IDENTIFICATION AND CHARACTERISATION OF NOVEL GENES INVOLVED IN SKELETAL MUSCLE HYPERTROPHY

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B. Appl. Sci. (Hons)

A thesis presented in fulfilment of the Degree of Doctor of Philosophy

Supervisor: Dr. David Cameron-Smith

December 2004

School of Exercise and Nutrition Sciences
Faculty of Health and Behavioural Sciences
Deakin University, Melbourne
I certify that the thesis entitled:

**Identification and characterisation of novel genes involved in skeletal muscle hypertrophy**

submitted for the degree of:

**Doctor of Philosophy**

is the result of my own research, except where otherwise acknowledged, and that this thesis in whole or in part has not been submitted for an award, including a higher degree, to any other university or institution.

Full Name:  **Kate Alison Carey**

Signed: ........................................................................................................................................

Date: ........................................................................................................................................
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DECLARATION

This thesis reports original previously unpublished studies conducted in the School of Exercise and Nutrition Sciences, Deakin University, Melbourne, Australia. All of the work conducted in completion of the studies discussed in this thesis is solely the work of the author with the following exceptions. The clone sequencing discussed in chapter 3 and chapter 5 were performed by Reuben Klein at the Metabolic Research Unit, Deakin University. The skeletal muscle biopsies were performed by Dr. Andrew Garnham.

Kate A. Carey
December 2004

School of Exercise and Nutrition Sciences
Deakin University
Burwood, Australia
There is mounting evidence in support of the view that skeletal muscle hypertrophy results from the complex and coordinated interaction of numerous signalling pathways. Well characterised components integral to skeletal muscle adaptation include the transcriptional activity of the members of the myogenic regulatory factors, numerous secreted peptide growth factors, and the regenerative potential of satellite cells. Whilst studies investigating isolated components or pathways have enhanced our current understanding of skeletal muscle hypertrophy, our knowledge of how all of these components react in concert to a common stimulus remains limited. The broad aim of this thesis was to identify and characterise novel genes involved in skeletal muscle hypertrophy. We have created a customised human skeletal muscle specific microarray which contains ~11,000 cDNA clones derived from a normalised human skeletal muscle cDNA library as well as 270 genes with known functional roles in human skeletal muscle. The first aspect of this thesis describes the production of the microarray and evaluates the robustness and reproducibility of this analytical technique.

Study one aimed to use this microarray in the identification of genes that are differentially expressed during the forced differentiation of human rhabdomyosarcoma cells, an in vitro model of skeletal muscle development. Firstly using this unique model of aberrant myogenic differentiation we aimed to identify genes with previously unidentified roles in myogenesis. Secondly, the data from this study permitted the examination of the performance of the microarray in detecting differential gene expression in a biological system. We identified several new genes with potential roles in the myogenic arrest of rhabdomyosarcoma and further characterised the expression of muscle specific genes in rhabdomyosarcoma differentiation.

In study two, the molecular responses of cell cycle regulators, muscle regulatory factors, and atrophy related genes were mapped in response to a single bout of resistance exercise in human skeletal muscle. We demonstrated an increased expression of MyoD, myogenin and p21, whilst the expression of myostatin was decreased. The results of this study contribute to the existing body of knowledge on the molecular regulation skeletal muscle to a hypertrophic stimulus.
In study three, the muscle samples collected in study two were analysed using the human skeletal muscle specific microarray for the identification of novel genes with potential roles in the hypertrophic process. The analysis uncovered four interesting genes (TXNIP, MLP, ASB5, FLJ 38973) that have not previously been examined in human skeletal muscle in response to resistance exercise. The functions of these genes and their potential roles in skeletal muscle are discussed.

In study four, the four genes identified in study three were examined in human primary skeletal muscle cell cultures during myogenic differentiation. Human primary skeletal muscle cells were derived from the vastus lateralis muscle of 8 healthy volunteers (6 males and 2 females). Cell cultures were differentiated using serum withdrawal and serum withdrawal combined with IGF-1 supplementation. Markers of the cell proliferation, cell cycle arrest and myogenic differentiation were examined to assess the effectiveness of the differentiation stimulus. Additionally, the expressions of TXNIP, MLP, ASB5 and FLJ 38973 measured in an attempt to characterise further their roles in skeletal muscle. The expression of TXNIP changed markedly in response to both differentiation stimuli, whilst the expression of the remaining genes were not altered. Therefore it was suggested that expression of these genes might be responsive to the mechanical strain or contraction induced by the resistance exercise. In order to examine whether these novel genes responded specifically to resistance type exercise, their expression was examined following a single bout of endurance exercise. The expression of TXNIP, MLP, and FLJ 38973 remained unchanged whilst ASB5 increased 30 min following the cessation of the exercise.
ACKNOWLEDGMENTS

First and foremost, my sincerest gratitude is due to my supervisor, Dr. David Cameron-Smith, for firstly providing the opportunity to pursue this research, but more importantly for his enthusiastic encouragement, support and invaluable advice. I would also like to thank Professor Greg Collier, Dr. David Segal and the team from the Metabolic Research Unit at Deakin University for the resources and tireless assistance necessary for the development of the microarray and analysis undertaken. Special thanks go to Janelle Mollica, Reuben Klein, Andrew Sanagorski, Mary Malakellis for their outstanding technical assistance.

Thank you to Dr. Andrew Garnham for his excellent medical assistance during trials and to all those people who gave their time to take part in these studies – this work would not have been possible without your willing participation.

Thanks to everyone in the Exercise Muscle and Metabolism Unit who has assisted in various aspects of my research and has contributed to a thoroughly enjoyable work environment.

I also wish to record to sincere appreciation to my family for their unwavering love and continuing support. Above all, I tender my heartfelt love and thanks to my husband Peter for his love and care. I deeply appreciate his encouragement and support and he has been, and remains, a profound source of inspiration in my life.

Melbourne, December 2004

Kate Carey
Results reported in this thesis have been submitted for publication as follows:

*Manuscripts in review or preparation:*


*Publications from collaborative studies conducted during PhD candidature:*


Murphy RM, Tunstall RJ, **Mehan KA**, Cameron-Smith D, McKenna MJ, Spriet LL, Hargreaves M, Snow RJ. Human skeletal muscle creatine transporter mRNA and...


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5.1 Real-Time PCR Primer Details

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6.1 Real-Time PCR Primer Details

7.1 Real-Time PCR Primer Details
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bar represents the mean ± SEM of eight independent, replicate experiments.

6.6 mRNA expression analysis of the FLJ38973, ASB5, MLP, and TXNIP during serum withdrawal (DM; solid bars) or serum withdrawal combined with 30ng/ml R3IGF-1 (DM+IGF-1; white bars). Each bar represents the mean ± SEM of eight independent, replicate experiments.

7.1 Examination of the mRNA expression of the endogenous control β-actin (A) and cyclophilin (B) following an acute bout of resistance exercise. Statistical analysis revealed no change in the expression of this gene at any time point following the exercise intervention. Therefore, this was considered an appropriate endogenous control for this study.

7.2 The effects of a single bout of endurance exercise on the expression of resistance exercise sensitive genes. Real-time PCR analysis was performed on, TXNIP, MLP, ASB5 and FLJ38973. Values are means ± SEM of 8 subjects.

7.3 The effects of a single bout of endurance exercise on the expression of the Muscle Regulatory Factors. Real-time PCR analysis was performed on, Myf5, MyoD, myogenin, and Myf6. Values are means ± SEM of 8 subjects.

7.4 The effects of a single bout of endurance exercise on the expression of PDK4. Values are means ± SEM of 8 subjects.
## ABBREVIATIONS

<table>
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<tr>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASB5</td>
<td>ankyrin repeat and SOCs box-containing 5</td>
</tr>
<tr>
<td>ATP5I</td>
<td>ATP synthase, H⁺ transporting mitochondrial F0 complex, subunit E</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>CCT7</td>
<td>chaperonin containing t polypeptide 1 eta</td>
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<tr>
<td>Cdk</td>
<td>cyclin dependent kinase</td>
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<td>cDNA</td>
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<td>Ct</td>
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<td>DLAT</td>
<td>dihydrioloapamide S-acetyltransferase</td>
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<td>DM</td>
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<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<td>extracellular signal-related kinase</td>
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<td>HNRPH1</td>
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<td>leukaemia inhibitory factor</td>
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<td>mitogen-activated kinase</td>
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<td>RNAi</td>
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<td>sodium dodecyl sulfate</td>
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<td>signal transducer and activator of transcription 3</td>
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<td>tris buffered saline plus tween</td>
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<tr>
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<td>transforming growth factor-β</td>
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<tr>
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<td>translocase of inner mitochondrial membrane</td>
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<td>vascular endothelial growth factor</td>
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CHAPTER ONE
REVIEW OF THE LITERATURE

I. INTRODUCTION

Skeletal muscle comprises 50-60% of total body mass and is one of the major tissues involved in regulating metabolism, locomotion and strength. Skeletal muscle mass and contractile characteristics are readily modified by increased loading (e.g. resistance exercise). Similarly, conditions of disuse (e.g. bed rest, microgravity), starvation, aging, and some disease states (e.g. cancer cachexia, AIDS) results in protein breakdown and considerable skeletal muscle atrophy. Significant loss of muscle mass results in numerous deleterious consequences including muscle weakness and frailty, increased risk of falls and associated morbidity, reduction in or prevention of ambulation, and in severe cases, mortality. Recent developments in molecular biology have begun to provide some insights into the subtle interactions and complex events which control these adaptations. Understanding the fundamental mechanisms underlying hypertrophy has the potential to provide new therapies to treat muscle disease. Importantly, the molecules and signalling pathways that induce hypertrophy might be manipulated or mimicked to directly arrest the atrophic process, or indeed augment the growth of skeletal muscle in these populations. In this review, current understandings of the cellular and molecular mechanisms of skeletal muscle hypertrophy are presented.
II. CELLULAR & MOLECULAR REGULATION OF SKELETAL MUSCLE HYPERTROPHY

Skeletal muscle hypertrophy results from a set of complex and highly orchestrated signalling events which result in increased protein synthesis and the addition of new contractile filaments to pre-existing fibres, ultimately enabling greater force production. It has become evident that there are two primary processes mediating hypertrophy. The first is increased protein synthesis within the myofibre leading to appropriate cellular adaptations. The second involves the proliferation, differentiation and fusion of satellite cells, providing additional myonuclei for the enlarging fibre. Equally as important are the many critical mechanisms by which the cell senses increased loading and transduces this information into intracellular adaptations (Figure 1.1).

A. Signalling Pathways Mediating Myofibre Adaptation

Strenuous, growth-inducing muscle activity is associated with changes in one or more variables including myofibre integrity or damage, calcium concentrations, energy demand, growth factors and cytokines, intramuscular temperature and oxygen concentrations, as well as mechanical stretch and contraction of the muscle itself (Rennie et al. 2004). Alterations of an appropriate magnitude in these variables have the capacity to activate numerous parallel signalling pathways. The involvement of these signalling pathways in the sensation and transduction of cellular environmental stimuli is widely recognised as critical in the activation of specific gene expression and cellular reprogramming (Martineau and Gardiner 2001; Nader and Esser 2001). Several signalling pathways involving cytoplasmic protein kinases and nuclear encoded transcription factors have been identified as possible master regulators of skeletal muscle adaptation. These pathways encompass both the sensing of mechanical and environmental perturbations as well as the transduction of these signals into intracellular responses.
Figure 1.1. Overview of the main events during resistance exercise leading to skeletal muscle hypertrophy. (1) Muscular overload is detected by the muscle through numerous perturbations in the cellular environment. Via receptor binding and other cellular signals, a network of signal transduction pathways (2) results in the activation of transcription factors. Active nuclear transcription factors alter the expression of numerous genes (3) which ultimately result in increased synthesis and assembly of myofibrillar proteins (4). Simultaneously, growth factors, cytokines released from injured cells, and other unknown mechanisms mediate the activation of satellite cells (5). The activated satellite cells subsequently proliferate, differentiate and then fuse to existing myofibres to promote muscle repair and enlargement via increased production of myofibrillar proteins (6).
1. Mechanotransduction & the Integrin Signalling Pathway

Mechanotransduction is the mechanism by which mechanical stress initiates intracellular signalling. Striated muscle cells are particularly responsive to mechanical stress as evidenced by the fact that cellular size is in large part dictated by physical forces. A fundamental unanswered question concerns how skeletal muscle cells sense mechanical loading and transduce this information into cellular level adaptations.

One potential candidate for influencing load-induced changes in skeletal muscle protein synthesis through the regulation of gene transcription is the integrin-mediated signalling pathway. Initially, integrins were considered solely as membrane-associated proteins necessary for maintaining adhesive interactions with the extracellular matrix, cell growth and cell motility (Carson and Wei 2000). More recently however, it is believed that integrins constitute a critical component of the signalling pathways by which the cell integrates physical or mechanical signals from the surrounding environment (Carson and Wei 2000). Activation of cellular signalling through extracellular matrix-integrin interactions initially occurs by the formation of focal adhesion complexes (FACs). Within the FACs, signalling proteins are brought into close proximity - thereby facilitating signal transduction.

Importantly, the formation of FACs appears sensitive to mechanical forces. The focal adhesion protein focal adhesion kinase (FAK) is activated by the mechanical stretch of muscle and both FAK and paxillin (a down-stream target of FAK) protein concentrations are increased in hypertrophied skeletal muscle (Flück et al. 1999; Gordon et al. 2001). Equally, the expression of a down stream effector molecule of integrins, RhoA, is increased following functional overload and during the recovery from disuse atrophy (McClung et al. 2003; McClung et al. 2004). Indeed, RhoA can regulate the expression of the muscle specific transcription factors, MyoD and myogenin and is critical for skeletal muscle differentiation (Carnac et al. 1998; Takano et al. 1998; Wei et al. 1998).

1.1. Signal Integration

Whilst integrins may be important for transducing mechanical stimuli into cellular level signalling, there is some suggestion that integrins may also be excellent candidates for integrating many of the signalling cascades activated during hypertrophy (Carson and Wei 2000). Muscle overload and contraction alter innumerable variables which, in turn activate many signalling pathways simultaneously. It appears to be that the combinatorial activation of these signal transduction pathways as well as the relative
magnitude of the signals ultimately result in the specific adaptations of the cell. Integrins may provide a mechanism by which all of these signalling pathways work together to give a unified response resulting in appropriate cellular adaptations. Signalling pathways that have been shown to be connected to integrin signalling include phosphatidylinositol-3 kinase (PI3k), mitogen-activated protein kinase (MAPK), and calcium signalling pathways (Clarke and Brugge 1995; Goel and Dey 2002; Zhang et al. 2003; Kim et al. 2004; Velling et al. 2004).

2. PI(3)k/Akt Signalling Pathway

Numerous studies have identified critical roles for the PI3k/Akt pathway in modulating the intracellular response to resistance exercise. Most notably, the growth factor insulin-like growth factor I (IGF-1), a powerful stimulator of muscle hypertrophy (Vandenburgh et al. 1991; Coleman et al. 1995), has recently been shown to act via the PI3k/Akt signalling pathway (Bodine et al. 2001b; Rommel et al. 2001). IGF-1 binding to the IGF-1 receptor stimulates a cascade of events which ultimately result in the activation of PI3k and further downstream, Akt. Akt in turn phosphorylates an ever increasing set of substrates amongst which are proteins that induce protein synthesis, block apoptosis, activate cell proliferation, and mediate gene transcription, steps essential for the development of skeletal muscle hypertrophy (Glass 2003; Matsui et al. 2003). Targeted activation of PI3k has been shown to induce skeletal muscle hypertrophy (Murgia et al. 2000). Indeed, pharmacological inhibition of PI3k is sufficient to block IGF-1 stimulated hypertrophy (Rommel et al. 1999). Equally, the over-expression of a constitutively active form of Akt plays critical roles in both the promotion of muscle hypertrophy and the inhibition of muscle atrophy in vivo (Bodine et al. 2001b). Furthermore, specific inhibition of mTOR, a downstream target of Akt, with rapamycin leads to a 95% inhibition of hypertrophy. Conversely, another downstream target of Akt, glycogen synthase kinase-3β (GSK-3β) appears to be a negative regulator of skeletal muscle hypertrophy. GSK-3β phosphorylation and inactivation by Akt has been observed during hypertrophy in animals (Bodine et al. 2001b) and in culture (Rommel et al. 2001; Vyas et al. 2002). Collectively, these studies identify a crucial role for the PI3k-Akt signalling pathway in regulating the development of skeletal muscle hypertrophy.

3. MAPK Signalling Pathway

The involvement of the MAPK cascades in mechanically induced signalling from the cytosol to the nucleus is receiving increasing attention. This function is compatible with the well known role of the MAPKs as points of convergence for various signalling cascades regulating gene expression (Martineau and Gardiner 2001). The MAPK superfamily can be separated into distinct parallel pathways including the extracellular
signal-related kinase (ERK) 1/2, the stress activated protein kinase cascades (SAPK1/JNK and SAPK2/p38) and ERK5. Cellular stresses can elicit signal transduction of the MAPK signalling pathways through protein phosphorylation on serine, threonine, and tyrosine residues. Many factors associated with exercise, including growth factors and their cell surface receptors, cytokines, hypoxia, changes in intracellular calcium and mechanical stresses have been shown to stimulate MAPK signal cascades (Widegren et al. 2001). Thus MAPK signal cascades are a likely candidate system that may convert the mechanical and biochemical stimuli associated with muscle contraction into appropriate intracellular responses (Widegren et al. 2001). Importantly, recent evidence suggests that exercise and muscle contraction can induce phosphorylation and activation of components of the ERK, JNK and p38 MAPK cascades in both human and rat skeletal muscle (Aronson et al. 1997a; Aronson et al. 1997b; Aronson et al. 1998; Boppart et al. 1999; Boppart et al. 2000; Osman et al. 2000; Ryder et al. 2000; Wretman et al. 2000; Carlson et al. 2001; Martineau and Gardiner 2001; Thompson et al. 2003). Furthermore, several of these studies (Aronson et al. 1997a; Aronson et al. 1998) demonstrated a rapid and concurrent induction of c-jun and c-fos mRNA levels, early response genes which have been implicated in the activation of genetic events leading to the differential regulation of muscle specific genes (Williams and Neufer 1996). Thus muscular contraction activates MAPK signalling cascades that ultimately lead to the activation of different transcription factors responsible for initiating muscle specific gene and protein expression.

4. Intracellular Calcium Signalling

Changes in intracellular calcium regulate many important cellular processes, including motility, neurotransmitter release, proliferation and gene expression (Bading et al. 1997). Calcium has been shown to regulate gene expression via multiple signalling pathways by activating calcium sensitive kinases such as calmodulin kinases, and mitogen-activated protein kinases. Although the essential role of intracellular calcium concentrations in the regulation of skeletal muscle contraction has been established, its role in the regulation of muscle growth is less well understood.

Changes in intracellular calcium have been implicated in regulating diverse processes in skeletal muscle, including differentiation, hypertrophy and fibre type determination. The calcium/calmodulin-depdendant protein phosphatase, calcineurin plays an important role in mediating downstream calcium-depdendant signalling. Calcineurin is a serine/threonine phosphatase that is activated in response to sustained increases in intracellular calcium (Dolmetsch et al. 1997). In skeletal muscle, calcineurin activation has been shown to be necessary for hypertrophic growth in response to insulin-like
growth factor-1 (Musarò et al. 1999), or during compensatory overload (Dunn et al. 1999). Considerable controversy exists however as to the relative importance of calcineurin in this process. Whereas some groups have reported that cyclosporin A, an inhibitor of calcineurin, blocks hypertrophy (Dunn et al. 1999), others have seen no effect (Bodine et al. 2001b; Musarò et al. 2001; Rommel et al. 2001; Dupont-Versteegden et al. 2002). Calcineurin dependent pathways have also been implicated in myoblast differentiation (Delling et al. 2000; Friday et al. 2000) through the activation of MyoD and MEF2 (Friday et al. 2003) as well as skeletal muscle regeneration (Sakuma et al. 2003). More recently, the hypothesised roles of calcineurin have been extended to include the regulation of skeletal muscle fibre type switching (Mitchell et al. 2002; Parsons et al. 2004). Indeed, calcineurin has been shown to selectively activate the expression of the slow MHC isoform (Delling et al. 2000).

B. Contribution of Satellite Cells to Muscle Adaptation

Each myonucleus controls the production of mRNA and proteins over a finite volume of cytoplasm, known as the myonuclear domain. mRNA encoding proteins do not freely mix along the fibre length but remain concentrated around their myonuclei of origin (Hall and Ralston 1989; Pavlath et al. 1989). Therefore, any increase in the size of a muscle fibre, in response to increased mechanical load for example, would necessitate the incorporation of additional myonuclei (Yan 2000). Yet, mature myofibres within adult skeletal muscle are terminally differentiated and therefore incapable of mitotic division to produce additional myonuclei in times of increased protein synthesis and muscle growth. Satellite cells, myogenic precursor cells located between the basal lamina and sarcolemma of multinucleated myofibres, provide the additional pool of myonuclei necessary for muscle growth (Hawke and Garry 2001). In normal mature fibres, satellite cells are quiescent. Rates of RNA and protein synthesis are low but the cell retains the ability to divide. Upon activation, they re-enter the cell cycle, proliferate, and differentiate to form myofibres by fusing with themselves or existing fibres (Hawke and Garry 2001).

There is very strong evidence to suggest that skeletal muscle remodelling and regeneration is dependent, to a large extent upon the activity of satellite cells (Rosenblatt and Parry 1992; Rosenblatt and Parry 1993). Such that, upon mild $\gamma$-radiation induced inhibition of satellite cell proliferation, overload induced hypertrophy was obliterated (Rosenblatt and Parry 1992; Rosenblatt and Parry 1993). The exact contribution of satellite cells to the hypertrophic response remains unclear however, as
the induction of hypertrophy has been observed despite the elimination of satellite cell activity (Lowe and Alway 1999). Similarly, studies have reported an enlargement of myofibre size before an increase in satellite derived myonuclear number had occurred (Allen et al. 1995a; Roy et al. 1999). Collectively, these observations indicate that although the activity of satellite cells are an integral component of skeletal muscle hypertrophy, existing myonuclei must also act in concert with satellite cells to regulate the adaptation of the entire myofibre.

