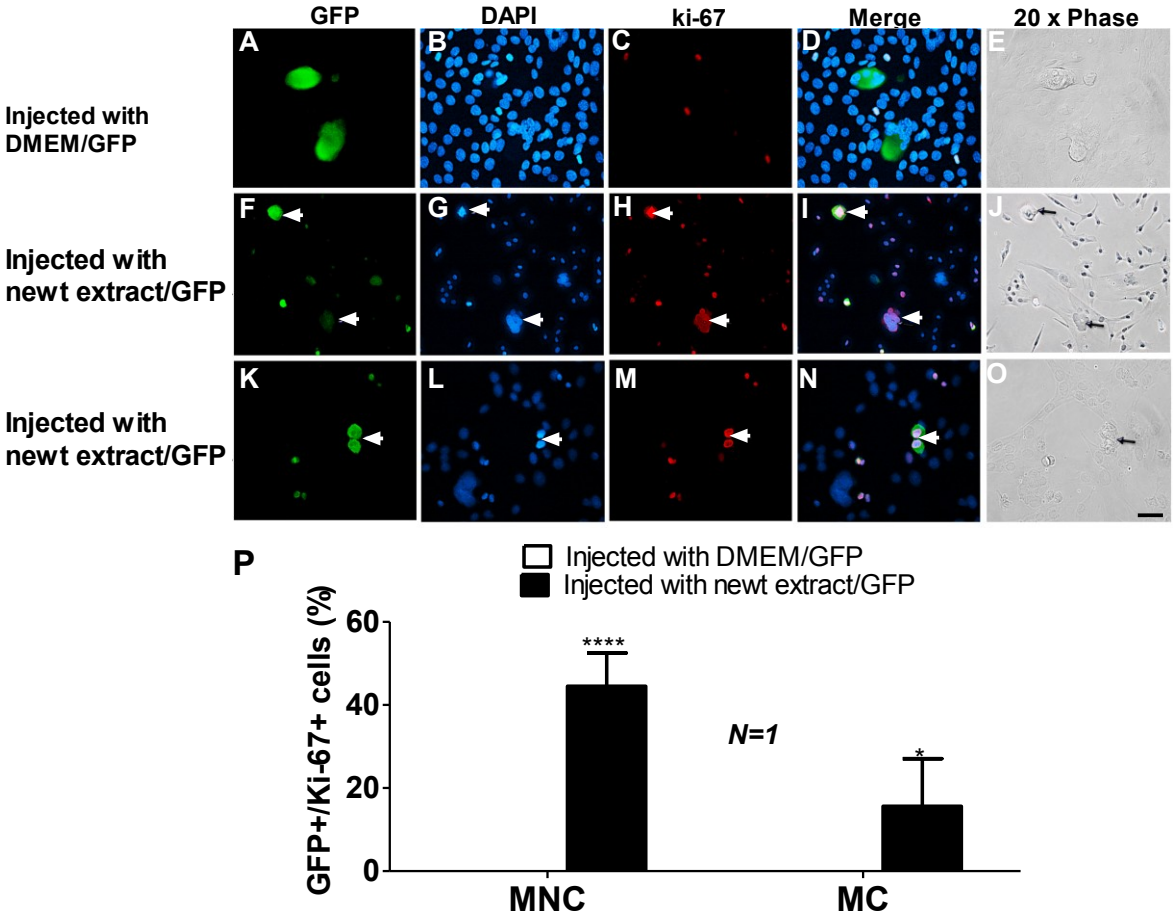


Figure 23. Cell cycle re-entry in C2C12 cells plated on non-coated dishes that were microinjected with both 2^0 newt extract (protein fraction of > 30 kDa) and GFP. C2C12 cells were stained with Ki-67 (red), DAPI (blue), and GFP (green). (A-E) Control myotubes injected with DMEM/GFP. There is no Ki-67 staining of injected myotubes. (F-J) Arrows identify GFP positive myotubes that were injected with the newt extract/ GFP undergoing cell cycle re-entry. (K-O) Arrows identify for GFP mononucleated cells that were injected with the newt extract/ GFP and are positive for Ki-67. (P) Cell counts show the percentage of multinucleated and mononucleated cells positive for both GFP and Ki-67 in cultures that were injected with newt extract/GFP. Each experiment was performed in triplicate. Asterisks indicate significant difference (*$p < 0.5$, ****$p < 0.0001$) between the cells injected with DMEM/GFP and newt extract/GFP. Scale bar = 100 μm.

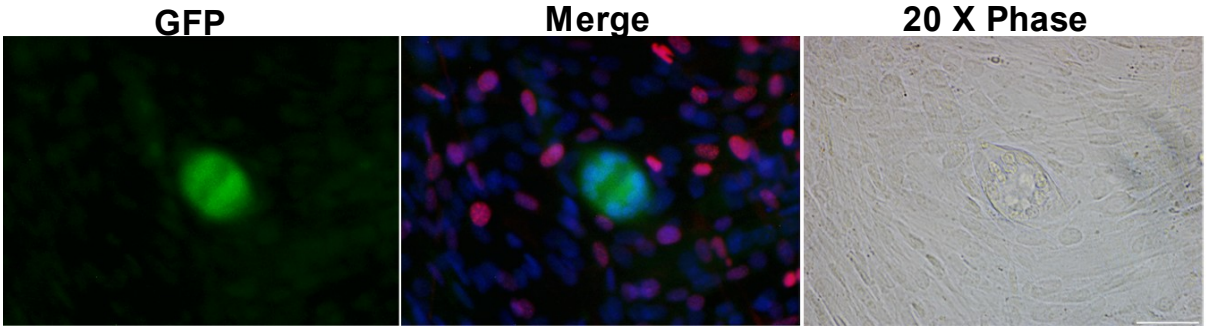


Figure 24. Immunostaining of myotubes injected with MEF extract/GFP. No cell cycle re-entry was seen in C2C12 myotubes plated on non-coated dishes that were microinjected with both MEF extract (protein fraction of > 30 kDa) and GFP. C2C12 cells were stained with Ki-67 (red), DAPI (blue), and GFP (green). There is no Ki-67 staining of injected myotubes. The experiment was performed in triplicate and repeated twice. Scale bar = 200 μm.

3.1.vii. Effect of the extracellular matrix on cell cycle re-entry and myotube fragmentation

The extracellular matrix has been implicated in influencing the behaviour of cells undergoing dedifferentiation. For example, it has been shown that some of the components of the extracellular matrix such as hyaluronic acid, tenascin-c and fibronectin can increase DNA synthesis, migration, and fusion in newt and mammalian myoblasts (Calve et al., 2010). This group also discovered that hyaluronic acid and tenascin-c are required for newt myotube fragmentation. Type I collagen was found to have no effect on myotube fragmentation.

Sodium hyaluronate coated plates were used to determine whether they can influence cell cycle re-entry, survival, or fragmentation of C2C12 multinucleated cells injected with the greater than 30 kDa protein fraction. Day 3 C2C12 myotubes were filtered through the 100 and 40 μm sieves and seeded on plates coated with 0.4% sodium hyaluronate. The following day, multinucleated cells in DM were microinjected with both the newt extract and the GFP plasmid in DM. After injection, the medium was switched to GM for 3 days followed by immunostaining with GFP and Ki-67. The control C2C12 myotubes were injected with DMEM/GFP. Figure 25 indicates that multinucleated cells cultured on sodium hyaluronate that were injected with newt extract can undergo cell cycle re-entry as indicated by Ki-67 staining of their nuclei. Approximately 23% of the nuclei in GFP +ve multinucleated cells displayed cell cycle re-entry, and this was significant in comparison to control multinucleated cells injected with DMEM/GFP. With this particular experiment, fewer cells re-entered the cell cycle in comparison to cells that were cultured on non-coated plates. Cell cycle re-entry was examined after 3 days (as opposed to 4 days in the previous experiment) and this could be the reason why fewer Ki-67+ve cells were detected. The only

difference that was observed between cells cultured on sodium hyaluronate and cells cultured on non-coated dishes was that cells plated on sodium hyaluronate looked healthier than cells on non-coated plates. It is possible that extracellular matrix components such as sodium hyaluronate influence cell survival. Cells injected with the greater than 30 kDa protein fraction that were plated on collagen were observed to undergo fragmentation into mononucleated cells and underwent cell cycle re-entry. This will be further discussed in section *3.2.ii*.

3.1.viii. Assessing cell cycle re-entry in microinjected primary myotubes

Primary myotubes of 3 nuclei or more were microinjected with 2° newt extract > 30 kDa protein fraction and GFP. The primary myoblasts were isolated from the transgenic mice (MCK-Cre/lox-Rosa-YFP). As mentioned above, upon differentiation, the primary myotubes derived from MCK-Cre/lox-Rosa-YFP mice express YFP. However, over time the expression of YFP could not be detected in these myotubes. Therefore, the myotubes were also injected with GFP and the newt extract that induced cell cycle re-entry in C2C12 myotubes as described above. The control myotubes were injected with DMEM and GFP. After injection, the cells were placed in GM for 4 days.

Newt extract did not induce cell cycle re-entry in primary myotubes plated on collagen dishes (figure 26A). Cell cycle re-entry was also not observed in the control cells which were injected with DMEM and GFP. However, we observed that cells injected with newt extract and GFP had an increase in the number of GFP positive cells in comparison to the control cells that were injected with DMEM and GFP (figure 26B). This would suggest that newt extract, although not able to induce cell cycle re-entry, might influence fragmentation of primary multinucleated cells into mononucleated cell

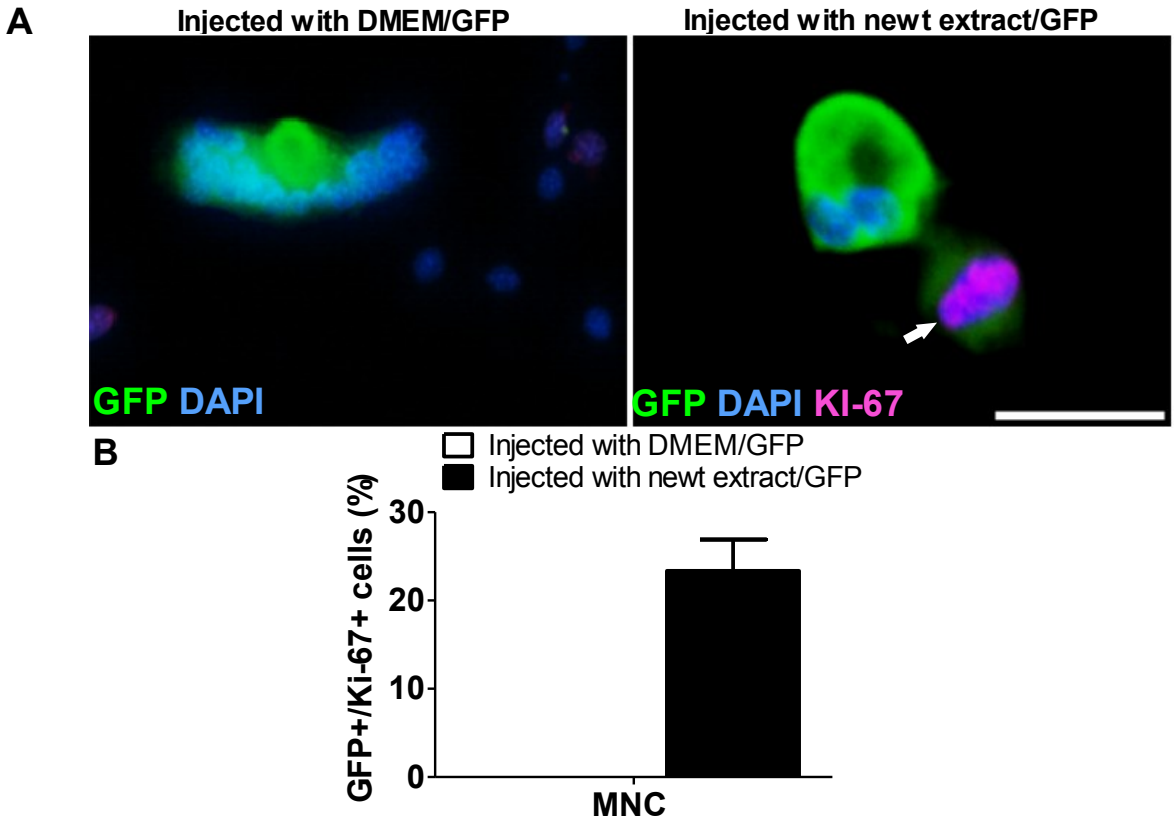


Figure 25. Cell cycle re-entry in C2C12 multinucleated cells plated on sodium hyaluronate that were microinjected with both 2^0 newt extract (protein fraction of > 30 kDa) and GFP. (A) C2C12 cells stained with Ki-67 (red), DAPI (blue), and GFP (green) that were injected with newt extract/GFP or DMEM/GFP and grown in GM for 3 days. Note the Ki-67 +ve myotubes in the extract treated cultures. (B) Cell counts show the percentage of multinucleated cells (MNC) positive for both GFP and Ki-67 in cultures that were injected with newt extract/GFP. Each experiment was performed in triplicate. Scale bar = 100 µm.

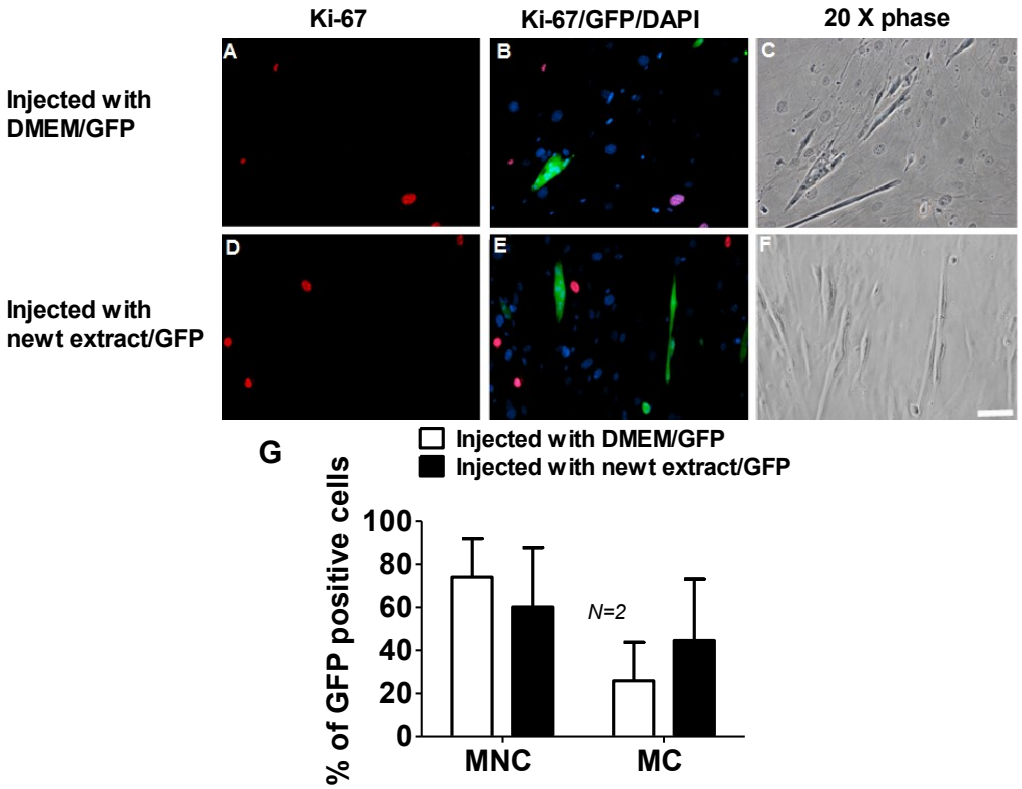


Figure 26. Primary myotubes from Mck-cre/lox-Rosa-YFP mice do not undergo cell cycle re-entry following injection with 2° newt extract. (A-C) show control myotubes that were injected with DMEM/GFP. (E-G) show myotubes that were injected with newt extract/GFP. (G) Percentage of GFP positive multinucleated (MNC) and mononucleated (MC) cells. Each experiment was performed in triplicate. Ki-67 (red), DAPI (blue), GFP (green). Scale bar = 100 μm. *N=2* represents two biological samples with three replicates in each sample.

3.2. Fragmentation of multinucleated myotubes into mononucleated cells

3.2.i. Fragmentation of ara-C treated myotubes following treatment with newt extract

In order to assess whether newt extract can induce mammalian myotubes to undergo fragmentation, 5 day old C2C12 differentiated myotubes that were treated with ara-C (as described in the Materials and Methods section) were treated with 1° newt extract at 0.3 mg/ml. The C2C12 treated cells were followed with Zeiss live imaging microscopy. As shown in figure 27, the initial sign of C2C12 myotube fragmentation into two small myotubes was observed after 63 h. After a further 9 hrs in culture, the fragmented cells were still present in the culture, though they now resembled a "myosac" phenotype (eg round and disorted) instead of the original elongated form. Furthermore, out of the 21 myotubes that were followed, only 4 were seen to undergo fragmentation into small myotubes.

Primary cells were also treated with ara-C and newt extract and followed with light microscopy. The first sign of primary myotube cleavage into mononucleated cells was observed at 28 h as shown in figure 28. By day 2 and 3 the number of mononucleated cells increased. This may indicate that newt extract induces fragmentation in multinucleated cells.

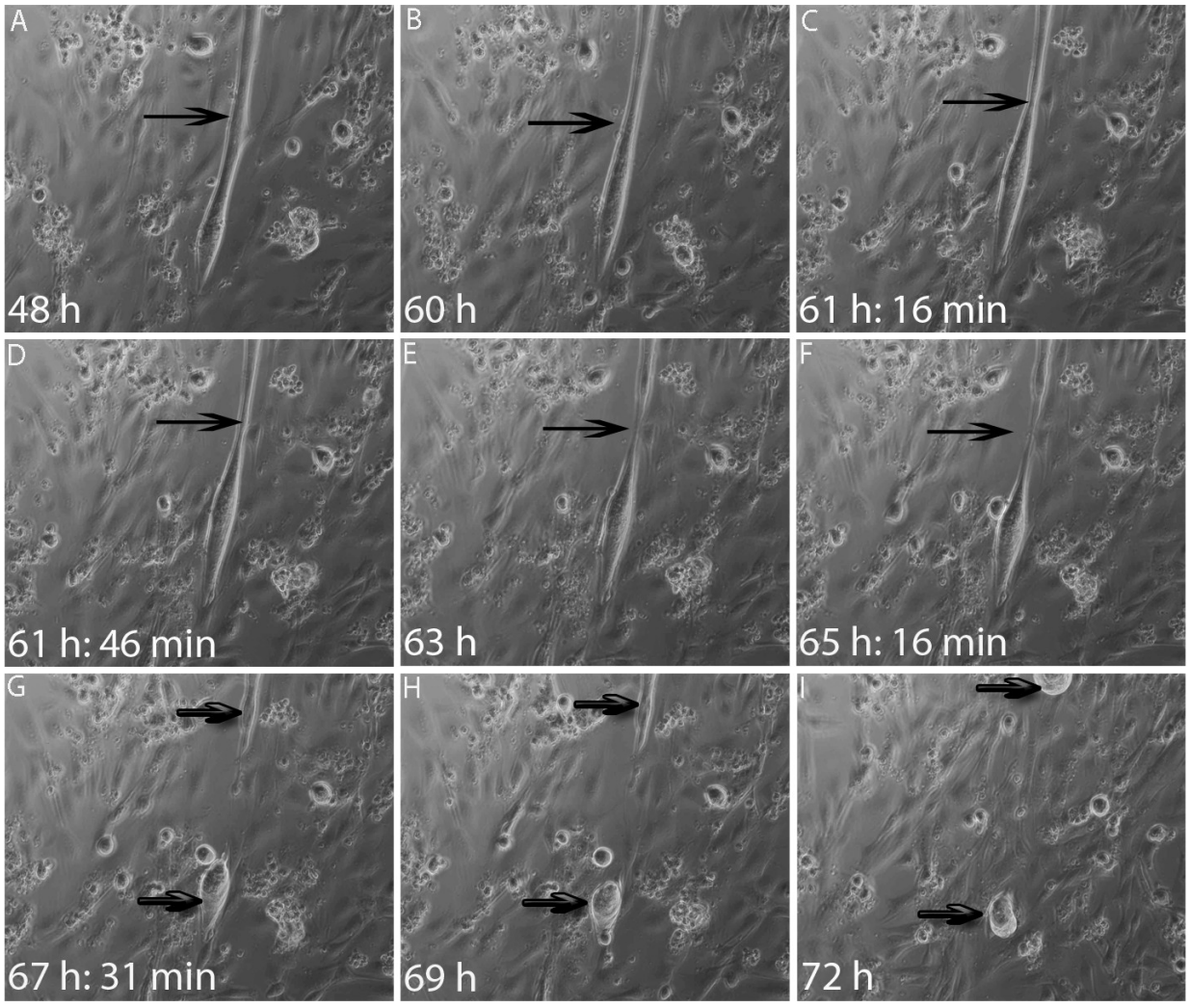


Figure 27. Ara-C-treated C2C12 myotube treated with newt extract at a concentration of 0.3mg/ml (1°) in GM was photographed using Zeiss live imaging microscopy. Images were taken even 15 mins, but only representative images are shown at the times indicated. The arrows identify a myotube as it is fragmenting.

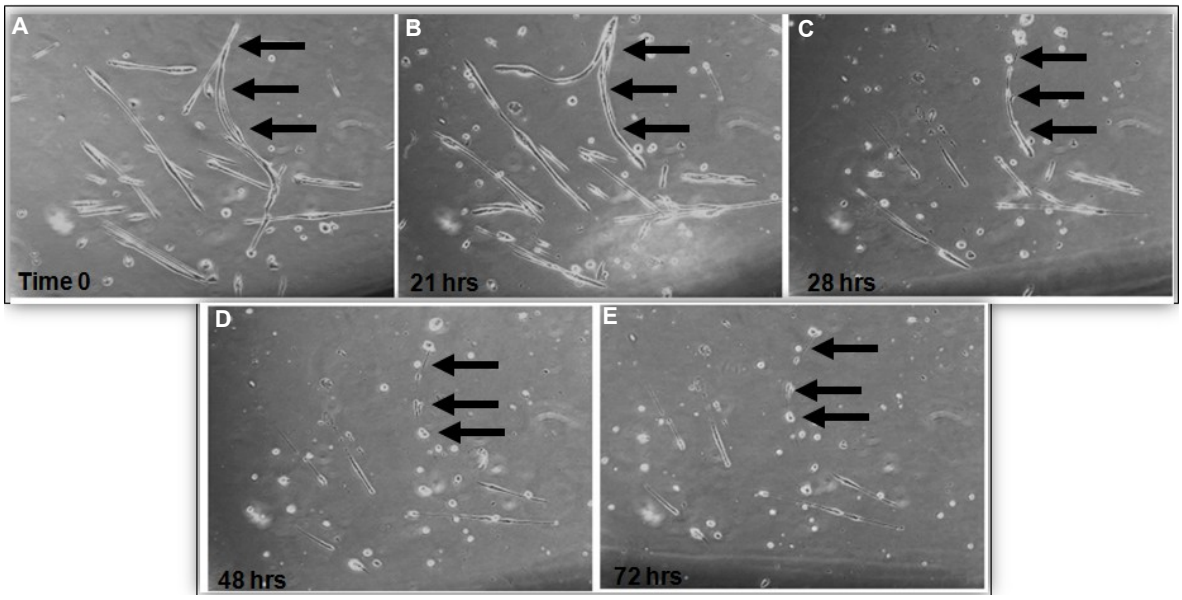


Figure 28. Ara-C treated primary myotubes treated with newt extract at a concentration of 0.3 mg/ml (1°) in DM were photographed daily using an Axiovert microscope. Arrows are pointing to the same myotube as it is breaking down into mononucleated cells.

3.2.ii. Fragmentation of newt extract-microinjected myotubes

To assess whether myotubes microinjected with newt extract can undergo fragmentation and proliferation, C2C12 and primary myoblasts were differentiated for 3 days followed by sieving the cells through a 100 μm and 40 μm filter. The filtered cells that were retained on the filter were plated back on 35 mm plates coated with 0.4% collagen. The following day the cells were injected with the >30 kDa newt extract fraction and the GFP plasmid. The control cells were injected with DMEM/GFP. The expression of GFP in myotubes was not detected until at least 24 hrs after the injection. For that reason, the cells were followed by Zeiss live imaging microscopy starting at 48 h, and images were taken every 15 mins.

As shown in figure 29, the initial sign of C2C12 myotube (with four nuclei) fragmentation was observed after 81 h. The fragmented cells are noticeable as two separate cells after 82 h: 50 min (figure 29). The cells continued to grow in culture and after 144 h : 16 min (figure 30I); one of the fragmented cells was found to fragment again into 2 cells. Out of 6 myotubes that were GFP positive, only 1 was found to breakdown into mononucleated cells. It is unclear whether this was further fragmentation or cell division. This demonstrated that the fragmented myotubes following injection with newt extract do survive and are able to divide again.

In primary cells, the initial sign of fragmentation was seen after 112 h: 35 min (figure 31). By 121 h: 26 min, the cells were fully separated from one another. The cells continued to survive in culture until the experiment was stopped at 263 h: 26 min. Primary myotubes were also examined for their ability to undergo fragmentation into mononucleated cells

following injection with DMEM/GFP. As shown in figure 32, myotubes injected with DMEM/GFP, which were followed with Nikon microscopy, do not undergo fragmentation.

3.2.iii. Fragmentation of primary myotubes using myoseverin

Myoseverin has been shown to induce fragmentation of skeletal muscle fibres into mononucleated cells (Kim et al., 2012). Further treatment of the fragmented myofibres with glycogen synthase-3 kinase inhibitor induced proliferation. As a positive control for the fragmentation process, we assessed fragmentation of primary myotubes into small fragments or mononucleate cells using myoseverin.

Primary myoblasts were placed in DM for 3 days leading to formation of multinucleated myotubes. Subsequently, the myotube cultures were treated with myoseverin at various concentrations. A 10 μM concentration of myoseverin was found to be less toxic to the cells so this was the concentration used for subsequent experiments. A 24 h treatment of myotube cultures with myoseverin resulted in fragmentation into smaller multinucleated cells or into mononucleated cells (figure 33). Additionally, the cells treated with myoseverin for 24 h, when put back in DM for 48 h, were able to re-fuse into multinucleated cells (figure 33). This had never been previously shown in primary myotubes.

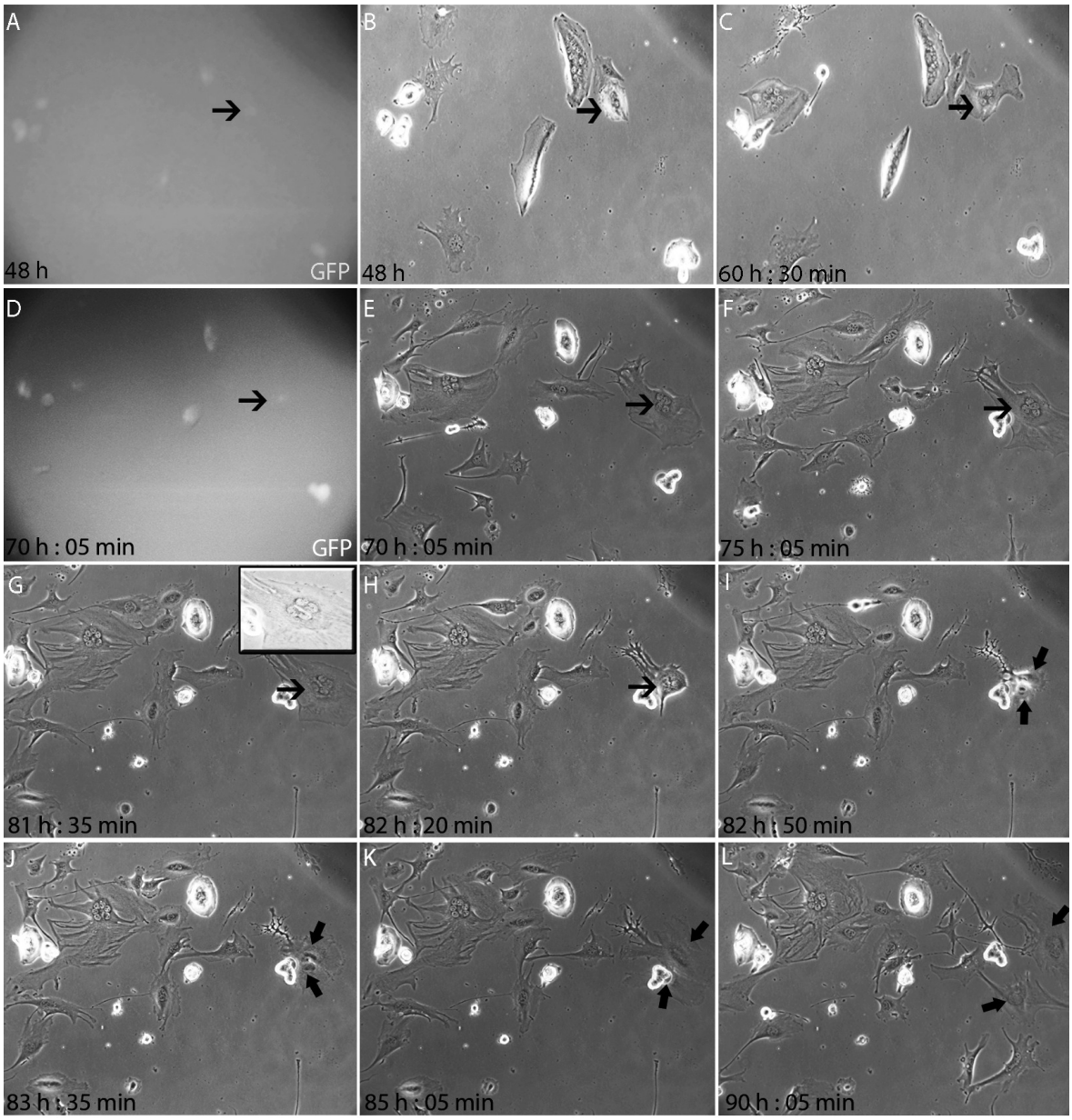


Figure 29. C2C12 myotube (with 4 nuclei) injected with both newt extract and the GFP plasmid can undergo fragmentation. (A and D) shows GFP (green) positive cells in the 10 X phase (grey) images, arrows are pointing to the same myotube as it is breaking down into two cells. The thick arrows point to two cells that have resulted from the fragmention. The images were taken even 20 mins using a Zeiss live imaging microscope. The time shown is when the image was taken. (G, inset) An enhanced image showing a cell with 4 nuclei starting to separate.