Mechanical loading, has been shown to induce augmented activation and proliferation of satellite cells in the muscle of humans (Kadi and Thornell 2000; Crameri et al. 2004; Kadi et al. 2004b) and adult rats (Darr and Schultz 1987; Rosenblatt et al. 1994; Smith et al. 2001). Increased myonuclei numbers have been reported in high-level resistance trained subjects as the result of years of intensive training (Kadi et al. 1999) and more recently in response to 10 weeks of strength training in women (Kadi and Thornell 2000). Since the importance of satellite cells to the remodelling and regenerative potential of skeletal muscle was described, much research has focused on understanding the molecular and cellular mechanisms regulating their functions in muscle.

1. Overload Induced Activation of Satellite Cells

In mature muscle, satellite cells are normally quiescent and require appropriate signals to become activated, proliferate and differentiate as required. As mentioned previously, resistance exercise has the capacity to generate numerous, parallel signalling pathways within skeletal muscle that ultimately result in cellular level adaptations. A variety of extracellular signalling pathways have been found to govern satellite cell activities, most prominently, cytokines from inflammatory cells and growth factors released from muscle and surrounding tissues (Vierck et al. 2000). Initially, strenuous or unaccustomed resistance exercise may result in muscle damage ranging from micro-tears to large tears in the sarcolemma and basal lamina as well as within the contractile proteins of the myofibre (Vierck et al. 2000). Whether muscle damage is causally linked to the hypertrophic process remains unclear. However, eccentric muscular contractions induce more muscle damage than concentric exercise and the hypertrophic response is blunted when the eccentric phase is omitted from resistance training (Hather et al. 1991).

One proposed theory suggests that resistance exercise resulting in myotrauma may initiate the release of signalling factors that stimulate and attract immune cells to the injury site. Cytokines from the activated immune cells, along with muscle-derived
growth factors, initiate a sequence of events that ultimately results in satellite cell proliferation and differentiation (Vierck et al. 2000), see Figure 1.2.

Integral to the proliferative response are the growth factors insulin-like growth factor (IGF-1), hepatocyte growth factor, basic fibroblast growth factor (bFGF), transforming growth factor β (TGF-β) and growth hormone as well as interleukin-6 (IL-6), and leukaemia inhibitory factor (LIF) (Vierck et al. 2000). Such factors differ in their effects on myogenesis: IL-6 and LIF only stimulate myoblast proliferation (Austin and Burgess 1991), whereas bFGF stimulates myoblast proliferation but also inhibits muscle differentiation (Gospodarowicz et al. 1976) and TGF-β inhibits proliferation. IGF-1 is unique amongst growth factors in that it is capable of stimulating both proliferation and differentiation of satellite cells (Allen and Boxhorn 1987).

Although there is no general consensus on the exact contribution of cytokines and muscle derived growth factors in overload induced hypertrophy, it is generally accepted that some, if not all of these factors contribute to the regulation of satellite cell activity within muscle.
Figure 1.2. Overview of overload induced myotrauma and satellite cell activation. (A) Myofibre damage occurs. (B) Inflammatory response releases cytokines and muscle derived growth factors which in turn activate satellite cells to (C) proliferate, differentiate and fuse to existing myofibres, resulting in (D) myofibre hypertrophy.
2. Proliferation & Differentiation of Satellite Cells

The effective capacity for regeneration and remodelling of muscle via satellite cell activity is remarkable considering that satellite cells constitute an estimated 2.5-6% of myofibre nuclei (Zammit et al. 2002). Once activated, satellite cells re-enter the cell cycle and proliferate until signalled to withdraw from the cell cycle and differentiate or return to a state of quiescence. Much research has focused on delineating the molecular mechanisms necessary for entire process of myogenesis including the regulation of the satellite cell cycle, myoblast determination, differentiation and subsequent fusion and maturation.

2.1. Cell Cycle Regulation

The decision of an activated satellite cell to progress through a new division appears primarily regulated late in the first gap phase (G₁), before the initiation of DNA synthesis (S phase). Critical to this process is the activity of positive cell cycle regulators, the G₁ cyclins (D1, D2, D3, A and E) and the regulatory subunits for the G1 cyclin-dependant kinases (Cdks) (Cdk4, Cdk6 and Cdk2) which together, promote progression into successive phases of the cell cycle (Sherr 1994). Conversely, permanent cell cycle withdrawal, necessary for the terminal differentiation and maturation of satellite cells, requires that the major positive cell cycle regulators are inhibited. The major negative regulators comprise the Cdk-inhibitors (p21cip, p27kip, p57kip), the product of the retinoblastoma susceptibility gene (Rb protein) and the two related Rb family proteins p107 and p130 (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995; Novitch et al. 1996).

Central to cell cycle regulation is the activity of the Rb protein. Active Cdk4/6-cyclin D1 phosphorylates Rb during early G₁; this leads to the up-regulation of cyclin E, its assembly with Cdk2, activation of the Cdk2-cyclin E complex and subsequent hyperphosphorylation of Rb. Hyperphosphorylated Rb releases, and subsequently activates the transcription factor E2F-1, allowing expression of genes necessary for G₁/S progression, and mitosis (Ishida et al. 2001). Conversely, dephosphorylation of Rb protein by Cdk-inhibitors results in Rb binding and inactivating E2F-1, contributing to permanent cell cycle withdrawal (Sherr and Roberts 1999; Mal et al. 2000).

2.2. Myogenic Regulatory Factors

Whilst the activity of the Cdks and Cdk-inhibitors are important in the proliferative response in many cell types, the specification of the skeletal muscle phenotype is dependent on the activity of the basic-loop-helix family of muscle regulatory factors (MRFs). The MRFs comprise four members: MyoD, Myf5, myogenin and Myf6 (MRF4,
Herculin) that bind to specific consensus sites that are functionally important in the transcription of muscle-specific genes. Despite potentially overlapping roles of the MRFs, current understanding suggests specification of muscle cell fate is reliant on the activation of the core network of myogenic regulatory factors, rather than on the independent action of a single myogenic factor. In all known skeletal muscle lineages, MRF expression follows essentially the same pattern. MyoD and Myf5 are expressed in actively proliferating cells prior to differentiation, followed, at the beginning of differentiation by increased myogenin expression (Montarras et al. 1991; Lassar et al. 1994). Lastly, Myf6 is transiently expressed during myofibre maturation (Montarras et al. 1991).

It is well known that the MRFs are functionally important in regulating the expression of muscle specific genes (Piette et al. 1990; Lin et al. 1991; Wentworth et al. 1991; Muscat et al. 1992; Lassar et al. 1994; Li and Capetanaki 1994). More recently however, it has been suggested that the MRFs (in particular MyoD) interact with cell cycle regulators to promote cell cycle arrest and thus differentiation of myogenic cells. MyoD is capable of inducing the expression of p21, p27, p57, and Rb (Martelli et al. 1994; Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995; Reynaud et al. 2000). As mentioned previously, the induction of these Cdk-inhibitors activate Rb protein and contribute to permanent cell cycle arrest.

Numerous studies have indicated that cells must exit the cell cycle to terminally differentiate. Central to the differentiation of skeletal myoblasts is myogenin, the absence of which results in the failure of myoblasts to attain the differentiated phenotype (Hasty et al. 1993). Myogenin, in conjunction with MyoD and the myocyte enhancer family protein, MEF-2C subsequently regulate the late phase of myogenesis by inducing the expression of myofibrillar and muscle specific proteins (Gu et al. 1993; Olson and Klein 1994).

3. Satellite Cell Fusion

The last critical step in terminal differentiation of satellite cells is the fusion of the differentiated myoblasts, either to themselves to form syncytial muscle fibres or to existing myofibres to promote repair and/or hypertrophy. Cell-surface molecules such as cadherins, ADAMs, EMC receptors, and the Ig superfamily molecules N-CAM and VCAM, have all been implicated in the regulation of myoblast fusion (Mege et al. 1992; Charlton et al. 2000; Cooper 2001; Fischer-Lougheed et al. 2001; Kang et al. 2003). Additionally, β1 integrins have been implicated in myoblast fusion and sarcomere incorporation (Li et al. 1999). Furthermore, the inactivation of Rho/ROCK signalling
and subsequent nuclear translocation of the transcription factor FKHR is required for myoblast fusion (Bois and Grosveld 2003; Nishiyama et al. 2004).

4. Satellite Cell Self-Renewal

Central to the continued regenerative and adaptive potential of muscle is the self-renewal of satellite cells without which recurrent demands for increased myonuclei would rapidly deplete the satellite cell pool. Several scenarios have been proposed to explain how the satellite cell pool is replenished. Firstly, it has been suggested that two populations of satellite cells exist; one rapidly dividing population responsible for the provision of myonuclei and a slowly dividing pool of ‘reserve cells’, thought responsible for the replenishment of the quiescent satellite cell population (Rantanen et al. 1995; Yoshida et al. 1998; Zammit et al. 2004). Alternatively, satellite cells might represent an intrinsically homogenous pool of cells, capable of rapid proliferation and yet able, as required, to withdraw from the differentiation program and return to quiescence. Furthermore, renewal of the satellite cell pool may not rely exclusively on the satellite cell compartment. Indeed, several recent studies have shown that other cells, isolated both from muscle (side population cells) and a diverse range of tissues (bone marrow in particular) are capable of adopting a myogenic phenotype, albeit at a reduced frequency (Ferrari et al. 1999; Asakura et al. 2002; LaBarge and Blau 2002; Camargo et al. 2003; Corbel et al. 2003). The relative importance of these cell types to normal muscle repair and remodelling is unknown.
III. TRANSCRIPTIONAL REGULATION OF MUSCLE HYPERTROPHY

Skeletal muscle adaptation involves the modification of cellular (myofibrils, mitochondria etc.) and extracellular compartments (capillaries, connective tissue etc.) such that the muscle is appropriately modified to suit its functional demands. Alterations in the molecular components of muscle tissue during adaptation are largely dependant on modulations in the protein make-up of the myofibres. The change in protein content and protein constituents can potentially be controlled at many steps, from RNA synthesis (transcription) to the synthesis of new proteins (translation) and post translational modifications. Translational regulation has been identified as a critical modulator of increased protein synthesis during skeletal muscle hypertrophy, however it is beyond the scope of this review and the reader is directed to several excellent reviews in the area (Jefferson and Kimball 2001; Nader et al. 2002; Bolster et al. 2003). Equally, it has long been recognised that increased transcription is essential for skeletal muscle hypertrophy (Goldberg and Goodman 1969; Sobel and Kkaufman 1970), although the genes and regulatory mechanisms responsible are only just beginning to be elucidated.

A. Muscle Derived Growth Factors

Changes in the availability of muscle derived growth factors appears to be a central regulatory process in muscle hypertrophy. These factors are produced locally within the muscle and act both in an autocrine and paracrine fashion. Muscle contraction or damage activates signal transduction pathways, ultimately stimulating the production of these growth factors most likely via increased transcription. In vitro, numerous growth factors have been implicated in muscle proliferation and differentiation including members of the FGF and TGF-β families, IGF-1, and HGF. However, physiological functions in vivo have only been described for relatively few of these factors, most notably IGF-1 and myostatin.

1. IGF-1/MGF

The role of IGF-1 in regulating growth and development in many tissues is widely acknowledged. More recently, the autocrine/paracrine regulation of IGF-1 within muscle tissue has become apparent with numerous studies identifying increased production and release of this factor following exercise or overload (Adams et al.
1999; Bamman et al. 2001; Psilander et al. 2003). Moreover, an alternate splice variant of IGF-1, called mechano-growth factor (MGF) or IGF-IEc, has been identified as being expressed exclusively in muscle in response to contraction or stretch (Yang et al. 1996; McKoy et al. 1999; Owino et al. 2001; Bickel et al. 2003; Hameed et al. 2003). Whilst the mechanisms by which IGF-1 and MGF promote muscle hypertrophy are not entirely clear, it is evident that they act by signalling to both satellite cells (promoting both proliferation and differentiation) and the mature myofibre (activating signalling pathways and ultimately increasing protein synthesis).

1. Myostatin

Myostatin (growth/differentiation factor 8), a member of the TGB-β family (McPherron et al. 1997) is a powerful endogenous negative regulator of muscle hypertrophy (Sharma et al. 2001). Myostatin is synthesised and secreted as an inactive complex of the N-terminus propeptide and active C-terminus. The latent form of myostatin circulates bound to and inhibited by follistatin. Mature myostatin exerts its effects via interaction with the activin/TGF-β ActIIIb receptor and subsequent activation of the Smad family to control gene transcription (Rebbapragada et al. 2003; Rios et al. 2004). Further studies have confirmed that myostatin signals satellite cell quiescence by up-regulating p21 thus reducing the differentiation potential of satellite cells, and subsequent capacity for hypertrophy (Carlson et al. 1999; Joulia et al. 2003). Mice, cattle and humans with inactive or absent myostatin proteins exhibit a gross hypermuscular phenotype (Grobet et al. 1997; Kambadur et al. 1997; McPherron and Lee 1997; Schuelke et al. 2004). Furthermore, reductions in circulating myostatin (Walker et al. 2004), and myostatin gene expression (Roth et al. 2003) have been observed following resistance exercise training in humans. Collectively, these studies suggest a model in which myostatin expression is responsive to muscle loading and plays a key role in regulating muscles adaptation to this increased load.

1. HGF & FGFs

Both HGF and FGF family members have been described as potent regulators of satellite cell activity (Allen et al. 1995b; Bischoff 1997; Sheehan and Allen 1999; Yablonka-Reuveni et al. 1999), however the importance of their expression in vivo during muscle overload is not well understood. To date only one study has investigated the expression of these growth factors following increased muscle loading. FGF family members, and HGF mRNAs were increased following compensatory overload of rat plantaris muscle, suggesting prominent roles in the early proliferative response of satellite cells during muscle adaptation (Sheehan and Allen 1999).
B. Expression of structural/contractile/enzymatic genes

Resistance or strength exercise, characterised by 'low repetition, high load' muscular contractions, induces specific and distinct structural and functional modifications in muscle fibres. Increases in structural and contractile proteins (e.g. desmin, troponin I) as well as muscle specific enzymes (e.g. muscle creatine kinase) are necessary for muscle to hypertrophy and remain functional. Numerous studies have observed increased structural, contractile and enzymatic gene expression following acute and chronic resistance exercise in humans as well as overload models of hypertrophy in animals. Both acute (1 session) and chronic (12 weeks) heavy resistance exercise results in an increased expression of myosin heavy chain isoforms, type I, type IIA and type IIx (Willoughby and Rosene 2001; Willoughby and Nelson 2002). In rats, compensatory overload has been associated with increased expressions of β-tubulin, cardiac troponin T and atrial myosin light chain 1 (Carson et al. 2002). Similarly, increased desmin and creatine kinase expression has been observed during quail muscle hypertrophy (Lowe et al. 1998).

C. Myogenic Regulatory Factors

The altered contractile characteristics and increased myofibre size is a generally excepted adaptation of skeletal muscle to increased loading. Importantly, research is beginning to elucidate the molecular mechanisms required for these cellular adaptations. The MRFs are likely candidates as regulators of this process considering the importance of their expression during myogenesis, and because of their regulation of the transcription of muscle specific genes including α-actin (Muscat et al. 1992), desmin (Li and Capetanaki 1994), nicotinic Ach receptor (Piette et al. 1990), muscle creatine kinase (Lassar et al. 1994), troponin I (Lin et al. 1991) and myosin light chain (Wentworth et al. 1991). Load mediated increases in the expression of the MRFs have been observed in animals models of hypertrophy (Carson and Booth 1998; Lowe et al. 1998; Lowe and Alway 1999; Adams et al. 2002; Carson et al. 2002; Haddad and Adams 2002). Similarly, alterations in MRF expression patterns have been demonstrated in response to resistance exercise training (Willoughby and Rosene 2003) as well as acute resistance exercise bouts (Willoughby and Nelson 2002; Bickel et al. 2003; Psilander et al. 2003; Bamman et al. 2004; Bickel et al. 2004). Importantly, the exact roles the MRFs play in mature muscle adaptation are yet to be established. Whilst their temporal patterns of expression and driving roles in myogenesis have been well described in vitro, it is yet to be determined whether these expression patterns and functions are mimicked in vivo. Equally, the relative contributions of the
MRFs to adaptation within the mature myofibre compared with their roles in satellite cell activity are yet to be fully characterised.

IV. PERSPECTIVES

Much progress has been made in recent years into understanding the molecular mechanisms by which skeletal muscle adapts to changes in physiological stimuli. Substantial information about the interrelationships between signalling pathways and the impact on transcriptional and translational processes has been accrued. Similarly, the importance of satellite cells and their mechanisms of action during skeletal muscle hypertrophy and remodelling are being established. Nevertheless, there are considerable gaps in our understanding. We still have no clear idea of the commonality of, or indeed distinct nature of the pathways governing satellite cell activity versus pathways necessary for mature myofibre adaptation. The common thread in all of these processes is that they rely on transcriptional reprogramming. Importantly, only a handful of genes have been isolated to date that have been shown to be uniquely responsive to resistance exercise and increased loading. The recent advancement in molecular biological techniques, particularly the use of high-throughput transcriptional profiling should enable the identification of previously unknown molecules. Similarly, the use of more sophisticated in vitro tools such as human primary skeletal muscle cell cultures provide a more physiologically revelent model to examine novel gene expression and function.

A. cDNA Microarray Technology in Studies of Skeletal Muscle

The complete identification of coding sequences in the human genome and advances in molecular biology have preceded the development of methods such as cDNA microarrays which allow the investigation of the transcriptional activity of thousands of genes or indeed the entire transcriptome simultaneously. cDNA microarrays can be used to compare the gene expression in two different cell types or tissue samples for example cancerous versus normal tissue. They can also be used to examine changes in transcriptional activity over time and following any number of potential interventions.

The use of microarray technology on human skeletal muscle samples following different exercise paradigms provides a new and exciting avenue for investigation. Microarrays have been used to characterise gene expression patterns in skeletal muscle in a variety of contexts including atrophy (Gomes et al. 2001; Jagoe et al. 2002; Lecker et al. 2004), treatment with thyroid hormone (Clement et al. 2002),
during a hyperinsulinemic clamp (Rome et al. 2003), in disease states such as diabetes (Yang et al. 2002; Hansen et al. 2004) and muscular dystrophy (Chen et al. 2000; Bakay et al. 2002; Noguchi et al. 2003), as well as the comparison of fast versus slow muscle tissue (Campbell et al. 2001).

The complex nature of the molecular processes regulating skeletal muscle development, adaptation and regeneration make it an ideal candidate for the development of a tissue specific microarray. Microarray analysis has also been used to examine the transcriptional response of skeletal muscle to various exercise interventions. Barash (Barash et al. 2003) observed the up-regulation of 36 known genes in eccentric exercised mouse muscle compared to contralateral and isometrically exercised controls. Similarly, Carson (Carson et al. 2002) used microarray analysis to identify 112 mRNAs differentially expressed in overload versus control rat muscle. To date, only three studies have employed microarray technology to examine global gene expression in skeletal muscle following exercise interventions. Roth (Roth et al. 2002) studied the role of age, sex and strength training on human muscle gene expression with 69 genes identified as differentially regulated by strength training, 200 genes differentially expressed between the sexes and 50 genes differentially expressed in relation to age. Zambon (Zambon et al. 2003) used microarrays to determine the effects of resistance exercise on gene regulation in biopsy samples of human quadriceps muscle obtained 6 and 18 hours after an acute bout of isotonic exercise with one leg. Diurnal gene regulation was also profiled at the same time points in the non-exercised leg. The results suggested that resistance exercise may directly modulate circadian rhythms in human skeletal muscle. Finally, Chen and colleagues (Chen et al. 2003) examined the effect of eccentric exercise on gene expression in human skeletal muscle. Volunteers performed 300 concentric contractions with one leg and 300 eccentric contractions with the opposite leg. Pronounced increases in inflammatory and vascular remodelling gene responses were observed following eccentric exercise.

B. Primary Human Skeletal Muscle Cell Culture

Both primary skeletal muscle cell cultures and established muscle cell lines have become important tools as model systems for skeletal muscle development. The dividing myoblasts provided by these systems provide great flexibility in studies of myogenesis as cultures are readily manipulated allowing the examination of distinct aspects of muscle development. Although many studies have been carried out on the
growth and differentiation of muscle cells, the vast majority use immortalised rat and mouse muscle cell lines. Considerable differences exist however, in the expression profiles (James et al. 1993; McCusker and Clemmons 1998; Crown et al. 2000), and physiological responses of these cell culture systems (Florini et al. 1991; Foulstone et al. 2003). Human primary skeletal muscle cell cultures are a more physiologically relevant \textit{in vitro} model of adult skeletal muscle because the cells retain the characteristics of the donor. For example, cells cultured from type 2 diabetic patients retain their characteristic insulin resistant skeletal muscle phenotype (reduced insulin-stimulated glucose uptake and glycogen synthesis) (Henry et al. 1995; Henry et al. 1996). Human primary skeletal muscle cultures have been used previously to examine the mechanisms of type 2 diabetes (Henry et al. 1995; Henry et al. 1996; Hansen et al. 2004), myoblast differentiation (Chazaud et al. 1998; Konig et al. 2004), the effect of IGF-1 treatment (Crown et al. 2000; Foulstone et al. 2003), mechanisms of muscular dystrophy (Merickel et al. 1981), and susceptibility to malignant hyperthermia (Girard et al. 2002).

Despite the complexity of the mechanisms governing skeletal muscle hypertrophy, advances in powerful molecular biological tools should begin to provide considerable insights into the regulation of this process, ultimately paving the way for the development of interventions and treatments for the myriad of diseases affecting skeletal muscle.
V. AIMS

The broad aims of the thesis are to examine the molecular regulation of skeletal muscle hypertrophy with a focus on the identification of novel genes involved in this process. This series of studies is critical to further elucidate the transcriptional events that contribute to the development of skeletal muscle hypertrophy.

The specific aims of this thesis are:

• To develop and test a human skeletal muscle specific cDNA microarray for global transcriptional profiling and the identification of novel gene transcripts.

• To further enhance current understanding of the process of myogenesis by examining the expression profile of rhabdomyosarcoma cells undergoing forced differentiation using the human skeletal muscle specific microarray.

• To further test the sensitivity and reliability of the microarray in identifying changes in gene expression in a biological system. Thus ensuring reliable and reproducible results would be attainable from the microarray in subsequent studies using less readily available human skeletal muscle samples.

• To investigate the expression of myogenic and atrogenic genes in human skeletal muscle following an acute bout of resistance exercise.

• To use the human skeletal muscle specific microarray to study the time-course of transcriptional changes and identify novel genes expressed following an acute bout of resistance exercise.