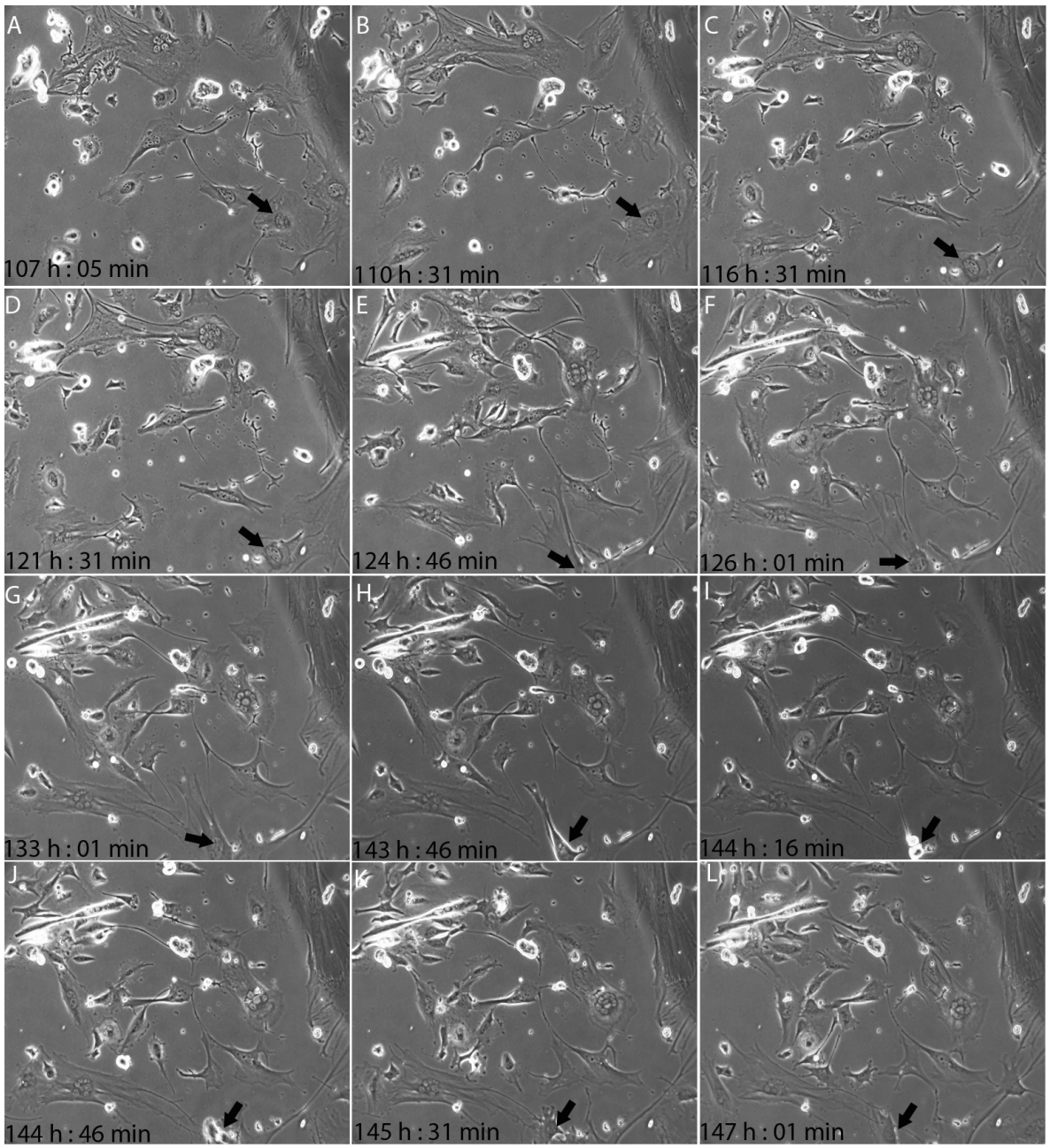


Figure 30. The C2C12 myotube that underwent fragmentation in figure 26, is followed with Zeiss live imaging microscopy as it undergoes further fragmentation or cell cycle re-entry and division into 2 cells. The time indicates when the image was captured.

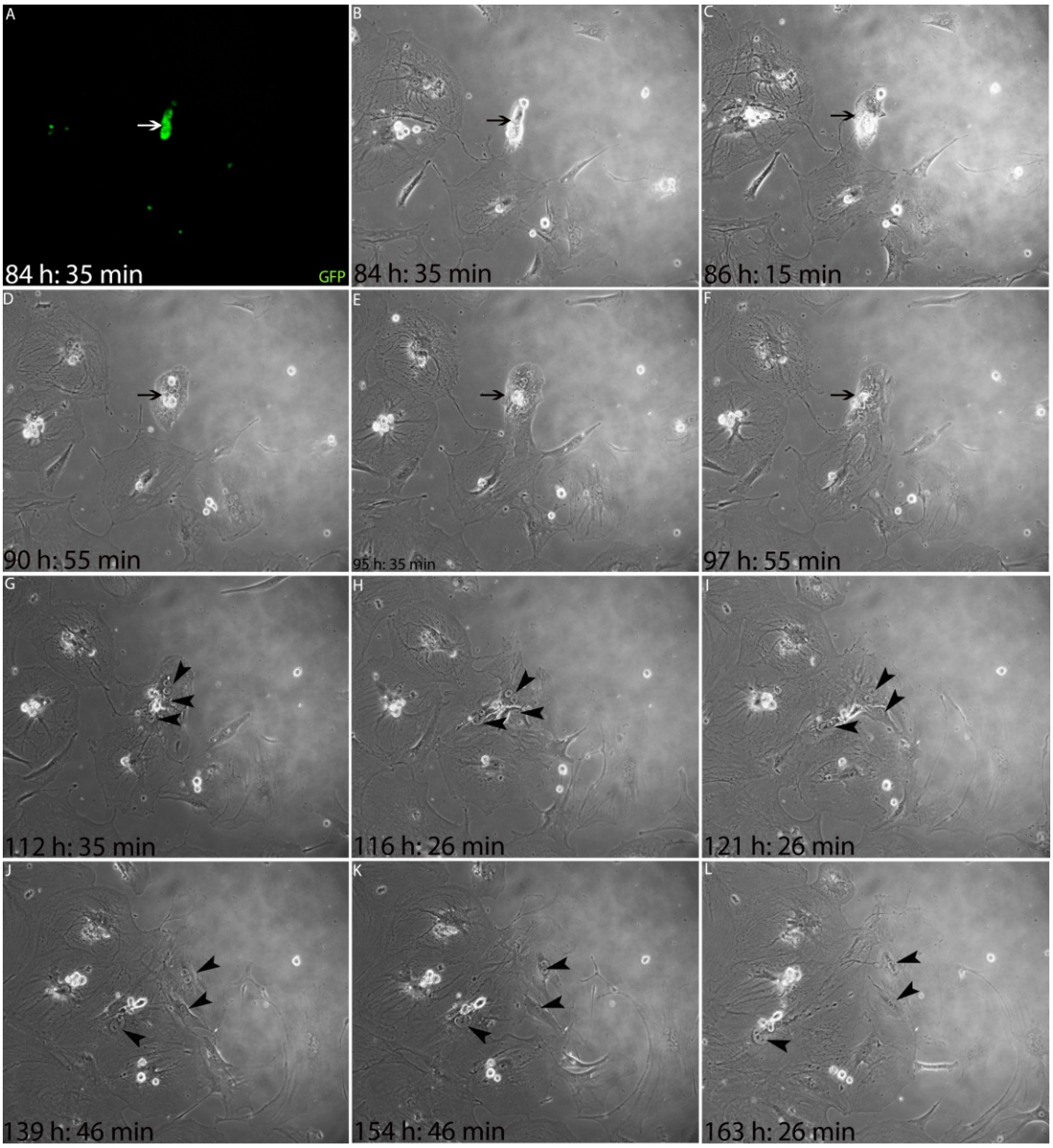


Figure 31. Primary myotube injected with newt extract can undergo fragmentation. Primary myotube injected with both newt extract protein fraction and the GFP plasmid. (A) Shows a GFP (green) positive myotube. (B-L) in the 10 X phase (grey) images, arrows are pointing to the same myotube as it is breaking down into three cells. The arrow heads point to the cells resulting from fragmentation. The images were taken even 20 mins using a Zeiss live imaging microscope, but only representative images are shown. The time indicates when the image was captured.

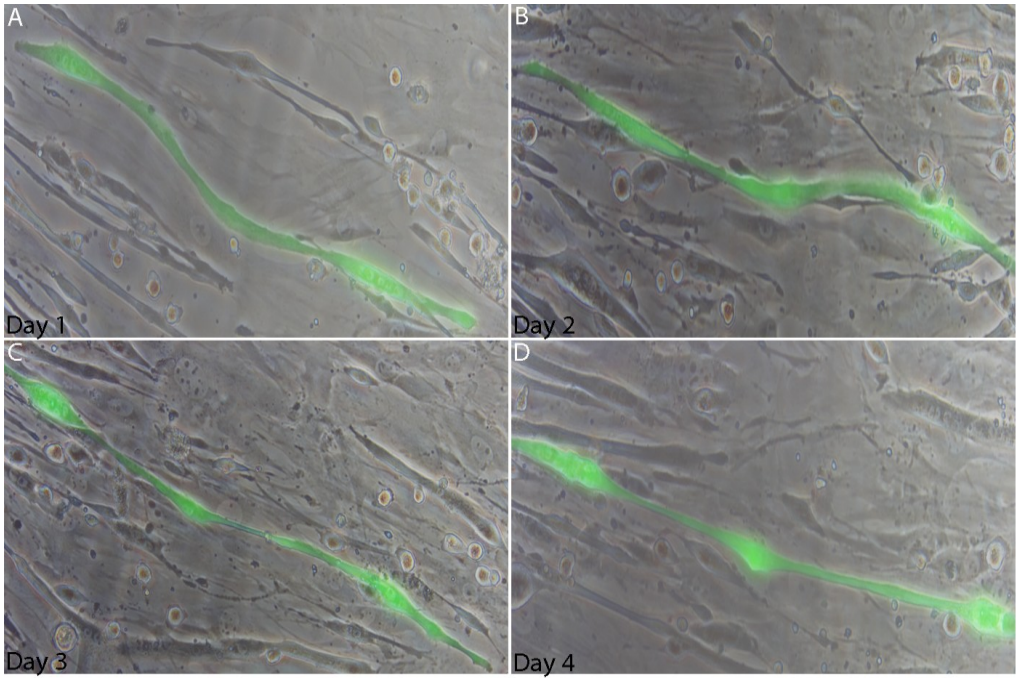


Figure 32. Primary myotubes injected with DMEM/GFP do not undergo fragmentation. Images were taken each day using a Nikon microscope for 4 days.

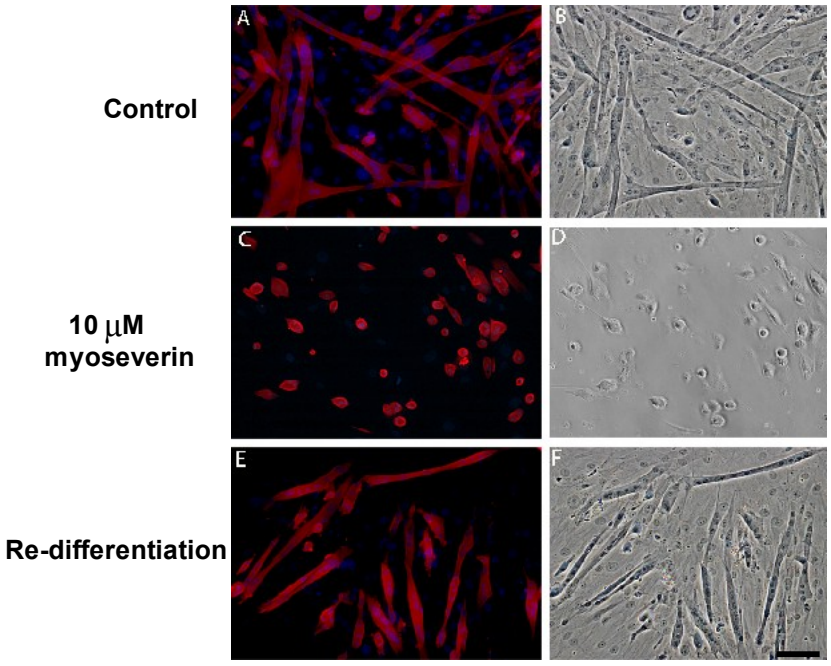


Figure 33. Myoseverin causes fragmentation of multinucleated myotubes into smaller multinucleated cells or into mononucleated cells in primary muscle cells derived from MCK-cre/lox-Rosa-YFP. Immunostaining shows primary myotubes stained with MHC (red) and DAPI (blue) that were treated with 10 μM myoseverin for 24 h undergoing breakdown into mononucleated cells (C-D). (E-F) Primary cells that were treated with myoseverin are able to re-fuse into multinucleated cells again when placed in DM for 48 h. (A-B) Control cells (not treated with myoseverin) in DM medium for 72 h. Scale bar = 100 μm.

3.3. Assaying down-regulation of differentiation-specific muscle markers

The dedifferentiation of myotubes involved cell cycle re-entry and fragmentation into mononucleated cells, as seen in the previous sections. However, dedifferentiation should also involve the down-regulation of muscle-specific markers such as myosin heavy chain (MHC). In order to study down-regulation of MHC, C2C12 myoblasts were differentiated for 3 days, and the cells were passed through a 75 μm sieve to allow contaminating myoblasts to be removed. The cells that were retained on the filter were plated back on dishes that were coated with 0.75% gelatin. On day 4, multinucleated cells were injected with the GFP plasmid. The following day, the cells were treated with the 2º newt extract at a concentration of 0.3mg/ml either in DM or GM for 3 to 6 days. The cells that were treated with newt extract in DM for 6 days were put back in GM for another 4 days. This was followed by fixation and staining of the cells with GFP and MHC.

Figure 34A shows an extract-treated C2C12 cell that is GFP^+ but MHC^-. This event was never seen in the control cells (figure. 34A). Control injected cells were always positive for both GFP and MHC. Since only multinucleated myotubes were injected with the GFP plasmid, the GFP +ve cell that was –ve for MHC could only have resulted from down-regulation of the MHC marker.

Figure 34B shows a decrease in MHC+/GFP- multinucleated cells. This may be explained by the following: Since the cells were in differentiation medium for a period of 6 days, it is possible that in the control dish the cells kept forming multinucleated cells which resulted in their increase. In the treated culture it is possible that the newt extract blocked differentiation as described below in section 3.7.

A decrease in multinucleated GFP +ve and MHC +ve cells was also observed in the treated culture. At the moment it is unknown if this decrease was a result of fragmentation or cell death. However it is interesting to note that the decrease in MHC+/GFP+ multinucleated cells was accompanied by a slight increase in the number of MHC+/GFP+ mononucleated cells. Although, the fragmentation seen in myotubes cultures injected with GFP and then treated with newt extract was not significant, this event was only seen in treated sample. Moreover, cells were observed each day and we did not see GFP +ve cells lifting off the plates.

3.4. The effect of newt extract on cell cycle regulators

Muscle differentiation is characterized by muscle specific gene expression and withdrawal from the cell cycle. p21 is a member of a family of cyclin-dependent kinase inhibitors and a regulator of cell cycle progression. During muscle differentiation the expression of p21 increases followed by cell cycle withdrawal in myocytes (Andres and Walsh, 1996). These myocytes eventually express mature muscle markers such as myosin heavy chain (MHC), and then start fusing, forming multinucleated myotubes (Andres and Walsh, 1996).

C2C12 myoblasts were allowed to differentiate for 5 days without ara-C. Newt extract in growth medium (GM) was added to the differentiated cells for 3 days followed by immunostaining for p21. A significant decrease in p21 expression was seen in treated cultures (Figure 35B) in MHC –ve mononucleated cells. There was also an increase in the percentage of p21-/MHC- mononucleated cells (figure 35A and B). This suggests that newt extract blocks differentiation by blocking p21 expression or may have the ability to down regulate p21 expression in myocytes and thereby block MHC expression.

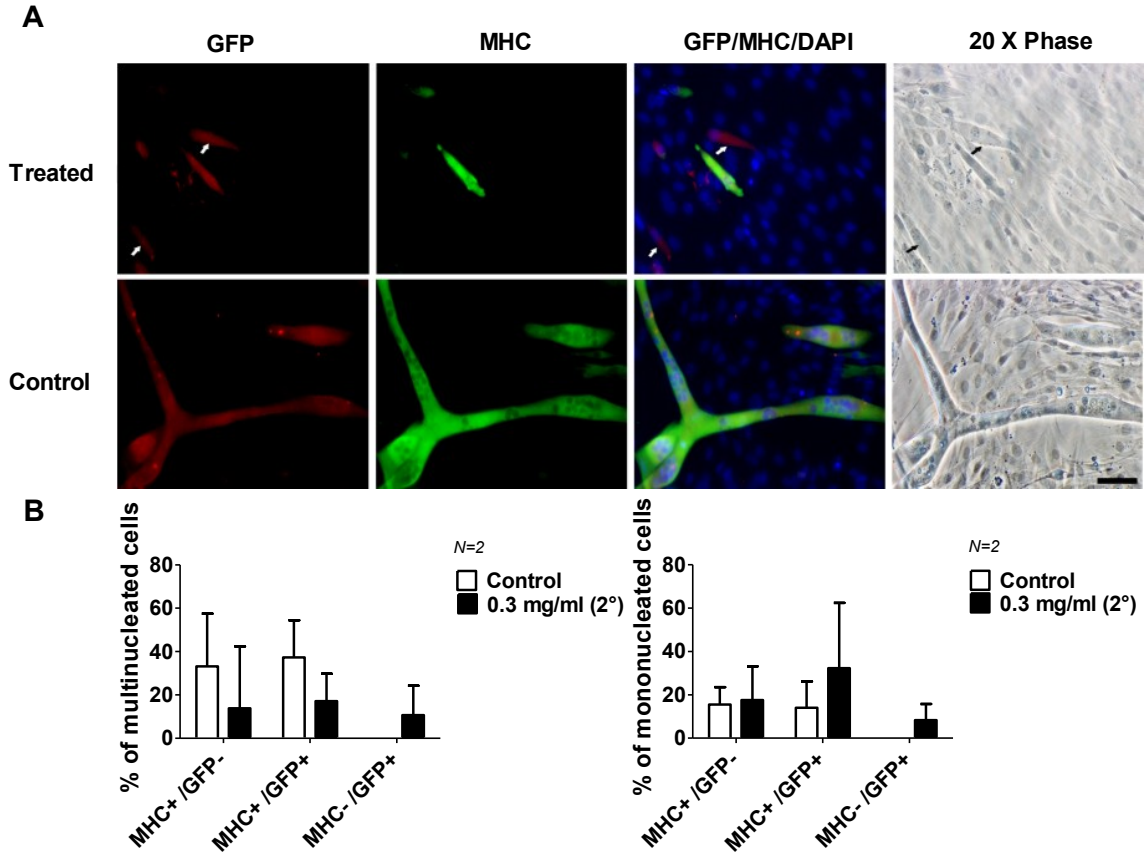


Figure 34. The effects of the secondary newt extract on GFP-microinjected C2C12 myotubes. (A) Immunostaining of C2C12 cells with GFP (red), DAPI (blue), MHC (green) of control cells and cells treated with newt extract for 6 days in differentiated medium and cultured back in growth medium for 4 days. The white arrows in the treated cultures show cells positive for GFP but negative for MHC. (B) Left graph shows the percentage of multinucleated cells positive for MHC and negative or positive for GFP and multinucleated cells negative for MHC and positive for GFP. The right graph shows the percentage of mononucleated cells positive for MHC and negative or positive for GFP, and mononucleated cells negative for MHC but positive for GFP. It is interesting to note that even in the control cultures, there were some GFP positive mononucleated cells (myocytes). This is likely due to the inadvertent microinjection of myocytes that may have been closely associated with myotubes. To correct for this, in subsequent experiments, only myotubes that were clearly separated from any surrounding myocytes or myotubes were injected. Each experiment was performed in triplicate. Scale bar = 100 μm

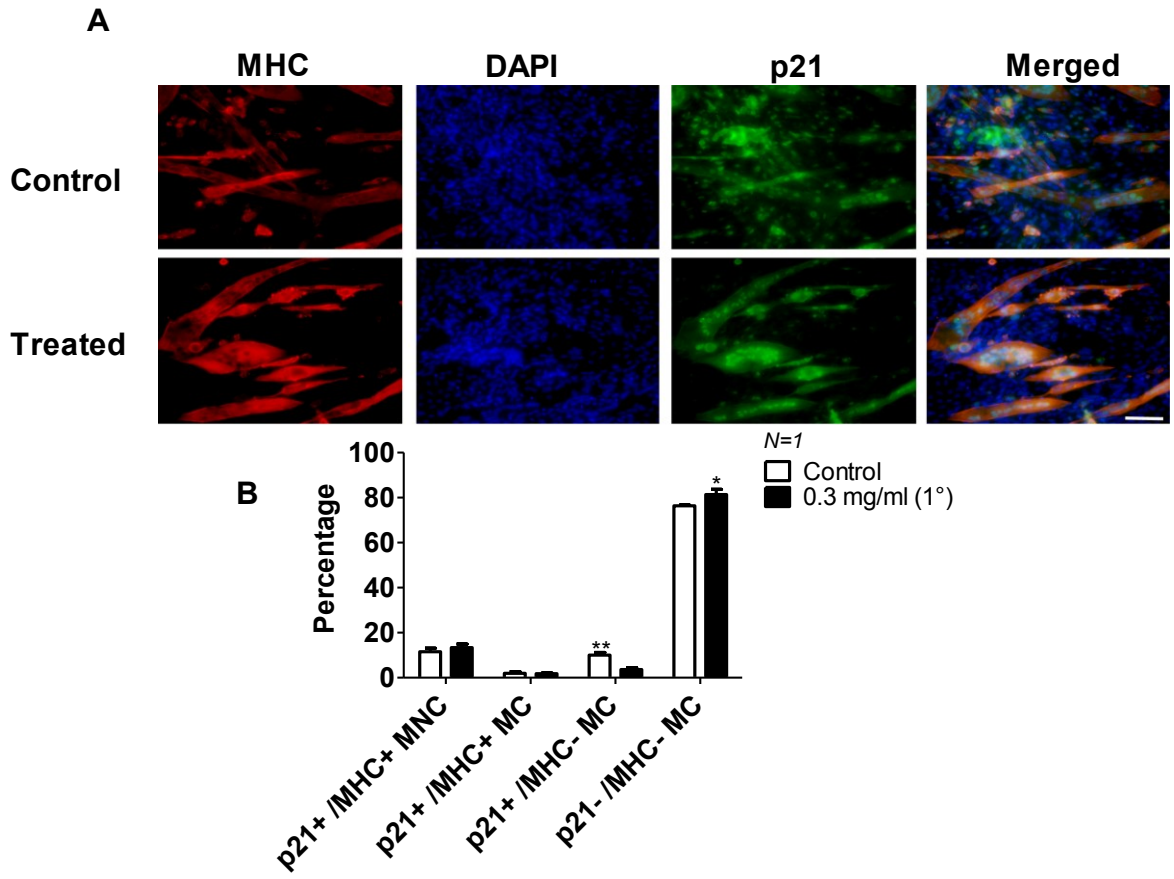


Figure 35. A decrease in p21 expression in cultures treated with the newt extract (without AraC treatment.) (A) Immunostaining of C2C12 differentiated culture with p21 (green) without (control) or with newt extract in GM for 3 days at a concentration of 0.3 mg/ml of the 1° extract. (B) Percentage of multinucleated cells (MNC) and mononucleated cells (MC) cells showing expression of p21 and MHC. Each experiment was performed in triplicate. Asterisks indicate significant difference (*$p<0.05$, **$p< 0.01$) between the control cells and newt extract treated cultures. Each experiment was performed in triplicate. Scale bar = 50 μm

3.5. Affymetrix gene expression analysis in ara-C treated C2C12 and primary differentiated cells following treatment with newt extract.

Techniques such as microarray analysis are commonly used to measure mRNA expression levels under different experimental conditions. We, therefore, conducted a microarray analysis to identify some of the factors expressed in differentiated myotubes following treatment with newt extract. Real-time PCR was also used to confirm the expression profile for some of the candidate genes.

C2C12 and primary differentiated cultures were treated with 1º newt extract for 24 hrs at a concentration of 0.3 mg/ml in DM. mRNA was isolated and used to probe the Mouse Genome 430 GeneChip as described in the Materials and Methods. Expression levels were determined and a cutoff of 1.8 fold was used to identify genes that were up or down-regulated by newt extract. Genes that were down-regulated or up-regulated in C2C12 and primary differentiated cultures following treatment with the newt extract are shown in Appendix 1. Analysis of the affected genes using the Gene Ontology (GO) gave a range of functional classes: GO analysis showed that genes involved in growth factor activity, DNA binding, unfolded protein binding and cation binding were up-regulated in either C2C12 or primary myotube cultures following treatment with newt extract. The newt extract seemed to affect zinc ion binding genes or proteins in both C2C12 and primary cultures. Out of 165 genes up-regulated by the newt extract in C2C12, 29 function in binding zinc ions (figure 36). In primary cultures, of 36 genes up-regulated by the newt extract, 6 are zinc ion binding genes or proteins (figure 36).

Genes involved in ion binding, cation ion binding, calcium ion binding and integrin binding were down-regulated in both C2C12 and primary myotubes cultures following

treatment with newt extract (figure 37 and 38). Figure 39, shows genes involved in extracellular matrix binding, heparin binding, chromatin binding, growth factor binding, actin filament binding and tubulin binding down-regulated in primary myotubes cultures.

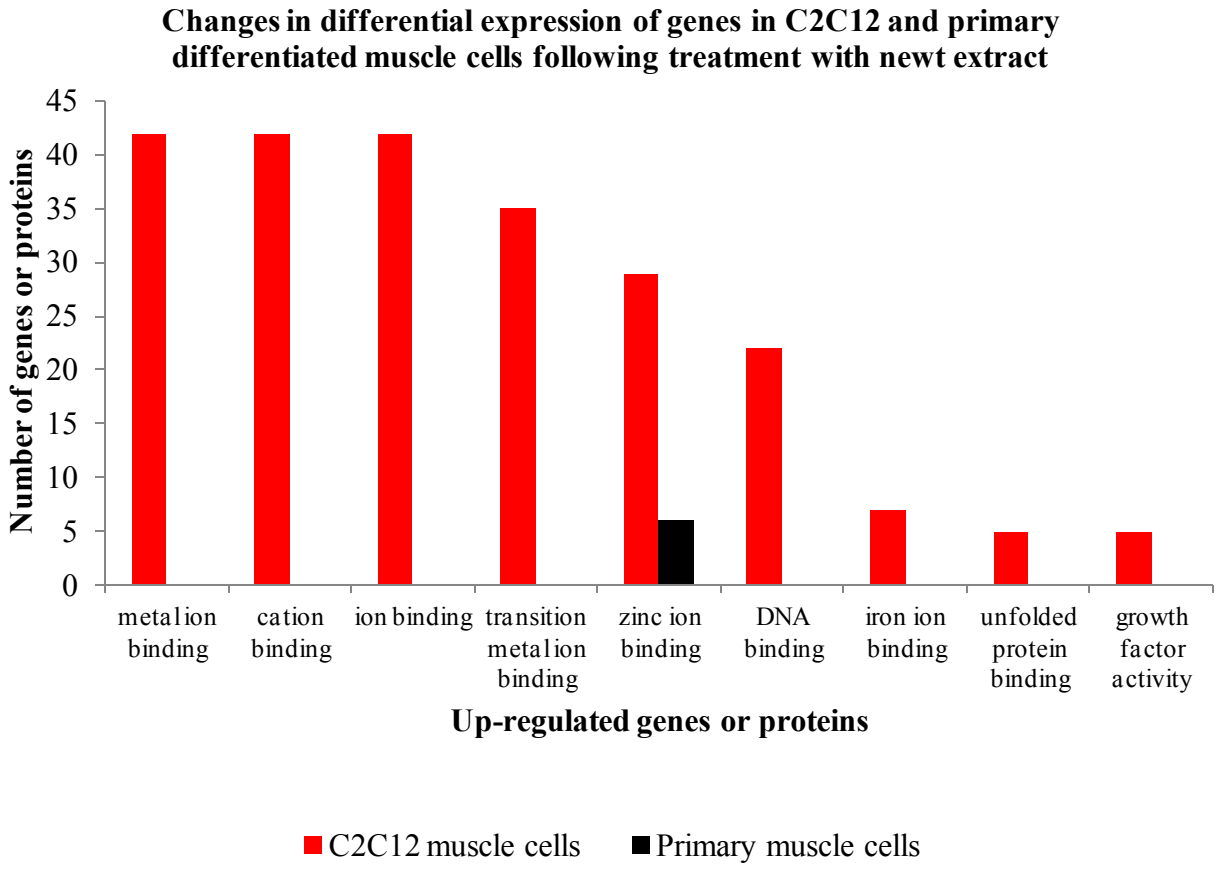


Figure 36. Analysis was performed with DAVID (The Database for Annotation, Visualization and Integrated Discovery version 6.7. GO analysis shows over-represented gene classes in primary and C2C12 differentiated myotube cultures following treatment with newt extract.