• To use human primary skeletal muscle cell cultures to identify whether the expression of the novel genes were (1) activated at any time during proliferation, cell cycle arrest or differentiation or (2) activated by the additional mitogenic and myogenic stimulation provided by exogenous IGF-1 peptide supplementation.

• To examine whether the novel genes are a unique response to resistance exercise or if they were responsive to skeletal muscle contraction or exercise in general.
VI. HYPOTHESES

It is hypothesised that:

- cDNA microarray analysis of the transcriptional profile of differentiating rhabdomyosarcoma cells will result in the identification of novel differentially expressed genes.

- An acute bout of resistance exercise will increase the expression of the MRFs and cell cycle regulators whilst reducing the expression of genes involved in skeletal muscle atrophy.

- The transcriptional analysis of human skeletal muscle following an acute bout of resistance exercise using a human skeletal muscle specific cDNA microarray will identify novel differentially expressed genes.

- The expression of ASB5, TXNIP, MLP, FLJ38973 and the MRFs will be altered during the differentiation of human skeletal muscle primary cell cultures.

- The expression of ASB5, TXNIP, MLP, FLJ38973 and the MRFs will not be altered following an acute bout of resistance exercise.
CHAPTER TWO
DEVELOPMENT AND VALIDATION OF A HUMAN SKELETAL MUSCLE SPECIFIC MICROARRAY

I. INTRODUCTION

New methods for the simultaneous assessment of the levels of expression of hundreds or thousands of mRNA levels in individual tissue samples are an attractive means by which biological processes can be studied. The analysis of gene transcript abundance is an important step in the determination of the functional roles of genes, how these genes and gene products interact as well as the comparison of gene expression patterns under different experimental conditions. Although mRNA is not the ultimate product of a gene, transcription is the first regulatory step in gene expression, and information about the mRNA levels is important for the understanding of gene regulatory networks.

The ability to monitor global gene expression at the transcript level has become possible due to the advent of cDNA microarray technologies. The power of microarray lies in its ability to simultaneously estimate the comparative level of gene expression of a large number of genes (up to 20,000 per array) in a single sample. Microarrays have been developed that allow a high throughput and provide identity and expression of both selected known genes and cDNAs representing uncharacterised genes between numerous biological samples.

Microarrays are constructed by "spotting" PCR-amplified cDNA clones at high density onto optically flat glass microscope slides in an orderly array (Hegde et al. 2000). RNA extracted from experimental samples can be fluorescently labelled and competitively hybridised to the microarray against a reference RNA sample, which is labelled with a different fluorophore (Figure 2.1).
Figure 2.1. Overview of microarray production and analysis. Initially cDNA clones are PCR amplified, purified and then spotted onto glass sides by microarray printing robots. RNA from samples of interest and a reference are extracted, reverse transcribed, and labelled with Cy3 (usually red) and Cy5 (usually green) fluorescent dyes. The two samples are then combined and competitively hybridised to the prepared microarray slides. Laser excitation of the incorporated fluorescent dyes yields an emission with a characteristic spectrum, which is measured using a scanning confocal laser microscope. Thus from the fluorescence intensities and colours from each spot, the relative expression of that particular RNA species in the experimental sample can be estimated. Laser excitation of the incorporated targets yields an emission with a characteristic spectrum which is measured using a scanning confocal laser microscope. Thus from the fluorescence intensities and colours for each spot, the relative expression level of that particular RNA species in the experimental sample can be estimated.
The complex nature of the molecular processes regulating skeletal muscle development, adaptation and regeneration make it an ideal candidate for the development of a tissue specific microarray. To this end we have created a customised microarray containing up to 11,000 uncharacterised cDNAs derived from a human skeletal muscle cDNA library as well as 270 genes with known functional roles in skeletal muscle. The genes chosen represent key regulatory steps in signal transduction pathways, various biochemical pathways including glycolysis and fatty acid oxidation, as well as a number of transcription factors, cytoskeletal and proteolytic proteins. The cDNA library used to construct the microarray was a human skeletal muscle 5'-STRETCH PLUS cDNA library. The mRNA source for this library was pooled from the quadriceps, iliopsoas and pectoralis major muscles from 8 male and female Caucasians, aged 29-60, who died following trauma. Furthermore, the cDNA library underwent extensive normalisation, a complex process where the cDNA copies of a primary library are equalized, so that each original mRNA transcript is represented in the normalized library to the same extent as all the rest of the clones. Subsequently, by increasing the frequency of occurrence of rare cDNAs whilst simultaneously decreasing the percentage of abundant genes, normalisation can expedite the discovery of novel transcripts.

The extraction of reliable information from the massive amounts of data generated by microarrays however, is dependant on optimal study design, array fabrication, data acquisition, data quality and data analysis. There are many process variables that will impact on the quality of the data generated by microarray platforms. Thus the parameters required for the effective manufacture of cDNA microarrays with highly reproducible performance characteristics, the quality and quantity of sample mRNA’s used to create the dye-labelled cDNA probes and the effects of these optimised procedures on the overall performance, accuracy, and precision are needed to be extensively tested to guarantee the reliability of generated expression data. Therefore, the aims of this study were firstly to describe the fabrication of the microarray and secondly, evaluate the robustness and reproducibility of this analytical technique.
II. METHODS

A. Microarray Fabrication

cDNA clones corresponding to the 270 known skeletal muscle genes were purchased from Incyte Genomics (Palo Alto, CA). The clones were grown overnight and inserts were PCR amplified using vector specific primers. Products were visualized by agarose gel electrophoresis, then excised and sequence verified using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). For the gene discovery aspect of the microarray, the Human Skeletal Muscle 5'-STRETCH PLUS cDNA Library was purchased from Clontech (Palo Alto, CA). The clones were grown overnight and picked by the Australian Genome Research Facility (Melbourne, Victoria, Australia) using a BioPick automated colony picker (BioRobotics Woburn, MA). The inserts were PCR amplified and the products visualized by agarose gel electrophoresis to confirm amplification of a single PCR product. Amplified cDNAs were then purified using an ArrayIT PCR Purification Kit (Telechem, Sunnyvale, CA) and resuspended in Spotting Solution Plus (Telechem) in preparation for arraying onto SuperAmine Microarray Substrates (TeleChem) using a ChipWriter Pro microarrayer (Virtek, Toronto, Canada). DNA adhesion to the glass was achieved by firstly baking the printed arrays for 1h at 80°C, followed by rehydration at 37°C and irradiation in a Stratalinker® UV Crosslinker (Stratagene, La Jolla, CA) at an energy output of 60mJ. To minimise any potential non-specific probe interactions with the glass the microarrays were washed for 2 min in 0.2% SDS (Invitrogen), followed by three rinses in H₂O for 2 minutes each with the last wash conducted at 95°C. They were washed again for 2 min in 0.2% SDS, and rinsed a further two times in H₂O for 2 min each. Finally, the arrayed slides were dried by brief centrifugation and stored in opaque plastic slide boxes at room temperature.

B. RNA Extraction

The RNA used for the homotypic hybridisations was a commercially available human skeletal muscle RNA (Ambion, Austin, TX)

RNA for the repeatability and reliability analysis was derived from differentiating rhabdomyosarcoma cells and formed part of a larger study (see chapter 3). Total RNA was extracted using the RNA-bee (Tel-Test, Friendswood, TX) reagent and purified
using RNeasy Columns (Qiagen, Mannhiem, Germany) as per the manufactures instructions (Qiagen 2001). Briefly, an appropriate volume of Trizol Reagent (Life Technologies, Invitrogen, Carlsbad, CA) was used to ensure complete cellular disruption and release of genetic material. Chloroform was added to the solution, thoroughly mixed, incubated on ice for 5 mins followed by centrifugation at 4°C for 15 min. The upper aqueous layer was removed and mixed with an equal volume of 70% ethanol before being added to the RNeasy spin column. After washing the column, the RNA was eluted as described in the manufacture’s protocol. RNA quality and concentration was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

C. Indirect labelling of cDNA

Fluorescently labelled cDNA was prepared from total RNA using an indirect labelling method. cDNA synthesis was performed in a 20µl reaction containing 5µg oligo-dT primer, 400U SuperscriptII (Invitrogen), 1X first strand buffer, 0.01M DTT, 0.5mM of each dATP, dCTP and dGTP, 0.150mM dTTP (Amersham, Buckinghamshire, UK) and 0.2mM aminoallyl-dUTP (Sigma, St Louis, MO). Synthesis was conducted in a GeneAmp PCR System 9700 (Applied Biosystems) at 42°C for 2 h. The reaction was stopped by addition of 5µl of 0.5M EDTA and RNA was hydrolysed by addition of 20µl of 1M NaOH at 70°C for 20 minutes. The reaction was neutralised with 25µl of 1M HEPES and the cDNA was purified using Qiaquick PCR purification kits (Qiagen) according to manufacturer’s instructions and eluted in nuclease-free water. The cDNA was concentrated using Microcon30 spin columns (Millipore, Bedford, MA). Following the addition of 0.09M sodium bicarbonate, the cDNA was coupled to Cy3 or Cy5 monofunctional N-hydroxysuccinimyl ester reactive dyes (Amersham). The coupling reaction was conducted in the dark for 1 h.

The fluorescently labelled cDNA was purified using Qiaquick PCR purification kits (Qiagen), combined and added to 10µg Human Cot1 DNA (Invitrogen). The cDNAs were again concentrated to the required volume with Microcon30 spin columns (Millipore). The cDNA were hybridised in a 40µl volume, containing the labelled cDNA, 5X SSC, 0.3% SDS, 4µg yeast tRNA, 8µg polydA, 2.5X Denhart’s solution. The cDNA was denatured at 98°C for 2 minutes and maintained at 60°C until required. 38µl of the hybridisation solution was applied to cover slips and then mounted onto the array slide. Hybridisation was conducted in humid hybridisation chambers in a hybridisation oven at 65°C for 16-20 hours.
D. Image Acquisition and Normalisation

Fluorescent images of the microarrays were acquired using the GenePix 4000B laser scanner (Axon Instruments, Inc., Union City, CA) and the images processed using Genepix Pro 3 software (Axon Instruments). Data files were entered into Acuity 4.0 software (Axon Instruments) where a global normalisation strategy was applied to normalise signal intensities across all arrays. Normalisation of signal intensities across all arrays is necessary in order to eliminate the effects of array-to-array variations in target preparation, hybridisation and scanning.
III. RESULTS & DISCUSSION

A. Microarray Validation

1. Quality of cDNA clones and Microarray Printing

The manufacture of high quality, reproducible arrays with 10,000 or more unique PCR products is an expensive and time consuming process. It requires considerable attention to the details and necessary procedures in each step to ensure the quality and reproducibility of the end product. Yue (Yue et al. 2001) state in their evaluation of the performance of cDNA arrays that low concentrations of DNA in the input printing solutions results in reduced amounts of arrayed DNA and this, in turn, reduces the dynamic signal range and produces an apparent underestimation of differential expression. Prior to being spotted onto optically flat microscope slides, the cDNA clones for both the known and discovery aspects of the microarray were PCR amplified, purified and visualised on agarose gels to verify amplification and presence of a single PCR product (Figure 2.2). Plates containing greater than 15% PCR failure or multiple products were not included in the array printing. Additionally, PCR products corresponding to the known genes were sequence verified as previous findings have identified incorrect sequences as a significant source of error (Knight 2001).

Representative microarrays from each manufactured batch were stained for DNA using SYBRGold (Molecular Probes) and scanned on a GenePix 4000A scanner (Axon Instruments) at 535 nm. The resulting image (Figure 2.3) demonstrated a high degree of DNA retention on the surface of the array, good spot morphology and a uniform printing pattern. The median diameter of the features on the array was 100 μm, with a preset spacing between the centres of adjacent features of 200 μm.
Figure 2.2. Representative agarose gel of amplified clones. PCR amplified cDNA clones were run on a 1% agarose gel to verify amplification and the presence of a single product.

Figure 2.3. A representative section of a SYBRGold stained microarray slide. Representative microarrays from each manufactured batch were stained for DNA using SYBRGold. The resulting image demonstrated a high degree of DNA retention on the surface of the array, good spot morphology and a uniform printing pattern.
2. Homotypic Response

An estimate of the accuracy and precision of microarray expression data was first made by performing a homotypic hybridisation. A competitive hybridisation of fluorescently labelled Cy3 and Cy5 cDNA, both prepared from the same mRNA, should theoretically give a ratio of 1 (i.e. Cy3 fluorescence divided by Cy5 fluorescence). Replicate hybridisations provide valuable information on the overall precision of the data by examining any deviations from the theoretical value (Yue et al. 2001).

Twenty µg of human skeletal muscle RNA was coupled to both Cy3 and Cy5 fluorophores and competitively hybridised to the skeletal muscle microarray. Figure 2.4 shows a false colour image of the hybridised microarray (A) with one 20 × 20 grid enlarged (B). Typically, an array composed of disparate RNA samples, each labelled with a different fluorophore, will exhibit varying degrees of expression typically represented as red and green spots on an array. Elements showing no difference in expression between the two samples are represented by yellow spots. As expected, a homotypic hybridisation such as the one shown in Figure 2.4 is composed almost exclusively of yellow spots.

A scatter plot of the Cy3 versus Cy5 fluorescent response and a histogram of intensity ratios from the skeletal muscle/skeletal muscle hybridisation is shown in Figure 2.5. Virtually all gene elements lie close to the diagonal line corresponding to the theoretical differential expression ratio of 1 (Log ratio of 0, r² = 0.99) The average calculated relative fluorescence ratios for all elements was 1.057 with the 99.5% confidence interval falling between 0.054 (lower bound) and 1.061 (upper bound). The average coefficient of variation for ‘differential expression’ of any element in this homotypic experiment is 11% over the entire signal range. These results demonstrate equal incorporation of Cy3 and Cy5 to the RNA and equal hybridisation to the microarray.
Figure 2.4. A false colour image of the muscle: muscle hybridisation (A) and an enlarged view of a single 20 × 20 grid (B). The array consists of PCR-amplified random cDNA clones spotted onto a glass slide. Equal quantities of skeletal muscle RNA was labelled with Cy3 and Cy5 fluorophores and competitively hybridised to the array. Yellow dots represent genes that show no difference in expression between the two sample RNAs.
Figure 2.5. Homotypic Hybridisations. (A) Scatter plot of the calibrated Cy5 versus Cy3 fluorescence response from a skeletal muscle/skeletal muscle hybridisation. Virtually all gene elements lie close to the diagonal line corresponding to the theoretical differential expression ratio of 1. (B) Data points from the homotypic hybridisations were used to construct a histogram, which shows the distribution of gene elements around the expected log ratio of 0.0.
3. Repeatability and Reliability

3.1 Within-array reproducibility

To examine the spot to spot reproducibility and reliability within arrays, a total of 155 genes printed in duplicate on each array were examined across 18 microarrays. The printing of replicate spots on the arrays allowed us to examine whether identical spots on the array produced comparable results. From the 18 arrays in this data set we calculated a coefficient of variation (CV) for each of the 155 duplicated genes. The average CV was observed to be 15% across the entire signal range, although there was slightly greater variation at low signal levels. Consequently, replicate genes showing large variation in expression were excluded from subsequent analysis.

3.2 Between-array reproducibility

To examine the influence on hybridisations conducted on different days, six identical hybridisations were performed: two performed on one day (repeat 1a and 1b), two performed one week later (repeat 2a and 2b) and two performed 4 weeks later (repeat 3a and 3b). Skeletal muscle RNA was coupled to Cy3 dye and RNA extracted from differentiated rhabdomyosarcoma cells was coupled to Cy5 dye. Disparate samples were chosen for this analysis to examine the reproducibility and reliability of the data over a range of expression values. Following normalisation and log transformation, clustering methods were utilised to examine the similarity between the replicate hybridisations. Algorithms utilized in clustering result in genes or groups whose members resemble each other, and thus provide a convenient way to illustrate the data. In this context, hierarchical clustering was performed to examine how similar or different the overall arrays were across the six replicate hybridizations. Hierarchical clustering partitions the data into a tree, so that substances that are not grouped together on one branch of the tree are grouped together at a higher branch. Substances occur only once in the tree. The distance between clusters was determined by the distance between 'average neighbours'. Figure 2.6 shows the hierarchical clustering dendrogram of the six replicate hybridisations. Pearson’s Correlation Coefficient was used to examine the similarities between clusters. The replicate hybridisations conducted on the same days showed a very high degree of similarity (r\(^2\) = 0.97, 0.91 and 0.99 for replicates 1, 2, and 3 respectively) indicating a very high degree of reproducibility. Greater variance was observed between arrays conducted on different days, however the highly positive correlation (r\(^2\) =0.84) indicated very good overall precision and reproducibility for the microarray.
Figure 2.6. Hierarchical clustering dendrogram of six identical hybridisations, two conducted on one day (repeat 1a and 1b), two conducted one week later (repeat 2a and 2b) and two conducted 4 weeks later. The closest correlations are indicated by low branches on the dendrogram. The overall high correlation (top branch on the dendrogram) across the six arrays indicated very good reproducibility of the microarray.
cDNA microarrays are a very attractive and powerful means of examining global gene expression. The extraction of reliable information from the massive amounts of data generated by microarrays however, is dependant on optimal study design, array fabrication, data acquisition, data quality and data analysis. An important first step in developing cDNA microarrays is the validation of the experimental and analytical techniques. It is critical to establish whether repeated hybridisations with identical RNA deliver comparable results before any attempt is made to quantitate biological variability. We have developed a skeletal muscle specific cDNA microarray from a normalised human skeletal muscle cDNA library which will allow expression analysis and identification of novel genes in a range of different experimental systems. The results presented demonstrate the robustness of the microarray in producing accurate and reliable data.
CHAPTER THREE
IDENTIFICATION OF NOVEL GENES IN DIFFERENTIATING RHABDOMYOSARCOMA CELLS

I. INTRODUCTION

Skeletal muscle differentiation is characterised by terminal withdrawal from the cell cycle, muscle-specific gene activation and subsequent fusion of myoblasts into multinucleated myotubes. It has been well documented that myogenic differentiation is under the control of a family of regulatory genes which belong to the basic helix-loop-helix family of transcription factors, also referred to as the myogenic regulatory factors (MRFs). The MRFs encode a family of transcription factors comprising four members, MyoD, Myf5, myogenin and Myf6 (MRF4, herculin) that bind to specific consensus sites that are functionally important in the transcription of muscle-specific genes (Megeney and Rudnicki 1995; Yun and Wold 1996). These factors are characterised by the ability, upon transfection, to convert nonmyogenic cells to the muscle phenotype. Although these transcription factors play a major role in the myogenic differentiation process, it is becoming evident that although their activity is necessary, multiple signalling pathways are required to act in concert for the execution of the differentiation program of muscle cells (Puri and Sartorelli 2000; Wei and Paterson 2001).

Rhabdomyosarcoma (RMS) are highly aggressive, malignant soft tissue sarcomas that primarily affect children and young adults. These tumours resemble primitive skeletal muscle-forming cells in appearance, suggesting that RMS may arise as a consequence of regulatory disruption of the growth and differentiation of skeletal muscle progenitor cells. Normal myogenesis is characterised by the expression of the MRFs (MyoD and Myf5 and then myogenin and Myf6), followed by the differentiation of myoblasts and formation of multinucleated myotubes. RMS cells however, are blocked on their way to terminal muscle differentiation. Paradoxically, this abortive myogenic differentiation occurs despite the expression of the MRFs, MyoD and myogenin, (Aguanno et al. 1990; Bouche et al. 1993). Most RMS cell lines of human and animal origin retain the ability to differentiate despite limited differentiation in vivo.
Treatment of RMS cells with phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) has been widely shown to induce growth arrest and myogenic differentiation, without affecting the expression of the myogenic transcription factors (Garvin et al. 1986; Mayer et al. 1988; Aguanno et al. 1990; Lollini et al. 1991; Bouche et al. 1993; Bouche et al. 1995; Bouche et al. 2000). Suggesting that the differentiation arrest is due to mechanisms that interfere with the MRFs; downstream of their expression (Bouche et al. 1995). However, the precise molecular mechanisms responsible for the disruption of the myogenic characteristics of RMS is not well understood.

The genetic regulation of RMS tumours is of great interest as the differentiation potential strongly influences metastatic ability and therefore prognosis of these tumours (Lollini et al. 1991). Furthermore, the aberrant myogenic differentiation program in RMS tumours provides a unique model for the identification of genes with previously unidentified roles in myogenesis. An extensive body of work has demonstrated the temporal and spatial roles of specific genes during myogenesis, however, limited knowledge exists about how broad the transcriptional control of this process may be or even which specific processes are the critical regulators of differentiation, determination and fusion of muscle cells. Given the complexity of myogenesis, clearly there are a host of genes, some known and some novel, involved in this process. Thus, identifying known molecules with unknown functions or novel molecules that play critical roles in the cascade of molecular events will help provide an essential foundation for our understanding of myogenesis.

Because of the wide spectrum of genes likely to be involved in myogenesis, cDNA microarray technology is well suited for the examination of genes expressed at key time points during proliferation and differentiation. Microarray technology is a very valuable tool, not only for the simultaneous examination of thousands of genes but also in its ability to examine the expression of novel genes. The aims of this study were two-fold. In chapter 2, the development of a skeletal muscle specific cDNA was discussed. The first aim of this study was to further enhance current understanding of the process of myogenesis by examining the expression profile of rhabdomyosarcoma cells undergoing forced differentiation using the human skeletal muscle specific microarray. The second aim was to further test the sensitivity and reliability of the microarray in identifying changes in gene expression in a biological system. Thus ensuring reliable and reproducible results would be attainable from the microarray in subsequent studies using less readily available human skeletal muscle samples.
II. METHODS

A. Cell culture

The rhabdomyosarcoma cell line RD-A was a kind gift from Dr. Bruce Thorley at the Victorian Infectious Diseases Reference Laboratory (North Melbourne, Australia). The cells were maintained in high glucose Dulbecco’s Modified Eagle Medium (Gibco, Invitrogen Corporation, Carlsbad, CA) containing 10% foetal bovine serum (Gibco) in a humidified atmosphere of 5% CO$_2$ in air at 37°C.

B. Study Design

RD-A cells were seeded at a density of 3000 cells/cm$^2$ in complete medium on the day prior to experimentation. After 24 h the medium was replaced with fresh medium containing 2% horse serum (Gibco) to reduce proliferative factors. At the same time 100nM of TPA (Sigma, St Louis, MO) in DMSO (Sigma) was added to the medium to induce differentiation. Cells were cultured for up to 10 days, with RNA and protein extracted for analysis at day 1, day 3, day 6, day 8 and day 10. The control condition for this experiment consisted of actively proliferating cells collected immediately before the addition of the differentiation medium. At least 3 independent replicate experiments were conducted.

C. Microarray Fabrication

The human skeletal muscle specific microarray was constructed as previously described.

D. RNA extraction

Total RNA was extracted using the RNA-bee (Tel-Test, Friendswood, TX) reagent and purified using RNeasy columns (Qiagen, Mannheim, Germany) as per the manufacturer’s instructions (Qiagen 2001). Briefly, an appropriate volume of Trizol Reagent was used to ensure complete cellular disruption and release of genetic material. Chloroform was added to the solution, thoroughly mixed, incubated on ice for 5 min followed by centrifugation at 4°C for 15 min. The upper aqueous layer was
removed and mixed with an equal volume of 70% ethanol before being added to the RNeasy spin column. After washing the column, the RNA was eluted as described in the manufacture’s protocol. RNA quality and concentration was determined using the RNA Nano 6000 kit (Agilent Technologies, Inc., Palo Alto, CA) on the Agilent 2100 Bioanalyzer (Agilent Technologies).

E. Indirect labelling of cDNA and hybridisation

Fluorescently labelled cDNA was prepared from 40 µg of total RNA using an indirect labelling method. The reference RNA for this study was pooled from both undifferentiated and differentiated rhabdomyosarcoma cells. Indirect labelling of the RNA and subsequent hybridisation was prepared as previously described. In all hybridisations, the reference RNA was labelled with Cy5 dye, whilst the experimental samples were labelled with Cy3 dye.

F. Image Acquisition and Data Analysis

Fluorescent images of the microarrays were acquired using the GenePix 4000B laser scanner (Axon Instruments, Inc., Union City, CA) and the images processed using GenePix Pro4 software (Axon Instruments). Data files were entered into Acuity 4.0 software (Axon Instruments) for subsequent data analysis. The microarrays were normalized using a linear, ratio-based normalization strategy. The normalization factor was calculated from the average ratio of all features with expression ratios between 0.1 and 10. Flagged spots and spots with less than 55% of feature pixels with intensities more than two standard deviations above the background pixel intensity were not retained for further analysis. Furthermore, genes had to be present in at least 70% of arrays to be considered in the analysis. The elements and time-points were averaged across replicate experiments and then organized by hierarchical clustering with the Pearson correlation metric and average linkage clustering (Eisen et al. 1998). Paired t-tests were used to identify ten elements with the greatest evidence of differential expression during the differentiation process.

G. Clone Identification

Selected cDNA clones were grown overnight and plasmid isolation was conducted using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. Inserts were PCR amplified using vector specific primers. Products were
visualized by agarose gel electrophoresis then excised and the sequence was
determined using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied
Biosystems). The basic local alignment search tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) was used to compare the cDNA clone
sequences with previously characterized genes (Altschul et al. 1990)

H. Real-time PCR Analysis

1. RNA Extraction

Total RNA was extracted as previously described (Chapter 3, section II, part D). The
RNA used for the real-time PCR analysis was derived from the same original stock
used in the microarray analysis.

2. Reverse Transcription

Prior to reverse transcription, the diluted RNA was heated to 65° C for 10 mins, then
quenched on ice for 5 mins. 1 µg of RNA was reverse transcribed to synthesise first
strand cDNA using the AMV reverse transcriptase kit (A3500; Promega, Madison, WI).
The RNA was added to a master mix containing a final concentration of 5 mM MgCl2,
10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1% Triton X-100, 1 mM of each dNTP, 20 U
Recombinant Rnasin Ribonuclease Inhibitor (40 U/µl) and 1 µg oligo (dT)15 Primer
per 1 µg RNA. Following the addition of 15 U AMV Reverse Transcriptase (20 U/µl)
(Promega, Madison, WI), the mixture was incubated at 42° C for 60 mins, before the
reaction was terminated by incubation at 99° C for 5 mins followed by 5 mins at 4° C
using the PCR Express Thermal Cycler (Hybaid, Middlesex, UK). The cDNA was
stored at -20° C for subsequent analysis. A reverse transcription (RT) negative was
obtained by not adding any RNA to an aliquot of the RT mix.

3. Primer Design

To perform PCR, specific primers were designed for all genes using Primer Express
software (Applied Biosystems, Foster City, CA) on sequences obtained from GenBank
(see Table 3.1 for details). Where possible, primers were designed spanning intron-
exon boundaries to prevent amplification of the target region from any contaminating
DNA. Sequence information, including the positions of intron-exon boundaries was
obtained from the Ensemble Genome Project (www.ensembl.org) (Hubbard et al.
2002). Primer specificity was confirmed using BLAST (www.ncbi.nlm.nih.gov/BLAST/).
Primers were purchased from GeneWorks (Adelaide, SA, Australia).
4. Real-time PCR Reaction

For the PCR step, reaction volumes of 20µl contained SYBR Green 1 Buffer (Applied Biosystems, Foster City, CA), forward and reverse primers (see Table 3.1) and cDNA template (diluted 1:40). All samples were run in duplicate. Real-time PCR was run for 1 cycle (50ºC 2 min, 95ºC 10 min) followed by 40 cycles (95ºC 15 s, 60ºC 60 s) and fluorescence was measured after each of the repetitive cycles. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was amplified. The expression of the gene of interest in a given sample was calculated by subtracting the CT of the target gene for a given sample, from the CT of the control sample. The relative expression of the gene of interest compared with control was then calculated using the expression \(2^{-\Delta CT}\).

5. PCR Efficiency

For each gene, the PCR efficiency of the primer pairs was determined using a dilution series of cDNA input into the PCR reaction. Linear regression was used to analyse the response of CT vs. the logarithm of the cDNA concentration. Using the slopes of the lines, we calculated the efficiency (E) of the target amplification with the equation \(E = \left(10^{1/slope}\right) - 1\) (Lekanne Deprez et al. 2002). PCR efficiencies indicated a linear detection of the cDNA for all genes (Table 3.1).

I. Western Blot Analysis

Rhabdomyosarcoma cells were cultured as described above. Control cells and cells collected at day 1, day 3, day 6, day 8 and day 10 were resuspended in lysis buffer (50mM Tris, pH7.6, 250mM NaCl, 5mM EDTA, 0.1% IGEPAL, Complete Protease Inhibitor Cocktail (Sigma) and passed through a syringe. The cell extracts were centrifuged to pellet cell debris, supernatants removed and analysed for total protein (BCA protein assay kit, Pierce, Rockford, IL) (Langley et al. 2002). Equal amounts of denatured total proteins from each sample were separated by electrophoresis on either a 10% (myogenin) or 6% (total myosin heavy chain (MHC)) SDS-polyacrylamide gel and transferred to nitrocellulose membrane by electroblotting. Membranes were blocked for 2 h with 5% skim milk in Tris-buffered saline (50 mM Tris-HCl, 750 mM NaCl, 0.25% Tween), and were incubated overnight at 4°C with a polyclonal anti-MHC (MY-32, Zymed, San Francisco, CA) or anti-myogenin antibody (F5D, Developmental Studies Hybridoma Bank, University of Iowa). Membranes were washed (4 x 5 min), followed by a 60 min incubation with anti-rabbit IgG (MHC) or anti-mouse IgG (myogenin) conjugated to horse-radish peroxidase (1 in 10,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), then washed again. Immunoreactive bands were
detected using enhanced chemiluminescence (Western Lightning Chemiluminescent
Reagent, Perkin-Elmer, Boston, MA). An internal control was used in each gel to
normalize for variation in signal observed across the membranes. The density of the
bands was analysed using Kodak 1D 3.5 image analysis software (Kodak Digital
Science, Rochester, NY).

J. Immunocytochemistry

Myosin heavy chain expression was evaluated on cells grown on cover slips under the
conditions described above. At different time points, the cells were fixed in cold 100%
methanol at -20°C for 10min. Non-specific binding sites were blocked with 1% BSA in
PBS followed by incubation in the primary antibody reactive to skeletal myosin heavy
chain (MY-32, Zymed, San Francisco, CA). Subsequently, the cells were incubated
with the fluorescent secondary antibody, anti-mouse AlexaFluor 488 (Molecular
Probes, Oregon, USA). Nuclei were stained by incubating the cells in the DNA binding
dye Bisbenzimide Hoechst 33285 (Sigma). Immunostained cells were visualised with
an Olympus IX70 fluorescent microscope (Olympus, Australia) and digital images
collected (Spot RT slider camera: Image-Pro® Plus Software).

K. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software,
San Diego, CA). Unless stated, means were compared using a one-way ANOVA (for
normally distributed data) and any significant differences analysed using a Dunnet's
Multiple Comparison and using a Kruskal-Wallis test (for non-Gaussian distributions)
and any significant differences analysed using a Dunn’s post test. Data is presented
as mean ± standard error of the mean (SEM) unless otherwise stated. P<0.05 was
considered statistically significant.
Table 3.1. Real-Time PCR Primer Details.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Forward Primer (5'-3')</th>
<th>Conc, µM</th>
<th>Reverse Primer (5'-3')</th>
<th>Conc, µM</th>
<th>Exon Span</th>
<th>Amplicon Size, bp</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCT7</td>
<td>NM_006429</td>
<td>AGAGATTGCTGTGACCGTGAAG</td>
<td>2</td>
<td>CGGTCATGGCACACTTTTCC</td>
<td>2</td>
<td>4 &amp; 5</td>
<td>74</td>
<td>0.8</td>
</tr>
<tr>
<td>CKM</td>
<td>NM_001824</td>
<td>GGCATCTGGCACAATGACAA</td>
<td>2</td>
<td>GATGACCCCGAGGATGCTCT</td>
<td>2</td>
<td>4 &amp; 5</td>
<td>69</td>
<td>0.8</td>
</tr>
<tr>
<td>MYF5</td>
<td>NM_005593</td>
<td>TTCTACGACGGCTCTGCATA</td>
<td>2</td>
<td>CCACTCGCGGCACAAACT</td>
<td>2</td>
<td>---</td>
<td>67</td>
<td>1.2</td>
</tr>
<tr>
<td>MYF6</td>
<td>NM_002469</td>
<td>CACCCTGCACGAAG</td>
<td>2</td>
<td>CACAGTGCCGCTCTCAGT</td>
<td>2</td>
<td>---</td>
<td>70</td>
<td>1.1</td>
</tr>
<tr>
<td>MYL1</td>
<td>NM_079420</td>
<td>TGCTGAACTCCCGGCTGTT</td>
<td>2</td>
<td>CCATCAGGGCTCCACTCTTT</td>
<td>2</td>
<td>4 &amp; 5</td>
<td>71</td>
<td>0.9</td>
</tr>
<tr>
<td>MYOD</td>
<td>NM_002478</td>
<td>CCGCCTGAGCAGTTAAATGA</td>
<td>4</td>
<td>GCAACCGCTGGTTGGATT</td>
<td>4</td>
<td>---</td>
<td>74</td>
<td>1.0</td>
</tr>
<tr>
<td>myogenin</td>
<td>NM_002479</td>
<td>GGTGCCCAGGGAAATGC</td>
<td>2</td>
<td>TGATGCTGTCCAGATGGA</td>
<td>2</td>
<td>2 &amp; 3</td>
<td>155</td>
<td>0.9</td>
</tr>
<tr>
<td>PTMA</td>
<td>NM_002823</td>
<td>CTCAGACGCAGCTCTAGACA</td>
<td>2</td>
<td>CTGCTCTCCACAACCTCTT</td>
<td>2</td>
<td>1 &amp; 2</td>
<td>80</td>
<td>0.9</td>
</tr>
<tr>
<td>Tim10</td>
<td>NM_012456</td>
<td>TCCTCTACGGCCACACAG</td>
<td>2</td>
<td>GGTGATCATATCGCCATCTTC</td>
<td>2</td>
<td>---</td>
<td>64</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Primer sequences were designed using Primer Express Version 2 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search (http://www.ncbi.nlm.nih.gov/BLAST/). Exon information was obtained from the Ensembl Genome Database (http://www.ensembl.org/). The concentrations of forward and reverse primers are indicated along with the intron-exon divide the primers were designed around (if possible) and amplicon size in base pairs. PTMA, Prothymosin alpha; CCT7, chaperonin containing t polypeptide 1 eta; CKM, creatine kinase, muscle; MYL1, myosin, light polypeptide 1; Tim10, translocase of inner mitochondrial membrane 10 homolog;.
III. RESULTS

A human skeletal muscle specific microarray was used to examine the gene expression of rhabdomyosarcoma cells undergoing a forced differentiation. TPA was used to induce growth arrest and myogenic differentiation of a derivative of the embryonal rhabdomyosarcoma cell line RD, RD-A. As expected, differentiation of the cells was accompanied by a significantly increased expression (p<0.01) of myosin heavy chain protein (Figure 3.1) and an increased appearance of some multinuclear, myotube-like structures (Figure 3.2).

Figure 3.1. Western blot analysis of MHC protein expression over 10 days of differentiation with TPA containing medium. A representative blot from one replicate experiment is shown above the histogram. The order of samples on the blot corresponds with the order of bars in the histogram below. The cell extracts were electrophoresed through a 6% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and probed with the myosin antibody (MY-35). The arbitrary density of the bands was analysed using Kodak 1D 3.5 image analysis software and the average of three independent replicate experiments is presented.

* Significantly different from control (p<0.05)
** Significantly different from control (p<0.01)
Figure 3.2. Immunofluorescence analysis of RMS cells over the 10 days of TPA induced differentiation. Cells were reacted to the MHC antibody MY-35 (green). Nuclei were counterstained using the DNA binding dye Bisbenzimide Hoechst 33285 (blue). (A), Control; (B), Day 1; (C), Day 3; (D), Day 6; (E), Day 8; (F), Day 10. Bar represents 100µM.
The microarray used for the experiments was fabricated from a human skeletal muscle cDNA library and contained approximately 11,000 elements. Due to the marked differences in gene expression between normal human skeletal muscle and rhabdomyosarcoma tissue, hybridization of rhabdomyosarcoma RNA to the microarray resulted in only 4042 elements showing adequate expression levels following normalization. Using hierarchical clustering and paired t-tests, genes were grouped according to common patterns of expression over the time-course of differentiation (Figure 3.3). Analysis was focused on the identification of genes with evidence for either increased or decreased expression during the differentiation process. 105 elements demonstrated consistent up-regulation of expression throughout the differentiation in all three of the replicate experiments. Conversely, 190 elements showed a reproducible reduction in expression.
Figure 3.3. Hierarchical clustering analysis of expression profiles. Hierarchical clustering was used to group genes based on their expression profiles. Each gene element is represented on the vertical axis, and the time points are shown on the horizontal axis. The expression ratios for each gene at each time point are colour coded: red indicates increased expression while green indicated reduced expression relative to the reference expression level. Two distinct clusters are evident, an up-regulated pattern of expression (highlighted in blue) and a down-regulated pattern of expression. The averages of three independent replicate experiments are presented.
Ten elements showing the greatest evidence of altered expression during differentiation were selected for sequencing and further analysis (Table 3.3). These ten elements were found to consist of seven genes that were differentially expressed during the differentiation of RMS cells. Muscle creatine kinase (CKM) and several cytoskeletal proteins including myosin light chain 1 (MLY1) and myosin light chain 2 (MLY2) were markedly up-regulated during differentiation (Figure 3.4). In contrast prothymosin alpha (PTMA), chaperonin containing t-complex polypeptide 1, eta subunit (CCT7) and translocase of inner mitochondrial membrane 10 (Tim10) showed reduced expression during differentiation.

**Table 3.3.** List of genes identified following clone sequencing.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Gene</th>
<th>Clone ID</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>M15n5</td>
<td>Creatine kinase, muscle</td>
<td>M17c4</td>
<td>Prothymosin, alpha</td>
</tr>
<tr>
<td>M11a3</td>
<td>Creatine kinase, muscle</td>
<td>M34l16</td>
<td>Translocase of inner mitochondrial membrane 10</td>
</tr>
<tr>
<td>M12l14</td>
<td>Creatine kinase, muscle</td>
<td>M07d22</td>
<td>Chaperonin containing t-complex polypeptide 1, eta subunit</td>
</tr>
<tr>
<td>M52e6</td>
<td>Myosin, light polypeptide 2, regulatory, cardiac, slow</td>
<td>M52f20</td>
<td>Myosin, light polypeptide 1, alkali; skeletal, fast</td>
</tr>
<tr>
<td>M52f20</td>
<td>Myosin, light polypeptide 3, alkali; ventricular, skeletal, slow</td>
<td>M02i7</td>
<td>Myosin, light polypeptide 1, alkali; skeletal, fast</td>
</tr>
<tr>
<td>M06e16</td>
<td>Creatine kinase, muscle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4. Microarray expression profiles of selected differentially expressed genes. Each bar represents the mean ± SEM of three independent, replicate experiments.

* Significantly different from control (p<0.05)

** Significantly different from control (p<0.01)
To confirm the results of the microarray experiments, the changes in mRNA levels of 5 genes were quantified using real-time RT-PCR (Figure 3.5). The changes measured by real-time PCR were very similar to that of the microarray with the exception of one gene, CCT7, whose expression was not changed over the time course of differentiation as measured by real-time PCR. CKM and MYL1 demonstrated a significant ~12-fold (p<0.01) and ~16-fold (p<0.01) increase in gene expression respectively following 3 days of differentiation which continued to increase after 6 days (although not significantly) and then slowly decreased. Tim10 mRNA tended to decrease during differentiation, but this did not reach statistical significance (p=0.09). PTMA expression was significantly reduced (p<0.01) following 3 days (2.5-fold), 6 days (2.5 fold), 8 days (4.7 fold) and 10 days (5.4 fold) of TPA induced differentiation of rhabdomyosarcoma cells.

Since the muscle regulatory factors are considered critical in the process of normal myogenic differentiation, we examined their expression over the time course of RMS differentiation. MyoD and Myf5 are expressed early on in the differentiation cascade, showing a significant reduction in expression at days 8 and 10 (MyoD, p=0.01) and days 6, 8 and 10 (Myf5, p<0.01) as shown in Figure 3.6. Myf6 (p=0.03) and myogenin (p=0.04) mRNA expression both increased in response to differentiation, however due to quite marked variability, the ~40-fold increase in Myf6 expression was only significant at day 6. Myogenin mRNA showed a marked (~6-fold) increase in expression which remained elevated above control at day 10. Again due to variability in expression, significant differences (p= 0.05) were only evident at day 8 (3 fold increase). Myogenin protein expression was not detectable in proliferating cells and increased maximally (p<0.01) at day 3 (Figure 3.6B).
**Figure 3.5.** Real-time PCR validation of the genes identified from the microarray analysis. Data is presented as arbitrary units normalized to the control value. Bars show the mean ± SEM of six replicate experiments.

* Significantly different from control (p<0.05)

** Significantly different from control (p<0.01)
Figure 3.6. Expression of the myogenic basic helix-loop-helix transcription factors.

(A) mRNA expression of the MRFs over 10 days of differentiation. Data is presented as arbitrary units normalized to the control value. Bars show the mean ± SEM of six replicate experiments.

(B) Western blot analysis of myogenin protein expression over 10 days of differentiation. A representative blot from one replicate experiment is shown above the histogram. The order of samples on the blot corresponds with the order of bars in the histogram below. The cell extracts were electrophoresed through a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and probed with the myogenin antibody (F5D). The average of three independent replicate experiments is presented.

* Significantly different from control (p<0.05)

** Significantly different from control (p<0.01)
IV. DISCUSSION

Differentiation of skeletal muscle involves the withdrawal of myoblasts from the cell cycle and the subsequent expression of muscle-specific genes. We used an in vitro model of myogenesis and microarray technology to identify novel genes involved in time course of RMS proliferation arrest and progression towards terminal differentiation. RMS differentiation was induced by the addition of a phorbol ester and assessed by the appearance of myosin heavy chain, a widely used marker of differentiation. As expected, differentiation of the cells was accompanied by an increased expression of myosin heavy chain protein. Interestingly, the maximal rate of differentiation appeared evident following 6 days of treatment with TPA and subsequently declined. Aguanno (Aguanno et al. 1990) reported the presence of elongated and multinucleated myofibre-like structures in TPA-treated rhabdomyosarcoma cultures in addition to mononucleated and extremely elongated spindle cells, polygonal or star-shaped cells and small round cells. The small round cells were shown to rapidly proliferate and eventually reach confluence when the medium was replaced, even in the continued presence of TPA. The length of treatment in the present study necessitated the replacement of culture medium to maintain cell viability and is likely to have contributed to the increased percentage of mono-nucleated, undifferentiated cells observed and subsequent reduction in myosin heavy chain expression at 8 and 10 days post differentiation. Similarly, multinucleated cells may have detached during the extended culture times and possibly contributed to the reduction in myosin positive cells post 8 days of differentiation.

Using microarray analysis of gene expression, we identified a cluster of genes which demonstrated marked up-regulation throughout the forced differentiation of the RMS cells. In addition, a number of genes were dramatically reduced in their expression following differentiation. The majority of genes that showed increased expression were markers of terminal skeletal muscle differentiation such as myosin light chain, desmin and creatine kinase. Among the genes showing decreased expression were markers of cell proliferation, consistent with proliferation arrest, which is necessary for progression along the myogenic pathway. Of particular interest are two genes whose expression has not, to our knowledge, been investigated in RMS, including prothymosin alpha (PTMA), and translocase of inner mitochondrial membrane 10 (Tim10).

PTMA is an acidic, nuclear protein which is highly conserved and ubiquitously expressed in a wide variety of tissues. Despite the number of effects described for this protein, none are accepted as its physiological role and thus its function remains
elusive. Of its many reported functions, the relationship between PTMA and cellular proliferation and survival appears to be the mostly widely acknowledged. Expression of PTMA is generally correlated with cellular proliferation and its over-expression has been shown to stimulate cell division due to shortening of the G1 phase of the cell cycle (Wu et al. 1997b) as well as inhibit cell differentiation (Rodriguez et al. 1999). It was reported that terminal differentiation signals in HL-60 cells lead to a pronounced decrease in PTMA expression (Dosil et al. 1993), and a subsequent study confirmed that this decrease was related to the signal for the cessation of proliferation, rather than for the start of differentiation (Smith et al. 1993). Whilst PTMA appears to play a critical role in the process of proliferation and differentiation in a wide range of cells, the mechanism of action remains unclear.