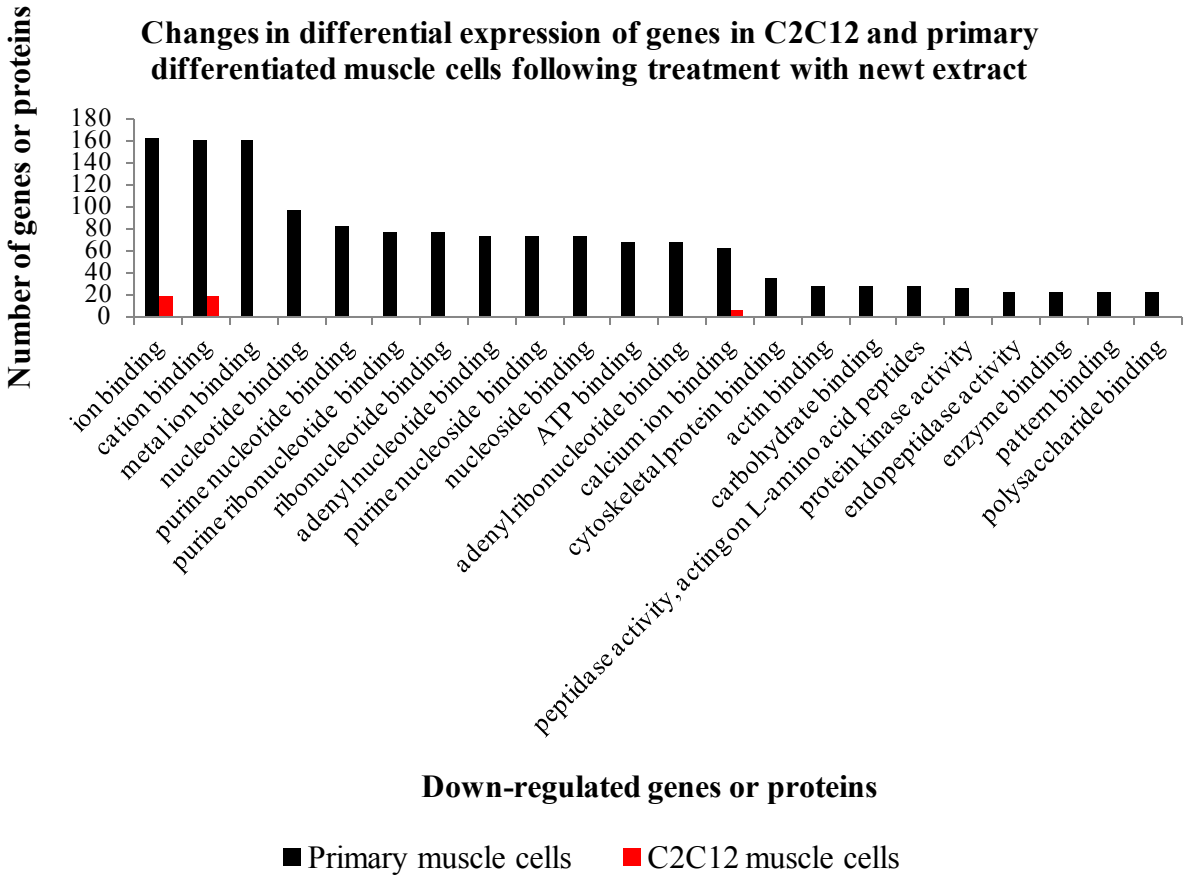


Figure 37. Analysis was performed with DAVID version 6.7. GO analysis shows a number of down-regulated gene classes in both primary and C2C12 differentiated myotube cultures following treatment with newt extract.

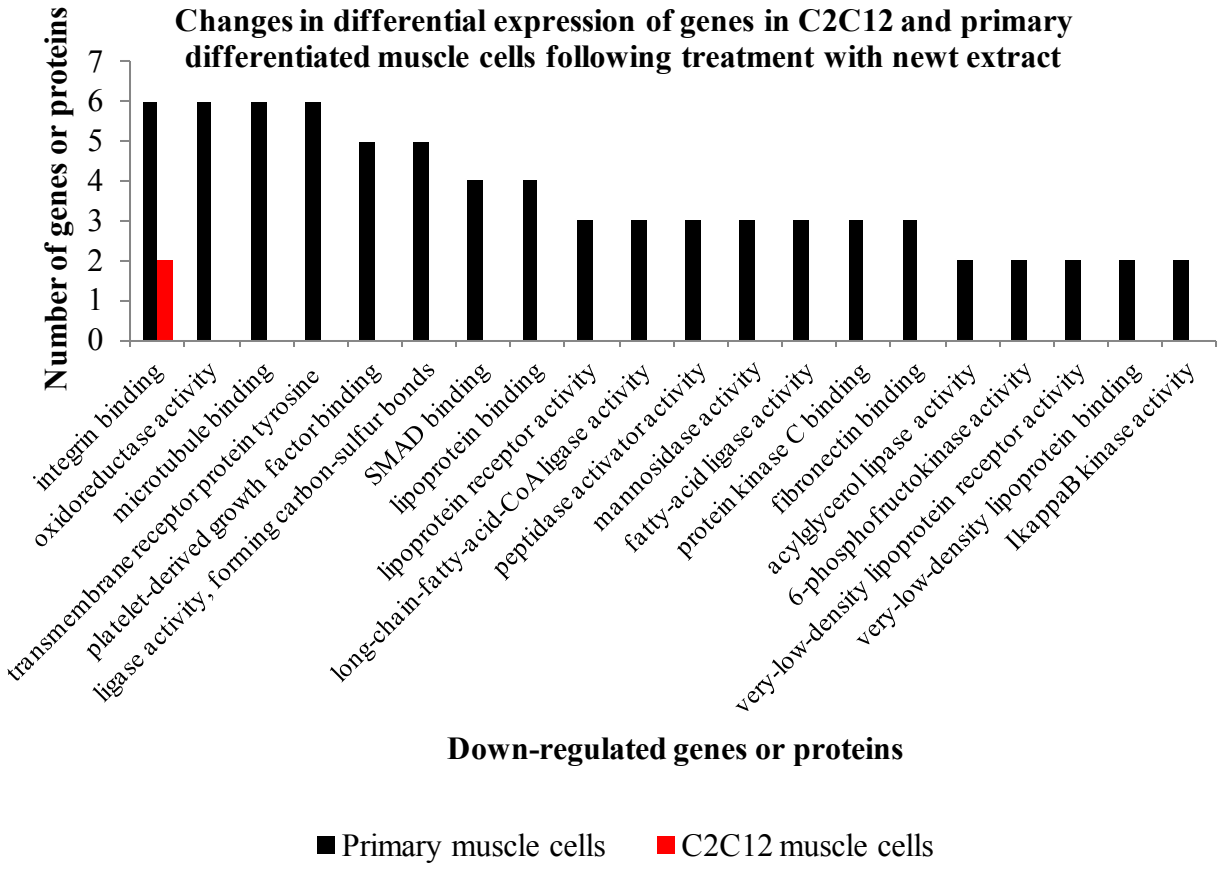


Figure 38. Analysis was performed with DAVID version 6.7. GO analysis shows down-regulated gene classes in primary and C2C12 differentiated myotube cultures following treatment with newt extract.

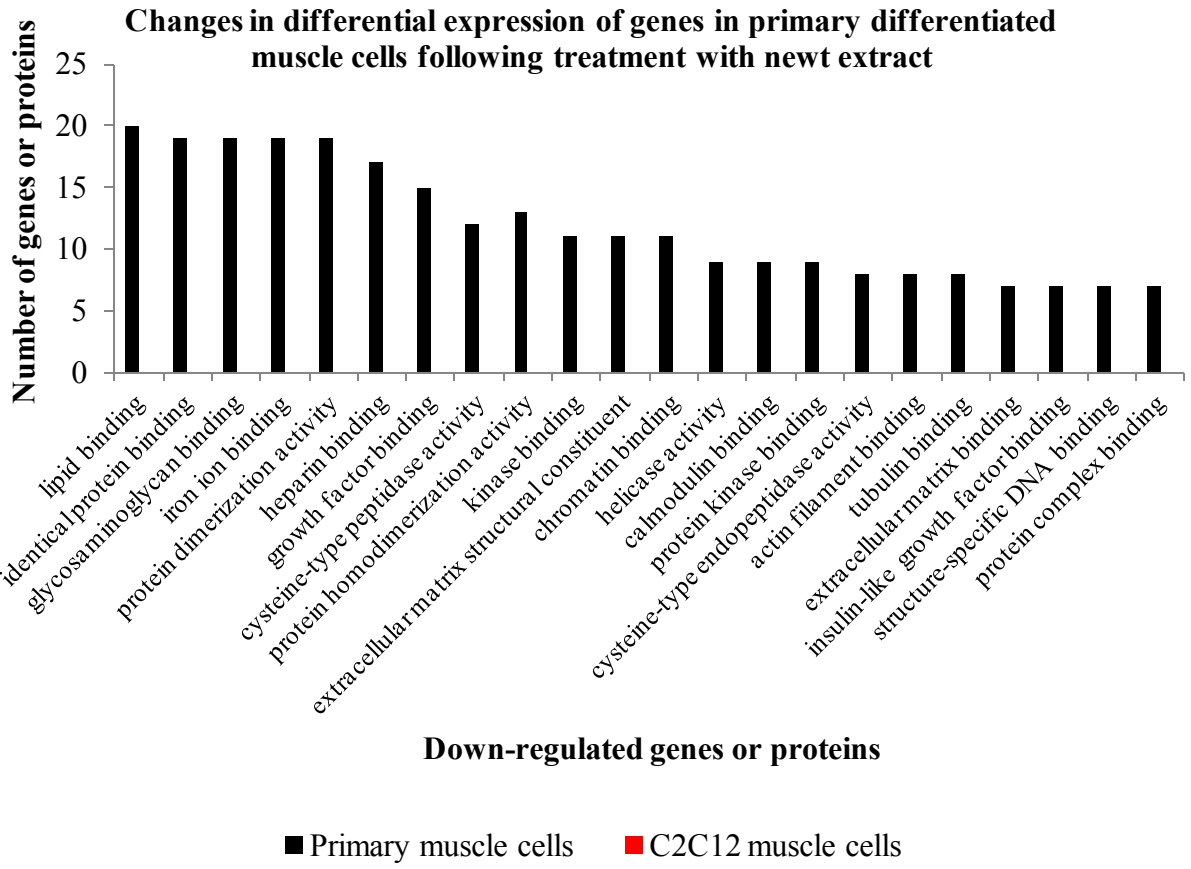


Figure 39. Analysis was performed with DAVID version 6.7. GO analysis shows down-regulated gene classes in primary differentiated myotube cultures following treatment with newt extract.

3.6. Real-time PCR analysis in ara-C treated C2C12 and primary differentiated cells following treatment with newt extract.

Real-time PCR analysis was performed on selected genes involved in muscle specification, determination, differentiation, and cell cycle. Figure 40 shows a significant increase in expression levels of *msx-1* in extract-treated primary cultures at 12 h (figure 40A), and a significant 2.9-fold increase at 24 h in treated C2C12 cultures (figure 40B). This upregulation of *msx-1* by newt extract makes sense since *msx-1* is thought to be involved in repressing muscle differentiation and inducing dedifferentiation.

Figure 41A shows the expression levels of *pax-3* significantly increased by a fold change of 2.5 in primary cultures treated with newt extract for 48 hrs. In C2C12 cells, the expression levels of *pax-3* were increased by a fold change of 4.3 and 3.5 in newt extract treated differentiated cultures at 24 h and 48 h, respectively (figure 41b), but there was a large variability in the means of the samples and thus the increase did not reach significance. *Pax-3* has previously been found to inhibit myoblast differentiation (Epstein et al., 1995). We noticed that the differentiated myotubes cultures had both myotubes and myoblasts cells. Myoblasts may have escaped the ara-C treatment and responded to extract by upregulating *pax-3*.

Cyclin D1, which promotes progression through the G1 to S phase of the cell cycle was also analysed using real-time PCR. The expression of *cyclin D1* was decreased in primary myotube cultures that were treated with newt extract, as indicated by microarray and confirmed by real-time PCR. Real time PCR showed a significant decrease at time points 8, 12 and 48 h in primary cultures treated with newt extract (figure 42A). In C2C12 cells, the

expression of *cyclin D1* was significantly increased at 12 h by a fold change of 1.8 (figure 42B).

The proliferating cell nuclear antigen (PCNA) gene, which is implicated in cell cycle control as well as in DNA replication, did not change in newt extract- treated primary cultures in comparison to the control (figure 43A). *PCNA* was significantly increased in treated C2C12 cultures with newt extract by a fold of 2.4 at 24 h (figure 43B). These results are in general agreement with the cell cycle re-entry studies described earlier which showed that C2C12 myotubes re-entered the cell cycle following treatment with newt extract, but primary myotubes did not. Myogenic transcription factor 5 (*myf5*) is a member of the basic helix-loop helix (BHLH) family. The expression of *myf5* has been shown in proliferating myoblasts in culture. Real-time PCR shows that newt extract increases *myf5* expression in treated primary cultures by a significant fold change of 2.5 at 4 h and 2 at 8 h (figure 44A). A significant fold change of 3.7 at 12 h, 3 at 48 h and 4.6 at 96 h was exhibited in C2C12 cultures treated with newt extract (figure 44B). Microarray analysis also showed the expression of *myf5* to go up in C2C12 myotube cultures that were treated with newt extract. This might suggests that newt extract is reverting differentiated muscle cells to a more "progenitor-like" state.

Myogenin, a muscle specific transcription factor belonging to the BHLH family, has been shown to be expressed in differentiating muscle cells. Previous work has shown that during dedifferentiation of C2C12 cells, the expression of *myogenin* decreases as shown by immunohistochemistry (McGann et al., 2001). Real-time PCR, however, did not show the expression of *myogenin* decreasing in treated C2C12 and primary myotubes (figure 45A and B). It is possible that the decrease of *myogenin* may occur at later time points. *Myogenin*

expression was unchanged in newt extract treated primary cultures (figure 45A). In C2C12 cultures that were treated with newt extract, *myogenin* expression was significantly increased by 2.3 fold at 48 h (figure 45B). It is difficult to explain these results since they do not appear to be consistent with the gene expression profiles found in the other genes tested. It is possible that the decrease of *myogenin* may occur at later time points.

Many of the results obtained by the real-time PCR analysis suggest that treatment with newt extract causes dedifferentiation through up regulation of progenitor markers (such as *msx-1*, *pax-3* or *myf-5*) and in C2C12 cells also affects cell cycle re-entry markers such as *PCNA*. However, there is another possible explanation for these results. If treatment with newt extract (in the presence of Ara-C) causes cell death in myotubes, then this would give similar expression profiles. By reducing the myotube population in the cultures, the myoblasts would predominate, and this would be reflected in the expression pattern seen. In order to eliminate the contribution of cell death to the expression patterns, the expression of *myogenin* and *msx-1* were also analyzed on C2C12 myotubes that were injected with both newt extract and GFP. C2C12 myoblasts were differentiated for 3 days, followed by filtration to remove myoblasts on day 4. The cells that were retained on the filter were plated on non-coated 35 mm plates. On day 5 the myotubes were injected both with the newt extract protein fraction of greater than 30 kDa and GFP in DM and then transferred to GM for 4 days. In these cultures, the expression of *myogenin* was decreased, and *msx-1* unchanged at 96 h (figure 46). This may suggests that the newt extract causes down-regulation of muscle differentiation markers in mammalian muscle cells.

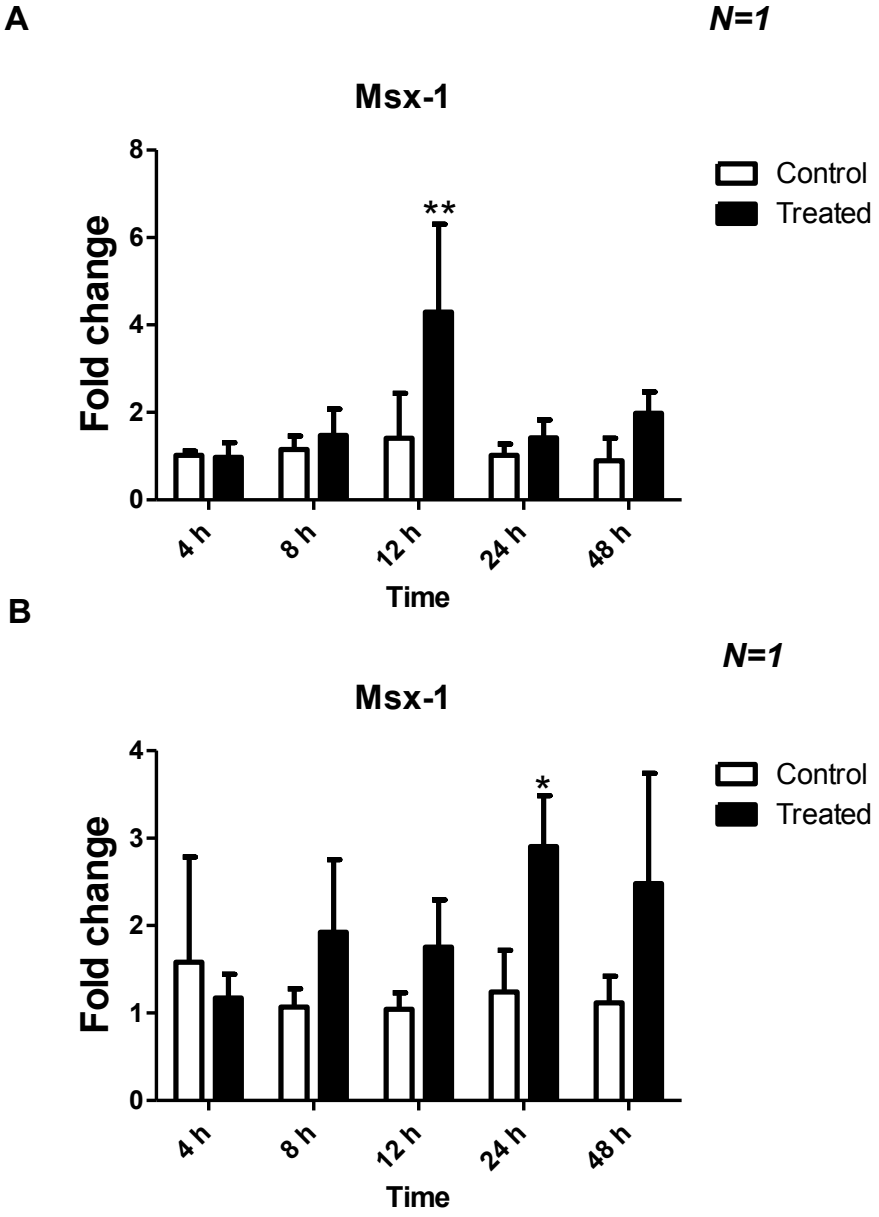


Figure 40. The expression level of *msx-1* in primary (A) and C2C12 (B) differentiated cultures. Treated cultures received 1º newt extract at a concentration of 0.3 mg/ml. Control cultures were not treated with extract. The data shows an average of three replicates in one experiment. The Y-axis shows the fold change and the X axis shows the time points. Newt extract causes significant increase in the expression of *msx-1* in primary cultures (A) at 12 h after treatment and a slight significant increase at 24 h in C2C12 cultures (B). Asterisks indicate significant difference (*$P < 0.05$, and **$P < 0.01$) between the control and treated groups.

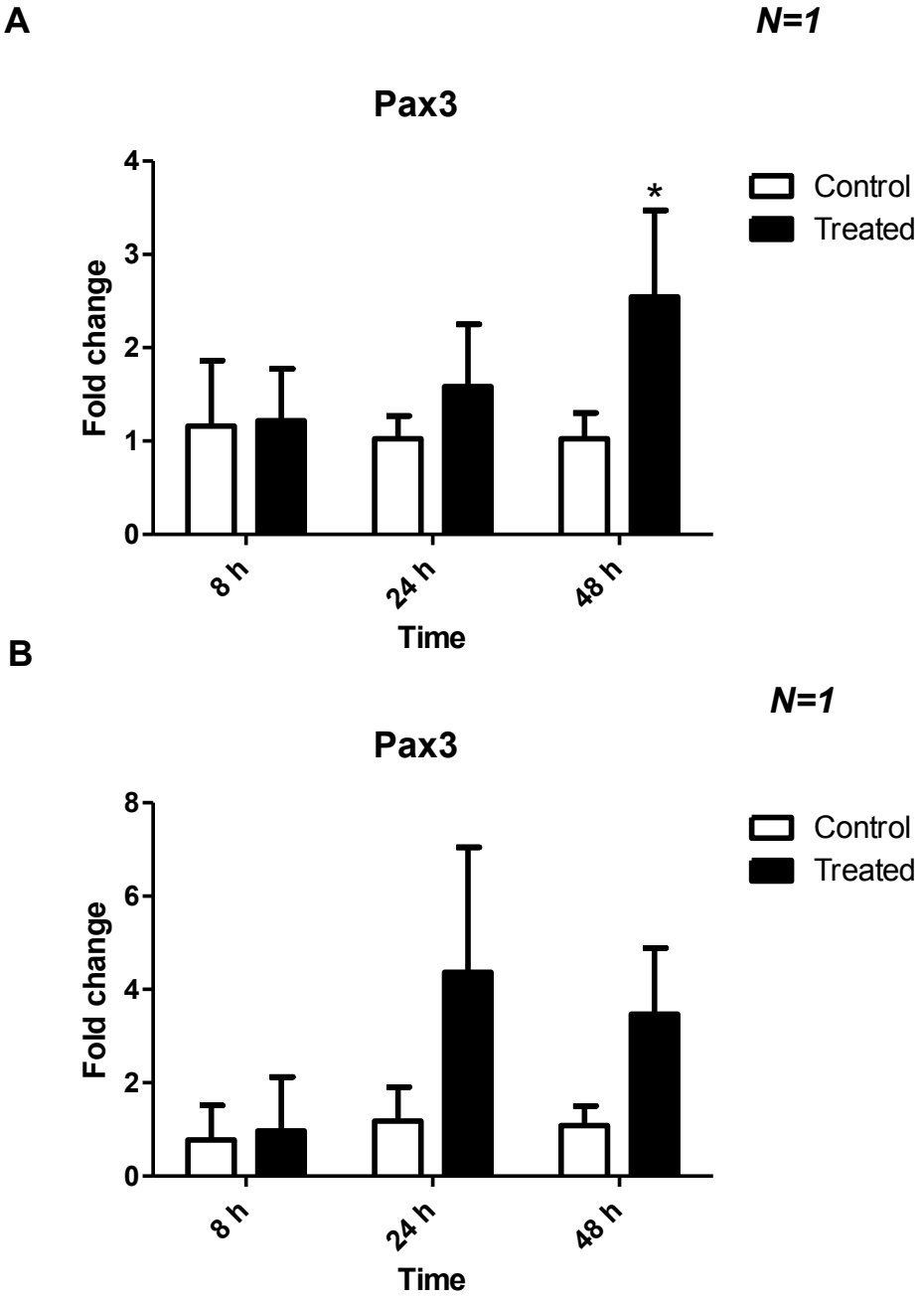


Figure 41. The expression level of *pax-3* in primary (A) and C2C12 (B) differentiated cultures. Treated cultures received 1º newt extract at a concentration of 0.3 mg/ml. Control cultures were not treated with extract. The data shows an average of three replicates in one experiment. Newt extract significantly increases the expression of *pax-3* in primary cultures (A) at 48 h after treatment. C2C12 cultures (B) showed increases at 24 and 48 h, but these did not reach significance. Asterisks indicate significant difference (*$P < 0.05$) between the control and treated groups.

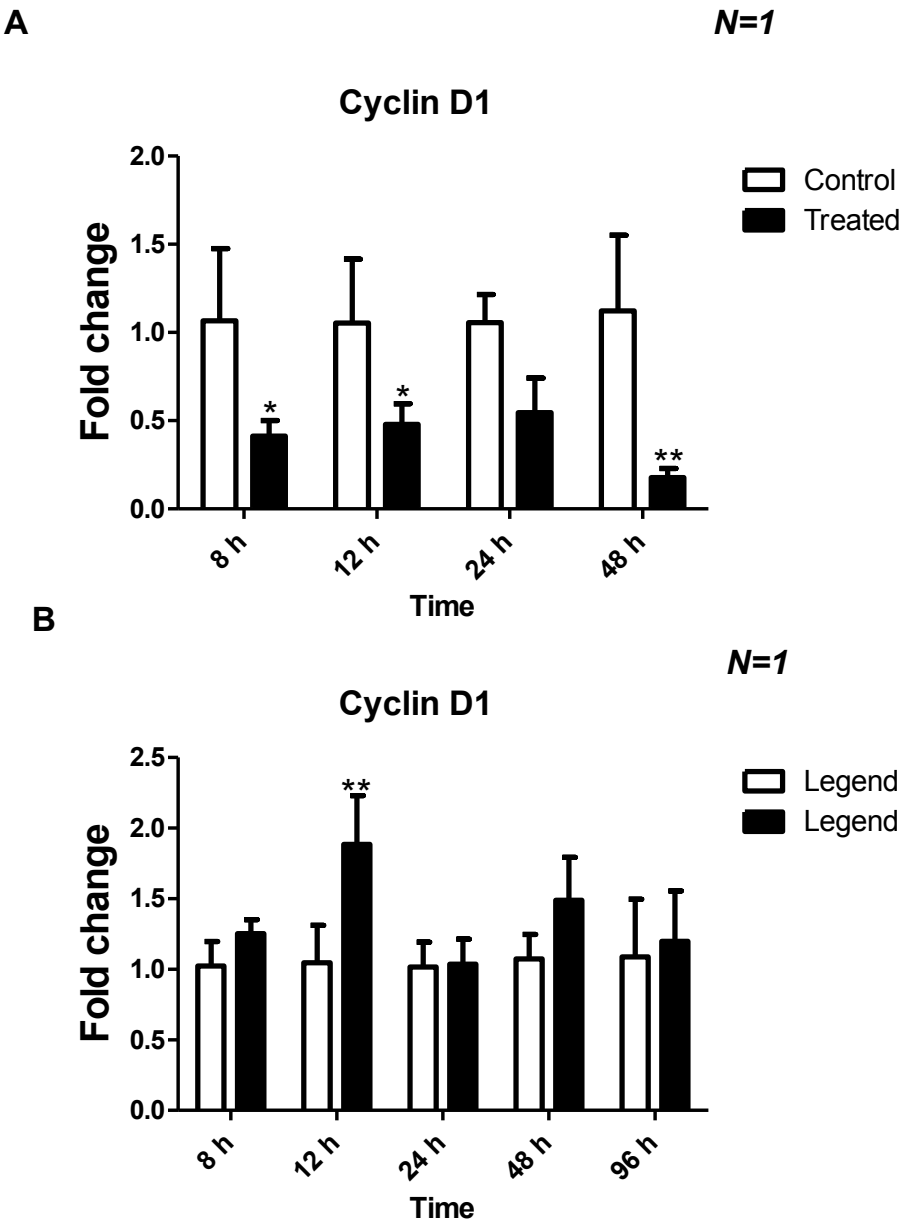


Figure 42. The expression level of *cyclin D1* in primary (A) and C2C12 (B) differentiated cultures. Treated cultures received 1º newt extract at a concentration of 0.3 mg/ml. Control cultures were not treated with extract. The data shows an average of three replicates in one experiment. Newt extract decreases the expression of *cyclin D1* in primary cultures (A) after treatment. In C2C12 cells (B), the expression of *cyclin D1* was significantly increased at 12 h. Asterisks indicate significant difference (*$P < 0.05$, and $P < 0.01$) between the control and treated groups.

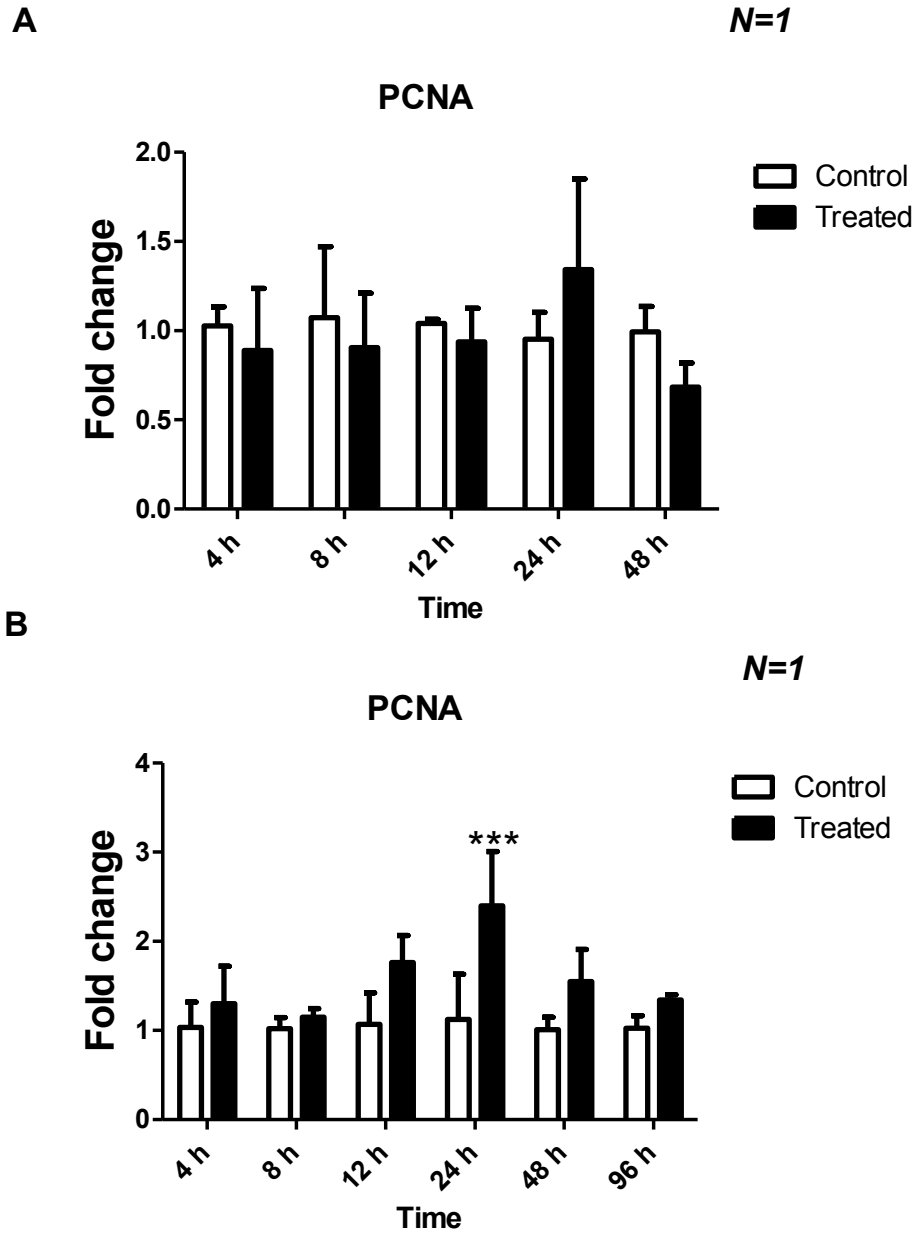


Figure 43. The expression level of *PCNA* in primary (A) and C2C12 (B) differentiated cultures. Treated cultures received 1º newt extract at a concentration of 0.3 mg/ml. Control cultures were not treated with extract. The data shows an average of three replicates in one experiment. Newt extract causes no change in expression of *PCNA* in primary cultures (A) and in C2C12 cultures (B) the expression is significantly increased at 24 h. Asterisks indicate significant difference (*P < 0.001) between the control and treated groups.

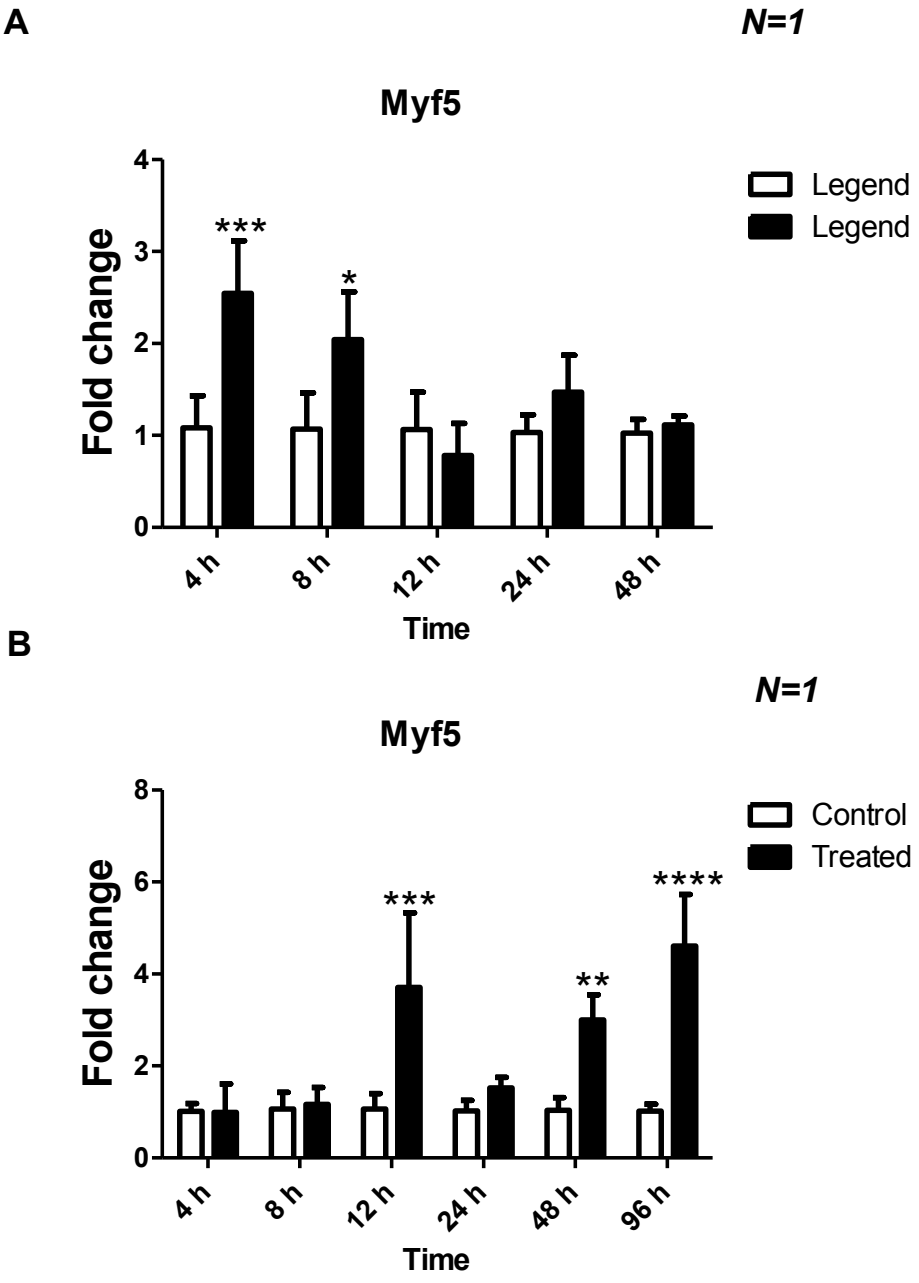


Figure 44. The expression level of *myf-5* in primary (A) and C2C12 (B) differentiated cultures. Treated cultures received 1º newt extract at a concentration of 0.3 mg/ml. Control cultures were not treated with extract. The data shows an average of three replicates in one experiment. Newt extract increases the expression of *myf-5* in primary cultures (A) at 4 and 8 after treatment and in C2C12 cultures (B) at 12, 48 and 96 h. Asterisks indicate significant difference (*P < 0.05, **P < 0.01, and ***P < 0.001) between the control and treated groups.

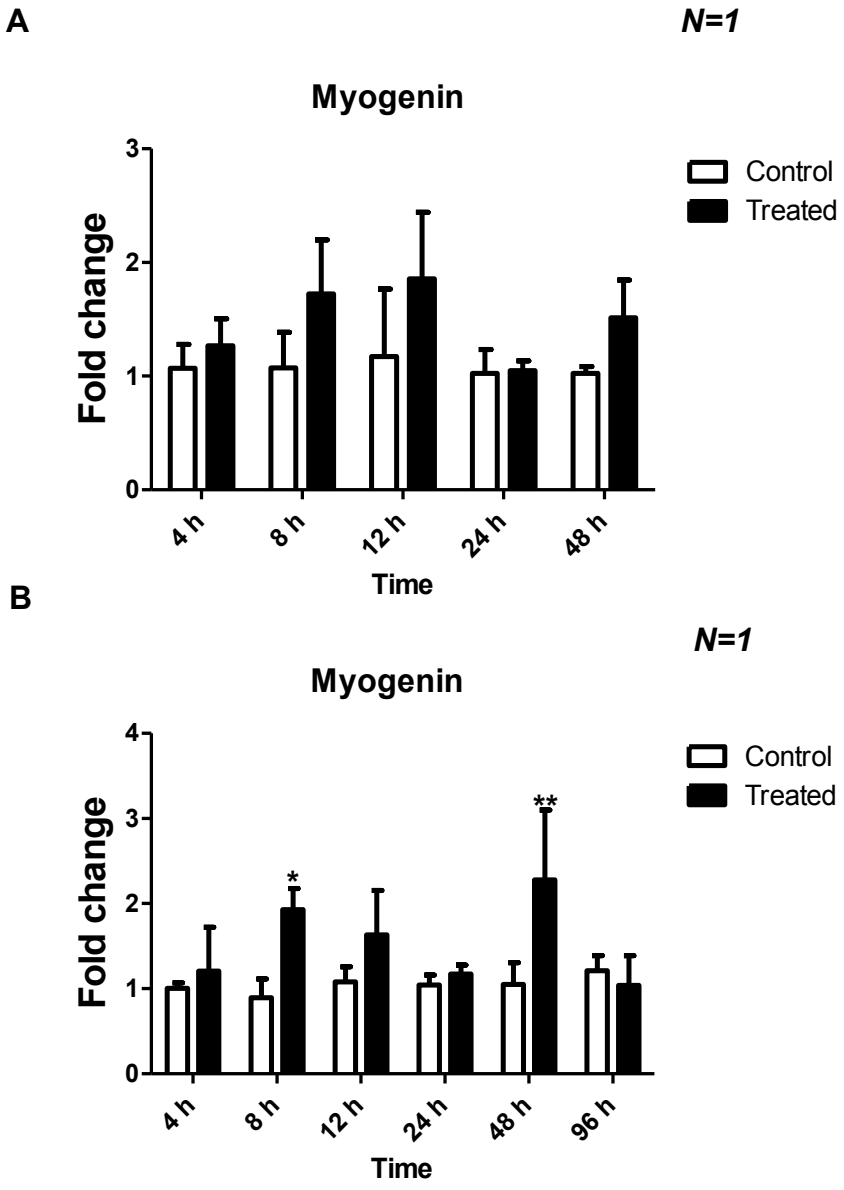


Figure 45. The expression level of *myogenin* in primary (A) and C2C12 (B) differentiated cultures. Treated cultures received 1° newt extract at a concentration of 0.3 mg/ml. Control cultures were not treated with extract. The data shows an average of three replicates in one experiment. There was no change in expression of *myogenin* in primary (A) and a slight increase in C2C12 (B) cultures at 48 h after treatment with newt extract. Asterisks indicate significant difference (**P < 0.01)

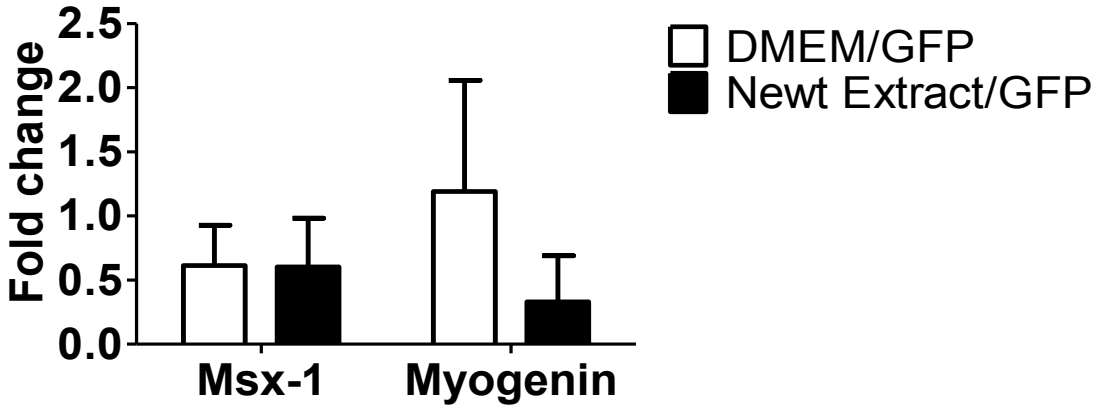


Figure 46. The expression levels of *myogenin* and *msx-1* in C2C12 myotubes following injection with newt extract. The graph shows myotubes injected with DMEM/GFP and myotubes injected with both newt extract and GFP and transferred to GM for 4 days. The data shows an average of three independent experiments. Newt extract causes no change in expression of *msx-1*. However, *myogenin* expression was found decreased in myotubes injected with newt extract/GFP, but this decrease did not achieve significance.

3.7. Effect of newt extract on myoblast differentiation

Myogenic differentiation is typically studied *in vitro* by the fusion of myoblasts into multinucleated myotubes. To examine the effect of newt extract on myoblast differentiation, the newt extract at a concentration 0.3 mg/ml was added to confluent C2C12 myoblasts cultured in DM or GM for 4 days; then the cells were analyzed for their ability to undergo myogenic differentiation. After confluent myoblasts were cultured without newt extract in DM for 4 days, the cells normally underwent myogenic differentiation to form multinucleated myotubes (figure 47A). In contrast, the newt extract treated cells had fewer multinucleated cells than the control (figure 47A) and were much smaller in size. A significant decrease in the expression of myosin heavy chain (MHC) was observed in the treated culture compared to the control in GM and DM (figure 47B). The effect of newt extract on primary myoblast differentiation was also assessed. Primary myoblasts were grown to 70% confluency in GM, followed by switching to DM in the absence or presence of 1^0 newt extract. Within 24 h multinucleated myotubes formation was observed in the control cultures (figure 48A). Myoblast fusion was reduced in the cultures that were treated with newt extract (figure. 48B). In addition myoblasts that were treated with newt extract showed a significant decrease in MHC positive cells in comparison to the control cells (figure 48C). This suggests that newt extract inhibits myogenic differentiation.

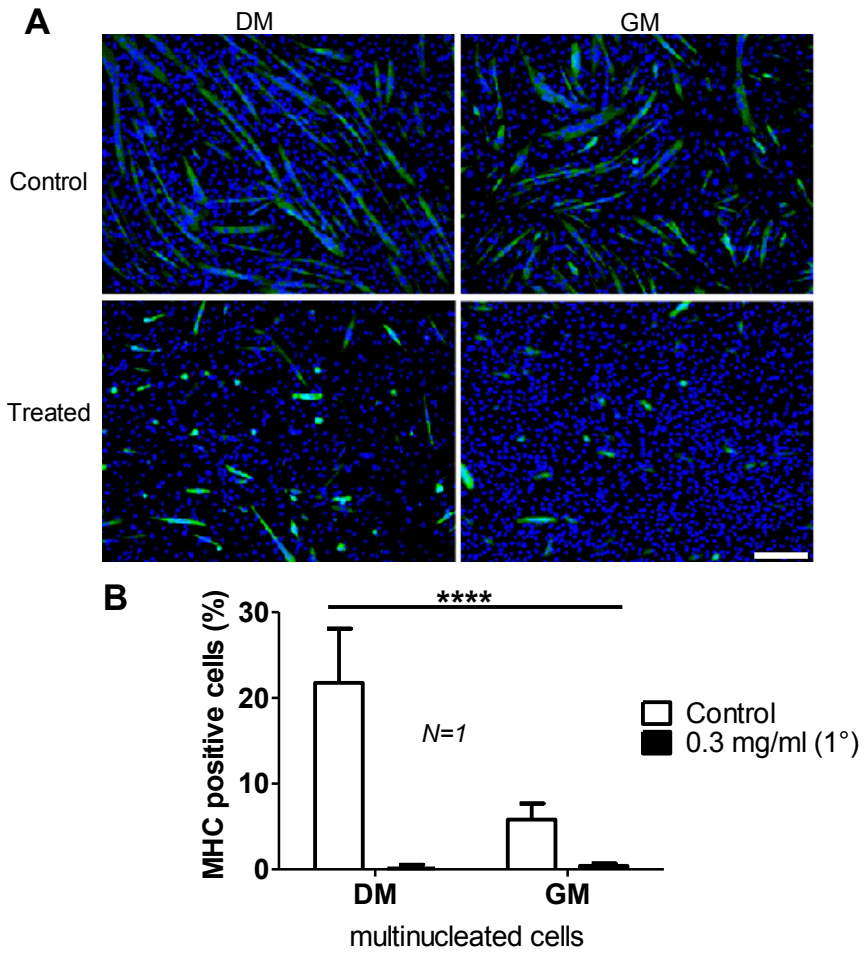


Figure 47. Inhibition of muscle differentation in C2C12 cells treated with newt extract (A) Staining of confluent C2C12 myoblasts with MHC (green) and DAPI (blue) treated with 0.3 mg/ml of 1° newt extract for 4 days. The control represents cells that were not treated with newt extract. (B) Percentage of cells positive for MHC in the control (non-treated) and newt-extract treated cultures in growth (GM) and differentiation medium (DM) for 4 days. Each experiment was performed in triplicate. Asterisk indicate significant differences (****$p < 0.0001$) between the control cells and newt extract treated cultures. Scale bar = 200 μm.

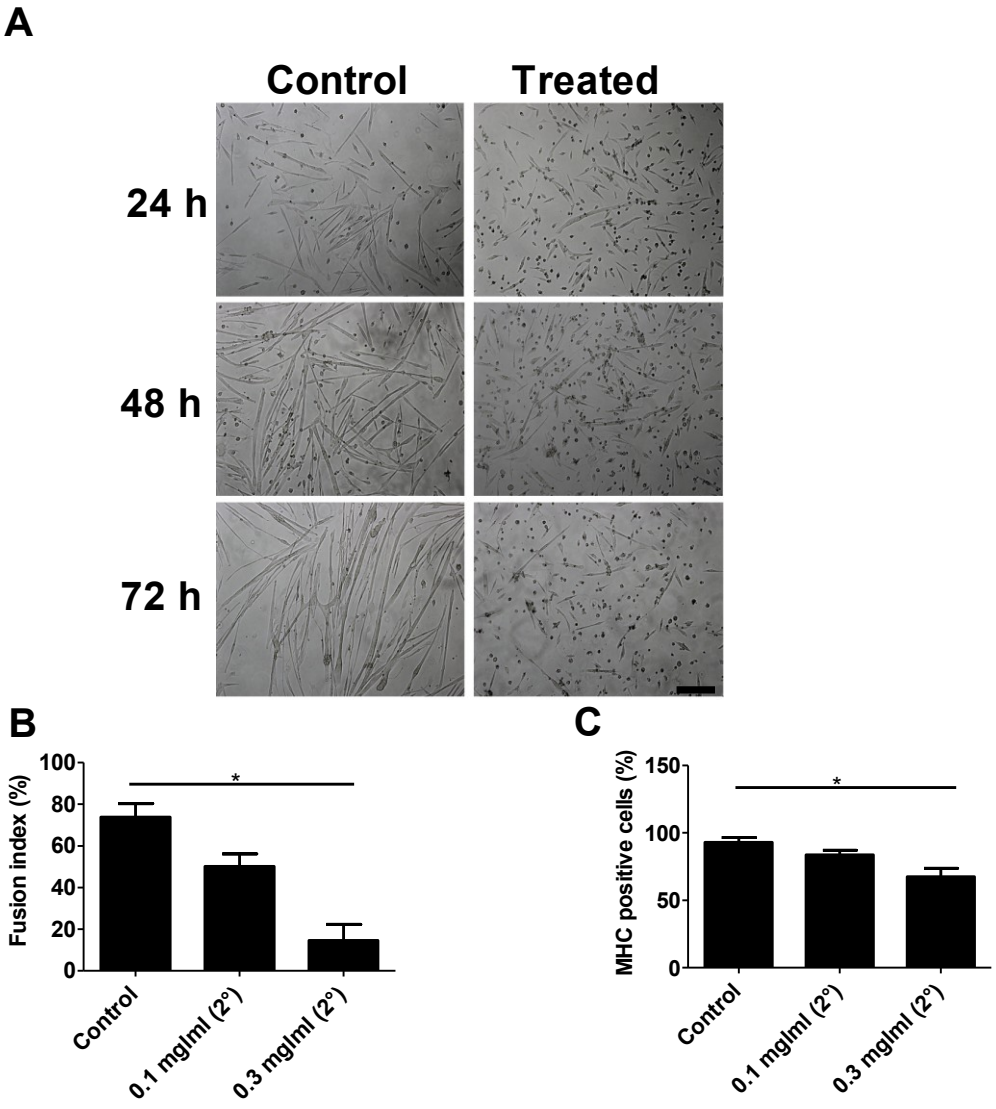


Figure 48. Inhibition of muscle differentation in primary muscle cells derived from MCK-cre/lox-Rosa-YFP mice that were treated with 1^0 newt extract. (A) Fewer myotubes are observed in newt extract treated cultures in comparison to control (not treated). (B) Fusion index in cells in the control (non-treated) and newt-extract treated cultures in differentiation medium (DM) for 3 days. Fusion index (%) was calculated as (number of nuclei in multinucleate/number of multinucleate cells) × 100. (C) Percentage of cells positive for MHC in the control (non-treated) and newt-extract treated cultures in differentiation medium (DM) for 3 days. Each experiment was performed in triplicate. Asterisk indicates significant difference (*$p < 0.5$) between the control cells and newt extract treated cultures. Scale bar = 200 μm.

3.8. The effect of newt extract on proliferation of myoblasts

The results presented have shown that newt extract causes cell cycle re-entry in C2C12 myotubes, but in some cases, increases in BrdU incorporation were also seen in both MHC +ve and MHC –ve mononucleated cells (figure 6, 17). For this reason, we decided to assess the effect of newt extract on myoblast proliferation. Cell proliferation was analyzed in C2C12 semi-confluent cells following treatment with newt extract using flow cytometry. Equal numbers of cells were plated in both control and extract treated cultures. After 48 h the cells were harvested followed by flow cytometry analysis. Figure 49A shows no effect on cell cycle in myoblasts that were treated with 1° newt extract at 0.3 mg/ml in comparison to the control. Cell proliferation was also analyzed using a hemocytometer. Semi-confluent myoblasts were plated with the same number of cells (ie. 69.25 X 10^4 in 1 ml of GM) either in the presence or absence of newt extract. After 48 h in culture, the cells were re-counted using the hemocytometer. As shown in Figure. 49B, there was no difference seen between the control and the extract-treated culture. This indicates that the newt extract does not increase proliferation in myoblasts cultures.

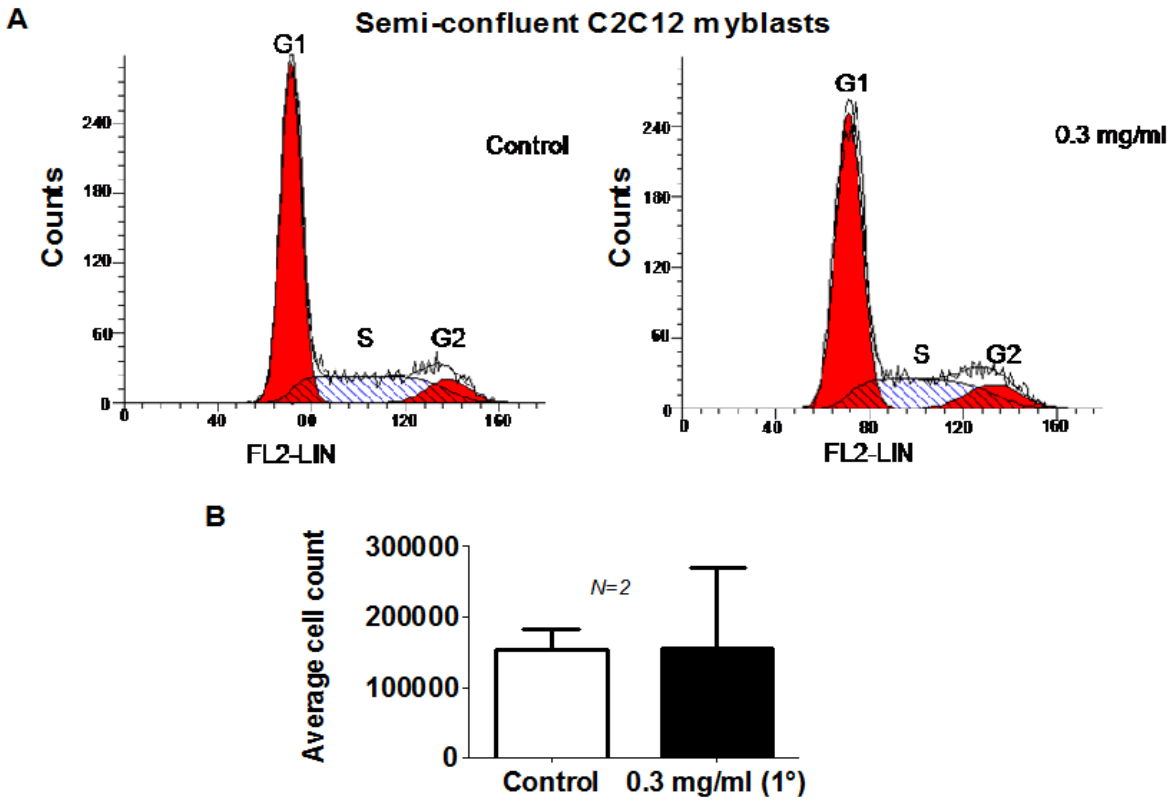


Figure 49. Treatment of semi-confluent C2C12 myoblasts with newt extract. (A) Graph for control represents myoblasts cells that were not treated with newt extract. 0.3 mg/ml represent the concentrations of the newt extract that was added to proliferating myoblasts. The G1-phase of the cell cycle prepares the cell for DNA synthesis. The S-phase of the cell cycle is when DNA synthesis occurs and the G2-phase of the cell cycle is when cells are preparing for mitosis. There are no discernible differences seen between control and extract-treated cells. (B) Cell counts using the hemocytometer of myoblasts not treated (control) or treated with newt extract in growth medium. There was no difference seen between the control and extract treated culture.

Chapter 4: Discussion

The purpose of this thesis was to further investigate dedifferentiation in mammalian skeletal muscle tissues, particularly in primary myotubes following treatment with newt extract. Most studies that investigate muscle dedifferentiation in mammals use C2C12 cells as a model for muscle differentiation. Given that C2C12 cells are an immortal cell line, it is possible that they have obtained mutations during serial passaging in culture. Due to these possible mutations, C2C12 myotubes may re-enter the cell cycle given the right stimulus. In addition, there is clear skepticism in the regeneration community about the efficacy and validity of McGann's original results concerning the effect of newt extract on mammalian muscle cells. For that reason, the research focus of this study was to repeat and further characterize some of the results obtained by McGann et al (2001), as well as examine dedifferentiation in primary cultures following treatment with newt extract.

Our results showed that dedifferentiation was induced in mammalian myotubes following treatment with newt extract, particularly in C2C12 cells. However, it is noteworthy that some batches of newt extract had little effect on cell cycle re-entry in myotube cultures, while other batches were able to induce cell cycle re-entry. In fact, we noted that the batches of extract that had little effect on cell cycle re-entry also appeared to be more toxic to the cultures (see Figure 11), and induced a greater degree of cell death, despite the fact that the concentration of the extract was kept constant. This shows that there is variability in the extract from one batch to the next. This can possibly be attributed to the variability in the health of the animals, the potentially different rates of regeneration (which is impacted by health), or the potentially small differences that can occur in tissue sampling from one animal to the next. It is interesting to note that the 2º newt extract was more

effective than the 1º newt extract in inducing cell cycle re-entry in MHC-positive cells. The 2º newt extract is derived from limbs that were previously amputated and allowed to re-grow for 1 or 3 months. Even though 3 month old regenerating limbs appeared fully regenerated, the bones were not completely calcified, and the skin pigmentation was not as dark as the original limb. Previous studies had shown that spinal cord extract isolated from young *Ambystoma mexicanum* salamanders was more effective in inducing blastema cell proliferation than extract isolated from older salamanders (Boilly and Albert, 1988). In our study, it is possible that the tissues from the 3 month old re-amputated limb were similar to a young limb, and so they may have had more mitogenic activity than the original limb.

4.1. Cell cycle re-entry in C2C12 differentiated cells treated with ara-C and newt extract

Ara-C, which interferes with DNA synthesis, was used to eliminate dividing cells (myoblasts) from a differentiated muscle culture and to yield a purer population of myotubes. In this study, ara-C treatment was followed by treating the cells with newt extract for 3 days. Cell cycle re-entry was mostly found in C2C12 myosin heavy chain (MHC) positive mononucleated cells cultured in growth medium (figure 6). Cell cycle re-entry was rarely detected in differentiated cells that were cultured in differentiation medium (DM). This is in contrast to the study by McGann et al. (2001), which detected cell cycle re-entry in C2C12 myotube cultured in DM following treatment with newt extract. Interestingly, we found that treatment of myotubes with ara-C (especially in the presence of newt extract) induced significant cell death of the differentiated myotubes. This may not be very surprising, since it has been shown that treatment of post-mitotic cells with ara-C can induce cells to undergo apoptosis (Wallace and Johnson, 1989). DNA nicking is a process that occurs during the

start of terminal differentiation in myotubes, followed by DNA repair (Dawson and Lough, 1988). Treatment of 2 to 3 day old myotubes with ara-C for a period of 2 days was found to increase DNA nicking (Masuck et al., 1990). The increase in DNA breaks in myotubes was found irreversible even after removing ara-C.

As mentioned above, the combined treatment of myotubes with ara-C and newt extract led to more pronounced apoptosis than ara-C treatment alone. The explanation for this may be as follows: If newt extract caused cell cycle re-entry in myotubes and there were any residual levels of ara-C in the myotube cultures, this would cause cell death in the newly dividing nuclei. It is interesting to note that TUNEL positive signals were mostly detected in larger myotubes suggesting that they are more sensitive to ara-C induced cell death.

4.2. Cell cycle re-entry in non-ara-C treated C2C12 myotube cultures

Repeating the assay in the non-ara-C-treated myotube cultures, we were able to detect BrdU incorporation in myotubes treated with 2^0 newt extract in growth medium. In non-filtered cultures, we noticed that myotubes that incorporated BrdU were smaller in size (with 2 to 9 nuclei). Filtered cultures had mostly larger myotubes, and this could be why only about 5 % of myotubes re-entered the cell cycle after treatment with 2^0 newt extract. In addition, not all nuclei within a myotube exhibited cell cycle re-entry. McGann et al. (2001) did not detect size differences between myotubes that underwent cell cycle re-entry following treatment with newt extract (McGann et al. 2001).

There may be a reason why smaller myotubes responded better to treatment with newt extract. Terminal differentiation of skeletal muscle cells involves down-regulation of cell division kinases such as $p34^{CDC2}$, a protein necessary for promoting G1 to S, and G2 to M phase transition during the cell cycle (Bailly et al., 1989; Jahn et al., 1994). The

expression of $p34^{CDC2}$ can be detected in a few nascent myotubes with less than 5 nuclei (Jahn et al., 1994), which might imply that small myotubes are still capable of synthesizing DNA. However, established myotubes are not able to up regulate this gene unless they have been transfected with SV40 T antigen (Ohkubo et al., 1994). Interestingly, microarray gene profiling showed the gene *cdc2* up-regulated in C2C12 differentiated cells following treatment with newt extract. This might explain why smaller myotubes re-entered the cell cycle following newt extract treatment.