Due to its proliferative activity, PTMA has also been extensively studied in tumoral processes. PTMA expression is evident at higher concentrations in malignant tumours than normal or surrounding tissues (Tsitsiloni et al. 1993) and has been established as a marker for breast cancer (Dominguez et al. 1993; Tsitsilonis et al. 1998; Magdalena et al. 2000), hepatocarcinoma (Wu et al. 1997a) and lung cancer (Sasaki et al. 2001). Thus the possibility remains that PTMA might also be an effective marker of RMS, and as in the case of breast cancer, has the potential to be of prognostic value in determining metastatic capacity and also the risk of death (Tsitsilonis et al. 1998).

Mitochondria import the vast majority of their proteins from the cytosol. To cope with the variety of these precursors, they have dedicated translocation machineries that facilitate importation through the outer (TOM translocase) and inner (TIM translocase) membrane. The carrier import pathway also depends on the function of a soluble 70 kDa complex made of Tim9 and Tim10 (the TIM10 complex). The passage of proteins across the intermembrane space is specifically mediated by the TIM10 complex which facilitates the transfer of carriers from the outer membrane to the outer surface of the inner membrane. This complex appears to be important as both the Tim9 and Tim10 genes are essential in yeast (Koehler et al. 1998; Leuenberger et al. 1999; Tokatlidis and Schatz 1999). In the present study, the Tim10 mRNA expression was markedly reduced during differentiation of rhabdomyosarcoma cells. However, its role in this context is unclear. No studies have previously identified differential expression of the Tim10 gene in either the cell cycle or associated with tumoral processes. However, it remains a possibility that proliferation arrest may be associated with a reduced mitochondrial proliferation and therefore a reduced need for associated mitochondrial proteins. Regardless of the functions of this protein, the change in its expression is more likely to be a consequence of the differentiation stimulus, rather than as a critical regulator of the process.
In addition to the hypothesis generating approach of microarray, we also used a more informed approach to examine the expression of the myogenic regulatory factors (MRFs) during the time course of rhabdomyosarcoma differentiation. Previous studies that have examined the MRF expression in rhabdomyosarcoma differentiation, have done so only at single time points (Bouche et al. 1993; Wasserman et al. 1996; Astolfi et al. 2001; Sirri et al. 2003) and did not observe any detectable level of Myf5 expression (Bouche et al. 1993; Wasserman et al. 1996), contradicting the results presented here. It is possible however, due to its very limited expression, that the methodologies used in the previous studies were not sensitive enough to detect the lowly expressed transcript.

Although the expression of the MRFs in rhabdomyosarcoma has been reported previously, no studies to date have examined the expression of all four of the basic helix-loop-helix factors over the time course of rhabdomyosarcoma differentiation. The expression of each of the MRFs observed reflects that previously described in normal myogenic differentiation (Megeney and Rudnicki 1995). MyoD and Myf5 were more highly expressed in proliferating cells and very early in the differentiated state, supporting their roles in the determination and/or maintenance of myogenic identity (Braun et al. 1992; Rudnicki et al. 1992), however their continued expression during myogenic differentiation may be required (Bergstrom and Tapscott 2001). Whereas, myogenin and Myf6 expression increased during differentiation, again consistent with their known roles in myogenic differentiation (Hasty et al. 1993; Sumariwalla and Klein 2001) and fusion (Dedieu et al. 2002).

The data presented here confirm and extend previous research by not only identifying the expression of the MRFs in RMS, but also mapping the temporal pattern of their expression throughout differentiation. These results again support the theory that the suppression of differentiation in rhabdomyosarcoma cells is due to mechanisms that interfere with the activity of the MRFs, rather than with their expression (Bouche et al. 1995).

As a secondary aim, this study sought to test the sensitivity and reliability of the microarray in detecting changes in gene expression in a biological system. The gene changes observed over the differentiation time-course were appropriate and occurred in a chronologically appropriate manner. Furthermore, quantitative real-time PCR confirmed the results of the microarray thus ensuring confidence in the microarray as a robust, reliable and sensitive measure of global changes in gene expression.
The microarray data presented here identify new information related to the genetic regulation of myogenesis in differentiating human rhabdomyosarcoma cells. Our current understanding of the differentiation block evident in rhabdomyosarcomas however remains limited. We also examined the temporal pattern of MRF expression over the time-course rhabdomyosarcoma differentiation identifying a remarkably similar pattern of expression to that exhibited in normal myogenesis.
CHAPTER FOUR

MYOGENIC AND ATROGENIC GENE EXPRESSION IN HUMAN SKELETAL MUSCLE FOLLOWING ACUTE RESISTANCE EXERCISE

I. INTRODUCTION

Skeletal muscle size is determined by the complex interplay between hypertrophic and atrophic stimuli ensuing remarkable adaptability to environmental challenges. Central to this adaptive process is the activation of satellite cells which are responsible for postnatal muscle growth. Increased loading, stretch, and muscular damage is accompanied by a myriad of growth signals that trigger normally mitotically quiescent satellite cells to re-enter the cell cycle and replicate (Rosenblatt et al. 1994; Kadi and Thornell 2000; Hill et al. 2003; Wozniak et al. 2003). The decision for the cell to divide occurs when a cell passes a restriction point late in the G1 phase of the cell cycle, after which it commits to the autonomous progression to divide. Critical to this G1 to S transition is the activity of a family of cyclin/cyclin-dependant kinases (Cdks) (eg. Cdk4, cyclin D1, cyclin E). Proliferation is also regulated by a number of Cdk-inhibitors (eg. p21^{cip}, p27^{kip}, p57^{kip}), that inhibit the activity of the Cdks and thus promote cell cycle arrest and progression towards terminal differentiation.

Whilst the activity of the Cdks and Cdk-inhibitors are important in the proliferative response in many cell types, the specification of the skeletal muscle phenotype is dependant on the activity of the basic-loop-helix family of MRFs. The MRFs comprise four members: MyoD, Myf5, myogenin and Myf6 (MRF4, Herculin) that bind to specific consensus sites that are functionally important in the transcription of muscle-specific genes. The critical role the MRFs play in muscle development (Braun et al. 1992; Rudnicki et al. 1992; Rudnicki et al. 1993; Ramakers et al. 2003) and their temporal expression during myogenic proliferation and differentiation has been extensively described (Montarras et al. 1991; Smith et al. 1994; Barjot et al. 1995). More recently, the role of the MRFs has been expanded to included the control of regenerating adult muscle (Cooper et al. 1999; Sakuma et al. 1999; Mendler et al. 2000; Launay et al. 2001) as well as the adaptive response of skeletal muscle to hypertrophic stimuli (Lowe et al. 1998; Adams et al. 1999; Alway et al. 2001; Carson et al. 2002; Bickel et
al. 2003; Psilander et al. 2003; Willoughby and Rosene 2003; Bamman et al. 2004; Bickel et al. 2004).

Since satellite cell regulation and specification of the muscle phenotype constitute critical components of skeletal muscle hypertrophy, several recent studies have begun to investigate the expression patterns of MRFs and cell cycle regulators in response to both acute resistance exercise (Haddad and Adams 2002; Willoughby and Nelson 2002; Psilander et al. 2003; Bickel et al. 2004) and following resistance exercise training (Bamman et al. 2004) in human skeletal muscle. To our knowledge however, no study has systematically investigated the temporal expression of all members of the MRF family in conjunction with both stimulators and inhibitors of the satellite cell cycle in human skeletal muscle. Furthermore, it has yet to be established whether acute loading of muscle through resistance exercise is sufficient to activate the entire process of satellite cell activation and differentiation. Indeed, understanding of the coordinated regulation of the multiple signalling pathways and molecular responses currently recognized in hypertrophying skeletal muscle is limited. In the present study, the expression of markers of the cell cycle and MRFs following a single bout of resistance exercise was investigated.

It has been established that many signalling pathways operate concurrently in skeletal muscle to elicit muscle adaptation. Although much interest has focused on the regulation of the anabolic response of skeletal muscle, the inhibition of the protein degradation pathways must also constitute an important part of the hypertrophy pathway. A secondary aim of the present study was to investigate the expression of recently identified downstream members of the protein degradation pathway following resistance exercise. It was hypothesized that a single bout of resistance exercise would increase the expression of members of the MRF and cell cycle regulator families whilst the expression of genes involved in skeletal muscle atrophy would be reduced.
II. METHODS

A. Subjects

Ten healthy male subjects volunteered to participate in this study. Informed written consent was obtained from each subject before participation in the study, after the nature, purpose and risks of the study were explained. Age, height and weight were on average 27.1 ± 2 years, 179.3 ± 1.6 cm, and 78.4 ± 2.7 kg (mean ± SEM). Concentric and eccentric maximal voluntary contraction of the quadriceps was 229.6 ± 10 Nm and 273.4 ± 9.5 Nm, respectively. Exclusion criteria included resistance training within the past six months or previous history of a diagnosed condition or illness that would endanger the subjects during strenuous resistance exercise. All experimental procedures involved in this study were formally approved by the Deakin University Ethics Committee.

B. Familiarisation

Each subject completed a familiarization session on a Cybex NORM dynamometer (Cybex International Inc. UK) to become familiar with the execution of the exercise. Isokinetic maximal voluntary contraction (MVC) strength was assessed during knee extension during the familiarization session. MVC was determined at 60°.sec-1 over five maximal leg extensions and the peak torque (Nm) for both concentric and eccentric contractions was recorded. Subjects were instructed to contract as hard as possible and were verbally encouraged throughout the test. Familiarization and strength testing was completed at least 7 days before the trial to prevent residual effects of the familiarization routine.

C. Experimental Design

Subjects arrived at the laboratory in the fasted state for a resting muscle biopsy. For the 24 h preceding, and the days of the trial, subjects were provided with and consumed a standard diet of 20% fat, 14% protein, 66% carbohydrate and abstained from alcohol, caffeine, tobacco and additional exercise. Macronutrient intake was assessed using FoodWorks 3.0 (Xyris Software, Australia). Following the resting biopsy, subjects completed an acute bout of concentric and eccentric isokinetic leg
extension exercise. All exercise was completed on the non-dominant limb using the Cybex NORM dynamometer at a constant speed of 60°.sec⁻¹. Subjects completed 3 sets of 12 repetitions of maximal single-legged knee extension exercise with 2 minutes rest between each set. The system provided visual graphical feedback for both subject and investigator. Subjects were instructed to contract as hard as possible and were verbally encouraged throughout each set. Further muscle samples from the recovering leg were obtained 30 min, 4h, and 24 h post exercise.

D. Muscle Biopsy Procedure

The vastus lateralis muscle of the non-dominant leg was sampled by percutaneous needle biopsy technique (Bergström 1962) modified to include suction (Evans et al. 1982). Excised muscle tissue from each biopsy was immediately frozen and stored in liquid nitrogen for subsequent analysis. Sampling muscle by needle biopsy will inevitably damage tissue and stimulate muscle regeneration in the immediate area. To minimise the impact of muscle damage from previous biopsies site on the response of muscle to the exercise serial muscle biopsies were collected from separate incisions made at least 2cm from previous biopsy sites. Repeat biopsies have been shown previously to have a negligible effect on the MRF gene expression in human skeletal muscle (Psilander et al. 2003). Additionally, fibre type variability across the vastus lateralis muscle may impact on variations in gene expression observed. Care was taken to ensure that muscle biopsies were collected from a similar anatomical position for all subjects. Considerable inter-human variability evident in skeletal muscle gene expression precluded a comparison of intra-muscular variation with an untreated control population of subjects.

E. Real-Time PCR Analysis

1. RNA Extraction

RNA was extracted from skeletal muscle samples (5-10mg) using the FastRNA™ Kit-Green (BIO 101, Vista CA) protocol and reagents (Dana et al. 1995). RNA quality and concentration were determined by UV spectrometry. (Helios, Unicam, Cambridge, U.K). RNA concentration was determined using the equation OD260 / 0.0625 µg/µl (Sambrook et al. 1989). RNA purity was determined by the equation OD260/OD280, with values required to be >1.7 to be accepted as non-contaminated. An aliquot of the RNA was stored at -80° C for subsequent reverse transcription.
2. cDNA Synthesis

cDNA synthesis was performed as described previously (Chapter 3, section II, part H.2)

3. Primer Design

Primer design was performed as previously described (Chapter 3, section II, part H.3)

4. Real-time PCR Reaction

Real-time PCR was performed using the GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). For the PCR step, reaction volumes of 20µl contained SYBR Green 1 Buffer (Applied Biosystems), forward and reverse primers (see Table 4.1) and cDNA template (diluted 1:40). All samples were run in duplicate. Real-time PCR was run for 1 cycle (50ºC 2 min, 95ºC 10 min) followed by 40 cycles (95ºC 15 s, 60ºC 60 s) and fluorescence was measured after each of the repetitive cycles. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was amplified.

5. PCR Efficiency

RNA extracted from a single human muscle sample was used to examine the dynamic range of responses for a series of dilutions of cDNA generated from the RT step. Linear regression was used to analyse the response of CT vs. the logarithm of the cDNA concentration. Using the slopes of the lines, we calculated the efficiency (E) of target amplification with the equation $E = (10^{-1/slope})-1$ (Table 4.1).

6. PCR Analysis and Endogenous Control Selection

Data was analysed using the comparative critical threshold (Ct) method where the amount of target normalised to the amount of the endogenous control is given by $2^{-\Delta Ct}$. β-actin has previously been identified as an appropriate endogenous control for resistance exercise studies (Mahoney et al. 2004) and as such was selected for the endogenous control for this study. The appropriateness of this gene as an endogenous control was confirmed by examining the $2^{-\Delta Ct}$ normalised to control values (Figure 4.1).
Figure 4.1. Examination of the mRNA expression of the endogenous control β-actin following an acute bout of resistance exercise. Statistical analysis revealed no change in the expression of this gene at any time point following the exercise intervention (p<0.05). Therefore, this was considered an appropriate endogenous control for this study.

G. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA). Means were compared using a one-way ANOVA and any significant differences analysed using a Dunnet’s multiple comparison test. Data is presented as mean ± standard error of the mean (SEM). A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.
Table 4.1. Real-Time PCR Primer Details.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Forward Primer (5' → 3')</th>
<th>Conc, µM</th>
<th>Reverse Primer (5' → 3')</th>
<th>Conc, µM</th>
<th>Intron-Exon Divide</th>
<th>Amplicon Length</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>NM_058229</td>
<td>CATCCTTATGTACACTGGTCCAAAGA</td>
<td>6</td>
<td>TCCGATACACCACATGTTAATG</td>
<td>6</td>
<td>---</td>
<td>74</td>
<td>0.8</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_001101</td>
<td>AAGCCACCCACCTCTCTCTAAT</td>
<td>2</td>
<td>AATGCTATACACTCTCTCTGTT</td>
<td>2</td>
<td>---</td>
<td>141</td>
<td>1.1</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>NM_053056</td>
<td>GCAATGTCGGTGGCCTCTAAGA</td>
<td>2</td>
<td>CGGTGTAGATGCAGCTTCTC</td>
<td>2</td>
<td>---</td>
<td>69</td>
<td>1.2</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>NM_032588</td>
<td>GGCATGGCTCTACATTTT</td>
<td>6</td>
<td>TCTCAAGTCTCCATGATTG</td>
<td>6</td>
<td>---</td>
<td>81</td>
<td>0.7</td>
</tr>
<tr>
<td>Myf5</td>
<td>NM_005593</td>
<td>TTCTACGACGGCTCCTGCATA</td>
<td>4</td>
<td>GCAACTCGGGACAAACT</td>
<td>4</td>
<td>---</td>
<td>67</td>
<td>1.2</td>
</tr>
<tr>
<td>Myf6</td>
<td>NM_002469</td>
<td>CACCTGCACGAAAG</td>
<td>2</td>
<td>CACAGTTGCGCCGCTTCA</td>
<td>2</td>
<td>---</td>
<td>70</td>
<td>1.1</td>
</tr>
<tr>
<td>MyoD</td>
<td>NM_002478</td>
<td>CCGCCCTGAGCAAGTAAATGA</td>
<td>4</td>
<td>GCAACCGCTGTGGTGGAT</td>
<td>4</td>
<td>---</td>
<td>74</td>
<td>1.0</td>
</tr>
<tr>
<td>Myogenin</td>
<td>NM_002479</td>
<td>GGTGCCCCAGCGATGCG</td>
<td>2</td>
<td>TGATGCTGTCCACAGTGA</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>155</td>
<td>0.9</td>
</tr>
<tr>
<td>Myostatin</td>
<td>NM_005259</td>
<td>CCAGAGAGAGAGCTGCAA</td>
<td>2</td>
<td>CAAGACCAAAATCCCCCTGGA</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>82</td>
<td>0.8</td>
</tr>
<tr>
<td>p21</td>
<td>NM_000389</td>
<td>GGCAGACCAGCATGACAGATTT</td>
<td>6</td>
<td>GGCAGATTGGGTTCCCTCT</td>
<td>6</td>
<td>---</td>
<td>73</td>
<td>0.9</td>
</tr>
<tr>
<td>p27</td>
<td>NM_004064</td>
<td>CGGTGAGACGAAAGATG</td>
<td>2</td>
<td>GGCTGGACTCCTCCATGTC</td>
<td>2</td>
<td>---</td>
<td>66</td>
<td>0.9</td>
</tr>
<tr>
<td>PCNA</td>
<td>NM_002592</td>
<td>CTAAAATGCACGCAAGGT</td>
<td>2</td>
<td>GCTCAAAATACTACGCGCGAAGGT</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>155</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Primer sequences were designed using Primer Express Version 2 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search (http://www.ncbi.nlm.gov/BLAST/). Exon information was obtained from the Ensembl Genome Database (http://www.ensembl.org/). The concentrations of forward and reverse primers are indicated along with the intron-exon divide the primers were designed around (if possible) and amplicon size in base pairs. MuRF-1, muscle ring finger protein 1; PCNA, proliferating cell nuclear antigen.
### III. RESULTS

10 male subjects performed 3 sets of 12 maximal concentric and eccentric isokinetic exercises with 2 minutes rest between each set. Peak torque was 229.6 ± 10.1 Nm and 273.4 ± 9.5 Nm for the concentric and eccentric contractions respectively. To ensure that the subjects were contracting maximally over the three sets, both concentric and eccentric torque was recorded for each of the 12 contractions in all three sets. Figure 4.2 shows that there was no significant difference in either concentric or eccentric average torque over the three exercise sets. This indicates that the two minute rest between each set provided sufficient recovery to allow subjects to contract at or near maximal force throughout the exercise session.

![Figure 4.2](image_url)  
*Figure 4.2. Average concentric (white bars) and eccentric (black bars) torque across the three exercise sets. The data represented is the average of 12 contractions from 10 subjects, ± SEM.*
A. Cell Cycle Regulators

The markers of cell proliferation, cyclin D1 and PCNA demonstrated no statistically significant change in their expression patterns however, the expression of both genes appeared to decrease somewhat, 30 min post exercise (Figure 4.3). p21, a Cdk inhibitor which blocks progression through the cell cycle, increased dramatically (8.5-fold) at 4 hours and remained elevated (7.5-fold) at 24 hours post exercise (p<0.01). The expression of p27, however, another member of the Cdk inhibitor family, was not altered at any point following exercise.

**Figure 4.3.** The effects of a single bout of heavy resistance exercise on the expression of cell cycle regulatory factors. Real-time PCR analysis was performed on Cyclin D1, PCNA, p21, and p27. Values are means ± SEM.

**Significantly different from control (p<0.01)**
B. Myogenic Regulatory Factors

The single bout of resistance exercise induced a small but significant increase (1.8-fold; p=0.01) in MyoD mRNA expression at 4 h although no change in the expression of Myf5 was observed (Figure 4.4). Myogenin mRNA expression increased 2-fold at 4 hours following exercise (p<0.01) with no change in the expression of Myf6.

*Figure 4.4. The effects of a single bout of heavy resistance exercise on the expression of the Muscle Regulatory Factors. Real-time PCR analysis was performed on Myf5, MyoD, myogenin, and Myf6. Values are means ± SEM.  
*Significantly different from control (p<0.05)  
**Significantly different from control (p<0.01)
C. Myostatin

In addition to examining the cell cycle regulators and MRFs, the expression of a powerful negative regulator of muscle growth, Myostatin, was investigated (Figure 4.5). As expected, Myostatin showed a marked reduction in expression during the recovery from exercise with approximately a 2-fold reduction at 4 and 24 hours post exercise (p<0.01).

![Myostatin](image)

**Figure 4.5.** The effects of a single bout of heavy resistance exercise on the expression of myostatin mRNA. Values are means ± SEM. **Significantly different from control (p<0.01)**
D. Protein Degradation Pathway

Two ubiquitin ligases, atrogin-1 and muscle ring finger protein 1 (MuRF-1), whose expression is increased significantly during multiple models of muscle atrophy were also examined as potential negative regulators of muscle hypertrophy (Figure 4.6). Interestingly, MuRF-1 expression appeared to increase (2.4-fold) although not significantly (p=0.07) at 4 hours post exercise. Conversely, atrogin-1 expression appeared to decrease slightly at 4 hours post exercise, although again not significantly.

![Graph of Atrogin-1 expression](image1)

![Graph of MURF-1 expression](image2)

**Figure 4.6.** The effects of a single bout of heavy resistance exercise on the expression of atrogin-1 and MuRF-1 mRNA. Values are means ± SEM. No statistical significance was observed (p>0.05).
IV. DISCUSSION

Whilst the central role that satellite cells play in skeletal muscle repair and adaptive hypertrophy has been well established, our understanding of the mechanisms necessary for, and regulating the satellite cell response is limited. In the present study, the aim was to investigate the expression of both activators and repressors of the cell cycle as well as the expression of critical drivers of the muscle phenotype, the MRFs, following a single bout of resistance exercise.

A. Cell cycle regulators

Crucial to the hypertrophic response is the progression of muscle precursor cells, or satellite cells through the cell cycle. Whilst it has been clearly established that satellite cells are activated in response to extensive damage to muscle cells (Vierck et al. 2000) and following heavy resistance exercise training (Kadi and Thornell 2000), it remains unclear whether a single bout of resistance exercise is capable of stimulating the entire process of satellite cell activation and differentiation. Therefore the expression of two markers of cellular proliferation, PCNA (involved in DNA replication) and cyclin D1 was investigated. Somewhat surprisingly, neither PCNA nor cyclin D1 expression increased following the resistance exercise bout. Whilst the expression of PCNA, to our knowledge, has not previously been investigated following resistance exercise, two previous studies (Haddad and Adams 2002; Bickel et al. 2004) found similar results for cyclin D following a single bout of resistance exercise. Interestingly, these studies demonstrated that two bouts of exercise seemed sufficient to elicit a significant increase in the expression of this gene. Collectively, these results seem to suggest that a single bout of resistance exercise is not sufficient to generate a measurable increase in the gene expressions of markers of satellite cell proliferation.