4.3. Cell cycle re-entry in labeled C2C12 differentiated cells

Cell cycle re-entry was detected in extract-treated C2C12 myotube cultures that were transfected with the two-plasmid system; the pCMV-EGFP/RFP plasmid, and the MCK-Cre plasmid. A number of studies have been able to track living cells using green fluorescent protein (GFP) or red fluorescent protein (RFP) (Lippincott-Schwartz and Patterson, 2003). The two-plasmid system enabled us to distinguish between undifferentiated cells and differentiated muscle cells. The expression of GFP was only detected in proliferating myoblasts, and upon differentiation GFP was switched to RFP, which was detected in myocytes and myotubes (figure 17 to 19). This system allowed tracking of RFP cells in culture followed by treatment with newt extract. The extract treated RFP+ve myocytes, which were followed with microscopy for 96 h, incorporated BrdU in their nuclei.

This two-plasmid system could be ideal for tracking dedifferentiating cells in culture, but the method still has limitations. Subsequent to transfecting myoblasts with pCMV-GFP/RFP plasmid, GFP was detected in almost 40 % of the myoblasts. However, within approximately one week, GFP was only detected in 5 % of cells. RFP expressing cells were detected, however there were very few in culture. Additionally, cell growth seemed altered

in the cultures which may indicate a toxic effect of the fluorescent proteins. It has previously been found that elevated expression levels of red fluorescent proteins can be toxic to Hela cells (Strack et al., 2008). For that reason, we decided to label myotubes by the microinjection procedure.

Microinjection is a technique used to deliver materials into living cells (Zhang, 2012). It has been applied to study dedifferentiation in mammalian myotubes (Meech et al., 2010). We initially injected C2C12 myotubes with both the crude newt extract and GFP, and found that it was able to induce 1.8 % of nuclei within myotubes to undergo cell cycle re-entry (figure 22). However, not all nuclei in the myotubes exhibited cell cycle re-entry. By fractionating the newt extract, we found that the protein fraction > than 30 kDa induced cell cycle re-entry in approximately 45% of the nuclei of microinjected myotubes (figure 23P). This suggests that the molecular weight of the proteins involved in inducing cell cycle re-entry in myotubes is equal to or greater than 30 kDa. The results clearly show that C2C12 myotubes are capable of undergoing cell cycle re-entry following treatment with newt extract, in support of the work of McGann et al. (2001).

In addition, this work provides further evidence indicating that the effect seen on myotubes was caused by proteins found in the regeneration extract and not proteins in the proliferating cells. Proteins fractions isolated from proliferating MEF cells were found to have no effect on myotube cell cycle re-entry. This indicates that the proteins in the regenerating extract induce cell cycle re-entry in myotubes.

4.4. Primary myotube cultures do not undergo cell cycle re-entry following treatment or injection with newt extract

Cell cycle re-entry was rarely found in primary multinucleated cells treated or injected with newt extract. A study by Pajcini et al. (2010) found that C2C12 myotubes can undergo cell cycle re-entry subsequent to inactivation of the retinoblastoma (Rb) tumor suppressor protein, which regulates the progression of proliferating myoblasts to terminal differentiation (Huh et al., 2004). Inactivation of Rb in primary myotubes did not result in cell cycle re-entry. However, cell cycle re-entry in primary myotubes was induced when both the Rb and $p19^{ARF}$ tumor suppressor are inactivated (Pajcini et al., 2010). $p19^{ARF}$ is one of the genes that encode the INK4a/ARF locus. $p19^{ARF}$ has been implicated in controlling cell growth, arrest and survival (Sharpless and DePinho, 1999). Some cell lines are found to have deletions in the INK4a/ARF locus, which allows the cells to continue to grow in culture (Ivanchuk et al., 2001). In primary culture cells, which do not divide indefinitely, the expression level of ARF is usually found to be quite high as cells reach their limited life span (Ivanchuk et al., 2001). Pajcini et al. (2010) found that C2C12 cell lines had a deletion in the $p19^{ARF}$ gene. Given that newt extract does not cause cell cycle re-entry in primary myotubes, it appears that the extract is lacking a factor which may inactivate ARF to allow primary myotubes to undergo cell cycle re-entry.

The intrinsic properties of primary myotubes may also prevent them from undergoing cell cycle re-entry. Our lab was able to establish a primary newt A2 muscle cell line from the limb of the newt *Notophthalmus viridescens (*unpublished work). Jason Vanstone (a Ph.D student) found that early passages of myotubes derived from newt A2 cells do not undergo cell cycle re-entry in the presence of elevated serum. Myotubes of passage twelve and above were able to synthesize DNA subsequent to serum stimulation. This raises the question as to whether cells that undergo cell cycle re-entry have accumulated mutations as a

result of being in culture for a longer period of time. Since primary myotubes used in these studies were newly established cultures, it is possible that this is the reason why they did not show cell cycle re-entry following extract treatment.

4.5. Fragmentation of C2C12 myotubes into "myosacs" or mononucleated cells following treatment with newt extract

According to McGann et al. (2001) C2C12 myotubes treated with newt extract are able to give rise to mononucleated cells that are able to proliferate in culture. Since myotube cultures are never free of contaminating myoblasts, the "proliferation" seen by McGann could possibly have been from these contaminating myoblasts. We decided to evaluate fragmentation of myotubes using live imaging microscopy. Following myotubes with the live imaging microscope, we were able to track myotubes in culture over a period of time as the cells underwent fragmentation. Ara-C treated myotubes fragmented into distorted round small myotubes after 63 h following treatment with newt extract (figure 24). The unusual distorted myotubes are referred to as "myosacs" by Palmer et al. (2001). The round myosacs were still present in culture even after 72 h. The newt extract seems to cause myotubes to retract (including myotubes that did not fragment) and form myosacs in cultures. However, placing cells back in differentiation medium without the newt extract causes the myosacs to regain their original elongated structure.

During myoblast differentiation, microtubule structures assemble forming elongated myotubes (Gundersen et al., 1989). Disruption of microtubules with drugs such as colchicine results in myotube fragmentation and changing shape into myosac myotubes (Bischoff and Holtzer, 1968). Myosacs being present in cultures treated with newt extract

may indicate that newt extract contains factors involved in disrupting the microtubules of mammalian myotubes.

C2C12 myotubes that were injected with newt extract protein fraction > than 30 kDa and GFP, and tracked with live imaging microscopy, were found to undergo fragmentation. The C2C12 myotube that underwent fragmentation following injection with newt extract was tracked further and found to undergo further fragmentation (or potentially cell division). This demonstrates that the fragmented cells following injection with newt extract do survive, and are able to fragment again or to proliferate. This data agrees with McGann et al. (2001) that found C2C12 myotubes are able to break down into mononucleated cells, and synthesize DNA following treatment with newt extract.

4.6. Fragmentation of primary myotubes following treatment with newt extract

Cell cycle reentry was not detected in primary myotube cultures following treatment or injection with newt extract. However, the newt extract appeared to induce fragmentation of primary multinucleated myotubes. This might indicate that the mechanisms involved in cell cycle re-entry and fragmentation in myotubes following treatments with newt extract are separate. Ara-C treated primary myotube cultures were found to undergo fragmentation into mononucleated cells following treatment with newt extract. At the moment, it not clear if the ara-C treated myotubes that fragmented went on to die by apoptosis. However, in subsequent experiments, primary myotubes that were injected with the newt extract protein fraction greater than 30 kDa and GFP underwent fragmentation into mononucleated cells, and survived for at least another 51 h in culture. This suggests that fragmentation does not necessarily result in apoptosis. Additional work is needed to determine whether the

fragmented cells continue to survive in culture and are able to re-differentiate again into multinucleated myotubes.

In order to determine if primary myotube fragmentation leads to apoptosis, we treated the myotubes with myoseverin. Myoseverin is a microtubule binding protein, and it has previously been used to treat C2C12 myotubes. In C2C12s, myoseverin induced fragmentation into mononucleated cells but the mononucleated cells were then capable of refusing into multinucleated myotubes (Rosania et al., 2000). We decided to test whether a similar process occurs in primary myotubes. We found that myoseverin is able to induce fragmentation of primary myotubes into smaller myotubes or mononucleated cells. When the fragmented cells were placed back in differentiation medium without myoseverin, they were able to re-fuse into multinucleated myotubes. This shows that fragmentation does not lead to cell death.

4.7. Newt extract affects the expression of myosin heavy chain in muscle cells

Myotube dedifferentiation is linked to down-regulation of differentiated muscle markers. Markers such as myosin heavy chain (MHC) are expressed in myotubes in culture. In C2C12 myotubes injected with the GFP plasmid, and treated with the 2º newt extract the expression of MHC was not present in GFP positive multinucleated cells. Furthermore, GFP positive mononucleated cells, which were negative for MHC, were detected in cultures that were treated with newt extract. Since only multinucleated cells were injected, the GFP mononucleated cells were most likely produced from fragmentation of multinucleated myotubes. This particular experiment was not carried out on primary myotubes. Nevertheless, reduction of MHC was never observed in primary cultures that were treated with newt extract. Cell cycle re-entry was only detected in C2C12 differentiated cells and

not in primary cells. Reduction of MHC might be a prerequisite to cell cycle re-entry in myotubes.

4.8. Differential gene expression in C2C12 and primary myotube cultures following treatment with newt extract.

The data that supports dedifferentiation in mammalian muscle cells suggests that factors including cell cycle regulators, transcription factors or ECM remodeling genes play a role in dedifferentiation (Pajcini et al., 2010; Rosania et al., 2000). The newt extract seems to up-regulate genes involved in transcription, protein folding, growth, response to drugs, positive regulation of gene expression, wound healing, chromatin modification, etc. in C2C12 myotubes cultures. Genes involved in skeletal muscle development, cell adhesion, and ossification were down-regulated in C2C12 myotube cultures. Surprisingly, genes involved in growth, G1/S transition of the mitotic cell cycle, wound healing, chromatin modification and cell proliferation were down-regulated in primary myotubes cultures. Genes involved in cell adhesion, extracellular matrix, ossification, muscle tissue development, positive regulation of the immune system, actin cytoskeleton reorganization, tissue remodeling, etc. were also found down-regulated in primary myotube cultures.

Myf5 is a marker for satellite cells and myoblasts but is downregulated in myocytes and myotubes. *Myf5* was up-regulated in C2C12 myotube cultures following treatment with newt extract. Since, we have found that the newt extract does not increase myoblast proliferation, the increase in *myf5* following extract treatment may have resulted from dedifferentiation of myotubes and not increased proliferation of contaminating myoblasts.

Genes encoding stress proteins were also up-regulated in C2C12 myotube cultures following treatment with newt extract. Heat shock proteins play a role in protein folding and unfolding. Their function is to regulate the internal environment of the living cell under

stress conditions (Feder and Hofmann, 1999). The expression of these proteins becomes elevated as cells are exposed to stress conditions (Michels et al., 1997). Up regulation of stress related genes has previously been implicated in dedifferentiation. During limb regeneration in urodeles, the expression level of *Hsp-70* was elevated when the dedifferentiation process was occurring (Levesque et al., 2005). In our study, DnaJ (Hsp40) homolog, subfamily B, member 1, and DnaJ (Hsp40) homolog, subfamily C, member 18 were up regulated in C2C12 differentiated cells following treatment with newt extract. Other genes affected by newt extract in C2C12 myotube cultures included H2-K region expressed gene 2, chaperonin containing Tcp1, subunit 4 (delta), heat shock protein 1 (chaperonin 10), and prefoldin 2. Interestingly, the newt extract did not affect genes encoding stress proteins in primary myotube cultures. Up-regulation of the stress proteins might protect C2C12 cells from undergoing apoptosis. The expression of these genes was not detected in primary cultures. If the up regulation of stress genes follows cell cycle re-entry, then primary cells, which did not re-enter the cell cycle following extract treatment would also not upregulate stress genes.

The newt extract also seems to affect genes involved in controlling cell proliferation in C2C12 myotubes cultures. E2F transcription factor, which functions to regulate cell-cycle progression from G1 to S phase, was up-regulated in C2C12 myotube cultures following treatment with newt extract. Metallothioneins which are also linked to cell proliferation, were up-regulated in C2C12 differentiated cells following treatment with newt extract. Expression of metallothionein has been detected in rat developing liver and regeneration tissues and in cancer cells (Andrews et al., 1987; Cai et al., 1998; Tsujikawa et al., 1994). These results suggest that the newt extract affects genes that control cell

proliferation in C2C12 myotube cultures. In primary myotube cultures, though, several genes that are implicated in cell proliferation were down-regulated by newt extract. An example is cyclin D1, which forms a complex with CDK4 to regulate cell cycle G1/S transition.

Newt extract also affects genes implicated in muscle atrophy in primary myotubes cultures. *Tumor necrosis factor alpha-induced protein 3* (TNFAIP3) was up-regulated in primary cultures following treatment with newt extract. TNFAIP3 has been implicated in muscle atrophy (Bhatnagar et al., 2010). The events involved in muscle wasting also include disruption of the ECM (Maria, 2006). The ECM genes down-regulated in primary muscle cells following treatment with newt extract included collagen 1alpha, collagen III alpha 1, collagen IV alpha 1, collagen IVA2, collagen IVA5, collagen VA1, collagen VA2, collagen XVIIIA1, as well as laminin B1 subunit 1. In C2C12 cells, the ECM genes affected by the newt extract included collagen IV alpha 5, collagen VIII alpha 1, collagen XII alpha 1, decorin, fibulin 5 and nidogen 2. Myofibril genes including PDC and LIM domain 5, matrix metallopeptidase 2, or nebulin were also down-regulated by newt extract in C2C12 or primary differentiated muscle cultures. Genes associated with the sarcolemma, actomyosin and cytoskeleton were largely affected by the newt extract in primary differentiated muscle cultures.

Actin binding including caldesmon, calponin, dystrophin, destrin, myosins, synaptopodin, trophomyosin, and utrophin genes were also down regulated in primary muscle cells following treatment with newt extract. Furthermore, microtubule binding genes including myosin VA, microtubule-associated protein, RP/EB family, member 2 and 3, and cytoskeleton associated protein 5 were found down-regulated in primary myotube cultures

subsequent to treatment with newt extract. Disruption of the microtubules by myoseverin induces fragmentation of multinucleated cells (Rosania et al., 2000) . This may indicate that newt extract affect genes that play a role in myotube fragmentation. However, fragmentation of myotubes following treatment with newt extract usually is detected after 2 days in culture, but the gene expression profiling was done in cultures that were treated with newt extract for 24 hrs. It is likely that the gene profiling in primary myotubes is linked to muscle atrophy or cell death rather than dedifferentiation.

The hepatoma-derived growth factor (HDGF), which is a DNA binding gene, was one of the genes that was up-regulated in both C2C12 and primary differentiated muscle cells following treatment with newt extract. The expression of HDGF has been coupled to myoblast differentiation. Over-expression of HDGF was found to repress a myogenic activator gene SMYD1, which is involved in myoblast differentiation and myotube formation in C2C12 cells (Li et al., 2009 and Yang and Everett, 2010). We observed inhibition of differentiation in both C2C12 and primary myoblasts following treatment with newt blastema extract. It is possible that the increase in expression of HDGF was the effect of newt extract on myoblasts or myocytes. This may indicate that newt extract has an effect on negative regulators of myoblast differentiation. This also indicates the newt extract has an effect on myoblasts, myocytes and myotubes.

4.9. RT-PCR analysis of gene expression in C2C12 and primary myotube cultures following treatment with newt extract

The data generated from GeneChip analysis did not show genes involved in myogenesis affected by the newt extract with the exception of *myf5* in C2C12 myotube cultures. However, since the GeneChip analysis was only conducted at 24 hrs after extract

treatment it is possible that some of the genes may be expressed at later time points. For that reason, we decided to analyze a few of the genes using real-time PCR. In addition, we validated the results for *cyclin D1*, which was found to be down-regulated in primary myotube cultures. Furthermore, we assayed the effect of newt extract on genes implicated in dedifferentiation.

Real-time PCR confirmed that *Myf5* expression is up-regulated in C2C12 myotubes cultures treated with newt extract. In primary myotube cultures treated with newt extract, a rapid response of *myf5* gene expression was detected after 4 and 8. Myoseverin, which promotes fragmentation of myotubes into mononucleated cells, seems to increase *myf5* levels (Rosania et al., 2000). Thus the increase in expression level of *myf5* in both C2C12 and primary myotube cultures treated with newt extract may be related to the fact that newt extract promotes fragmentation of myotubes. The *myf5* increase in extract treated myotubes cultures could also suggest that newt extract affects genes that negatively regulate myoblast differentiation.

In the presence of ara-C, the newt extract appears to cause no change in *myogenin* expression in primary and a slight increase in C2C12 myotube cultures at 48 h (figure 42), which is in disagreement with McGann et al. (2001). This group indicated that newt extract decreases the expression of *myogenin* in myotubes. Our subsequent experiments in microinjected (non ara-C) myotubes confirmed that *myogenin* was indeed decreased in the presence of extract. The contradictory nature of the effects on myogenin in the presence or absence of ara-C may be attributed to the toxic effects of ara-C on myotubes. In the microinjection studies the newt extract was found also to induce cell cycle re-entry and fragmentation in myotubes. This may suggest that a decrease in *myogenin* leads to

dedifferentiation in myotubes, which was also indicated by Mastroyiannopoulos et al. (2012).

Msx-1 is implicated in inducing fragmentation of myotubes as well as inhibiting myoblast differentiation (Hu et al., 2001; Odelberg et al., 2000). The expression level of *msx-1* was up-regulated in both primary and C2C12 myotube cultures that were treated with newt extract. The newt extract seems to inhibit differentiation in both C2C12 and primary myoblasts. This suggests that the newt extract affects negative regulators of muscle differentiation and genes involved in dedifferentiation. Pax-3 belongs to the family of paired box transcription factors. The expression level of this gene was significantly up-regulated in primary myotube cultures that were treated with newt extract. Pax-3 is also implicated in repressing myoblast differentiation (Epstein et al., 1995).

Expression levels of proliferating markers including cyclin D1 and proliferating cell nuclear antigen (PCNA) were evaluated following treatment with newt extract. *PCNA* and *cyclin D1* were increased in C2C12 myotube cultures following treatment with newt extract. In primary cultures, the expression levels of *PCNA* did not change in comparison to the control. Moreover the expression of *cyclin D1* was decreased in primary myotube cultures following treatment with newt extract, as shown by microarray analysis and real-time PCR. This makes sense considering that primary myotubes did not re-enter the cell cycle following extract treatment. The importance of cyclin D1 in cell cycle progression was shown by Pajcini et al. (2010). Cell cycle re-entry in primary myocytes by inactivation of Rb and $p19^{ARF}$ was accompanied by upregulation of proliferating markers.

4.10. Newt extract inhibits myogenic differentiation

Newt extract suppressed the differentiation of confluent myoblasts. The blastema extract of *Sternopygus macrurus* were also found to inhibit differentiation of mammalian myoblast (Kim et al., 2008). Myogenic differentiation involves induction of myogenic markers, cell cycle exit, up regulation of mature muscle markers (eg. myosin heavy chain) and fusion of myoblasts into multinucleated myotubes. Proliferating myoblasts do not express myogenic markers such as myogenin, a muscle specific transcription factor (Andres and Walsh, 1996). Placing cells in low mitogen serum causes the differentiating cells to express myogenin. Mononucleated cells expressing myogenin begin to express p21, a cyclin-dependent kinase inhibitor, resulting in cell cycle exit (Andres and Walsh, 1996). Myoblast differentiation can be repressed by factors including fibroblast growth factor, high serum medium and transforming growth factor beta (Massague et al., 1986; Spizz et al., 1986). Fibroblast growth factor suppresses myoblast differentiation via the protein kinase C pathway, which inhibits myogenin (Li et al., 1992). Unfortunately, the effect of newt extract on myogenin expression in differentiating myoblasts was not examined. However, the transcriptional level of *myogenin* was found reduced in myotubes injected with newt extract suggesting that newt extract has an effect on myogenin expression. The newt extract also could affect myoblast differentiation by up regulating genes which are involved in suppressing myogenic differentiation.

Msx-1, a homeobox gene family member is implicated in inhibiting myoblast differentiation. It is suggested that Msx-1 interacts with histone enzymes such as histone methyltransferase that play a role in chromatin modification, and this interaction leads to repression of myoD, an initiator for myogenic differentiation (Wang and Abate-Shen, 2012). The newt extract seems to affect the expression of *msx-1* in C2C12 myotube cultures.

Overexpression of Msx-1 in myotubes also reduces the expression of p21, a marker for myoblast differentiation (Odelberg et al., 2000). The newt extract also seems to affect p21expression. The protein levels were found reduced in C2C12 myotubes cultures following treatment with newt extract. Knowledge on how Msx-1 functions in repressing myoblast differentiation is beginning to emerge. However, more work needs to be done to have a better understanding on the mechanisms involved in dedifferentiation.

The newt extract also appears to affect myoblast fusion. Cell adhesion proteins including integrins are important in myoblast fusion (Chen and Olson, 2004). Microarray analysis showed a number of cell adhesion genes down-regulated in both C2C12 and primary myotubes cultures, and this may affect myoblast differentiation.

4.11. Newt extract has no effect on myoblast proliferation

Mitogenic factors including insulin-like growth factor 1 can stimulate myoblast proliferation via activation of cyclin D1 and D2 (Engert et al., 1996). Cyclin D1, which makes a complex with CDK4/6, phosphorylates and inactivates the retinoblastoma gene (pRb) (Connell-Crowley et al., 1997). As a result, pRb dissociates from E2F, a transcriptional factor, which activates cyclin E and A, and this pushes the cell through the cell cycle (Sherr and Roberts, 2004). Although, the newt extract seems likely to have mitogenic factors which may induce cell cycle progression, an increased effect on myoblast proliferation was not observed. This may have been because myoblasts were already maximally proliferating in the absence of newt extract.

5. Overall conclusion

The ultimate goal of understanding dedifferentiation is to one day apply it to mammalian cells to improve their regeneration potential. It may be that similar genes in newts and mammals may have slightly different functions of expression patterns that allow regeneration in one organism but do not have the same effect in the other. Most genes have similar profiles in muscle differentiation in newts and mammals. However, myf5 appears to be an exception. For example, myf5 has been shown in differentiated newt myotubes (Imokawa et al. 2004), whereas this gene is only expressed in less differentiated muscle or myoblasts in mammals. If this is the case, by understanding the role of these genes in regeneration and their expression pattern in newt versus mammalian cells, we may be able to alter their normal pattern and improve regeneration in higher vertebrates

Nevertheless, the work presented in this thesis shows that mammalian myotubes can be induced to dedifferentiate. Differentiated muscle markers (eg. myosin heavy chain (MHC) and myogenin) were down-regulated in C2C12 myotube cultures following treatment with newt extract. Furthermore, the newt extract induced cell cycle re-entry in C2C12 myotubes by affecting genes involved in cell proliferation, and chromatin remodeling. The newt extract did not induce cell cycle re-entry in primary multinucleated myotubes. Both C2C12 and primary myotubes can undergo fragmentation following treatment with newt extract. Genes implicated in fragmentation such as *msx-1* and *myf5* were affected in primary and C2C12 myotube cultures treated with extract. We also observed additional genes involved in fragmentation such as microtubule binding proteins. However, the expression of some of these genes may have been associated with myotube atrophy rather than dedifferentiation, in particular in primary myotube cultures.

A major question which remains to be answered is: what happens to the cells that have undergone cell cycle re-entry? Are they capable of continued proliferation and redifferentiation? Seeing that cell cycle re-entry was observed in C2C12 cells but not in primary myotubes, it is important to determine which cell lines are good models to use for understanding dedifferentiation. More work in the dedifferentiation field will need to be done to achieve the ultimate goal of enhancing the regenerative process in mammals.

References

Alitalo K., Kuismanen E., Myllyla R., Kiistala U., Asko-Seljavaara S., Vaheri A., 1982. Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells. Journal of Cell Biology 94, 497–505

Andres, V., Walsh, K., 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. The Journal of cell biology 132, 657-666.

Andrews, G.K., Gallant, K.R., Cherian, M.G., 1987. Regulation of the ontogeny of rat liver metallothionein mRNA by zinc. European journal of biochemistry / FEBS 166, 527-531.

Arellano, M., Moreno, S., 1997. Regulation of CDK/cyclin complexes during the cell cycle. The international journal of biochemistry & cell biology 29, 559-573.

Bailly, E., Doree, M., Nurse, P., Bornens, M., 1989. p34cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. The EMBO journal 8, 3985-3995.

Bendall, A.J., Ding, J., Hu, G., Shen, M.M., Abate-Shen, C., 1999. Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. Development (Cambridge, England) 126, 4965-4976.

Bentzinger, C.F., Wang, Y.X., Rudnicki, M.A., 2012. Building muscle: molecular regulation of myogenesis. Cold Spring Harbor perspectives in biology 4.

Bhatnagar, S., Panguluri, S.K., Gupta, S.K., Dahiya, S., Lundy, R.F., Kumar, A., 2010. Tumor necrosis factor-alpha regulates distinct molecular pathways and gene networks in cultured skeletal muscle cells. PloS one 5, e13262.

Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., Engler, J.A., 1993. Matrix metalloproteinases: a review. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists 4, 197-250.

Bodemer, C.W., Everett, N.B., 1959. Localization of newly synthesized proteins in regenerating newt limbs as determined by radioautographic localization of injected methionine-S35. Developmental biology 1, 327-342.

Boilly, B., Albert, P., 1988. Blastema cell proliferation in vitro: effects of limb amputation on the mitogenic activity of spinal cord extracts. Biology of the cell / under the auspices of the European Cell Biology Organization 62, 183-187.

Boilly, B., Cavanaugh, K.P., Thomas, D., Hondermarck, H., Bryant, S.V., Bradshaw, R.A., 1991. Acidic fibroblast growth factor is present in regenerating limb blastemas of axolotls and binds specifically to blastema tissues. Developmental biology 145, 302-310.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., Arnold, H.H., 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. The EMBO journal 8, 701-709.

Brockes, J.P., Kumar, A., 2002. Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nature reviews. Molecular cell biology 3, 566-574.

Bruning, J.C., Michael, M.D., Winnay, J.N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L.J., Kahn, C.R., 1998. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Molecular cell 2, 559-569.

Cai, L., Wang, G.J., Xu, Z.L., Deng, D.X., Chakrabarti, S., Cherian, M.G., 1998. Metallothionein and apoptosis in primary human hepatocellular carcinoma (HCC) from northern China. Anticancer research 18, 4667-4672.

Call, M.K., Tsonis, P.A., 2005. Vertebrate limb regeneration. Advances in biochemical engineering/biotechnology 93, 67-81.

Calve, S., Odelberg, S.J., Simon, H.G., 2010. A transitional extracellular matrix instructs cell behavior during muscle regeneration. Developmental biology 344, 259-271.

Calve, S., Simon, H.G., 2011. High resolution three-dimensional imaging: Evidence for cell cycle reentry in regenerating skeletal muscle. Developmental dynamics : an official publication of the American Association of Anatomists 240, 1233-1239.

Calve, S., Simon, H.G., 2012. Biochemical and mechanical environment cooperatively regulate skeletal muscle regeneration. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 26, 2538-2545.

Camarda, G., Siepi, F., Pajalunga, D., Bernardini, C., Rossi, R., Montecucco, A., Meccia, E., Crescenzi, M., 2004. A pRb-independent mechanism preserves the postmitotic state in terminally differentiated skeletal muscle cells. The Journal of cell biology 167, 417-423.

Carlson, B.M., 2005. Some principles of regeneration in mammalian systems. Anatomical record. Part B, New anatomist 287, 4-13.

Charge, S.B., Rudnicki, M.A., 2004. Cellular and molecular regulation of muscle regeneration. Physiological reviews 84, 209-238.

Chen, E.H., Olson, E.N., 2004. Towards a molecular pathway for myoblast fusion in Drosophila. Trends in cell biology 14, 452-460.

Chen, L., Huang, H.W., Gu, S.H., Xu, L., Gu, Y.D., Xu, J.G., 2011. The study of myogenin expression in denervated human skeletal muscles. The Journal of international medical research 39, 378-387.

Chen S., Choo A., Chin A., Oh SK., 2006. TGF-beta2 allows pluripotent human embryonic stem cell proliferation on E6/E7 immortalized mouse embryonic fibroblasts. Journal of Biotechnology. 122, 341–361.

Chen, Z.L., Yu, W.M., Strickland, S., 2007. Peripheral regeneration. Annual review of neuroscience 30, 209-233.

Chin, L., Pomerantz, J., DePinho, R.A., 1998. The INK4a/ARF tumor suppressor: one gene--two products--two pathways. Trends in biochemical sciences 23, 291-296.

Cooper, R.N., Tajbakhsh, S., Mouly, V., Cossu, G., Buckingham, M., Butler-Browne, G.S., 1999. In vivo satellite cell activation via Myf5 and Myod in regenerating mouse skeletal muscle. Journal of cell science 112, 2895-2901

Collins, C.A., 2006. Satellite cell self-renewal. Current opinion in pharmacology 6, 301-306.

Connell-Crowley, L., Harper, J.W., Goodrich, D.W., 1997. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Molecular biology of the cell 8, 287-301.

Cornelison, D.D., Olwin, B.B., Rudnicki, M.A., Wold, B.J., 2000. MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. Developmental biology 224, 122-137

Cossu, G., Tajbakhsh, S., Buckingham, M., 1996. How is myogenesis initiated in the embryo? Trends Genet 12, 218-223.

Dawson, B.A., Lough, J., 1988. Immunocytochemical localization of transient DNA strand breaks in differentiating myotubes using in situ nick-translation. Developmental biology 127, 362-367.

Dinsmore, C.E., 1996. Urodele limb and tail regeneration in early biological thought: an essay on scientific controversy and social change. The International journal of developmental biology 40, 621-627.

Endo, T., Bryant, S.V., Gardiner, D.M., 2004. A stepwise model system for limb regeneration. Developmental biology 270, 135-145.

Engert, J.C., Berglund, E.B., Rosenthal, N., 1996. Proliferation precedes differentiation in IGF-I-stimulated myogenesis. The Journal of cell biology 135, 431-440.

Epstein, J.A., Lam, P., Jepeal, L., Maas, R.L., Shapiro, D.N., 1995. Pax3 inhibits myogenic differentiation of cultured myoblast cells. The Journal of biological chemistry 270, 11719-11722.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual review of physiology 61, 243-282.

Ferretti, P., 2001. Regeneration of Vertebrate Appendages, eLS. John Wiley & Sons, Ltd.

Ferretti, P., Brockes, J.P., 1988. Culture of newt cells from different tissues and their expression of a regeneration-associated antigen. The Journal of experimental zoology 247, 77-91.

Francetic, T., Li, Q., 2011. Skeletal myogenesis and Myf5 activation. Transcription 2, 109-114.

Garfield AS., 2010. Derivation of primary mouse embryonic fibroblast (PMEF) cultures. Methods in Molecular Biology 633, 19-27

Geraudie, J., Ferretti, P., 1998. Gene expression during amphibian limb regeneration. International review of cytology 180, 1-50.

Greising, S.M., Gransee, H.M., Mantilla, C.B., Sieck, G.C., 2012. Systems biology of skeletal muscle: fiber type as an organizing principle. Wiley interdisciplinary reviews. Systems biology and medicine 4, 457-473.

Gulati, A.K., Reddi, A.H., Zalewski, A.A., 1983. Changes in the basement membrane zone components during skeletal muscle fiber degeneration and regeneration. The Journal of cell biology 97, 957-962.

Gundersen, G.G., Khawaja, S., Bulinski, J.C., 1989. Generation of a stable, posttranslationally modified microtubule array is an early event in myogenic differentiation. The Journal of cell biology 109, 2275-2288.

Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., Beach, D., Lassar, A.B., 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 267, 1018-1021.

Hay, E.D., 1958. The fine structure of blastema cells and differentiating cartilage cells in regenerating limbs of Amblystoma larvae. The Journal of biophysical and biochemical cytology 4, 583-591.

Hay, E.D., 1959. Electron microscopic observations of muscle dedifferentiation in regenerating Amblystoma limbs. Developmental biology 1, 555-585.

Hay, E.D., Fischman, D.A., 1961. Origin of the blastema in regenerating limbs of the newt Triturus viridescens. An autoradiographic study using tritiated thymidine to follow cell proliferation and migration. Developmental biology 3, 26-59.

Hjiantoniou, E., Anayasa, M., Nicolaou, P., Bantounas, I., Saito, M., Iseki, S., Uney, J.B., Phylactou, L.A., 2008. Twist induces reversal of myotube formation. Differentiation; research in biological diversity 76, 182-192.

Hu, G., Lee, H., Price, S.M., Shen, M.M., Abate-Shen, C., 2001. Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. Development (Cambridge, England) 128, 2373-2384.

Huh, M.S., Parker, M.H., Scime, A., Parks, R., Rudnicki, M.A., 2004. Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. The Journal of cell biology 166, 865-876.

Imokawa, Y., Gates, P.B., Chang, Y., Simon, H., Brockes, J.P., 2004. Distinctive expression of *Myf5* in relation to differentiation and plasticity of newt muscle cells. International Journal of developmental biology 48, 285-291

Iten, L., Bryant, S., 1973. Forelimb regeneration from different levels of amputation in the newt,Notophthalmus viridescens: Length, rate, and stages. W. Roux' Archiv f. Entwicklungsmechanik 173, 263-282.

Ito, M., Hayashi, T., Kuroiwa, A., Okamoto, M., 1999. Lens formation by pigmented epithelial cell reaggregate from dorsal iris implanted into limb blastema in the adult newt. Development, growth & differentiation 41, 429-440.

Ivanchuk, S.M., Mondal, S., Dirks, P.B., Rutka, J.T., 2001. The INK4A/ARF locus: role in cell cycle control and apoptosis and implications for glioma growth. Journal of neuro-oncology 51, 219-229.

Jahn, L., Sadoshima, J., Izumo, S., 1994. Cyclins and cyclin-dependent kinases are differentially regulated during terminal differentiation of C2C12 muscle cells. Experimental cell research 212, 297-307.

Johnson, D.G., Walker, C.L., 1999. Cyclins and cell cycle checkpoints. Annual review of pharmacology and toxicology 39, 295-312.

Juliano, R.L., Haskill, S., 1993. Signal transduction from the extracellular matrix. The Journal of cell biology 120, 577-585.

Kaczmarczyk, S.J., Green, J.E., 2001. A single vector containing modified cre recombinase and LOX recombination sequences for inducible tissue-specific amplification of gene expression. Nucleic acids research 29, E56-56.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G., Sherr, C.J., 1997. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell 91, 649-659.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., Tajbakhsh, S., 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature 431, 466-471.

Kherif, S., Lafuma, C., Dehaupas, M., Lachkar, S., Fournier, J.G., Verdiere-Sahuque, M., Fardeau, M., Alameddine, H.S., 1999. Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: a study in experimentally injured and mdx muscles. Developmental biology 205, 158-170.

Kim, H.J., Archer, E., Escobedo, N., Tapscott, S.J., Unquez, G. A., 2008. Inhibition of mammalian muscle differentiation by regeneration blastema extract of Sternopygus macrurus. Developmental dynamics 237, 2830-48

Kim, W.H., Jung, D.W., Kim, J., Im, S.H., Hwang, S.Y., Williams, D.R., 2012. Small molecules that recapitulate the early steps of urodele amphibian limb regeneration and confer multipotency. ACS chemical biology 7, 732-743.

Kintner, C.R., Brockes, J.P., 1985. Monoclonal antibodies to the cells of a regenerating limb. Journal of embryology and experimental morphology 89, 37-55.

Kitzmann, M., Fernandez, A., 2001. Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. Cellular and molecular life sciences : CMLS 58, 571-579.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H.H., Tanaka, E.M., 2009. Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature 460, 60-65.

Kruger, M., Mennerich, D., Fees, S., Schafer, R., Mundlos, S., Braun, T., 2001. Sonic hedgehog is a survival factor for hypaxial muscles during mouse development. Development (Cambridge, England) 128, 743-752.

Kuang, S., Rudnicki, M.A., 2008. The emerging biology of satellite cells and their therapeutic potential. Trends in molecular medicine 14, 82-91.

Kumar, A., Velloso, C.P., Imokawa, Y., Brockes, J.P., 2000. Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. Developmental biology 218, 125-136.

Lallemand, Y., Nicola, M.A., Ramos, C., Bach, A., Cloment, C.S., Robert, B., 2005. Analysis of Msx1; Msx2 double mutants reveals multiple roles for Msx genes in limb development. Development (Cambridge, England) 132, 3003-3014.

Levesque, M., Guimond, J.C., Pilote, M., Leclerc, S., Moldovan, F., Roy, S., 2005. Expression of heat-shock protein 70 during limb development and regeneration in the axolotl. Developmental dynamics : an official publication of the American Association of Anatomists 233, 1525-1534.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M.P., Olson, E.N., 1992. FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell 71, 1181-1194.

Lippincott-Schwartz, J., Patterson, G.H., 2003. Development and use of fluorescent protein markers in living cells. Science 300, 87-91.

Lo, D.C., Allen, F., Brockes, J.P., 1993. Reversal of muscle differentiation during urodele limb regeneration. Proc Natl Acad Sci U S A 90, 7230-7234.

Maden, M., 1999. Axolotl/newt. Methods in molecular biology (Clifton, N.J.) 97, 415-428.

Mailman, M.L., Dresden, M.H., 1976. Collagen metabolism in the regenerating forelimb of Notophthalmus viridescens: synthesis, accumulation, and maturation. Developmental biology 50, 378-394.

Markiewski, M.M., Mastellos, D., Tudoran, R., DeAngelis, R.A., Strey, C.W., Franchini, S., Wetsel, R.A., Erdei, A., Lambris, J.D., 2004. C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. Journal of immunology (Baltimore, Md. : 1950) 173, 747-754.

Massague, J., Cheifetz, S., Endo, T., Nadal-Ginard, B., 1986. Type beta transforming growth factor is an inhibitor of myogenic differentiation. Proceedings of the National Academy of Sciences of the United States of America 83, 8206-8210.

Mastroyiannopoulos, N.P., Nicolaou, P., Anayasa, M., Uney, J.B., Phylactou, L.A., 2012. Down-regulation of myogenin can reverse terminal muscle cell differentiation. PloS one 7, e29896.

Masuck, T.M., Taylor, A.R., Lough, J., 1990. Arabinosylcytosine-induced accumulation of DNA nicks in myotube nuclei detected by in situ nick translation. Journal of cellular physiology 144, 12-17.

McGann, C.J., Odelberg, S.J., Keating, M.T., 2001. Mammalian myotube dedifferentiation induced by newt regeneration extract. Proceedings of the National Academy of Sciences of the United States of America 98, 13699-13704.

Meech, R., Gomez, M., Woolley, C., Barro, M., Hulin, J.A., Walcott, E.C., Delgado, J., Makarenkova, H.P., 2010. The homeobox transcription factor Barx2 regulates plasticity of young primary myofibers. PloS one 5, e11612.

Michalopoulos, G.K., 2007. Liver Regeneration. Journal of cellular physiology 213, 286-300.

Michels, A.A., Kanon, B., Konings, A.W., Ohtsuka, K., Bensaude, O., Kampinga, H.H., 1997. Hsp70 and Hsp40 chaperone activities in the cytoplasm and the nucleus of mammalian cells. The Journal of biological chemistry 272, 33283-33289.

Miller, J.B., 1990. Myogenic programs of mouse muscle cell lines: expression of myosin heavy chain isoforms, MyoD1, and myogenin. The Journal of cell biology 111, 1149-1159.

Morrison, J.I., Loof, S., He, P., Simon, A., 2006. Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population. The Journal of cell biology 172, 433-440.

Moscatelli D., Presta M., Joseph-Silverstein J., Rifkin D.B., 1986. Both normal and tumor cells produce basic fibroblast growth factor. Journal of cellular physiology 129, 273–276

Myers, T.K., Andreuzza, S.E., Franklin, D.S., 2004. p18INK4c and p27KIP1 are required for cell cycle arrest of differentiated myotubes. Experimental cell research 300, 365-378.

Odelberg, S.J., Kollhoff, A., Keating, M.T., 2000. Dedifferentiation of mammalian myotubes induced by msx1. Cell 103, 1099-1109.

Ohkubo, Y., Kishimoto, T., Nakata, T., Yasuda, H., Endo, T., 1994. SV40 large T antigen reinduces the cell cycle in terminally differentiated myotubes through inducing Cdk2, Cdc2, and their partner cyclins. Experimental cell research 214, 270-278.

Ott, M.O., Bober, E., Lyons, G., Arnold, H., Buckingham, M., 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development (Cambridge, England) 111, 1097-1107.

Pajcini, K.V., Corbel, S.Y., Sage, J., Pomerantz, J.H., Blau, H.M., 2010. Transient inactivation of Rb and ARF yields regenerative cells from postmitotic mammalian muscle. Cell stem cell 7, 198-213.

Palmer, S., Groves, N., Schindeler, A., Yeoh, T., Biben, C., Wang, C.C., Sparrow, D.B., Barnett, L., Jenkins, N.A., Copeland, N.G., Koentgen, F., Mohun, T., Harvey, R.P., 2001. The small muscle-specific protein Csl modifies cell shape and promotes myocyte fusion in an insulin-like growth factor 1-dependent manner. The Journal of cell biology 153, 985-998.

Parker, M.H., Perry, R.L., Fauteux, M.C., Berkes, C.A., Rudnicki, M.A., 2006. MyoD synergizes with the E-protein HEB beta to induce myogenic differentiation. Molecular and cellular biology 26, 5771-5783.

Parkinson, D.B., Bhaskaran, A., Arthur-Farraj, P., Noon, L.A., Woodhoo, A., Lloyd, A.C., Feltri, M.L., Wrabetz, L., Behrens, A., Mirsky, R., Jessen, K.R., 2008. c-Jun is a negative regulator of myelination. The Journal of cell biology 181, 625-637.

Parrales, A., Lopez, E., Lopez-Colome, A.M., 2011. Thrombin activation of PI3K/PDK1/Akt signaling promotes cyclin D1 upregulation and RPE cell proliferation. Biochimica et biophysica acta 1813, 1758-1766.

Peault, B., Rudnicki, M., Torrente, Y., Cossu, G., Tremblay, J.P., Partridge, T., Gussoni, E., Kunkel, L.M., Huard, J., 2007. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Molecular therapy : the journal of the American Society of Gene Therapy 15, 867-877.

Repesh, L.A., Oberpriller, J.C., 1980. Ultrastructural studies on migrating epidermal cells during the wound healing stage of regeneration in the adult newt, Notophthalmus viridescens. The American journal of anatomy 159, 187-208.

Rosania, G.R., Chang, Y.T., Perez, O., Sutherlin, D., Dong, H., Lockhart, D.J., Schultz, P.G., 2000. Myoseverin, a microtubule-binding molecule with novel cellular effects. Nature biotechnology 18, 304-308.

Sanchez, I., Dynlacht, B.D., 2005. New insights into cyclins, CDKs, and cell cycle control. Seminars in cell & developmental biology 16, 311-321.

Sassoon, D., Lyons, G., Wright, W.E., Lin, V., Lassar, A., Weintraub, H., Buckingham, M., 1989. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature 341, 303-307.

Schneider, J.W., Gu, W., Zhu, L., Mahdavi, V., Nadal-Ginard, B., 1994. Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. Science 264, 1467-1471.

Sharpless, N.E., DePinho, R.A., 1999. The INK4A/ARF locus and its two gene products. Current opinion in genetics & development 9, 22-30.

Sherr, C.J., Roberts, J.M., 2004. Living with or without cyclins and cyclin-dependent kinases. Genes & development 18, 2699-2711.

Shimizu-Nishikawa, K., Tsuji, S., Yoshizato, K., 2001. Identification and characterization of newt rad (ras associated with diabetes), a gene specifically expressed in regenerating limb muscle. Developmental dynamics : an official publication of the American Association of Anatomists 220, 74-86.

Simon, H.G., Nelson, C., Goff, D., Laufer, E., Morgan, B.A., Tabin, C., 1995. Differential expression of myogenic regulatory genes and Msx-1 during dedifferentiation and redifferentiation of regenerating amphibian limbs. Developmental dynamics : an official publication of the American Association of Anatomists 202, 1-12.

Spicer, D.B., Rhee, J., Cheung, W.L., Lassar, A.B., 1996. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science 272, 1476-1480.

Spizz, G., Roman, D., Strauss, A., Olson, E.N., 1986. Serum and fibroblast growth factor inhibit myogenic differentiation through a mechanism dependent on protein synthesis and independent of cell proliferation. The Journal of biological chemistry 261, 9483-9488.

Springer, M.L., Rando, T.A., Blau, H.M., 2002. Gene delivery to muscle. Current protocols in human genetics / editorial board, Jonathan L. Haines ... [et al.] Chapter 13, Unit13 14.

Strack, R.L., Strongin, D.E., Bhattacharyya, D., Tao, W., Berman, A., Broxmeyer, H.E., Keenan, R.J., Glick, B.S., 2008. A noncytotoxic DsRed variant for whole-cell labeling. Nature methods 5, 955-957.

Tajbakhsh, S., Rocancourt, D., Cossu, G., Buckingham, M., 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 89, 127-138.

Tanaka, E.M., 2003. Cell differentiation and cell fate during urodele tail and limb regeneration. Current opinion in genetics & development 13, 497-501.

Tanaka, E.M., Gann, A.A., Gates, P.B., Brockes, J.P., 1997. Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. The Journal of cell biology 136, 155-165.

Tapscott, S.J., Davis, R.L., Thayer, M.J., Cheng, P.F., Weintraub, H., Lassar, A.B., 1988. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. Science 242, 405-411.

Tassava, R.A., Nace, J.D., Wei, Y., 1996. Extracellular matrix protein turnover during salamander limb regeneration. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 4, 75-81.

Tiainen, M., Pajalunga, D., Ferrantelli, F., Soddu, S., Salvatori, G., Sacchi, A., Crescenzi, M., 1996. Terminally differentiated skeletal myotubes are not confined to G0 but can enter G1 upon growth factor stimulation. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research 7, 1039-1050.

Tsonis, P.A., Madhavan, M., Tancous, E.E., Del Rio-Tsonis, K., 2004. A newt's eye view of lens regeneration. The International journal of developmental biology 48, 975-980.

Tsujikawa, K., Suzuki, N., Sagawa, K., Itoh, M., Sugiyama, T., Kohama, Y., Otaki, N., Kimura, M., Mimura, T., 1994. Induction and subcellular localization of metallothionein in regenerating rat liver. European journal of cell biology 63, 240-246.

Velloso, C.P., Kumar, A., Tanaka, E.M., Brockes, J.P., 2000. Generation of mononucleate cells from post-mitotic myotubes proceeds in the absence of cell cycle progression. Differentiation; research in biological diversity 66, 239-246.

Vinarsky, V., Atkinson, D.L., Stevenson, T.J., Keating, M.T., Odelberg, S.J., 2005. Normal newt limb regeneration requires matrix metalloproteinase function. Developmental biology 279, 86-98.

Wallace, T.L., Johnson, E.M., Jr., 1989. Cytosine arabinoside kills postmitotic neurons: evidence that deoxycytidine may have a role in neuronal survival that is independent of DNA synthesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 9, 115-124.

Wang, J., Abate-Shen, C., 2012. The MSX1 homeoprotein recruits G9a methyltransferase to repressed target genes in myoblast cells. PloS one 7, e37647.

Wang, J., Walsh, K., 1996. Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science 273, 359-361.

Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B., Miller, A.D., 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proceedings of the National Academy of Sciences of the United States of America 86, 5434-5438.

Yokoyama, H., 2008. Initiation of limb regeneration: the critical steps for regenerative capacity. Development, growth & differentiation 50, 13-22.

Zabludoff, S.D., Csete, M., Wagner, R., Yu, X., Wold, B.J., 1998. p27Kip1 is expressed transiently in developing myotomes and enhances myogenesis. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research 9, 1-11.

Zhang, Y., 2012. Single-cell microinjection technologies. Methods in molecular biology (Clifton, N.J.) 853, 169-176.

Table 2a: ***Up-regulated genes in C2C12 myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1418380_at	Terf1	308.5125	177.865	**1.80**
1435595_at	1810011O10Rik	121.7551	66.9058	**1.80**
1427213_at	Pfkfb1	56.3523	31.6481	**1.80**
1449042_at	Ctcf	711.747	401.804	**1.80**
1442427_at	---	44.39405	20.73845	**1.81**
1454928_at	E130307D12	148.9775	85.352	**1.81**
1419964_s_at	Hdgf	2142.76	1305.851	**1.82**
1439200_x_at	D14Wsu89e	1178.55	667.9315	**1.82**
1452146_a_at	Cox15	124.7075	69.0411	**1.82**
1452621_at	Pcbd2	420.647	241.531	**1.82**
1455108_at	Eif4el3	871.8465	477.9655	**1.83**
1452406_x_at	Erdr1	557.81	312.339	**1.83**
1417408_at	F3	140.45	82.4574	**1.84**
1439794_at	Ntn4	442.731	260.7575	**1.84**
1450464_at	E4f1	65.50605	35.5695	**1.84**
1430020_x_at	Hnrpa1	2651.935	1499.045	**1.84**
1437828_s_at	Bing4	382.9805	208.111	**1.84**
1440029_at	St8sia3	18.14035	9.93191	**1.84**
1416804_at	LOC114601	795.9185	525.447	**1.85**
1419572_a_at	Abcd4	149.9765	85.91395	**1.85**
1421425_a_at	Dscr1l1	279.8095	167.9927	**1.85**
1424728_at	Rrp36	198.0615	119.5833	**1.85**
1424954_a_at	Pip5k1c	151.6525	81.1792	**1.85**
1420757_at	Myf5	4724.395	2547.62	**1.85**
1426564_at	Zfp553	219.8005	118.772	**1.86**
1428942_at	Mt2	2326.04	1245.77	**1.86**
1417654_at	Sdc4	5489.64	3155.805	**1.86**
1433747_at	Lnpep	7853.115	4419.4	**1.86**
1456531_x_at	Prpf19	16165.3	9482.84	**1.86**
1447577_x_at	Pcca	32.2693	17.27975	**1.86**
1436833_x_at	Ttll1	691.224	393.886	**1.86**
1437069_at	Osbpl8	224.6715	129.9553	**1.87**
1436813_x_at	Khsrp	582.993	337.9895	**1.87**
1442150_at	---	18.80265	11.19316	**1.87**
1429893_at	Il17rd	385.4405	227.1645	**1.87**
1447936_at	2410006H16Rik	745.6805	398.586	**1.87**
1433758_at	Nisch	160.9025	85.57845	**1.87**
1435340_at	Jmjd2	861.2555	488.0335	**1.88**
1420905_at	Il17r	702.4705	385.9605	**1.88**
1455436_at	Diras2	28.76815	17.11455	**1.88**
1451339_at	Suox	559.1405	326.946	**1.89**
1420411_a_at	Pi4k2b	146.4865	95.04175	**1.89**
1448400_a_at	Smarcd2	3571.835	2044.265	**1.89**
1429775_a_at	Tm7sf1	341.017	212.9232	**1.89**
1447612_x_at	Kdm6b	1000.518	584.2235	**1.89**
1416633_a_at	Fam96a	1527.385	908.291	**1.89**
1437585_x_at	Zfp161	289.249	153.968	**1.89**
1416184_s_at	Hmga1	786.329	433.7045	**1.90**
1452351_at	Parp4	31.59875	16.4647	**1.90**
1432004_a_at	Dnm2	21.9304	11.40705	**1.90**

Table 2b: *Up-regulated genes in C2C12 myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1433962_at	Trmt61a	1170.815	643.0365	**1.90**
1437334_x_at	Parn	341.909	182.986	**1.90**
1455075_at	Pigv	54.4387	29.2263	**1.91**
1440826_s_at	2610002I17Rik	67.2179	35.3442	**1.91**
1419209_at	Cxcl1	122.397	66.48615	**1.91**
1415744_at	H2-Ke2	777.326	437.1165	**1.91**
1427390_at	Bloc1s3	118.6385	71.97095	**1.91**
1424916_x_at	Zfp764	138.6356	72.3224	**1.92**
1441967_at	Pddc1	39.96225	21.32745	**1.92**
1449178_at	Pdlim3	2088.305	1199.251	**1.92**
1426260_a_at	Ugt1a1	404.51	210.337	**1.92**
1439516_at	2610201A13Rik	61.7092	35.50195	**1.93**
1420159_at	Myo1e	20.81225	11.86139	**1.93**
1436125_at	D16Ertd472e	241.4065	132.4758	**1.93**
1445843_at	Chd2	229.983	118.5855	**1.94**
1437638_at	Srrm2	258.3215	132.5575	**1.94**
1418258_s_at	Arhgdia	3397.215	1778.12	**1.94**
1457468_at	---	48.0627	25.1953	**1.94**
1456393_at	Pdcd4	4930.17	2732.44	**1.94**
1417790_at	Dok1	131.051	68.8313	**1.95**
1416755_at	Dnajb1	3680.815	2054.86	**1.95**
1440755_at	---	318.2895	171.531	**1.95**
1419308_at	Invs	51.49545	26.0627	**1.95**
1437594_x_at	Pigt	69.7917	35.4622	**1.95**
1440076_at	---	286.543	145.4115	**1.95**
1452063_at	Zbtb8a	21.54155	14.85282	**1.96**
1436546_at	Lix1l	482.3035	302.8915	**1.96**
1442223_at	Enah	46.38315	26.70995	**1.96**
1439965_at	Slc43a2	1968.645	1054.48	**1.97**
1452378_at	Malat1	1667.955	834.619	**1.97**
1451214_at	Kbtbd2	2025.865	1015.345	**1.98**
1456662_at	AA386476	47.2092	24.5046	**1.99**
1445229_at	Dgat1	99.52005	50.93935	**1.99**
1450686_at	Pon2	1366.555	742.2695	**1.99**
1417878_at	E2f1	239.4255	120.6073	**2.00**
1434040_at	Appbp2	107.4385	57.59055	**2.00**
1421122_at	Cbll1	210.1745	105.5097	**2.00**
1416862_at	Stam	727.5875	395.287	**2.00**
1455964_at	Cdk12	272.551	136.9575	**2.01**
1438755_at	C80068	419.483	219.587	**2.01**
1436459_at	---	24.9457	12.4281	**2.02**
1427049_s_at	Smo	219.3305	112.3693	**2.02**
1417584_at	Slc11a2	222.709	116.0001	**2.02**
1419728_at	Cxcl5	165.828	80.63725	**2.03**
1450881_s_at	Tm7sf1	1527.65	745.0445	**2.03**
1457459_at	---	22.5965	11.10605	**2.04**
1433920_at	Sema4c	2751.755	1431.121	**2.04**
1419879_s_at	Trim25	2223.34	1119.324	**2.05**
1455556_at	Notch2	2486.93	1341.87	**2.05**
1423531_a_at	Hnrnpa1	1979.97	1032.764	**2.05**

Table 2c: *Up-regulated genes in C2C12 myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1434660_at	Alkbh1	620.8465	316.8965	**2.05**
1430019_a_at	Hnrpa1	1822.81	940.7265	**2.05**
1436175_at	Sca7	121.211	58.77245	**2.06**
1447706_at	---	66.22765	32.2346	**2.06**
1418134_at	Slc25a46	3529.935	1834.98	**2.07**
1451104_a_at	Snrp70	994.2015	526.7255	**2.07**
1421344_a_at	Jub	1051.18	613.8765	**2.07**
1440778_x_at	Zfp712	62.21975	36.8164	**2.07**
1433932_x_at	C030046I01Rik	933.5295	475.497	**2.07**
1429008_at	1810074P20Rik	24.21965	11.2405	**2.07**
1419403_at	Fn5	40.7801	21.1747	**2.08**
1415908_at	Tspyl	1475.88	755.4025	**2.09**
1433960_at	Isg20l2	154.99	75.9386	**2.09**
1444328_at	Clta	110.1828	60.11695	**2.09**
1445143_at	Vash1	149.042	71.31415	**2.10**
1433916_at	Vamp3	1061.225	591.0915	**2.12**
1456005_a_at	Bcl2l11	371.685	183.8985	**2.13**
1439255_s_at	Gpr137b-ps	2714.67	1249.905	**2.14**
1433931_at	C030046I01Rik	577.6855	286.691	**2.14**
1450668_s_at	Hspe1	1796.365	865.441	**2.14**
1424424_at	Slc39a1	1404.595	834.863	**2.14**
1416597_at	Hdgfrp2	72.80805	37.03155	**2.15**
1417470_at	Apobec3	225.375	106.7511	**2.15**
1447977_x_at	Gm14430	653.06	302.286	**2.15**
1434437_x_at	Rrm2	7068.27	4027.4	**2.16**
1450882_s_at	Tm7sf1	645.3405	313.9735	**2.17**
1417541_at	Hells	47.0844	22.12425	**2.18**
1424407_s_at	Nptxr	378.2235	177.685	**2.18**
1434471_at	BC003331	784.6485	423.13	**2.18**
1435616_at	Cyp20a1	2291.145	1315.987	**2.18**
1451070_at	Gdi1	5660.77	2841.205	**2.18**
1423251_at	Luc7l2	2238.995	1108.135	**2.20**
1424620_at	Nop16	221.908	111.3522	**2.20**
1428498_at	Rnf219	300.377	134.8315	**2.21**
1421950_at	Pfdn2	1538.46	790.295	**2.21**
1425617_at	Dhx9	311.0325	157.8745	**2.21**
1444806_at	---	123.5035	62.48935	**2.21**
1418008_at	Gcfc1	127.358	58.56085	**2.23**
1452690_at	Khsrp	177.847	82.74995	**2.25**
1441229_at	---	33.5017	15.5244	**2.26**
1443486_at	5.63E+30	2589.325	1352.512	**2.27**
1460608_at	Cacna1b	30.4548	13.96152	**2.28**
1423124_x_at	Rad54l	56.7564	24.724	**2.30**
1417409_at	Jun	362.252	159.59	**2.30**
1455111_at	Yipf6	988.0315	458.585	**2.33**
1455899_x_at	Socs3	1864.12	832.9845	**2.34**
1416949_s_at	Slc39a7	4703.545	2403.305	**2.34**
1417698_at	Gtf2f1	838.6305	406.5595	**2.34**
1425974_a_at	Trim25	646.195	275.646	**2.38**
1457002_at	Zfp408	554.0935	274.226	**2.40**

Table 2d: ***Up-regulated genes in C2C12 myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1445626_at	---	75.2251	32.42785	**2.43**
1416368_at	Gsta4	595.8995	246.8265	**2.43**
1429041_at	---	203.014	81.90315	**2.45**
1428081_at	Klhl21	1337.04	603.763	**2.46**
1440169_x_at	Ifnar2	48.9834	19.69765	**2.49**
1451121_a_at	Gltscr2	183.1875	77.45075	**2.49**
1441520_at	Calmbp1	39.3531	16.57845	**2.51**
1442569_at	---	131.0941	52.94115	**2.54**
1427882_at	Dnttip2	161.3695	64.07185	**2.55**
1449130_at	Cd1d1	504.0345	220.3005	**2.55**
1419839_x_at	Prpf19	1136.045	569.6945	**2.57**
1434863_at	Dnajc18	69.45365	26.77325	**2.59**
1449635_at	Prpf19	1112.57	541.0945	**2.60**
1440346_at	Kdm6b	1126.76	587.0325	**2.64**
1453007_at	3110082I17Rik	175.259	74.62985	**2.67**
1452719_at	Zdhhc24	314.169	143.2112	**2.72**
1436004_at	Usp27x	132.683	47.91795	**2.75**
1434326_x_at	Coro2b	76.30845	24.3881	**2.75**
1451286_s_at	Fus	2522.1	1260.306	**2.78**
1436403_at	Fam171a2	692.2645	366.4533	**2.84**
1451044_at	Sip1	120.99	43.1906	**2.89**
1448440_x_at	Il25	956.807	448.1725	**2.92**
1448439_at	Il25	1200.08	562.6875	**3.00**
1457692_at	---	91.4792	34.4005	**3.20**
1420725_at	Tmlhe	1234.665	581.176	**3.24**
1420726_x_at	Tmlhe	968.9985	463.5975	**3.30**
1443694_at	Rgs20	1137.638	423.303	**3.46**
1434427_a_at	Rnf157	2053.45	773.2625	**3.65**
1457246_at	Rai16	8317.44	3169.589	**3.70**
1442849_at	Lrp1	276.854	107.5876	**3.76**
1420904_at	Il17r	57.4429	16.57346	**3.95**
1451285_at	Fus	1245.905	569.2099	**4.18**
1427760_s_at	Plf	1409.36	326.3125	**4.35**