Considering the lack of observed changes in markers of proliferation, it was surprising to observe a marked increase the Cdk inhibitor and mediator of cell cycle arrest, p21. Although a closely related Cdk-inhibitor family member, p27, showed no changes in expression following resistance exercise. In view of the very early induction of p21 following the hypertrophic stimulus (4 hours), it seems unlikely that any activated and proliferating satellite cells (or indeed any other proliferating non-muscle cells) would have time to reach differentiation.
B. Myogenic Regulatory Factors

The results of the present study demonstrated an up-regulation of MyoD and myogenin expression 4 hours following an acute bout of resistance exercise whilst no change in the expression of Myf5 and Myf6 was observed. These results support the results reported previously in human skeletal muscle following a single bout of resistance exercise (Willoughby and Nelson 2002; Bickel et al. 2003; Psilander et al. 2003). However, the present study is the only study to date to examine the expression of all four members of the MRF family following a single bout of heavy resistance exercise.

In addition to the up-regulation of muscle specific genes, the MRFs (in particular MyoD) interact with cell cycle regulators to promote cell cycle arrest and differentiation of myogenic cells. Previous studies have indicated that MyoD is capable of inducing the expression of p21 (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995) as well as the other Cdk inhibitors, p27 and p57 (Reynaud et al. 2000) during myogenesis. Although numerous studies have identified an up-regulation of p21 following resistance exercise (Carson et al. 2002; Bickel et al. 2003; Welle et al. 2004), its interaction with the MRFs in regulating this adaptive response has not been investigated. The results of the present study, with MyoD and p21 both up-regulated 4 h post exercise might suggest cooperation between the Ckd inhibitors and MyoD in the differentiation signaling pathways in adult skeletal muscle. Similarly, myogenin mRNA expression, considered one of the earliest markers of differentiation and itself up-regulated by MyoD, was increased markedly at 4 h post exercise. As mentioned previously however, it seems highly unlikely that the observed increase in markers of satellite cell differentiation would be a result of satellite cell activation, proliferation, cell cycle arrest and differentiation within 4 hours of the exercise stimulus.

C. Possible Roles of p21, MyoD and Myogenin

The most likely source of the increased expression of p21, myogenin and MyoD at 4 h post exercise is the mature myofibres. Previously, the expression of p21, MyoD and myogenin has been localized in mature myonuclei early after the initiation of an overload stimulus (Ishido et al. 2004b). However the function of these proteins in myonuclei of adult myofibres has not been elucidated. It has been clearly established that myogenin and MyoD regulate the expression of muscle specific genes such as CKM and MYL. Therefore, it is quiet likely that the increased expression of these mRNAs in the immediate recovery period following heavy resistance exercise is
contributing to the early repair and/or adaptive response of the myofibre such as increased expression of the contractile filaments and fibre-type transitions. In contrast, following the exercise stimulus satellite cells would become activated and then from approximately 24 h undergo DNA synthesis and cell division (proliferation). MyoD and Myogenin expression would subsequently increase from up to 3 days later regulating the differentiation, fusion and hypertrophic events (McGeachie and Grounds 1987).

MyoD and p21 have also been considered as effectors of anti-apoptosis (Wang and Walsh 1996; Zhang et al. 1999; Ishido et al. 2004a). A number of studies have demonstrated that apoptosis can be induced in skeletal muscle during denervation, unloading and disease, suggesting that apoptosis may contribute to the loss of myonuclei and myofibre atrophy under these conditions (Allen et al. 1997; Allen et al. 1999; Yoshimura and Harii 1999; Schmalbruch and Lewis 2000). Similarly, hypertrophic stimuli such as stretch may elicit apoptotic cell death (Cheng et al. 1995). p21 contributes to protection against apoptosis by dephosphorylating the retinoblastoma protein (Rb). Dephosphorylated Rb inhibits the activation of members of the E2F family of transcription factors, which are involved in the induction of apoptosis (Wang et al. 1997; Wang 1997). Furthermore, mice lacking the p21 gene fail to form myotubes and demonstrate increased apoptotic rates in myoblasts (Walsh 1997; Zhang et al. 1999). Similarly, Rb deficient myotubes demonstrate increased rates of apoptosis (Zacksenhaus et al. 1996). Ishido (Ishido et al. 2004a) recently proposed that MyoD might contribute to protection against apoptosis by up-regulating the expression of p21 during denervation. A theory is proposed whereby MyoD up-regulates the expression of p21 in myofibres early in the recovery from heavy resistance exercise to help protect against apoptotic cell death.

D. Myostatin

In addition to examining the expression of cell cycle regulatory factors and MRFs, we sought to examine the expression of a powerful negative regulator of skeletal muscle, myostatin. Myostatin (growth and differentiation factor 8) has demonstrated a remarkable capacity to regulate skeletal muscle growth such that animals exhibiting null mutations in the myostatin gene result in a dramatic hypermuscular phenotype (Grobet et al. 1997; Kambadur et al. 1997; McPherron et al. 1997; McPherron and Lee 1997). Myostatin gene expression is suppressed in human muscle following 9 weeks of heavy resistance strength training, indicating that it is responsive to changes in the loading state of the muscle.
The results of the present study demonstrated that acute resistance exercise is indeed capable of a rapid and marked down-regulation of the myostatin gene. Myostatin appears to inhibit muscle hypertrophy via two independent pathways. Firstly, it inhibits the progression of myoblasts through the cell cycle via the up-regulation of p21 (Thomas et al. 2000; McCroskery et al. 2003). Secondly, myostatin can also inhibit differentiation through the up-regulation of Smad3; Smad3 subsequently binds and represses the transcriptional activity of MyoD. As a result, the expression of several regulatory factors is reduced (myogenin, p21, Myf5, Rb), which results in improper cell cycle arrest and inhibition of myoblast differentiation (Allen and Boxhorn 1989; Langley et al. 2002; Rios et al. 2002). Resistance exercise appears to regulate the transcriptional activity of myogenin such that its atrogenic effect is mitigated during recovery.

**E. Protein Degradation Pathway**

Several recent studies have indicated that muscle growth is not only reliant on anabolic processes but also on the suppression of protein breakdown (Sacheck et al. 2004; Stitt et al. 2004). During atrophy, there is an increase in the amount of components of the ubiquitin-protein conjugates and increased transcription of components of the ubiquitin degradation pathway (Bodine et al. 2001a; Haddad et al. 2003a; Haddad et al. 2003b). Two of the most sensitive markers of muscle atrophy are the mRNA expression of two muscle specific ubiquitin-ligases, atrogin-1 and muscle ring finger protein 1 (MuRF-1) (Sacheck et al. 2004). Rapid suppression of atrogin-1 and MuRF-1 mRNA has recently been demonstrated following a hypertrophic stimulus in vitro suggesting that inhibition of the protein degradation pathway is an important component of muscle growth (Sacheck et al. 2004).

The significance of the inhibition of the protein degradation pathways in human skeletal muscle hypertrophy has not, to our knowledge, been investigated. Surprisingly, atrogin-1 expression demonstrated only a small non-significant reduction in expression, whilst MuRF-1 mRNA levels appeared to increase, although again not significantly. The lack of reduction in the expression of these genes observed in this study contrasts with previous in vitro work (Sacheck et al. 2004). The hypertrophic stimulus (IGF-1 stimulation of myogenic cells) employed by Sacheck et al. is likely to promote a greater hypertrophic response to that induced by a single bout of resistance exercise. Therefore a pronounced reduction in the expression of these genes might be observed following a more powerful, prolonged hypertrophic stimulus in vivo (e.g. resistance exercise training).
The activation and proliferation of satellite cells has been identified as a critical component of adaptive skeletal muscle hypertrophy. Despite recent advances in our understanding of the importance of satellite cells, many questions remain unanswered. The early induction of p21, MyoD and myogenin following resistance exercise likely preclude the involvement of satellite cells in the very early adaptive response to resistance exercise. Therefore, it is possible that the increased expression of these mRNAs in the immediate recovery period following heavy resistance exercise is contributing to the early repair and/or adaptive response of the myofibre.
CHAPTER FIVE
IDENTIFICATION OF NOVEL GENES RESPONSIVE TO ACUTE RESISTANCE EXERCISE

I. INTRODUCTION

There is mounting evidence in support of the view that skeletal muscle hypertrophy results from the complex and coordinated interaction of numerous signalling pathways. Well characterised components integral to skeletal muscle adaptation include the IGF-1 mediated activation of the PI3K-Akt pathway, transcriptional activity of the members of the myogenic regulatory factors, numerous secreted peptide growth factors, and the regenerative potential of satellite cells. Whilst studies investigating isolated components or pathways have enhanced our current understanding of skeletal muscle hypertrophy, our knowledge of how all of these components react in concert to a common stimulus remains limited. Moreover, as it is estimated that approximately 40% of the 30,000 - 40,000 definitive genes in the human genome have undefined functions, it is likely that numerous genes integral in skeletal muscle hypertrophy are yet to be characterised (Blumenthal 2001). Receiving increasing focus has been the identification of novel genes that may play roles in the regulatory network through which muscle cells coordinate growth, adaptation and repair.

Gene expression profiling technology permits the parallel assessment of expression levels of thousands of genes and may lead to a better understanding of the signalling pathways activated in skeletal muscle in response to a hypertrophic stimulus. A number of recent studies have all employed microarray technology to examine global gene expression in skeletal muscle following exercise. Carson (Carson et al. 2002) examined gene expression following synergist ablation-induced functional overload in rats whilst Chen (Chen et al. 2002) and Barash (Barash et al. 2003) examined the transcriptional response of muscle to eccentric contractions in rats and mice respectively. Jozsi (Jozsi et al. 2000) used filter arrays to examine both the influence of age and exercise on skeletal muscle expression profiles, Zambon (Zambon et al. 2003) examined the effects of circadian rhythms and exercise on skeletal muscle gene expression and finally Chen (Chen et al. 2003) investigated the molecular response of human skeletal muscle to eccentric contractions. Whilst each study has provided
valuable progress towards the overall understanding of skeletal muscle adaptation to exercise, many questions remain unanswered.

The primary purpose of the present study was to use microarray technology to study the transcriptional changes during the 24 hours following an acute bout of resistance exercise. Importantly, a time-course approach was chosen for this analysis in order to capture a more thorough picture of the molecular changes occurring at different times throughout the recovery period. Specifically the time-points selected provide a glimpse into the early responding and transient genes as well as genes with more prolonged or delayed responses to resistance exercise.
II. METHODS

A. Subjects

Ten healthy male subjects volunteered to participate in this study. Informed written consent was obtained from each subject before participation in the study, after the nature, purpose and risks of the study were explained. Age, height and weight were on average 27.1 ± 2 years, 179.3 ± 1.6 cm, and 78.4 ± 2.7 kg (means ± SE). Concentric and eccentric maximal voluntary contraction of the quadriceps was 229.6 ± 10 Nm and 273.4 ± 9.5 Nm respectively. Exclusion criteria included resistance training within the past six months or previous history of a diagnosed condition or illness that would endanger the subjects during strenuous resistance exercise. All experimental procedures involved in this study were formally approved by the Deakin University Ethics Committee.

B. Familiarisation

Familiarisation was conducted as described previously (Chapter 4, section II, part B).

C. Experimental Design

The experiment was conducted as described previously (Chapter 4, section II, part C).

D. Muscle Biopsy Procedure

The muscle biopsy was performed as previously described (Chapter 4, section II, part D).

E. Microarray Production

The skeletal muscle specific microarray was prepared as described previously (Chapter 2, section II, part A).
F. RNA Extraction

Total RNA was extracted from skeletal muscle biopsy samples by a two-step procedure using Trizol Reagent followed by purification using RNeasy columns (Qiagen, Mannheim, Germany) according to the manufacturer’s instructions (Qiagen 2001). Briefly, frozen muscle was homogenised using a rotor-stator homogenizer in an appropriate volume of Trizol. Chloroform was added to the solution, thoroughly mixed, incubated on ice for 5 mins followed by centrifugation at 4°C for 15 min. The upper aqueous layer was removed and mixed with an equal volume of 70% ethanol before being added to the RNeasy spin column. After washing the column, the RNA was eluted as described in the manufacturer’s protocol. RNA quality and concentration was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

G. Indirect labelling of cDNA

Fluorescently labelled cDNA was prepared from equal quantities of total RNA using an indirect labelling method as described previously (Chapter 2, section II, part C). The reference RNA used for this study was a commercially available human skeletal muscle RNA (Ambion, Austin, TX).

H. Image Acquisition and Data Analysis

Fluorescent images of the microarrays were acquired using the GenePix 4000B laser scanner (Axon Instruments, Inc., Union City, CA) and the images processed using GenePix Pro3 software (Axon Instruments). Data files were entered into Acuity 4.0 software (Axon Instruments) where a global normalisation strategy was applied to normalise between arrays. Flagged spots and spots with less than 55% of feature pixels with intensities more than two standard deviations above the background pixel intensity were not retained for further analysis. Furthermore, genes had to be present in at least 70% of arrays to be considered in the analysis. Initially, self-organising map clustering analysis was performed to provide an overall picture of the pattern of gene expression. Clustering is mainly applied to microarray data in order to reduce the dimensions of the data and to provide visualisations of the complex microarray experiment, but are also particularly useful if current knowledge of the transcriptional patterns of the biological system is limited (Hautaniemi et al. 2003). Importantly, once the data is grouped, the investigator needs to visually identify and interpret the results of the clustering.
Following clustering analysis, the expression of genes contained within individual clusters was examined more closely. Statistical analysis revealed genes demonstrating consistent and marked changes in each of the 10 subjects, over the time-course of recovery from exercise. Using the statistical analysis tools provided within the microarray analysis package Acuity 4.0, we calculated paired t-tests to explore the changes in each time-point compared to control. However in order to minimise the false discovery rate and lack of power for appropriate statistical analysis, we chose to confirm all discussed novel gene changes with quantitative real-time PCR and more stringent statistical analysis.

I. Clone Identification

Selected cDNA clones were grown overnight and plasmid isolation was conducted using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Inserts were PCR amplified using 5'-amino modified vector specific primers. Products were visualized by agarose gel electrophoresis then excised and the sequence was determined using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The resultant sequence was entered into BLAST (www.ncbi.nlm.nih.gov/BLAST/) to ascertain its homology to known gene sequences.

J. Real-time PCR Analysis

1. RNA Extraction

RNA was extracted from skeletal muscle samples (5-10mg) as described previously (Chapter 4, section II, part E.1).

2. cDNA Synthesis

Reverse transcription was conducted as described previously (Chapter 3, section II, part H.2).

3. Primer Design

To perform PCR, specific primers were designed for all genes using Primer Express software (Applied Biosystems, Foster City, CA) as described previously (Chapter 3, section II, part H.3) See Table 5.1 for details.
4. Real-time PCR Reaction

The real-time PCR reaction was conducted as previously described (Chapter 3, section II, part H.4).

5. PCR Efficiency

PCR efficiency was conducted as previously described (Chapter 3, section II, part H.5). See table 5.1 for details.

6. PCR Analysis and Endogenous Control Selection

PCR analysis and analysis of the endogenous control was conducted as previously described (Chapter 4, section II, part H.6).

K. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA). Means were compared using a one-way ANOVA and any significant differences analysed using a Dunnet's multiple comparison test. Data is presented as mean ± standard error of the mean (SEM). A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.
### Table 5.1. Real-Time PCR Primer Details.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Conc, µM</th>
<th>Reverse Primer (5’ → 3’)</th>
<th>Conc, µM</th>
<th>Exon Divide</th>
<th>Amplicon Length</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASB5</td>
<td>AY057053</td>
<td>AAATGCAGTAACCTTAGACCATGTCA</td>
<td>2</td>
<td>ACATGCCACGTGATCTCCAA</td>
<td>2</td>
<td>---</td>
<td>67</td>
<td>0.8</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_001101</td>
<td>AAGCCACCCCATTTCTCTCTAA</td>
<td>2</td>
<td>AATGCTATCACCTCCCTGTGT</td>
<td>2</td>
<td>---</td>
<td>141</td>
<td>1.1</td>
</tr>
<tr>
<td>FLJ38973</td>
<td>NM_153689</td>
<td>GAATGGTTGCAAAGGAGAAAA</td>
<td>4</td>
<td>GCGTCATTTAGTGATGGGATAA</td>
<td>4</td>
<td>---</td>
<td>87</td>
<td>1.1</td>
</tr>
<tr>
<td>MLP</td>
<td>NM_003476</td>
<td>TCAGTCTATGCTGAGAAGGTT</td>
<td>2</td>
<td>GATGGACACCGGAAAACACG</td>
<td>2</td>
<td>exon 4 &amp; 5</td>
<td>72</td>
<td>0.8</td>
</tr>
<tr>
<td>Myf5</td>
<td>NM_005593</td>
<td>TTCTACGACGCCCTCTGCCATA</td>
<td>4</td>
<td>CCACTGGCAGCAAAACT</td>
<td>4</td>
<td>---</td>
<td>67</td>
<td>1.2</td>
</tr>
<tr>
<td>Myf6</td>
<td>NM_002469</td>
<td>CACCCTGCGGAAAG</td>
<td>2</td>
<td>CACAGTCCCGCCTTCAGT</td>
<td>2</td>
<td>---</td>
<td>70</td>
<td>1.1</td>
</tr>
<tr>
<td>MyoD</td>
<td>NM_002478</td>
<td>CGCCTCTGAGACAAAGTAAATGA</td>
<td>4</td>
<td>GCAACCCTGCGTTTGGAT</td>
<td>4</td>
<td>---</td>
<td>74</td>
<td>1.0</td>
</tr>
<tr>
<td>myogenin</td>
<td>NM_002479</td>
<td>GGTGCCACGGAATGC</td>
<td>2</td>
<td>TGATGCTGTCCACGATGGA</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>155</td>
<td>0.9</td>
</tr>
<tr>
<td>Myostatin</td>
<td>NM_005259</td>
<td>CCAGGAGAAGATGGCTGCAA</td>
<td>2</td>
<td>CAAGACAAATCCCTTCTGGAT</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>82</td>
<td>0.8</td>
</tr>
<tr>
<td>TTS2.2</td>
<td>XM_290535</td>
<td>CAGACGCGCADGATGTTTAT</td>
<td>4</td>
<td>TGACGACATTGGCCTTGGTA</td>
<td>4</td>
<td>exon 2 &amp; 3</td>
<td>69</td>
<td>0.7</td>
</tr>
<tr>
<td>TXNIP</td>
<td>NM_006472</td>
<td>AGATCGTGCTTACACAGCAACA</td>
<td>4</td>
<td>TCAGATCTACCCACTCTACAGA</td>
<td>4</td>
<td>---</td>
<td>73</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Primer sequences were designed using Primer Express Version 2 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search (http://www.ncbi.nlm.gov/BLAST/). Exon information was obtained from the Ensembl Genome Database (http://www.ensembl.org/). The concentrations of forward and reverse primers are indicated along with the intron-exon divide the primers were designed around (if possible) and amplicon size in base pairs. ASB5, ankyrin repeat and SOCs box-containing 5; MLP, muscle LIM protein; TXNIP, thioredoxin interacting protein; TTS2.2, transport secretion protein 2.2.
III. RESULTS

A. Microarray results

Gene expression profiling analysis was performed using a customised human skeletal muscle cDNA microarray during the recovery from an acute bout of resistance exercise. Since the majority of the spots on the microarray were derived from a human skeletal muscle cDNA library, specifically designed for the analysis of gene expression in human skeletal muscle, it was not surprising to see that 7,229 elements out of the 11,000 showed adequate expression levels, indicating a high degree of hybridisation to the array. Initially, self-organising map clustering analysis was performed to provide an overall picture of the pattern of gene expression changes during the recovery from resistance exercise. Clustering in microarray experiments utilise algorithms that typically result in groups of genes whose members exhibit similar expression profiles. Thus clustering methods offer convenient ways to illustrate data. Figure 5.1 shows a 3 × 2 self-organising map whereby genes were grouped together based on similar patterns of expression. Cluster A contains genes showing a reduction in expression following resistance exercise, whilst clusters B and C contain genes with increased expression levels following exercise. Subsequently, the expression patterns in each of these clusters was examined more closely in order to isolate elements that demonstrated the most statistically significant and dramatic changes in expression and importantly, consistent patterns of expression across all subjects.
Figure 5.1. 3 × 2 self organising map representation of the patterns of gene expression observed following a single bout of endurance exercise. Subjects completed an acute bout of resistance exercise consisting of 3 sets of 12 repetitions of maximal leg extension. Biopsies were taken before exercise (pre), at 30 min, 4 h, and 24 h post exercise. Self organising map was used to group genes based on their expression profiles. Each gene element is represented vertically, and the time points are shown horizontally. The expression ratios for each gene at each time point are colour coded: red, increased expression compared to reference RNA; green reduced expression compared to reference RNA. The average results of ten subjects are presented.
1. Known skeletal muscle genes

In addition to the unknown gene elements derived from the skeletal muscle cDNA library, 270 genes with known functional roles in skeletal muscle were selected for printing on to the microarray. Following hybridisation however, it appeared that only a fraction of the selected genes demonstrated adequate expression levels. The remainder of the known genes provided little or no expression data and were therefore unsuitable for further analysis. The reasons for the poor hybridisation levels in this subset of genes are unclear. The results of the remaining genes which demonstrated adequate expression must therefore be interpreted with a degree of caution. We identified 11 genes that demonstrated an increase in their mRNA expression following the acute bout of resistance exercise (Table 5.2). Whilst a further eight genes were identified as showing a reduction in expression following exercise (Table 5.3).

Table 5.2. List of genes with increased mRNA abundance in response to an acute bout of resistance exercise. The mRNA expression for each gene derived from the microarray analysis is presented as fold change from the resting (pre) value.