Table 3: ***Up-regulated genes in primary myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1449639_at	---	142.3448	74.3878	**1.80**
1420038_at	Atp6v1e1	79.91025	42.2469	**1.81**
1428196_a_at	Fam82a2	1566.78	864.745	**1.81**
1447591_x_at	A830082N09Rik	37.79545	20.63695	**1.82**
1420264_at	---	103.8876	49.77745	**1.82**
1424145_at	Prr3	101.1977	52.6073	**1.82**
1443184_at	Cdc14a	18.51431	8.51104	**1.85**
1420400_at	Sval1	18.2818	9.29516	**1.88**
1455203_at	A930003A15Rik	138.5765	74.2669	**1.88**
1438106_at	Pcdhb22	24.01655	10.51485	**1.89**
1437663_at	Pigv	19.50715	9.51919	**1.91**
1418913_at	Bhmt2	37.3452	19.0684	**1.94**
1433699_at	Tnfaip3	275.054	132.948	**1.96**
1439200_x_at	D14Wsu89e	1485.98	760.2175	**1.96**
1435909_at	C030034I22Rik	29.8346	13.9344	**1.97**
1460583_at	Golt1b	53.2358	27.16755	**1.97**
1427853_a_at	Hspb1	271.269	137.3541	**1.97**
1424490_at	Zfp428	57.54395	29.0589	**1.99**
1455899_x_at	Socs3	808.214	403.4335	**2.01**
1431281_at	Ppp1r27	332.836	161.4095	**2.02**
1459897_a_at	Sbsn	424.419	209.238	**2.03**
1427867_at	Myh1	1035.177	451.516	**2.04**
1457632_s_at	Mrg1	45.4114	17.6535	**2.06**
1432130_a_at	Ttc14	35.10475	17.038	**2.06**
1422562_at	Rrad	269.47	133.4749	**2.11**
1444981_at	Punc	35.23055	15.7271	**2.12**
1437256_at	Dcun1d5	97.1599	44.7399	**2.16**
1426928_at	Ccdc93	71.85405	32.097	**2.16**
1436716_at	Ppp1r14b	14.85935	6.666015	**2.20**
1454373_x_at	Ubc	333.0715	141.4515	**2.21**
1420074_at	D8Ertd738e	137.139	54.373	**2.21**
1452406_x_at	Erdr1	562.439	273.4315	**2.21**
1439630_x_at	Sbsn	146.23	65.42115	**2.24**
1433082_at	4930448K20Rik	45.2313	16.41685	**2.34**
1440171_x_at	1700049G17Rik	90.319	39.83095	**2.46**
1424067_at	Icam1	63.4309	23.98445	**2.70**
1449681_at	Hdgf	205.496	75.7221	**2.70**
1458299_s_at	Nfkbie	164	46.4286	**3.37**
1436936_s_at	Xist	2011.865	396.0735	**5.14**
1427262_at	Xist	882.6	138.6159	**6.70**

Table 4a: ***Down-regulated genes in C2C12 myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1439571_at	E230008J23Rik	20.88875	65.31095	**-3.10**
1421832_at	Twsg1	20.3592	59.7903	**-3.02**
1416164_at	Fbln5	216.684	648.8765	**-2.90**
1435831_at	Upk1b	11.70781	40.85295	**-2.77**
1448416_at	Mglap	1686.13	4466.39	**-2.58**
1428891_at	Parm1	12.5831	38.74195	**-2.54**
1434172_at	Cnr1	18.17515	48.94965	**-2.53**
1417262_at	Ptgs2	235.051	616.9865	**-2.51**
1449434_at	Car3	336.269	879.1565	**-2.51**
1423942_a_at	Camk2g	38.2839	132.867	**-2.46**
1448735_at	Cp	22.9748	54.94965	**-2.43**
1423153_x_at	Cfh	160.2085	387.0505	**-2.40**
1434411_at	Cox7a2	758.509	1881.89	**-2.39**
1455645_at	Mybpc1	421.578	1002.243	**-2.35**
1419024_at	Ptp4a1	11.4175	34.2128	**-2.33**
1456599_at	Nxt2	13.74405	34.24825	**-2.29**
1434243_s_at	Tomm70a	21.9647	61.91245	**-2.26**
1422983_at	Itgb6	21.91435	44.75245	**-2.24**
1416205_at	Glb1	94.1075	193.0175	**-2.15**
1460684_at	Tm7sf2	601.4645	1324.408	**-2.13**
1424232_a_at	Ftsjd1	7.714795	17.4944	**-2.11**
1454075_s_at	Nudt13	32.00175	74.2302	**-2.10**
1421413_a_at	Pdlim5	68.45065	153.8485	**-2.10**
1434381_at	Atmin	13.92965	32.0884	**-2.10**
1431980_a_at	As3mt	32.37195	57.2515	**-2.09**
1428895_at	Rftn2	20.0534	51.5526	**-2.08**
1450757_at	Cdh11	18.9518	42.06525	**-2.07**
1425476_at	Col4a5	188.5285	385.7205	**-2.06**
1439425_x_at	Mettl22	33.96035	73.5546	**-2.06**
1425789_s_at	Anxa8	84.18755	183.272	**-2.05**
1437467_at	Alcam	47.51025	96.0175	**-2.02**
1426913_at	Lss	42.96365	76.7536	**-2.01**
1424348_at	Mcmbp	17.0052	54.6661	**-2.00**
1418450_at	Islr	1040.963	2066.04	**-1.99**
1454699_at	Sesn1	29.0323	60.97055	**-1.98**
1448220_at	Ctrb1	48.9069	102.6017	**-1.97**
1430462_at	9430066l12Rik	23.6345	46.45085	**-1.96**
1455176_a_at	Syt11	11.29733	20.3217	**-1.95**
1423033_at	Itm1	48.42935	90.9003	**-1.95**
1437532_at	Rnf216	33.0361	71.8136	**-1.93**
1448364_at	Ccng2	7.343655	18.72728	**-1.92**
1451348_at	R75183	261.7885	510.6785	**-1.92**
1433943_at	Itprip	133.0735	260.483	**-1.91**
1423516_a_at	Nid2	193.701	383.653	**-1.89**
1451991_at	Epha7	51.7642	91.7882	**-1.88**
1416981_at	Foxo1	123.759	257.817	**-1.87**
1451203_at	Mb	2374.93	4459.97	**-1.87**
1449368_at	Dcn	860.852	1686.41	**-1.87**
1416190_a_at	Sec61a1	33.26535	64.73865	**-1.87**

Table 4b: ***Down-regulated genes in C2C12 myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1417732_at	Anxa8	556.067	1056.332	**-1.87**
1418155_at	Ttid	313.4315	585.022	**-1.87**
1420858_at	Pkia	42.1363	89.15685	**-1.87**
1423267_s_at	Itga5	19.96875	43.9059	**-1.87**
1437045_at	Mapk8	25.91285	63.38305	**-1.86**
1422902_s_at	Mgea5	18.32415	42.8113	**-1.86**
1416319_at	Adk	59.21285	109.3515	**-1.86**
1435355_at	Neb	1200.095	2354.41	**-1.85**
1416121_at	Lox	1431.04	2725.555	**-1.85**
1432001_at	Zmynd17	483.0925	891.467	**-1.84**
1431072_a_at	Ccdc50	71.6792	129.446	**-1.84**
1456661_at	---	19.37805	34.5443	**-1.84**
1452173_at	Hadha	75.6415	180.6677	**-1.84**
1438783_at	---	109.6892	199.93	**-1.83**
1423584_at	Igfbp7	3834.49	7007.71	**-1.83**
1428909_at	A130040M12Rik	48.69635	88.79575	**-1.83**
1453149_at	Slc25a32	17.7316	32.6622	**-1.82**
1421028_a_at	Mef2c	99.48155	241.5756	**-1.82**
1449048_s_at	Rab4a	128.0305	255.1525	**-1.82**
1422909_at	Smc6l1	24.2842	43.0723	**-1.81**
1435378_at	2210020M01Rik	109.5025	205.136	**-1.81**

Table 5a: Down-regulated genes in primary myotube cultures following treatment with newt extract

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1432466_a_at	Apoe	4.649115	187.5317	**-30.91**
1448591_at	Ctss	252.6931	3091.33	**-13.64**
1423547_at	Lyzs	94.9789	1264.599	**-12.26**
1439426_x_at	Lyzs	502.002	4736.685	**-11.51**
1436996_x_at	Lyz1	1344.904	12528.51	**-11.04**
1415823_at	Scd2	25.28504	197.5365	**-9.56**
1417262_at	Ptgs2	99.13865	932.8265	**-9.22**
1439768_x_at	Sema4f	11.2825	100.5811	**-8.53**
1436905_x_at	Laptm5	174.1075	1530.711	**-8.41**
1420699_at	Clecsf12	106.5776	780.835	**-7.28**
1423586_at	Axl	57.45125	224.2205	**-7.03**
1423091_a_at	Gpm6b	22.79105	180.0665	**-6.94**
1418440_at	Col8a1	10.6509	68.7668	**-6.17**
1452077_at	Ddx3y	210.3651	717.6345	**-5.99**
1450958_at	Tm4sf1	85.2307	492.9645	**-5.80**
1450678_at	Itgb2	10.34137	77.27445	**-5.70**
1423407_a_at	Fbln2	77.8925	458.3675	**-5.68**
1450781_at	Hmga2	31.68775	165.998	**-5.65**
1449164_at	Cd68	4.39575	40.42383	**-5.54**
1451718_at	Plp	24.08615	133.9247	**-5.29**
1449153_at	Mmp12	11.15798	77.4347	**-5.29**
1423606_at	Postn	156.5675	913.1505	**-5.26**
1455627_at	Col8a1	144.564	739.652	**-5.11**
1419706_a_at	Akap12	46.68555	241.7545	**-5.09**
1423505_at	Tagln	61.2164	305.663	**-4.92**
1416022_at	Fabp5	69.15545	361.095	**-4.82**
1434709_at	Nrcam	32.96085	160.2125	**-4.81**
1427076_at	Mpeg1	51.88655	238.464	**-4.66**
1436970_a_at	Pdgfrb	53.34025	284.561	**-4.65**
1416021_a_at	Fabp5	224.853	1085.121	**-4.61**
1431004_at	Loxl2	6.281965	33.3688	**-4.58**
1419149_at	Serpine1	272.588	1258.84	**-4.50**
1460302_at	Thbs1	46.9199	220.8935	**-4.49**
1416136_at	Mmp2	25.25095	121.8318	**-4.48**
1448303_at	Gpnmb	126.5976	569.815	**-4.37**
1416805_at	Fam198b	43.35165	197.177	**-4.30**
1448865_at	Hsd17b7	35.49825	139.4115	**-4.23**
1448228_at	Lox	231.434	997.643	**-4.21**
1426438_at	Ddx3y	384.3235	1222.37	**-4.14**
1427183_at	Efemp1	85.9982	352.844	**-4.13**
1449145_a_at	Cav	77.51935	318.2845	**-4.13**
1455494_at	Col1a1	361.0275	1098.378	**-4.02**
1457823_at	Cyr61	72.8007	291.149	**-4.01**
1422124_a_at	Ptprc	7.969745	34.74985	**-3.99**
1442340_x_at	Cyr61	82.08115	325.475	**-3.97**
1418252_at	Padi2	164.825	648.784	**-3.96**
1424701_at	Pcdh20	8.869185	35.5623	**-3.94**
1425466_at	Senp2	50.00258	96.4508	**-3.92**
1416953_at	Ctgf	1311.498	5076.28	**-3.89**
1450857_a_at	Col1a2	459.8955	1830.59	**-3.87**

Table 5b: *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1417210_at	Eif2s3y	530.0925	1592.6	**-3.86**
1422795_at	Cul3	67.6005	105.6627	**-3.84**
1423078_a_at	Sc4mol	162.3801	580.189	**-3.81**
1416529_at	Emp1	325.1035	1228.005	**-3.79**
1438133_a_at	Cyr61	78.9818	266.8145	**-3.78**
1433796_at	Endod1	40.6838	119.3975	**-3.74**
1416034_at	Cd24a	70.9734	178.3795	**-3.73**
1416808_at	Nid1	433.591	1658.955	**-3.72**
1416749_at	Prss11	105.7016	402.6755	**-3.69**
1415824_at	Scd2	64.4424	234.3495	**-3.69**
1433434_at	AW551984	97.09205	266.5725	**-3.68**
1423680_at	Fads1	64.18875	237.4645	**-3.64**
1437726_x_at	C1qb	87.57005	288.129	**-3.63**
1424186_at	Ccdc80	120.284	443.023	**-3.61**
1434520_at	Sc5d	42.67335	129.7675	**-3.57**
1423110_at	Col1a2	275.042	985.1605	**-3.57**
1434360_s_at	Ptprg	18.83286	57.27305	**-3.56**
1456250_x_at	Tgfbi	28.56145	93.4591	**-3.54**
1416637_at	Slc4a2	61.09545	205.617	**-3.52**
1428306_at	Ddit4	71.9904	252.664	**-3.48**
1420394_s_at	Gp49b	13.32075	66.33855	**-3.45**
1443801_at	---	29.32065	98.54565	**-3.45**
1451122_at	Idi1	908.564	3017.79	**-3.38**
1443017_at	Cpeb2	60.90405	205.7415	**-3.38**
1434423_at	Gulp1	24.4271	78.7051	**-3.37**
1426439_at	Ddx3y	144.3956	445.305	**-3.35**
1416530_a_at	Pnp	29.42565	99.3964	**-3.34**
1450085_at	Angptl2	44.50181	94.18075	**-3.34**
1428572_at	Basp1	215.3395	662.82	**-3.33**
1448259_at	Fstl1	137.7315	488.015	**-3.33**
1451726_at	Mtmr6	73.5168	124.1615	**-3.32**
1421840_at	Abca1	137.8464	389.333	**-3.32**
1437360_at	Pcdh19	82.10545	284.4345	**-3.32**
1457373_at	---	7.472695	37.2761	**-3.28**
1460208_at	Fbn1	120.63	397.8215	**-3.25**
1435484_at	Slc5a3	118.7635	386.3515	**-3.25**
1425896_a_at	Fbn1	50.35615	160.3105	**-3.20**
1452361_at	Rnf20	16.62122	47.86165	**-3.19**
1426936_at	---	13.31365	39.159	**-3.19**
1452035_at	Col4a1	502.119	1698.875	**-3.19**
1422842_at	Xrn2	91.7309	279.9465	**-3.14**
1452105_a_at	Tsc2	28.5791	87.40065	**-3.14**
1421811_at	Thbs1	803.434	2479.46	**-3.13**
1455304_at	Unc13c	39.1552	117.892	**-3.10**
1423331_a_at	Pvrl3	237.9272	467.273	**-3.10**
1421276_a_at	Dst	64.321	139.0877	**-3.09**
1422053_at	Inhba	226.9435	688.912	**-3.08**
1433770_at	Dpysl2	116.1115	364.3065	**-3.08**
1423804_a_at	Idi1	835.171	2540.495	**-3.06**
1428573_at	Chn2	39.9626	121.8427	**-3.06**

Table 5c: ***Down-regulated genes in primary myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1447329_at	---	152.5369	370.9985	**-3.05**
1424768_at	Cald1	26.49465	80.3668	**-3.05**
1451263_a_at	Fabp4	25.73565	72.0938	**-3.04**
1434877_at	Nptx1	33.9492	76.3026	**-3.03**
1436329_at	Egr3	478.295	1455.24	**-3.02**
1424704_at	Runx2	11.12289	34.23215	**-3.02**
1448340_at	Cox7a2	66.30505	172.537	**-3.01**
1421821_at	Ldlr	577.295	1742.775	**-3.00**
1424603_at	Rpl36	36.71035	86.96915	**-2.99**
1455978_a_at	Matn2	27.53875	85.7985	**-2.99**
1415904_at	Lpl	588.6415	1756.075	**-2.98**
1419872_at	Csf1r	105.6369	325.7455	**-2.97**
1437231_at	Slitrk6	9.056045	27.45785	**-2.95**
1450652_at	Ctsk	33.4521	96.09505	**-2.94**
1459885_s_at	Cox7c	71.2835	141.636	**-2.94**
1450646_at	Cyp51	379.5505	1117.08	**-2.93**
1454671_at	Insig1	559.3645	1636.24	**-2.93**
1459601_at	Snf1lk	40.9427	110.4099	**-2.93**
1434003_a_at	Dhps	43.74065	96.86235	**-2.92**
1417303_at	Mvd	23.5105	64.84595	**-2.92**
1415935_at	Smoc2	7.70464	22.4714	**-2.91**
1426955_at	Col18a1	80.1657	262.687	**-2.90**
1416164_at	Fbln5	43.30135	126.228	**-2.90**
1416039_x_at	Cyr61	487.483	1378.585	**-2.89**
1429637_at	Fam198b	10.92368	32.45955	**-2.89**
1425476_at	Col4a5	61.93015	172.9805	**-2.88**
1456174_x_at	Ndr1	38.14555	110.8249	**-2.87**
1452745_at	Trappc9	64.3457	163.0625	**-2.86**
1424051_at	Col4a2	164.1445	517.9805	**-2.86**
1454699_at	Sesn1	164.1658	345.304	**-2.85**
1431050_at	Rps6ka5	79.06775	179.986	**-2.85**
1420664_s_at	Procr	81.4238	204.396	**-2.84**
1417555_at	Atad1	32.55299	59.8356	**-2.82**
1426238_at	Bmp1	67.23125	187.2765	**-2.81**
1457296_at	Cilp	106.3615	292.39	**-2.81**
1416121_at	Lox	286.2855	826.057	**-2.81**
1427475_a_at	Pdlim5	142.6295	400.358	**-2.81**
1450065_at	Adcy7	37.7858	106.3602	**-2.81**
1431033_x_at	Agl	1693.465	4724.22	**-2.80**
1455812_x_at	Dnaja3	118.2695	337.33	**-2.80**
1416226_at	Arpc1b	83.4261	231.6215	**-2.78**
1440911_at	Col23a1	62.52765	192.273	**-2.78**
1451827_a_at	Nox4	16.2153	46.1675	**-2.78**
1423584_at	Igfbp7	645.9935	1811.62	**-2.78**
1426758_s_at	Gtl2	37.75025	93.22275	**-2.77**
1452008_at	Ttc39b	41.24675	93.37305	**-2.76**
1431032_at	Agl	1784.495	4927.185	**-2.76**
1418350_at	Dtr	220.322	576.0835	**-2.75**
1439364_a_at	Mmp2	54.78585	161.9603	**-2.74**
1429240_at	Stard4	33.5941	91.5313	**-2.74**

Table 5d: ***Down-regulated genes in primary myotube cultures following treatment with newt extract***

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1444422_at	Pcdh19	17.55885	48.6229	**-2.74**
1456214_at	Pcdh7	103.9183	242.52	**-2.73**
1433521_at	Ankrd13c	146.4677	319.2625	**-2.73**
1423828_at	Fasn	91.53585	262.7785	**-2.72**
1428909_at	A130040M12Rik	43.01545	117.0708	**-2.72**
1422437_at	Col5a2	1755.36	4915.21	**-2.71**
1449010_at	Osp94	89.1238	205.885	**-2.70**
1431057_a_at	Prss23	1825.755	4747.825	**-2.70**
1420505_a_at	Stxbp1	34.10375	86.4402	**-2.70**
1426590_at	Gfm2	46.4725	74.2823	**-2.70**
1415822_at	Scd2	4939.69	13042.75	**-2.69**
1424917_a_at	Wipi1	141.2271	342.7335	**-2.68**
1446835_at	---	48.3575	126.507	**-2.68**
1435637_at	Itfg1	138.8896	278.689	**-2.68**
1416221_at	Fstl1	157.202	431.0505	**-2.68**
1416342_at	Tnc	139.8915	377.6585	**-2.67**
1448558_a_at	Pla2g4a	95.2368	255.738	**-2.66**
1448914_a_at	Csf1	39.44335	113.4187	**-2.65**
1454903_at	Ngfr	106.3855	323.4135	**-2.65**
1452179_at	Jade1	31.96525	87.8693	**-2.65**
1449292_at	Rb1cc1	137.2135	364.2165	**-2.64**
1424495_a_at	Cklf	14.3368	32.01725	**-2.64**
1437467_at	Alcam	24.8913	62.3832	**-2.64**
1424348_at	Mcmbp	44.6013	75.95715	**-2.63**
1453796_a_at	Ergic2	58.5289	141.778	**-2.63**
1426642_at	Fn1	1082.57	3023.745	**-2.62**
1436778_at	Cybb	4.471165	15.98187	**-2.62**
1451666_at	Acly	137.7345	362.8815	**-2.62**
1437635_at	Dcbld2	157.922	402.392	**-2.62**
1415993_at	Sqle	253.2975	667.0445	**-2.61**
1434840_at	Agfg1	59.3984	127.732	**-2.61**
1439148_a_at	Pfkl	169.8621	405.1885	**-2.61**
1455180_at	Myzap	130.6774	308.624	**-2.61**
1448594_at	Wisp1	92.75715	243.8225	**-2.60**
1448698_at	Ccnd1	235.4925	609.222	**-2.59**
1423303_at	Paxip1	24.5071	54.91195	**-2.58**
1451828_a_at	Facl4	199.476	482.6685	**-2.57**
1458716_at	Dusp27	108.4327	188.6625	**-2.56**
1448214_at	Pdhb	51.45105	97.7738	**-2.56**
1450872_s_at	Lip1	75.29195	214.15	**-2.56**
1423100_at	Fos	673.071	1683.8	**-2.56**
1419599_s_at	Ms4a11	16.37925	48.91945	**-2.55**
1460573_at	AI848100	58.38235	103.5408	**-2.55**
1418129_at	Dhcr24	128.334	344.9615	**-2.55**
1448797_at	Elk3	19.85335	49.4779	**-2.55**
1458368_at	Myh4	472.971	1132.49	**-2.53**
1415964_at	Scd1	918.8145	2296.47	**-2.53**
1434920_a_at	Evl	51.2026	112.717	**-2.52**
1427760_s_at	Plf	41.33635	101.0876	**-2.52**
1448229_s_at	Ccnd2	149.126	376.5525	**-2.51**

Table 5e: Down-regulated genes in primary myotube cultures following treatment with newt extract

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1423418_at	Fdps	129.3262	308.968	**-2.51**
1416814_at	Tia1	162.8519	319.041	**-2.51**
1426300_at	Alcam	50.88005	121.6175	**-2.50**
1442082_at	C3ar1	13.6771	38.4022	**-2.50**
1451127_at	Vopp1	82.19365	134.431	**-2.50**
1435972_at	Arts1	47.7298	114.5951	**-2.49**
1451169_at	Nomo1	21.45345	48.8035	**-2.49**
1423516_a_at	Nid2	50.7618	143.0354	**-2.49**
1450010_at	Hsd17b12	44.67575	108.902	**-2.49**
1433776_at	Lhfp	234.6175	579.879	**-2.49**
1420850_at	Crnkl1	130.7654	240.539	**-2.49**
1451457_at	Sc5d	125.4195	292.5095	**-2.49**
1429148_at	Nfic	47.12655	98.617	**-2.48**
1415971_at	Marcks	106.3809	250.3605	**-2.47**
1455160_at	2610203C20Rik	156.0965	377.6925	**-2.47**
1417476_at	Fbxw5	26.8221	55.1911	**-2.47**
1422799_at	Bat2	108.1258	248.699	**-2.47**
1449315_at	Odz3	132.651	319.7235	**-2.46**
1417196_s_at	Wwc2	100.1263	223.774	**-2.45**
1419376_at	Fibin	35.56215	77.90525	**-2.45**
1435890_at	Atl3	12.40215	30.8646	**-2.45**
1423790_at	Dap	41.08875	90.5804	**-2.44**
1437874_s_at	Hexb	66.3053	161.448	**-2.44**
1434745_at	Ccnd2	269.0345	657.4695	**-2.44**
1425340_a_at	Ptpra	55.37425	121.8146	**-2.44**
1433474_at	Edil3	115.983	302.5455	**-2.44**
1416405_at	Bgn	761.021	1888.955	**-2.44**
1426561_a_at	Npnt	136.2607	353.573	**-2.43**
1455011_at	Stard4	33.0405	77.33495	**-2.42**
1450355_a_at	Capg	70.9249	173.0625	**-2.42**
1426503_a_at	Rnf121	19.41255	42.46485	**-2.42**
1435254_at	Plxnb1	45.28455	96.43005	**-2.42**
1451483_s_at	1700054N08Rik	34.90205	63.884	**-2.41**
1425795_a_at	Map3k7	51.12135	76.312	**-2.41**
1451413_at	Cast	145.0627	303.8355	**-2.40**
1455061_a_at	Acaa2	55.7786	85.1863	**-2.40**
1418152_at	Nsbp1	101.5414	236.4965	**-2.40**
1426892_at	Utrn	105.203	254.0285	**-2.40**
1416923_a_at	Bnip3l	43.271	83.03035	**-2.39**
1419211_s_at	4933424B01Rik	62.14835	122.8935	**-2.39**
1417492_at	Ctsb	80.54935	194.737	**-2.38**
1439096_at	Ddo	86.1266	185.865	**-2.38**
1426600_at	Slc2a1	37.8016	86.45905	**-2.38**
1441971_at	---	94.6091	224.1505	**-2.37**
1426260_a_at	Ugt1a1	32.1267	72.03645	**-2.37**
1418349_at	Dtr	111.766	264.8045	**-2.37**
1428383_a_at	2310021P13Rik	138.4933	338.278	**-2.37**
1419431_at	Ereg	33.48275	81.50775	**-2.36**
1460396_at	Ddx54	154.1169	257.7805	**-2.36**
1419821_s_at	Idh1	95.42125	186.814	**-2.36**

Table 5f: *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1456130_at	Thsd7a	50.3676	116.5895	**-2.35**
1454268_a_at	Cyba	67.5411	164.87	**-2.35**
1416687_at	Plod2	120.4282	258.696	**-2.35**
1429146_at	Gas2	101.6105	203.9125	**-2.35**
1450035_a_at	Fnbp3	78.5366	141.014	**-2.34**
1435222_at	Foxp1	13.85307	30.36315	**-2.34**
1448732_at	Ctsb	336.268	704.6285	**-2.33**
1439793_at	Gja3	105.1697	238.747	**-2.33**
1448383_at	Mmp14	215.46	517.931	**-2.33**
1451989_a_at	Mapre2	53.92295	117.225	**-2.32**
1427915_s_at	Tceb1	33.1151	77.17845	**-2.32**
1429775_a_at	Tm7sf1	15.06583	34.41715	**-2.32**
1451943_a_at	Ppm1a	105.0717	178.7615	**-2.31**
1454937_at	B630005N14Rik	234.654	478.677	**-2.31**
1427258_at	Trim24	76.9868	152.0785	**-2.31**
1456413_at	Pde4dip	46.3035	102.4538	**-2.30**
1423237_at	Galnt1	29.8428	63.87195	**-2.30**
1420093_s_at	Hnrpdl	285.634	555.8305	**-2.30**
1429178_at	Odz3	69.34955	158.341	**-2.29**
1427314_at	Tmed7	190.248	404.779	**-2.29**
1419456_at	Dcxr	75.97385	160.4815	**-2.28**
1417900_a_at	Vldlr	281.959	570.1	**-2.28**
1417420_at	Ccnd1	23.4651	53.5664	**-2.28**
1436334_at	Synj1	55.9992	99.33495	**-2.28**
1417673_at	Grb14	101.0401	214.111	**-2.28**
1454768_at	Kcnf1	68.52965	146.753	**-2.28**
1429028_at	Dock11	146.5225	365.815	**-2.28**
1435893_at	Vldlr	412.9145	855.9895	**-2.27**
1455492_at	B330016D10Rik	15.45223	33.61925	**-2.27**
1438634_x_at	Lasp1	1704.865	3798.015	**-2.27**
1451717_s_at	Senp2	117.8233	202.695	**-2.27**
1448593_at	Wisp1	168.0215	393.88	**-2.27**
1437797_at	Atp2a2	67.05995	149.3865	**-2.27**
1460633_at	Prpf19	244.726	543.416	**-2.27**
1419089_at	Timp3	92.2061	223.9405	**-2.27**
1448928_at	Hdac6	16.62048	38.94465	**-2.26**
1425545_x_at	H2-D1	174.3695	407.0545	**-2.26**
1418746_at	Brp17	118.7661	219.663	**-2.24**
1460320_at	Becn1	48.00715	76.53965	**-2.24**
1437811_x_at	---	10.67033	26.35825	**-2.24**
1435353_a_at	---	55.81435	81.27185	**-2.24**
1428944_at	Uba6	45.098	84.3843	**-2.23**
1430357_at	H3f3b	13.80425	31.899	**-2.23**
1450743_s_at	Syncrip	52.31005	115.633	**-2.23**
1449249_at	Pcdh7	19.5119	43.7964	**-2.22**
1417491_at	Ctsb	53.3493	117.6009	**-2.22**
1418043_at	Abcc5	10.50843	19.38155	**-2.22**
1452767_at	Rrbp1	145.0364	332.975	**-2.22**
1423894_a_at	Dalrd3	48.3892	119.5832	**-2.22**
1437409_s_at	Gpr126	21.2282	47.8795	**-2.22**