<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>FOLD CHANGE</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 MINS</td>
<td>4 HOURS</td>
<td>24 HOURS</td>
<td></td>
</tr>
<tr>
<td>Intracellular signalling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC-like kinase 2</td>
<td>1.22</td>
<td>1.20</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX7c</td>
<td>1.24</td>
<td>1.38</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>DLAT</td>
<td>1.50</td>
<td>-1.11</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>GLUT4</td>
<td>-1.10</td>
<td>1.10</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>PDK4</td>
<td>3.21</td>
<td>1.54</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>Regulatory proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serpine1</td>
<td>2.03</td>
<td>1.81</td>
<td>2.29</td>
<td></td>
</tr>
<tr>
<td>SNAP 29</td>
<td>-1.25</td>
<td>1.02</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>Structural / contractile proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dystrophin</td>
<td>1.16</td>
<td>1.00</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>Transcription</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGC-1</td>
<td>1.00</td>
<td>1.34</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>HSP27</td>
<td>1.43</td>
<td>1.51</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>NFkB1</td>
<td>-1.14</td>
<td>1.27</td>
<td>1.32</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: COX7c, cytochrome c oxidase, subunits VIIc; DLAT, dihydrolipoamide S-acetyltransferase; PDK4, pyruvate dehydrogenase kinase, isoenzyme 4; SNAP 29, synaptosomal-associated protein, 29 kDa; PGC-1, peroxisome proliferators-activated receptor-gamma, coactivator 1; HSP27, heat shock 27-kDa protein; NFkB1, nuclear factor kappa-B, subunit 1
Table 5.3. List of genes with decreased mRNA abundance in response to an acute bout of resistance exercise. The mRNA expression for each gene derived from the microarray analysis is presented as fold change from the resting (pre) value.

<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>30 MINS</th>
<th>4 HOURS</th>
<th>24 HOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular signalling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIK3R1</td>
<td>-1.43</td>
<td>-1.25</td>
<td>1.01</td>
</tr>
<tr>
<td>STAT3</td>
<td>-1.52</td>
<td>-1.01</td>
<td>1.05</td>
</tr>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP5I</td>
<td>-1.54</td>
<td>-1.04</td>
<td>-1.09</td>
</tr>
<tr>
<td>Enolase 3</td>
<td>-1.10</td>
<td>-1.16</td>
<td>-1.28</td>
</tr>
<tr>
<td>GOT2</td>
<td>-1.14</td>
<td>-1.22</td>
<td>-1.25</td>
</tr>
<tr>
<td>UQCRC2</td>
<td>-1.49</td>
<td>-1.32</td>
<td>1.07</td>
</tr>
<tr>
<td>Structural / contractile proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decorin</td>
<td>-1.01</td>
<td>-1.19</td>
<td>-1.61</td>
</tr>
<tr>
<td>α-actinin 1</td>
<td>-1.05</td>
<td>-1.19</td>
<td>-1.43</td>
</tr>
</tbody>
</table>

Abbreviations: PIK3R1, phosphatidylinositol 3-kinase, regulatory 1; STAT3, signal transducer and activator of transcription 3; ATP5I, ATP synthase, \( H^+ \) transporting, mitochondrial F0 complex, subunit E; GOT2, glutamate oxaloacetate transaminase, mitochondrial; UQCRC2, ubiquinol-cytochrome c reductase core protein II.

2. Unknown cDNA clones

Due to the nature of the array construction, all cDNAs spotted from the cDNA library are unknown until sequencing analysis is performed. As it is impractical to sequence all differentially expressed genes, a subset of these genes were selected for sequencing. Only genes showing consistent expression across the majority of the ten subjects, and showing the greatest statistically significant fold change from the control biopsy were selected for sequencing. In order to obtain a good cross section of both early and late responding genes, elements were selected that showed differential expression at each, but not necessarily all time-points. In total, 19 elements were sequenced, with 15 individual genes identified as shown in Table 5.4.
Table 5.4. List of genes identified using microarray analysis with increased mRNA abundance in response to an acute bout of resistance exercise. The mRNA expression for each gene derived from the microarray analysis is presented as fold change from the resting (pre) value.

<table>
<thead>
<tr>
<th>GENE ID</th>
<th>GENE NAME</th>
<th>FOLD CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 MINS</td>
</tr>
<tr>
<td>M02e13</td>
<td>Crystallin, alpha B</td>
<td>1.32</td>
</tr>
<tr>
<td>M07d10</td>
<td>Leiomodin 2 (cardiac)</td>
<td>1.24</td>
</tr>
<tr>
<td>M11h23</td>
<td>HNRPH1</td>
<td>1.10</td>
</tr>
<tr>
<td>M13d6</td>
<td>Synaptopodin 2</td>
<td>1.26</td>
</tr>
<tr>
<td>M13e13</td>
<td>TXNIP</td>
<td>1.72</td>
</tr>
<tr>
<td>M17j10</td>
<td>MLP</td>
<td>-1.10</td>
</tr>
<tr>
<td>M17j8</td>
<td>MLP</td>
<td>1.15</td>
</tr>
<tr>
<td>M20a22</td>
<td>ASB5</td>
<td>-1.33</td>
</tr>
<tr>
<td>M20p18</td>
<td>Filamin C, gamma</td>
<td>1.09</td>
</tr>
<tr>
<td>M20k22</td>
<td>NADH dehydrogenase 1 β</td>
<td>1.05</td>
</tr>
<tr>
<td>M21h1</td>
<td>TXNIP</td>
<td>1.68</td>
</tr>
<tr>
<td>M23a7</td>
<td>LRP16 protein</td>
<td>1.34</td>
</tr>
<tr>
<td>M23m3</td>
<td>Troponin I</td>
<td>2.25</td>
</tr>
<tr>
<td>M23n22</td>
<td>RNA binding motif protein 8A</td>
<td>1.03</td>
</tr>
<tr>
<td>M12i11</td>
<td>MLP</td>
<td>1.13</td>
</tr>
<tr>
<td>M24n10</td>
<td>FLJ38973</td>
<td>1.62</td>
</tr>
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<td>M28p16</td>
<td>HSP27</td>
<td>1.39</td>
</tr>
<tr>
<td>M29p15</td>
<td>α-tropomyosin</td>
<td>-1.02</td>
</tr>
<tr>
<td>M31n6</td>
<td>COX6B</td>
<td>1.00</td>
</tr>
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<td>M32i7</td>
<td>PDK4</td>
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</tr>
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<td>M34j8</td>
<td>TTS2.2</td>
<td>-1.06</td>
</tr>
<tr>
<td>M46d2</td>
<td>ASB5</td>
<td>1.16</td>
</tr>
<tr>
<td>M52i5</td>
<td>HSP40</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Abreviations: HNRPH1, Heterogeneous nuclear ribonucleoproteins H1; TXNIP, thioredoxin interacting protein; MLP, muscle LIM protein; ASB5, ankyrin repeat and SOCS box-containing 5; HSP27, heat shock 27-KD protein; COX6B, cytochrome c oxidase subunit Vlb; PDK4, pyruvate dehydrogenase kinase; TTS2.2, transport secretion protein 2.2; HSP40 heat-shock 40-kDa protein 1.

Of the 15 genes identified, 5 were selected for further analysis. The genes selected, ankyrin repeat and SOCS box-containing 5 (ASB5), thioredoxin interacting protein (TXNIP), muscle LIM protein (MLP), unknown protein FLJ38973 and transport-secretion protein 2.2 (TTS2.2) all showed marked changes in expression and represented potentially interesting candidates for roles in the adaptive response to resistance exercise. Extensive bioinformatical analysis was performed on the selected genes to identify the gene sequence, transcript structure, amino acid profile in addition to known or predicted functions of the protein. The databases searched for this analysis included, Ensemble (http://www.ensembl.org/), GeneCards.
(http://bioinfo.weizmann.ac.il/cards/index.shtml) and the Entrez suit of databases (http://www.ncbi.nlm.nih.gov/Entrez/). Information on each of the selected genes was readily available from these databases and is presented in Appendices A-D.

Further analysis and confirmation of the microarray data was performed using quantitative real-time PCR on ASB5, TXNIP, MLP, FLJ38973 and TTS2.2 (Figure 5.2). The results of the real-time PCR analysis generally supported the microarray results with one exception, TTS2.2. FJL38973 gene expression increased markedly (~2-fold) although not significantly at 4 hours post exercise. ASB5 mRNA expression increased significantly at 4 hours (~3-fold increase, p<0.01) which remained elevated at 24 hours (~2.3-fold). MLP expression markedly increased (~4-fold increase, p<0.01) 24 hours following the exercise bout. Whilst TXNIP showed a marked (~10-fold increase, p<0.01) albeit transient increase at 30 minutes followed by a rapid return to resting levels by 4 hours.
Figure 5.2. Real-time PCR results for selected genes. Subjects completed an acute bout of resistance exercise consisting of 3 sets of 12 repetitions of maximal leg extension. Biopsies were taken before exercise (pre), at 30 min, 4 h, and 24 h post exercise. Bars represent mean ± SEM, average of 10 subjects.

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01
Gene expression profiling analysis was performed using a customised human skeletal muscle cDNA microarray during the recovery from an acute bout of resistance exercise. Muscle biopsies were taken from the vastus lateralis muscle before, 30 min, 4 h and 24 h after the exercise bout. An examination of the transcriptional events over the time-course of 24 hours was employed to monitor both early and transient responses to the exercise as well as delayed or sustained gene changes. Thus the chances of identifying novel genes with important roles in the response to resistance exercise was far greater than by simply examining a single time point post exercise. In addition to the examination of potentially novel genes, a selection of genes with known or potentially interesting roles in skeletal muscle was included in the development of the microarray.

Expression analysis of these known genes revealed 11 up-regulated genes. Of note was the increase in PDK4, previously recognised for its rapid increase during and after more prolonged endurance type exercise (Pilegaard et al. 2000; Hildebrandt et al. 2003; Pilegaard and Neufer 2004). As well as the increase in expression of HSP27 and NFκB1, two genes that have previously been implicated in mediating cellular stress responses (Kumar and Boriek 2003; Thompson et al. 2003). In contrast, nine genes demonstrated slight decreases in expression following the resistance exercise bout. Surprisingly, the magnitude of gene changes, were on the whole, somewhat smaller than expected although consistent with the degree of change in general that has been observed previously using microarray following acute resistance exercise (Zambon et al. 2003).

The second aspect to this study involved the examination of the expression patterns of genes derived from a human skeletal muscle cDNA library. A subset of genes demonstrating markedly increased expression following the resistance exercise bout was selected for sequencing and further analysis. Whilst only genes showing increased expression were sequenced for this analysis, it is anticipated that similar analysis will be conducted for genes exhibiting down-regulation expression. In total, 19 cDNA clones were sequenced with 15 individual genes identified following sequencing and bioinformatics analysis. Importantly, two of these genes, PDK4 and HSP27 were also identified as showing increased expression in the analysis of the known skeletal muscle genes. In addition to HSP27, the expressions of two further heat shock proteins (HSP40 and alpha-2-B crystallin) were also shown to increase following
resistance exercise. The increased expression of these proteins has been linked to the facilitation of muscle repair and remodelling following damaging exercise as well as providing considerable protection against subsequent periods of resistance type exercise (Neufer et al. 1998; Thompson et al. 2001; McArdle et al. 2002; Thompson et al. 2003).

From the 15 genes identified, five genes were selected for further analysis based on: (1) the statistically significant degree of change demonstrated by the microarray expression analysis, (2) their unique nature, and/or (3) their known or potentially important role in skeletal muscle. The genes selected were TTS2.2, hypothetical protein FLJ38973, ASB5, MLP, and TXNIP. Real-time PCR validation confirmed the expression of all but TTS2.2 which showed no change in expression following exercise and was subsequently excluded from further analysis. The reasons for the incongruent results from the two analytical techniques are unclear however it may reflect inadvertent measurement of the expression of a closely related unidentified gene or perhaps differential exon usage of the gene itself.

MLP (also referred to as CRP3) was originally discovered in denervated skeletal muscle and identified as a positive regulator of the myogenic program in myogenic cells (Arber et al. 1994). MLP belongs to the LIM superfamily of proteins and consists of two tandem LIM motifs each followed by a glycine-rich region that serve as points of interaction with other proteins (Arber et al. 1994; Bach 2000). Detected exclusively in skeletal and cardiac muscle, MLP has been shown to be up-regulated in fast muscle by enhanced contractile activity (Schneider et al. 1999) as well as during the transition to slower phenotypes (Willmann et al. 2001) in rats and mice respectively. Furthermore, MLP transcripts have been shown to be induced during \( C_2C_{12} \) myocyte differentiation (Arber et al. 1994; Shen et al. 2003) as well as being up-regulated during the recovery from eccentric contraction in the \textit{tibialis anterior} muscles of mice (Barash et al. 2003).

To our knowledge this is the first study to measure the up-regulation of MLP in human skeletal muscle following an acute bout of resistance exercise. Whilst the exact function of MLP in muscle adaptive response to contractile activity is not discernable from this study, mice homozygous null for MLP exhibit highly disorganised cytoskeletal architecture and decreased cell-cell interactions (Arber et al. 1997) suggesting critical roles in the development of muscle structure and function. Further to this MLP has been observed to interact with the structural proteins \( \beta_1 \)-spectrin, telethonin and \( \alpha \)-actinin suggesting that it may be involved in facilitating the assembly or stabilization of the myofibril apparatus (Flick and Konieczny 2000) in addition to a potential role in the
mechanical stretch sensor machinery (Knoll et al. 2002). The understanding of the mechanistic actions of MLP is further confounded however by its dual sub-cellular localization, initially expressed exclusively in the nucleus early in muscle differentiation and later translocating to the cytoplasm (Arber et al. 1994). Interestingly, nuclear MLP appears to positively regulate myogenesis through the interaction with the myogenic transcription factors, MyoD, Myf6 and myogenin (Kong et al. 1997). Collectively these data seem to suggest multiple complementary roles for MLP in sensing mechanical stretch, regulating the subsequent transcriptional response and ultimately facilitating the assembly or stabilization of the contractile apparatus. Thus it appears that in response to a stimulus like resistance exercise, MLP plays a critical and coordinated role in the repair and adaptation of skeletal muscle.

Thioredoxin-interacting protein (TXNIP), also known as vitamin-D3 up-regulating protein-1 or thioredoxin-binding protein-2, is the endogenous inhibitor of thioredoxin, a major regulator of cellular redox state. Several recent studies, through examination of the TXNIP-null mouse, have established a causal relationship between the loss of TXNIP and disregulated carbohydrate and lipid metabolism (Donnelly et al. 2004; Hui et al. 2004; Van Greevenbroek et al. 2004). Furthermore, TXNIP has been described as a stress responsive gene that regulates cardiac hypertrophy and cardiomyocyte viability through interaction with thioredoxin. In contrast to the present study however, expression of TXNIP was rapidly suppressed by biomechanical strain in cardiomyocytes (Wang et al. 2002) and by transverse aortic constriction in vivo (Yoshioka et al. 2004). Importantly, pathological cardiac hypertrophy is characterized by diminished contractility and ultimately congestive heart failure, whilst skeletal muscle hypertrophy is characterized by enhanced muscle contractility and strength gains. Although cardiac and skeletal muscle hypertrophy share elements of common signalling pathways (Molkentin and Olson 1996), it is likely that there exist numerous divergent regulatory mechanisms providing a potential explanation for these paradoxical results.

Whilst the mechanisms regulating TXNIP expression or the significance of its observed increase in response to exercise cannot be elucidated from this study, its marked, early induction is of interest. TXNIP is readily inducible by various stress stimuli including \( \text{H}_2\text{O}_2 \), TGF-\( \beta \) and heat shock (Junn et al. 2000). We have demonstrated here that an acute bout of resistance exercise is sufficient to increase the expression of several markers of cellular stress (HSP27, HSP40, alpha-2-B crystallin). As such, it is interesting to speculate that the very rapid and transient increase in TXNIP might constitute an early signalling response to the stress imposed
by the exercise. The exact function of TXNIP in skeletal muscle and its role in the response to resistance exercise however requires further investigation.

Ankyrin repeat and SOCS box-containing 5 (ASB5) is a member of the asb family, which is characterized by a non-conserved N-terminus, a various number of ankyrin repeats as well as a C-terminal SOCS box (Kile et al. 2000). To date only one study has investigated the expression of ASB5 which observed its up-regulation in growing collateral arteries following femoral ligation (Boengler et al. 2003). The authors noted an increased expression of ASB5 in both endothelial and smooth muscle cells which proliferate rapidly during collateral artery growth. Interestingly, these studies indicated that ASB5 is expressed predominately in skeletal muscle and to a lesser extent in cardiac muscle (Kile et al. 2000; Boengler et al. 2003). Moreover, ASB5 proteins have been localized in some satellite cells whilst mature myocytes themselves do not appear to express this protein (Boengler et al. 2003). These data, combined with the marked up-regulation in skeletal muscle presented here, suggest a role for ASB5 in the recovery and adaptation from resistance exercise. Since ASB5 has been shown to be up-regulated in actively proliferating cells, we speculate that this protein might constitute a novel molecule involved in the proliferation of satellite cells in response to a hypertrophic stimulus. Similarly, since skeletal muscle biopsies contain a mixture of cell types (e.g. myofibers, satellite cells, fibroblasts, endothelial cells, smooth muscle cells, and nerve cells), the increase in ASB5 might not reflect changes in muscle cells at all. Capillary proliferation is an important feature of skeletal muscle hypertrophy (Hather et al. 1991; McCall et al. 1996; Kadi et al. 1999; Chen et al. 2003; Degens et al. 2003a; Degens et al. 2003b). Moreover, elevated markers of capillarisation including vascular endothelia growth factor (VEGF) mRNA and protein expression are evident following compensatory hypertrophy before increased capillary growth is evident (Takahashi et al. 2002; Degens et al. 2003b). Since ASB5 expression has been identified during arteriogenesis, it is plausible that the increased ASB5 expression observed in the present study might be associated with capillary growth rather than a muscle specific adaptation. Therefore it would be of interest to further examine importance of ASB5 during the response of skeletal muscle to a hypertrophic stimulus, both within muscle cells and surrounding supportive structures.

The hypothetical protein FLJ38973, because of its novel character, represented a potentially interesting gene to investigate further. FLJ38973 was originally identified during a large-scale screen of ESTs for the purpose of identifying the full-open reading frame cDNA sequence for each human and mouse gene (Mammalian Gene Collection Program Team et al. 2002). Whilst no subsequent study has investigated FLJ38973, bioinformatics analysis using AceView (http://www.aceview.org/) revealed that it is
located on chromosome 2 and produces, by alternative splicing, 2 different transcripts (aDec03, bDec03) together encoding 2 different protein isoforms. PSORT II analysis (http://psort.nibb.ac.jp), trained on yeast data, predicts that the subcellular location of this protein is most likely in the nucleus (43%) or in the mitochondria (43%). Less likely possibilities are in the cytoplasm (13%). We report here, for the first time that the hypothetical protein FLJ38973, is found in skeletal muscle and that its mRNA levels increase following a bout of resistance exercise in human skeletal muscle. Much work however is necessary to fully describe the functional role of this protein.

In conclusion, this experiment used microarray technology to examine the transcriptional response of human skeletal muscle to a single bout of resistance exercise. The analysis uncovered four interesting genes that have not previously been examined in this context. The role of these genes in the biological response of muscle to hypertrophic stimuli such as resistance exercise remains to be determined. However, their identification constitutes a valuable contribution to our understanding of the molecular responses of skeletal muscle following resistance exercise.
CHAPTER SIX

EXPRESSION OF NOVEL GENES DURING THE DIFFERENTIATION OF HUMAN PRIMARY SKELETAL MUSCLE CELLS IN CULTURE

I. INTRODUCTION

Strenuous, growth-inducing exercise is associated with changes in numerous variables including growth factors, cytokines, cellular damage, sarcoplasmic calcium concentrations and energy demand. Alterations in any one or more of these factors is capable of stimulating signal transduction pathways that ultimately regulate the transcription of genes differentially expressed during muscle hypertrophy (Rennie et al. 2004). Insulin-like growth factor (IGF-1) is released during skeletal muscle stretch and overload, and is a potent regulator of muscular growth and repair (Frost and Lang 2003). The most pronounced examples of the anabolic effect of IGF-1 on muscle growth come from studies of transgenic animals whereby localised over-expression of IGF-1 results in a dramatic increase in skeletal muscle mass (Coleman et al. 1995; Musarò et al. 2001).

Much of our understanding of the signalling mechanisms mediating IGF-1 induced hypertrophy, and indeed hypertrophy in general have come from the study of myoblast proliferation and differentiation in culture. The process of muscle hypertrophy has been described as a recapitulation of the stages of embryonic muscle development with the proliferation, differentiation and fusion of muscle specific stem cells (satellite cells) integral to both (Grounds 1991; Chambers and McDermott 1996; Grounds et al. 2002). IGFs are unique among growth factors in that they are capable of stimulating both proliferation and differentiation of muscle cells in culture. Initially, IGF-1 stimulates proliferation via inhibition of the growth suppressor retinoblastoma (Rb) protein, and up-regulation of the mRNA expression of proliferative factors including proliferating cell nuclear antigen (PCNA) and cyclin-dependant protein 1 (cyclin D1) whilst simultaneously inhibiting the expression of myogenin (Rosenthal and Chen 1995). Following an unknown differentiation signal however, IGF-1 stimulates the expression of MyoD which in turn up-regulates the expression of the cyclin-dependent kinase inhibitor p21, ultimately resulting in cell cycle arrest, expression of myogenin...
and progression towards terminal differentiation (Lawlor and Rotwein 2000; Xu and Wu 2000).

Central to the terminal differentiation of myoblasts, which is characterised by cell cycle arrest, expression of muscle specific proteins, formation and maturation of myofibres, is the expression of the MRFs. The examination of the developmental sequence of myogenesis has clearly established the temporal pattern of MRF expression and action. As such the expression of the MRFs along with other muscle specific genes provides valuable molecular markers for the entire process of muscle development.

Both primary skeletal muscle cell cultures and established muscle cell lines have become important tools as model systems for skeletal muscle development. The dividing myoblasts provided by these systems provide great flexibility in studies of myogenesis in that they remain as activity proliferating cells until growth factors provided in the medium are withdrawn. Following the reduction of growth factors below a critical threshold, myoblasts readily cease proliferating and progress towards terminal differentiation with the expression of muscle specific genes and proteins and finally fuse with adjacent myoblasts to become syncytial myotubes. Thus these cultures are readily manipulated allowing the examination of distinct aspects of muscle development.

Many studies have been carried out on the growth and differentiation of muscle cells however the vast majority use immortalised rat and mouse muscle cell lines. Although these studies have highlighted the critical role of IGF-1 in the control of proliferation and differentiation, these muscle cell lines differ in which IGF binding proteins they express (James et al. 1993; McCusker and Clemmons 1998; Crown et al. 2000) and are not identical in their responses to IGFs (Florini et al. 1991). The great advantage of primary skeletal muscle cell cultures, readily obtainable from adult human muscle biopsies, is that they provide a more physiologically relevant in vitro model of the human skeletal muscle phenotype.