Table 5g: *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1433571_at	Serinc5	92.86955	214.958	**-2.21**
1450716_at	Adamts1	169.298	369.416	**-2.21**
1434376_at	Cd44	376.662	843.22	**-2.21**
1428877_at	Srp72	93.2658	167.559	**-2.21**
1436197_at	Cdc42bpg	111.1236	241.3885	**-2.21**
1429534_a_at	Immt	205.2839	353.7355	**-2.21**
1436188_a_at	Ndr4	4581.43	9643.375	**-2.21**
1418911_s_at	Facl4	62.7171	118.197	**-2.20**
1454741_s_at	Tmem164	42.20695	76.89775	**-2.20**
1424621_at	Krcc1	92.59745	146.2575	**-2.20**
1416915_at	Msh6	53.35945	102.1258	**-2.20**
1444429_at	Lrtm1	51.8394	126.8746	**-2.19**
1417124_at	Dstn	219.98	483.927	**-2.19**
1436917_s_at	Gpsm1	46.8705	79.12795	**-2.19**
1420922_at	Usp9x	219.3778	380.065	**-2.19**
1418202_a_at	Wiz	23.89829	43.5649	**-2.19**
1421033_a_at	Tcerg1	39.24475	68.8622	**-2.19**
1424914_at	2310044G17Rik	46.33415	86.44445	**-2.19**
1427468_at	Ppp3cb	143.7475	201.5645	**-2.19**
1443037_at	---	15.09455	32.7905	**-2.18**
1443847_x_at	Aff2	41.7369	88.46875	**-2.18**
1455470_x_at	Lasp1	110.0284	228.4995	**-2.18**
1452235_at	Man1b1	6.410025	19.98907	**-2.17**
1436870_s_at	Afap1l2	16.99145	37.25015	**-2.17**
1433736_at	Hcfc1	117.5972	218.7695	**-2.17**
1435514_at	Lztfl1	61.44645	76.9908	**-2.16**
1433883_at	Tpm4	330.145	720.3115	**-2.16**
1429348_at	Sema3c	279.453	586.7565	**-2.16**
1425911_a_at	Fgfr1	10.87444	22.31795	**-2.16**
1448617_at	Cd53	26.0708	64.7016	**-2.15**
1422526_at	Facl2	96.02005	206.1735	**-2.15**
1416442_at	Ier2	81.23	176.4255	**-2.15**
1438251_x_at	Prss11	168.77	362.301	**-2.15**
1454795_at	Cobll1	106.5794	216.0055	**-2.15**
1415959_at	Slc2a4	49.2336	91.8156	**-2.15**
1426528_at	Nrp2	65.6642	141.0725	**-2.15**
1416152_a_at	Sfrs3	113.1275	222.274	**-2.15**
1448123_s_at	Tgfbi	16.28435	43.27495	**-2.15**
1435315_s_at	3732409C05Rik	36.52405	75.94865	**-2.14**
1449335_at	Timp3	83.41565	199.07	**-2.14**
1433592_at	Calm1	302.0985	618.7935	**-2.14**
1455375_at	---	110.0491	234.407	**-2.14**
1434479_at	Col5a1	150.001	322.2025	**-2.14**
1417582_s_at	Dhodh	45.1742	88.62695	**-2.13**
1426709_a_at	Usp33	225.514	400.4905	**-2.13**
1427406_at	Trip11	86.15435	187.889	**-2.13**
1417379_at	Iqgap1	400.466	870.9315	**-2.13**
1455145_at	Pcdh19	97.6256	202.572	**-2.13**
1428387_at	Facl3	75.31145	154.987	**-2.13**
1418968_at	Rb1cc1	184.6421	329.3565	**-2.13**

Table 5h: *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1438633_x_at	Lasp1	1252.531	2597.505	**-2.12**
1425187_at	Sel1h	38.8164	75.30355	**-2.12**
1424114_s_at	Lamb1-1	166.4995	364.0745	**-2.12**
1417655_a_at	Ars2	205.8119	377.8185	**-2.12**
1419820_at	Pkhd1	10.90179	23.3328	**-2.12**
1448308_at	Ap3m1	166.0532	298.432	**-2.12**
1422414_a_at	Calm3	157.9355	332.1545	**-2.12**
1452520_a_at	Chrng	106.5663	194.3945	**-2.12**
1460328_at	Brd3	15.48323	31.40945	**-2.12**
1434707_at	Sbf1	52.72815	111.6682	**-2.11**
1429393_at	Dcaf12	95.57735	193.15	**-2.11**
1439496_at	Ston1	16.3584	34.4095	**-2.11**
1416406_at	Pea15	100.6705	213.5705	**-2.11**
1434178_at	Mll3	95.536	210.902	**-2.11**
1451348_at	Deptor	704.3515	1426.16	**-2.11**
1450928_at	Idb4	57.0986	112.6183	**-2.11**
1449151_at	Pctk3	69.05065	135.8624	**-2.11**
1418980_a_at	Cnp1	19.7227	42.8147	**-2.10**
1450786_x_at	Pdlim5	20.7648	46.49635	**-2.10**
1416122_at	Ccnd2	229.9325	479.7715	**-2.10**
1448323_a_at	Bgn	1310.88	2795.045	**-2.10**
1424570_at	Ddx46	17.2126	34.7948	**-2.10**
1421365_at	Fst	729.605	1504.455	**-2.10**
1451895_a_at	Dhcr24	267.978	561.7645	**-2.10**
1421891_at	Siat5	109.514	207.0435	**-2.10**
1419754_at	Myo5a	74.04015	149.1325	**-2.09**
1429145_at	Nhlrc2	288.0835	591.158	**-2.09**
1417404_at	Elovl6	39.50725	82.55775	**-2.09**
1417307_at	Dmd	172.2422	301.668	**-2.09**
1455403_at	Manea	84.23205	166.687	**-2.09**
1454806_at	Fam49a	62.33075	128.531	**-2.09**
1442150_at	---	12.11942	25.1646	**-2.09**
1415951_at	Fkbp10	15.4841	39.0675	**-2.09**
1450981_at	Cnn2	459.912	931.895	**-2.09**
1416607_at	4931406C07Rik	100.0167	171.863	**-2.09**
1454838_s_at	Pkdcc	19.33325	41.16535	**-2.09**
1422556_at	Gna13	12.56206	20.2737	**-2.09**
1452295_at	Pmepa1	53.52345	111.574	**-2.08**
1424737_at	Thrsp	122.547	251.869	**-2.08**
1453367_a_at	Abhd12	48.64375	93.0721	**-2.08**
1428107_at	Sh3bgrl	79.8844	167.8955	**-2.08**
1431098_at	Rsn	67.7828	137.2871	**-2.07**
1450757_at	Cdh11	72.4699	155.2665	**-2.07**
1417766_at	Cyb5b	227.3505	453.331	**-2.07**
1449876_at	Prkg1	86.8758	176.754	**-2.07**
1454604_s_at	Tspan12	603.5245	1213.41	**-2.07**
1419103_a_at	Abhd6	22.49505	41.862	**-2.07**
1451270_at	Dusp18	56.4211	121.7766	**-2.07**
1434768_at	Cln2	235.409	471.65	**-2.07**
1437101_at	Lats2	84.35105	157.0955	**-2.07**

Table 5i: *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1450731_s_at	Tnfrsf21	101.298	179.1515	**-2.07**
1434860_at	Dpy19l4	312.5835	570.3935	**-2.07**
1437475_at	BC028454	30.3795	60.72685	**-2.06**
1427294_a_at	Slc38a10	83.9368	175.98	**-2.06**
1438602_s_at	Masp1	14.89715	31.27625	**-2.06**
1457248_x_at	Hsd17b7	472.4215	969.2915	**-2.06**
1419917_s_at	Tmed7	446.839	841.2805	**-2.05**
1425599_a_at	Gatad1	224.0185	385.2515	**-2.05**
1429974_at	Tbx18	80.385	133.908	**-2.05**
1452905_at	Gtl2	191.443	391.6705	**-2.05**
1454763_at	Ankrd17	75.2718	120.0925	**-2.05**
1416262_at	Tmem19	56.197	107.1459	**-2.05**
1426871_at	Fbxo33	84.3943	140.4175	**-2.05**
1448392_at	Sparc	1823.98	3837.97	**-2.05**
1417409_at	Jun	151.791	306.307	**-2.05**
1419035_s_at	Csnk2a1	20.14035	37.28175	**-2.05**
1456623_at	Tpm1	69.6662	143.845	**-2.05**
1425658_at	Cd109	27.27255	56.81805	**-2.05**
1433702_at	Ermp1	162.577	315.977	**-2.05**
1440739_at	Vegfc	7.145165	14.53505	**-2.05**
1451620_at	Pter	259.465	465.425	**-2.04**
1424769_s_at	Cald1	843.6875	1705.855	**-2.04**
1452908_at	Dip2a	70.1719	126.4545	**-2.04**
1455482_at	Ap2a2	184.2813	297.8375	**-2.04**
1422453_at	Prpf8	283.092	544.73	**-2.04**
1435349_at	Nrp2	20.05845	40.25355	**-2.04**
1448272_at	Btg2	237.323	448.911	**-2.03**
1438945_x_at	Gja1	154.32	311.257	**-2.03**
1448870_at	Ltbp1	225.3085	444.688	**-2.03**
1442590_at	Tnfrsf22	646.5775	1317.425	**-2.03**
1416357_a_at	Mcam	40.18535	86.41215	**-2.03**
1416303_at	Litaf	133.858	272.668	**-2.03**
1420408_a_at	Abcc9	110.5997	223.4415	**-2.03**
1417953_at	Fam3c	192.8555	383.565	**-2.03**
1456482_at	Pik3r3	140.32	265.53	**-2.03**
1418547_at	Tfpi2	35.25095	74.7918	**-2.03**
1419238_at	Abca7	89.04725	175.8835	**-2.03**
1453744_a_at	Ankrd40	187.0441	310.859	**-2.02**
1426690_a_at	Srebf1	295.929	613.168	**-2.02**
1436909_at	Slc25a44	37.23685	60.82225	**-2.02**
1452163_at	Ets1	60.31465	108.3767	**-2.02**
1430127_a_at	Ccnd2	114.7685	233.297	**-2.02**
1441055_at	Palm2	57.8072	113.5405	**-2.02**
1460648_at	Nr2f6	68.62015	132.2506	**-2.02**
1434386_at	Atp2c1	95.4597	186.536	**-2.01**
1421871_at	Sh3bgrl	19.9961	37.14825	**-2.01**
1428449_at	Gtf3c2	67.33585	127.5116	**-2.01**
1423765_at	Athl1	32.21815	59.0903	**-2.01**
1425273_s_at	Emp2	40.95945	68.0366	**-2.01**
1434107_at	Spata2	66.28865	107.9047	**-2.01**

Table 5j: Down-regulated genes in primary myotube cultures following treatment with newt extract

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1447774_x_at	5730469M10Rik	87.0339	160.4455	**-2.01**
1426757_at	Ampd2	83.7805	161.751	**-2.01**
1425186_at	Lmbrd1	83.2022	139.2365	**-2.01**
1420897_at	Snap23	389.6375	675.6215	**-2.01**
1437627_at	Mex3d	85.02485	154.292	**-2.01**
1418237_s_at	Col18a1	78.6002	176.7285	**-2.01**
1435691_at	Agphd1	41.03265	73.0639	**-2.01**
1453299_a_at	Pnp	28.31285	57.29215	**-2.01**
1458236_at	Lox	29.98595	59.4657	**-2.00**
1457656_s_at	C230085N15Rik	22.05115	43.73115	**-2.00**
1417380_at	Iqgap1	113.4072	226.6105	**-2.00**
1435785_at	Ehd2	40.5996	79.3037	**-2.00**
1428883_at	Tmem57	183.9135	358.044	**-2.00**
1418011_a_at	Sh3glb1	308.924	617.125	**-2.00**
1433685_a_at	MGC66590	178.6321	318.8125	**-2.00**
1433930_at	Hpse	7.12598	14.23605	**-2.00**
1416030_a_at	Mcm7	25.02445	37.87975	**-2.00**
1437433_at	B3galt2	89.37235	169.313	**-1.99**
1421487_a_at	Nck1	332.5835	541.0885	**-1.99**
1458381_at	Clic5	19.9502	37.7587	**-1.99**
1439264_x_at	Lasp1	1316.575	2564.59	**-1.99**
1451683_x_at	H2-D1	9.59064	23.35337	**-1.99**
1450883_a_at	Cd36	151.8495	285.2015	**-1.99**
1451839_a_at	Pde7a	18.52835	36.79285	**-1.99**
1419097_a_at	Epb7.2	381.5715	717.6835	**-1.99**
1415972_at	Marcks	46.0363	90.6787	**-1.99**
1435384_at	Ube2n	106.3787	200.071	**-1.99**
1423449_a_at	Actn4	611.8895	1211.38	**-1.99**
1422533_at	Akap9	203.9515	403.4205	**-1.99**
1448623_at	Tmem123	486.9855	910.977	**-1.99**
1420911_a_at	Mfge8	696.851	1466.091	**-1.99**
1416457_at	Ddah2	69.4681	130.2435	**-1.98**
1454613_at	Dpysl3	143.0887	271.063	**-1.98**
1441111_at	Mylk4	584.276	1146.402	**-1.98**
1450625_at	Col5a2	267.7725	529.725	**-1.98**
1428759_s_at	Cwc25	71.5863	122.6845	**-1.98**
1451077_at	Rpl5	192.914	352.041	**-1.98**
1433587_at	Rgmb	100.2703	194.4985	**-1.98**
1460167_at	Aldh7a1	87.69135	158.21	**-1.97**
1426233_at	Map2k4	30.3514	61.67465	**-1.97**
1452253_at	Crim1	90.85375	179.5295	**-1.97**
1419502_at	D11Lgp1e	35.08875	55.7315	**-1.97**
1423144_at	Pik3ca	89.78905	153.4905	**-1.97**
1423299_at	Txnl	77.48775	133.358	**-1.97**
1419589_at	C1qr1	18.26505	38.66355	**-1.97**
1426657_s_at	Phgdh	183.513	319.0045	**-1.97**
1434444_s_at	Mcpr	110.0174	176.3275	**-1.97**
1423361_at	Yme1l1	327.5485	600.524	**-1.97**
1433501_at	Ctso	12.50225	23.6963	**-1.97**
1436272_at	MGC63429	70.2604	125.7255	**-1.96**

Table 5k: Down-regulated genes in primary myotube cultures following treatment with newt extract

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1438398_at	Rnpc2	207.0375	405.47	**-1.96**
1435538_at	0610030E20Rik	113.7646	176.7195	**-1.96**
1425617_at	Dhx9	103.3915	202.383	**-1.96**
1422766_at	Stau1	60.5144	94.0752	**-1.96**
1415773_at	Ncl	203.7685	399.7715	**-1.96**
1416007_at	Satb1	32.32645	65.6073	**-1.96**
1425373_a_at	Psmg2	236.3265	404.595	**-1.96**
1443632_at	Obscn	155.361	278.438	**-1.96**
1434282_at	Ibtk	106.0318	198.9465	**-1.95**
1450079_at	Nrk	161.4515	304.1315	**-1.95**
1420644_a_at	Sec61a2	193.3848	303.1575	**-1.95**
1430697_at	Ammecr1	46.4934	87.95005	**-1.95**
1426288_at	Lrp4	175.054	343.5785	**-1.95**
1429273_at	Bmper	38.7314	73.05095	**-1.95**
1416222_at	Nsdhl	497.153	962.653	**-1.95**
1422792_at	Pafah1b2	66.94325	89.06625	**-1.95**
1422902_s_at	Mgea5	100.952	181.4315	**-1.95**
1460220_a_at	Csf1	249.7375	495.7375	**-1.95**
1416340_a_at	Man2b1	62.63375	122.3255	**-1.95**
1426918_at	Itgb1	1053.634	1885.27	**-1.95**
1438258_at	Vldlr	269.407	507.146	**-1.94**
1440231_at	Mtap9	62.87495	92.85235	**-1.94**
1423184_at	Sh3d1B	52.09145	100.89	**-1.94**
1427962_at	Ccdc102a	24.2038	46.4031	**-1.94**
1448944_at	Nrp	113.4114	234.097	**-1.94**
1416794_at	Arl6ip2	55.9932	88.9288	**-1.94**
1427079_at	Mapre3	48.5057	97.4588	**-1.94**
1425350_a_at	Myef2	89.8671	188.463	**-1.94**
1426677_at	Flna	123.19	236.729	**-1.93**
1460455_at	Ubr3	1415.825	2520.895	**-1.93**
1451254_at	Ikbkap	760.4165	1475.315	**-1.93**
1433531_at	Facl4	1364.04	2596.895	**-1.93**
1416686_at	Plod2	167.6735	322.5335	**-1.93**
1434202_a_at	Fam107a	163.937	316.808	**-1.93**
1437502_x_at	Cd24a	1690.41	3170.845	**-1.93**
1423311_s_at	Tpbg	85.37865	164.3415	**-1.93**
1418963_at	Fam188a	60.19345	96.92485	**-1.93**
1450662_at	Tesk1	61.5817	119.1829	**-1.93**
1448501_at	Tm4sf6	77.78625	146.1185	**-1.93**
1456532_at	Pdgfd	71.43975	138.4515	**-1.93**
1424356_a_at	Metrnl	156.9755	285.16	**-1.93**
1416133_at	Efr3a	257.9645	418.4035	**-1.93**
1423389_at	Madh7	45.6645	91.02035	**-1.92**
1418042_a_at	Abcc5	118.838	228.696	**-1.92**
1415743_at	Hdac5	126.9409	212.8125	**-1.92**
1418532_at	Fzd2	168.115	312.27	**-1.92**
1435325_at	Usp46	82.0445	133.1815	**-1.92**
1417149_at	P4ha2	479.882	861.3065	**-1.92**
1436403_at	Fam171a2	285.806	547.5025	**-1.92**
1418579_at	Cetn2	370.452	692.831	**-1.92**

Table 51: Down-regulated genes in primary myotube cultures following treatment with newt extract

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1427059_at	Tmem184b	135.975	263.249	**-1.91**
1417133_at	Pmp22	121.648	240.4255	**-1.91**
1429009_at	Snrp70	200.3825	342.4625	**-1.91**
1423694_at	Kctd10	85.79735	151.654	**-1.91**
1451114_at	Cklfsf6	60.90885	117.0578	**-1.91**
1457672_at	Chd9	70.2728	112.1176	**-1.91**
1423678_at	BC017643	44.4986	74.5057	**-1.91**
1453304_s_at	Ly6e	40.84285	80.0496	**-1.91**
1419392_at	Pclo	21.08175	40.3865	**-1.91**
1415806_at	Plat	65.57355	125.3599	**-1.91**
1449674_s_at	Pdcd6ip	262.99	464.481	**-1.91**
1441165_s_at	Clstn2	159.8435	301.9615	**-1.91**
1417728_at	Mbd3	29.26065	61.46065	**-1.91**
1433445_x_at	Hmgcs1	1246.197	2283.35	**-1.91**
1450105_at	Adam10	32.9022	50.2742	**-1.90**
1429193_at	Ankib1	125.1944	198.9255	**-1.90**
1426240_at	Chmp4b	80.595	153.119	**-1.90**
1434089_at	Synpo	54.29635	103.3901	**-1.90**
1433444_at	Hmgcs1	1110.461	2045.575	**-1.90**
1417091_at	Chuk	84.8322	157.2645	**-1.90**
1426750_at	Flnb	54.85715	104.1991	**-1.90**
1418365_at	Ctsh	187.6315	346.8675	**-1.90**
1418117_at	Ndufs4	7.56418	13.2925	**-1.90**
1420886_a_at	Xbp1	213.8962	332.92	**-1.90**
1437992_x_at	Gja1	368.7185	681.5045	**-1.90**
1417533_a_at	Itgb5	321.214	588.683	**-1.90**
1416201_at	Crk	231.8345	378.594	**-1.90**
1427126_at	Hspa1a	47.49415	79.6689	**-1.90**
1429735_at	Qk	200.3405	351.7305	**-1.90**
1424903_at	Smcy	20.15865	37.75965	**-1.90**
1438672_at	Parvb	94.9375	184.2545	**-1.89**
1415840_at	Elovl5	276.623	497.677	**-1.89**
1438200_at	Sulf1	102.3204	198.107	**-1.89**
1424086_at	Oaf	31.11435	57.4711	**-1.89**
1423096_at	Capn7	371.0815	637.823	**-1.89**
1448553_at	Myh6	278.288	545.183	**-1.89**
1416069_at	Pfkp	29.5837	54.99845	**-1.89**
1426207_at	Ikbkb	436.8805	803.239	**-1.89**
1434013_at	Ablim3	1739.24	3291.7	**-1.89**
1455176_a_at	Syt11	50.85675	77.0379	**-1.89**
1422851_at	Hmga2	35.3583	67.16405	**-1.89**
1429369_at	Tnpo3	145.0231	199.675	**-1.89**
1434210_s_at	Slc25a6	550.4345	1006.696	**-1.89**
1417871_at	Hsd17b7	83.67355	161.5415	**-1.89**
1432094_a_at	Ccdc132	166.6787	277.0205	**-1.89**
1421116_a_at	Rtn4	606.544	1105.218	**-1.89**
1455206_at	Rps6ka3	793.497	1477.48	**-1.89**
1423152_at	Vapb	592.6275	1112.69	**-1.88**
1417868_a_at	Ctsz	1179.947	2202.105	**-1.88**
1420946_at	Atrx	36.96075	69.5404	**-1.88**

Table 5m: *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1437041_at	Sfrs18	351.2445	611.4765	**-1.88**
1460173_at	Lasp1	93.38535	174.2755	**-1.88**
1427544_a_at	Papola	34.65105	62.30665	**-1.88**
1423566_a_at	Hsp105	699.6145	1155.155	**-1.88**
1436989_s_at	Slc12a6	24.7462	44.2261	**-1.88**
1429321_at	Rnf149	241.0475	451.097	**-1.88**
1434070_at	Jag1	41.67	81.734	**-1.87**
1439459_x_at	Acly	2070.09	3871.96	**-1.87**
1449214_a_at	Opa1	142.2214	229.82	**-1.87**
1450889_at	Smarca3	100.9788	190.417	**-1.87**
1435647_at	Ikbkg	8.117705	12.56145	**-1.87**
1450903_at	Rad23b	1038.297	1839.435	**-1.87**
1434480_at	Pdpr	175.9775	324.907	**-1.87**
1424131_at	Col6a3	48.0929	87.8701	**-1.87**
1428229_at	Prkcn	228.938	354.7025	**-1.87**
1454236_a_at	Fam118b	145.1604	229.027	**-1.87**
1425567_a_at	Anxa5	1476.735	2758.29	**-1.87**
1450744_at	Ell2	692.3445	1146.18	**-1.87**
1450917_at	Myom2	1936.03	3614.12	**-1.87**
1426513_at	Rbm28	111.135	193.027	**-1.87**
1435828_at	Maf	93.96615	173.144	**-1.87**
1417572_at	Mpg	72.45515	160.1965	**-1.87**
1451285_at	Fus	598.372	1103.455	**-1.86**
1452360_a_at	Rbbp2	186.5791	308.865	**-1.86**
1453684_s_at	Zc3h15	161.3085	299.0975	**-1.86**
1453103_at	Ablim1	126.6019	219.72	**-1.86**
1451019_at	Ctsf	148.3735	264.7335	**-1.86**
1417065_at	Egr1	784.8475	1462.04	**-1.86**
1423756_s_at	Igfbp4	15.77195	29.88825	**-1.86**
1424353_at	Lrpprc	417.371	709.5015	**-1.86**
1437349_at	Ckap5	65.6613	111.4649	**-1.86**
1436048_at	Exoc8	166.624	273.542	**-1.86**
1423298_at	Add3	8.349485	14.88175	**-1.86**
1442226_at	Sema3e	309.821	527.5325	**-1.86**
1423649_at	Tmem68	24.0246	43.3511	**-1.86**
1426598_at	Uty	13.69932	24.26375	**-1.86**
1452882_at	Pgrmc2	109.8045	209.1335	**-1.86**
1431822_a_at	Azi2	141.9305	242.4505	**-1.86**
1454772_at	Snrnp200	122.2388	204.8625	**-1.86**
1454867_at	Mn1	108.2506	202.464	**-1.85**
1456326_at	Fndc3c1	518.406	930.1635	**-1.85**
1415892_at	Sgpl1	62.3183	128.4948	**-1.85**
1449219_at	Fads3	73.976	135.925	**-1.85**
1451222_at	Btf3l4	133.7464	215.02	**-1.85**
1434352_at	B630005N14Rik	231.1735	393.9715	**-1.85**
1448005_at	Sash1	129.3305	254.626	**-1.85**
1455188_at	Ephb1	38.0973	69.84375	**-1.85**
1437632_at	Med14	272.811	474.86	**-1.85**
1454780_at	Galntl4	32.52535	64.0382	**-1.85**
1450381_a_at	Bcl6	9.11421	16.3984	**-1.85**

Table 5n : *Down-regulated genes in primary myotube cultures following treatment with newt extract*

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold -Change
1422479_at	Acas2	188.676	348.6605	**-1.85**
1446324_at	---	20.2	34.41395	**-1.85**
1448575_at	Il7r	21.09925	39.42345	**-1.85**
1450969_at	Pccb	143.966	255.478	**-1.84**
1440966_at	Axot	18.19215	33.631	**-1.84**
1415812_at	Gsn	308.067	546.468	**-1.84**
1417540_at	Elf1	26.8008	46.8472	**-1.84**
1421425_a_at	Dscr1l1	83.8414	147.453	**-1.84**
1433545_s_at	Acad11	108.0724	186.132	**-1.84**
1452309_at	Cgnl1	89.35475	162.2505	**-1.84**
1433943_at	Itprip	21.2709	42.08815	**-1.84**
1415973_at	Marcks	203.8185	375.175	**-1.84**
1437532_at	Rnf216	108.8763	161.373	**-1.84**
1436838_x_at	Cotl1	78.9964	146.6675	**-1.84**
1434663_at	Mzt1	125.5088	212.4395	**-1.84**
1433782_at	Cldn12	100.4506	159.741	**-1.83**
1440153_at	App	84.28965	149.054	**-1.83**
1418500_at	Nap1l3	30.8536	55.7446	**-1.83**
1456028_x_at	Marcks	108.2604	196.403	**-1.83**
1436722_a_at	Actb	1507.02	2746.4	**-1.83**
1429987_at	Cyb5b	82.33825	154.016	**-1.83**
1417767_at	1810044O22Rik	131.7828	213.151	**-1.83**
1426541_a_at	Endod1	282.02	513.275	**-1.83**
1449443_at	Decr1	115.557	190.625	**-1.83**
1452207_at	Cited2	1111.607	1998.055	**-1.82**
1439755_at	Sipa1l1	111.351	202.7085	**-1.82**
1449059_a_at	Oxct	2364.165	4218.87	**-1.82**
1427884_at	Col3a1	22.40075	40.22745	**-1.82**
1451891_a_at	Dysf	159.6973	263.694	**-1.82**
1418355_at	Nucb2	12.85198	23.5456	**-1.82**
1417517_at	Plagl2	70.12805	121.455	**-1.82**
1460178_at	Lonp2	351.998	578.7755	**-1.82**
1428791_at	Ube2h	418.551	710.768	**-1.82**
1437889_x_at	Bgn	996.096	1798.85	**-1.82**
1453726_s_at	2810407C02Rik	316.979	550.685	**-1.82**
1439526_at	---	24.5466	42.94075	**-1.82**
1450008_a_at	Catnb	60.1751	101.0732	**-1.82**
1427229_at	Hmgcr	228.221	409.3025	**-1.81**
1450919_at	Mpp1	258.884	430.8015	**-1.81**
1452217_at	Ahnak	5208.465	9417.785	**-1.81**
1426999_at	Zc3h14	182.1183	285.0275	**-1.81**
1415787_at	G2an	104.8089	176.471	**-1.81**
1435133_at	Ugcg	173.474	313.436	**-1.81**
1438030_at	Rasgrp3	13.81542	23.35175	**-1.81**
1426460_a_at	Ugp2	177.5608	271.1075	**-1.81**
1436175_at	Sca7	44.8039	64.5218	**-1.81**
1441477_at	Calu	25.43625	49.99675	**-1.81**
1451286_s_at	Fus	885.477	1637.575	**-1.81**
1456195_x_at	Itgb5	755.656	1407.635	**-1.81**
1429219_at	Ikbip	92.92295	165.947	**-1.81**

Table 5o: Down-regulated genes in primary myotube cultures following treatment with newt extract

Probe Set ID	Gene Symbol	Experimental Signal	Control Signal	Affymetrix Fold-Change
1425711_a_at	Akt1	78.59085	129.2961	**-1.81**
1449080_at	Hdac2	654.9015	1038.185	**-1.81**
1437158_at	Nipbl	390.185	680.6855	**-1.81**
1416750_at	Oprs1	47.68525	83.1425	**-1.81**
1453055_at	Sema6d	118.2719	209.1675	**-1.81**
1458385_at	Osp94	21.60315	36.0298	**-1.81**
1423671_at	Dner	339.9115	576.728	**-1.81**
1448121_at	Wbp2	189.26	355.1475	**-1.81**
1417602_at	Per2	55.7873	92.43875	**-1.81**
1437671_x_at	Prss23	4502.455	8255.095	**-1.81**
1452225_at	2010106G01Rik	655.762	1178.905	**-1.81**
1426046_a_at	Rabggta	45.7352	72.16205	**-1.81**
1454826_at	Zbtb11	289.4715	448	**-1.81**
1428187_at	Cd47	81.2269	149.6775	**-1.80**
1423129_at	Shoc2	541.3325	903.583	**-1.80**
1460729_at	Rock1	114.1852	197.847	**-1.80**
1454901_at	Ypel2	300.593	533.3795	**-1.80**
1437371_at	Fam160a1	167.7555	287.59	**-1.80**
1455872_at	Fam167a	47.7544	87.398	**-1.80**
1438442_at	Sike1	69.22005	105.628	**-1.80**
1418432_at	Cab39	177.3145	313.7595	**-1.80**
1418815_at	Cdh2	1465.262	2495.805	**-1.80**
1438673_at	Slc4a7	386.109	659.1335	**-1.80**
1416928_at	Rbm12	224.309	386.815	**-1.80**
1426801_at	Sept8	94.77165	178.34	**-1.80**
1429712_at	Etohi1	55.14425	97.7699	**-1.80**
1456510_x_at	Mettl7a2	49.2496	87.07735	**-1.80**
1443881_at	Pofut1	100.3316	179.275	**-1.80**