Previous studies in this thesis have identified a number of genes that show increased expression in skeletal muscle following an acute bout of resistance exercise. Three of these genes: thioredoxin-interacting protein (TXNIP), ankyrin repeat and SOCS box-containing 5 (ASB5), and unknown protein FLJ38973, have not been previously investigated in skeletal muscle and as such provide unique targets for further investigation into the molecular regulation of hypertrophy. The fourth gene, muscle LIM protein (MLP), has previously been implicated in skeletal muscle hypertrophy however the exact functions of this protein in muscle are not completely understood.
In an effort to understand the functional roles of these genes in skeletal muscle a human primary skeletal muscle cell culture model was employed to examine the expression of the genes during the proliferation, cell cycle arrest and differentiation. Since IGF-1 is a potent stimulator of muscle hypertrophy, satellite cells in culture were stimulated to differentiate with serum withdrawal, and serum withdrawal combined with exogenous IGF-1 administration.

In this study the aim was to identify whether the expression of these genes were (1) activated at any time during proliferation, cell cycle arrest or differentiation or (2) activated by the additional mitogenic and myogenic stimulation provided by exogenous IGF-1. It was hypothesised that the expression of ASB5, TXNIP, MLP, FLJ38973 and the MRFs would be altered during the differentiation of human myoblasts in culture.
II. METHODS

A. Materials

All media and supplements were purchased from (Gibco, Invitrogen Corporation, Carlsbad, CA) unless otherwise stated. Primary skeletal muscle growth medium consisted of minimum essential medium (MEM)-alpha modification with 50 IU/ml penicillin, 5 µg/ml streptomycin and 10% FBS. Differentiation medium consisted of MEM-alpha modification with 50 IU/ml penicillin, 5 µg/ml streptomycin and 2% horse serum to reduce proliferative growth factors. The IGF-1 analogue Long\textsuperscript{R3}IGF-1 was purchased from GroPep, (Adelaide, SA). Long\textsuperscript{R3}IGF-1 is a variant of human IGF-I that contains an amino terminal extension peptide as well as glutamate-3 replaced by arginine, exhibits very weak binding to IGF-binding proteins and has been shown to exhibit greater biological activity than natural IGF-1 (Francis et al. 1992; Tomas et al. 1993).

B. Subjects

Human primary skeletal muscle cells were obtained from the vastus lateralis muscle of 8 healthy volunteers (6 males and 2 females, average age 25.1 ± 1.3 years). Informed written consent was obtained from each subject before participation in the study, after the nature, purpose and risks of the study were explained. All experimental procedures involved in this study were formally approved by the Deakin University Ethics Committee.

C. Human primary skeletal muscle cell cultures

Skeletal muscle samples were excised using the percutaneous needle biopsy technique (Bergström 1962) modified to include suction (Evans et al. 1982). Human primary skeletal muscle cells were cultured according to the technique of Gaster (Gaster et al. 2001). The excised muscle was immersed and extensively washed in ice-cold Hams F-10 medium before being minced in 0.5% Trypsin/EDTA. Minced tissue was then digested in 0.5% Trypsin/EDTA at room temperature with agitation for 20 minutes to release the myoblasts. The supernatant containing the myoblasts was then collected and the process repeated a further two times to break down any remaining tissue. FBS was subsequently added to the supernatant to a final
concentration of 10%. The supernatant was filtered through a 100µm filter to remove any connective tissue and then spun for 7 minutes at 1600rpm to collect the cells. The resulting cell pellet was resuspended in α-MEM medium. The cells were then seeded on to an uncoated flask and incubated at 37°C for 25 minutes to induce fibroblast attachment, leaving myoblasts suspended in the medium. The medium was aspirated and seeded on to an extra-cellular matrix-coated (Sigma, St Louis, MO) flask. The resulting primary cell cultures were maintained in α-MEM medium containing 10% FBS in humidified air at 37°C and 5% CO₂.

D. Experimental conditions

Primary skeletal myoblasts were plated onto plates coated with ECM containing laminin and entactin derived from Engelbroh-Holm Swarm murine sarcoma cells. In each experiment, the cells were initially plated in α-MEM medium containing 10% FBS until 80% confluency was attained. The plating medium was then removed, the cells were washed twice with PBS and the standard differentiation medium (DM) (α-MEM containing 2% horse serum) or IGF-1 treated medium (DM+IGF-1) (α-MEM containing 2% horse serum plus 30ng/ml Long™R³IGF-1) was added. Medium was changed every 48h throughout the 72 differentiation time-course. Following 2 washes with PBS, cells were extracted at 12h, 24h, 48h and 72h following the addition of the differentiation medium. The control for each condition consisted of actively proliferating cells grown in the maintenance medium and extracted immediately before the addition of the differentiation medium (0h).

E. Immunocytochemistry

The differentiation potential of our skeletal muscle cell cultures was initially determined using immunocytochemical staining of myosin heavy chain MHC. MHC expression was evaluated on cells grown on cover slips in the proliferative state and following 7 days of differentiation in DM. The cells were fixed in cold 100% methanol at -20°C for 10min. Non-specific binding sites were blocked with 1% BSA in PBS followed by incubation in the primary antibody reactive to skeletal myosin heavy chain (MY-32, Zymed, San Francisco, CA). Subsequently, the cells were incubated with the fluorescent secondary antibody, anti-mouse AlexaFluor 488 (Molecular Probes, Oregon, USA). Nuclei were stained by incubating the cells in the DNA binding dye Bisbenzimide Hoechst 33285 (Sigma). Immunostained cells were visualised with an
Olympus IX70 fluorescent microscope (Olympus, Australia) and digital images collected (Spot RT slider camera: Image-Pro® Plus Software).

F. Creatine kinase activity

The creatine kinase (CK) activity, an established marker of myoblast differentiation (Gaster et al. 2001), was determined in cell culture lysates. Cells were resuspended in lysis buffer (50mM Tris, pH7.6, 250mM NaCl, 5mM EDTA, 0.1% IGEPAL, Complete protease inhibitor cocktail) and passed through a syringe. The cell extracts were centrifuged to pellet cell debris and supernatants and analysed for total protein (BCA protein assay kit, Pierce, Rockford, IL). CK activity in aliquots of the supernatant was determined with a commercial assay kit (Randox Laboratories Ltd. Crumlin, UK) and automated clinical analyser (RX Daytona, Randox).

G. Western blot analysis

100 µg of denatured total proteins (extracted as described above) from each sample were separated by electrophoresis on a 6% (MHC) SDS-polyacrylamide gel and transferred to nitrocellulose membrane by electroblotting. Membranes were blocked for 2 h with 5% skim milk in Tris-buffered saline (50 mM Tris-HCl, 750 mM NaCl, 0.25% Tween), and were incubated overnight at 4°C with a polyclonal anti-MHC (MY-32, Zymed, San Francisco, CA). Membranes were washed (4 x 5 min), followed by a 60 min incubation with anti-rabbit IgG conjugated to horse-radish peroxidase (1 in 10,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), then washed again. Immunoreactive bands were detected using enhanced chemiluminescence (Western Lightning Chemiluminescent Reagent, Perkin-Elmer, Boston, MA). An internal control was used in each gel to normalize for variation in signal observed across the membranes.

H. Real-time PCR analysis

1. RNA Extraction

RNA was extracted as previously described (Chapter 3, section II, part D).

2. cDNA Synthesis

The cDNA synthesis reaction was conducted as previously described (Chapter 3, section II, part H.2).
3. Primer Design

Primers were designed as previously described (Chapter 3, section II, part H.3). See Table 6.1 for details.

4. Real-time PCR reaction

PCR was performed as previously described (Chapter 3, section II, part H.4).

5. PCR Efficiency

For each gene, the PCR efficiency of the primer pairs was determined as previously described (Chapter 3, section II, part H.5). See Table 6.1 for details.

I. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 4.1. Two-way ANOVAs with repeated measures were performed to examine differences in both treatment and time. Significant main effects were explored using Bonferroni post hoc tests. Data is presented as mean ± standard error of the mean (SEM). A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.
## Table 6.1. Real-Time PCR Primer Details.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Forward Primer (5’-3’)</th>
<th>Conc, µM</th>
<th>Reverse Primer (5’-3’)</th>
<th>Conc, µM</th>
<th>Exon Divide</th>
<th>Amplicon Size,</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASB5</td>
<td>AY057053</td>
<td>AAATGCAGTAACCTTAGACCATG</td>
<td>2</td>
<td>ACATGCCCCAGTGATCTCCA</td>
<td>2</td>
<td>---</td>
<td>67</td>
<td>0.8</td>
</tr>
<tr>
<td>FLJ38973</td>
<td>NM_153689</td>
<td>GAATGGTTGCAAAGGGAGAA</td>
<td>4</td>
<td>GCGTCATTAGTGATGGTGGA</td>
<td>4</td>
<td>---</td>
<td>87</td>
<td>1.1</td>
</tr>
<tr>
<td>MHC I</td>
<td>NM_000257</td>
<td>TGAAAGTGGCAATGACTA</td>
<td>2</td>
<td>TCCAGTTGAATCTCTCATACA</td>
<td>2</td>
<td>exon 10 &amp; 11</td>
<td>105</td>
<td>0.8</td>
</tr>
<tr>
<td>MHC IIx</td>
<td>NM_005963.2</td>
<td>GACTGATCGGAGATCAGCTATC</td>
<td>2</td>
<td>TCCCCAGTAACGGCAATTGTG</td>
<td>2</td>
<td>exon 3, 4, 5, &amp; 6</td>
<td>111</td>
<td>0.7</td>
</tr>
<tr>
<td>MLP</td>
<td>NM_003476</td>
<td>TCAGTCTATGCTGTAGAAGGT</td>
<td>2</td>
<td>GATGGCCACGCGAACACAG</td>
<td>2</td>
<td>exon 4 &amp; 5</td>
<td>72</td>
<td>0.8</td>
</tr>
<tr>
<td>Myf5</td>
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<td>TTCTACGCCAGCCTCTCAG</td>
<td>4</td>
<td>CCACCTCGGACAAACTA</td>
<td>4</td>
<td>---</td>
<td>67</td>
<td>1.2</td>
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<tr>
<td>Myf6</td>
<td>NM_002469</td>
<td>CACCCTGCAGCGAAAG</td>
<td>2</td>
<td>CACGTTTCGGCGCTAGT</td>
<td>2</td>
<td>---</td>
<td>70</td>
<td>1.1</td>
</tr>
<tr>
<td>MyoD</td>
<td>NM_002478</td>
<td>CCACCTACGAAAGTAAATGA</td>
<td>4</td>
<td>GCAACCCTGATTGGAT</td>
<td>4</td>
<td>---</td>
<td>74</td>
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<tr>
<td>myogenin</td>
<td>NM_002479</td>
<td>GGTGCCACCGAATGCG</td>
<td>2</td>
<td>TGATGCTTGCAGATGGA</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>155</td>
<td>0.9</td>
</tr>
<tr>
<td>Myostatin</td>
<td>NM_005259</td>
<td>CCGGAGAAGATGAGCTGA</td>
<td>2</td>
<td>CAAGACAAATACCTTCTGG</td>
<td>2</td>
<td>exon 2 &amp; 3</td>
<td>82</td>
<td>0.8</td>
</tr>
<tr>
<td>p21</td>
<td>NM_000389</td>
<td>GGCAGACCAGCATACAGATT</td>
<td>6</td>
<td>GGCGGGATTGGGCTCCTCT</td>
<td>6</td>
<td>---</td>
<td>73</td>
<td>0.7</td>
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<tr>
<td>PCNA</td>
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<td>GCTTCAATCTTGGGCGAAGGT</td>
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<td>exon 2 &amp; 3</td>
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<td>1.0</td>
</tr>
<tr>
<td>TXNIP</td>
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<td>TCAGATCTACCAAACATCTACAGA</td>
<td>4</td>
<td>---</td>
<td>73</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Primer sequences were designed using Primer Express Version 2 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search (http://www.ncbi.nlm.nih.gov/BLAST/). Exon information was obtained from the Ensembl Genome Database (http://www.ensembl.org/). The concentrations of forward and reverse primers are indicated along with the intron-exon divide the primers were designed around (if possible) and amplicon size in base pairs. ASB5, ankyrin repeat and SOCs box-containing 5; MLP, muscle LIM protein; TXNIP, thioredoxin interacting protein; MHC I, myosin heavy chain I, MHC IIx, myosin heavy chain IIx; PCNA, proliferating cell nuclear antigen.
III. RESULTS

In an effort to improve the understanding of the functional roles of several novel resistance exercise responsive genes a human primary skeletal muscle cell culture model was employed to examine the expression of the genes during the proliferation, cell cycle arrest and differentiation. Human primary skeletal muscle cell cultures were established from vastus lateralis muscles from 8 healthy young male and female volunteers. These cell lines were maintained in a proliferating state in medium containing 10% foetal bovine serum and all experiments were performed between the 2nd and 6th passages. Cells were stimulated to differentiate with serum withdrawal, and serum withdrawal combined with over-expression of exogenous IGF-1 (R3IGF-1). Since IGF-1 is a potent stimulator of muscle hypertrophy we sought to identify whether the expression of our novel genes were (1) activated at any time during proliferation, cell cycle arrest or differentiation or (2) activated by the additional mitogenic and myogenic stimulation provided by R3IGF-1.

A. Characterisation of human skeletal muscle cell cultures

Initially, the capacity of the human skeletal muscle cells lines to differentiate was examined using classical serum withdrawal induced differentiation. Cells actively proliferating in high serum media and cells exposed to low serum conditions were grown for up to 6 days on glass cover slips for immunocytochemical analysis of MHC protein expression. MHC expression is a widely used marker of differentiation in skeletal muscle cell cultures. No MHC expression was evident in proliferating cells (Figure 6.1, A), whereas by 7 days, extensive MHC expression was apparent with multi-nucleation and myotube formation evident (Figure 6.1, B).
Figure 6.1. Differentiation of primary human skeletal muscle cell lines. The differentiation potential of our cell lines was examined using immunocytochemical staining of MHC protein in proliferating cells (A) and following 7 days of serum withdrawal mediated differentiation (B). Cells were reacted to the MHC antibody MY-35 (green). Nuclei were counterstained using the DNA binding dye Bisbenzimide Hoechst 33285 (blue). Bar represents 100µm.
B. Assessment of Proliferation and Differentiation

To examine the relative effectiveness of our treatments on myogenesis, well established markers of progression through the cell cycle and myoblast differentiation were examined using real-time PCR (Figure 6.2). The expression of PCNA, necessary for cell cycle progression, was examined to assess cell proliferation. As expected, differentiation stimulated a reduction in the expression of PCNA for both the IGF-1 treated and untreated differentiating cells (significant main effect of time, p<0.01). No statistically significant difference was observed between treatments; however, DM+IGF-1 treatment appeared to initially increase the expression of PCNA (~1.4-fold) 12 h after the initiation of differentiation. The expression of p21, a prominent regulator of and molecular marker of cell cycle arrest, demonstrated a significant main effect of time (p<0.01); however no differences in expression were observed between DM and DM+IGF-1 treatment groups. Myostatin, a powerful negative regulator of muscle growth, has been shown to regulate cell cycle arrest through p21 (McCroskery et al. 2003). Differentiation appeared to decrease the expression of myostatin (main effect of time, p<0.01); although the decrease observed in the IGF-1 treated group failed to reach statistical significance. No differences between treatment groups were observed. In addition to the analysis of cell cycle regulators, we sought to examine the expression of several established markers of myogenic differentiation. As expected, the expression of myosin heavy chain type IIx (MHC IIx) was markedly increased by DM (~35-fold) and DM+IGF-1 (~27-fold) however no differences between treatment groups were observed (main effect of time, p<0.01). Conversely, the expression of the slow MHC isoform (MHC I) displayed marked differences between DM and DM+IGF-1 treatment groups. DM treatment alone significantly increased MHC I expression (~12-fold) whilst DM+IGF-1 treatment resulted in a comparably modest (~3-fold) and insignificant increase in MHC I expression. Furthermore, significant main effects of time were observed for CKM mRNA and activity, during DM and DM+IGF-1 treatments; however no differences between treatment groups were observed.

To further confirm effectiveness of differentiation stimuli, we examined both the expression of a muscle specific protein (MHC) as well as the activity of CMK. CKM activity increased following 48 h and remained elevated 72 h post differentiation (Figure 6.3). Finally, MHC protein expression was assessed to confirm the attainment of the differentiated state (Figure 6.4). Consistent with CKM activity, no difference in MHC protein expression was observed between treatment groups, although MHC protein expression increased over the time-course of differentiation (Figure 6.4).
Figure 6.2. mRNA expression analysis of markers of proliferation (PCNA), cell cycle arrest (p21, Myostatin) and differentiation (MHC IIx, MHC I, CKM) during serum withdrawal (DM; solid bars) or serum withdrawal combined with 30ng/ml R3IGF-1 (DM+IGF-1; white bars). Each bar represents the mean ± SEM of eight independent, replicate experiments.

* Significantly different from control (p<0.05)
Figure 6.3. CK activity increases with time in differentiating conditions. Human primary skeletal myoblasts were differentiated with serum withdrawal (DM) or serum withdrawal combined with 30ng/ml IGF-1 (DM+IGF-1). CK assay was performed on cell lysates and is expressed as mU/mg of total protein normalised to the 0h time-point. The average of eight independent primary cell cultures is presented, ± SEM.

* Significantly different from control.
Figure 6.4. Western blot analysis of MHC protein expression over time in differentiating conditions. The cell extracts were electrophoresed through a 6% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and reacted to the myosin antibody (MY-35). A: MHC expression analysed using densitometry and expressed as arbitrary density units. The average of four independent primary cell culture experiments is presented B: Representative blots from DM and DM+IGF-1 treated cells.
C. Myogenic regulatory factor expression

Since the temporal expression of the MRF transcription factors is critical to the appropriate development of the muscle phenotype, the expression of these factors was examined during the 72 h differentiation treatments (Figure 6.5). MyoD expression decreased significantly 72 h following DM and DM+IGF-1 treatments (p<0.05) however there were no differences in expression between treatments. In contrast, Myf5 showed no significant main effects of time or treatment; however a moderate decrease seemed evident at 48 h and 72 h post differentiation. Myogenin, considered the one of the earliest indicators of differentiation, demonstrated a dramatic increase at 24 h post differentiation which was sustained out to 72 h. Again, no differences were observed between groups. Myf6 expression demonstrated a significant main effect of time (p<0.01), with no significant difference evident between treatment groups. Myf6 expression was decreased by approximately 3-fold at both 48 h and 72 h in the DM treatment group. The expression of Myf6 was reduced by approximately 4-fold at 48 h and 72 h in the DM+IGF-1 treatment group.
Figure 6.5. mRNA expression analysis of the myogenic regulatory factors (MyoD, Myf5, myogenin, Myf6) during serum withdrawal (DM; solid bars) or serum withdrawal combined with 30ng/ml R3IGF-1 (DM+IGF-1; white bars). Each bar represents the mean ± SEM of eight independent, replicate experiments.

* Significantly different from control (p<0.05)
** Significantly different from control (p<0.01)
D. Expression of novel resistance exercise responsive genes

Once the temporal patterns of expression of markers of cell cycle and myogenic events that characterise the myogenic differentiation were established, we examined the expression of the novel genes previously identified as being responsive to resistance exercise (Figure 6.6). The expression of FLJ38973, ASB5 did not change with either the DM or DM+IGF-1 treatments, whilst MLP expression appeared to increase slightly, although not significantly different during differentiation. A significant main effect of time (p<0.05) was observed for TXNIP expression with early induction evident from 12 h post exercise, sustained out to 72 h. No differences between treatment groups were observed for any of these genes.
Figure 6.6. mRNA expression analysis of the FLJ 38973, ASB5, MLP, and TXNIP during serum withdrawal (DM; solid bars) or serum withdrawal combined with 30ng/ml R3 IGF-1 (DM+IGF-1; white bars). Each bar represents the mean ± SEM of eight independent, replicate experiments.

** Significantly different from control (p<0.01)
IV. DISCUSSION

Muscle differentiation is a multi-step process involving permanent cell cycle arrest, expression of muscle specific genes and proteins, and fusion of myoblasts to form multi-nucleated myotubes. Previous work has clearly delineated the sequential expression of numerous molecular markers of muscle differentiation. Using human primary skeletal muscle cell cultures derived from the *vastus lateralis* muscle of healthy volunteers, and the established knowledge of the temporal patterns of myogenesis, the expression patterns of several novel myogenic genes were investigated.

A. R<sub>3</sub>IGF-1 mediated differentiation

The differentiation of skeletal myogenic cells in culture is classically induced by serum deprivation. In an attempt to augment an *in vitro* hypertrophic response, the standard differentiation stimulus of serum withdrawal was combined with the added hypertrophic stimulus of exogenous IGF-1. The IGF-1 analogue we selected, Long<sup>TM</sup>R<sup>3</sup>IGF-1 is a variant of human IGF-I that contains an amino terminal extension peptide as well as glutamate-3 replaced by arginine. This form of IGF-1, exhibits very weak binding to IGF-binding proteins and has been shown to exhibit greater biological activity than natural IGF-1 (Francis et al. 1992; Tomas et al. 1993). Since IGF-1 has been reported to stimulate both the proliferation and differentiation of muscle cells in culture, several molecular markers were selected to compare the mitogenic and myogenic effects of DM+IGF-1 and DM alone on our human primary skeletal muscle cell cultures. Somewhat surprisingly, no obvious differences in the expressions of any of the chosen markers were observed between the two differentiation stimuli, with the possible exception of PCNA and MHC I. PCNA is a widely used marker of proliferating myoblasts with increased expression in the G<sub>1</sub> phase of the cell cycle, peak during S phase, and decline during G<sub>2</sub> (Bravo and Macdonald-Bravo 1987; Mesires and Doumit 2002). The moderate increase observed in PCNA expression at 12 hours post differentiation in the DM+IGF-1 group might indicate increased proliferative activity in these cells.

Despite this minor difference, DM+IGF-1 appeared to have very little if any effect on the differentiation potential of the human skeletal muscle cell cultures above DM alone. Although unexpected, the lack of an obvious increase in the differentiation potential of IGF-1 treated myoblasts might be explained by endogenous IGF-1
production. After serum withdrawal, spontaneous IGF-1 peptide production is observed in muscle cells (Tollefsen et al. 1989a; Tollefsen et al. 1989b; Rosenthal et al. 1991). Therefore, exogenous IGF-1 in the DM+IGF-1 treated cells may have had no added effect on differentiation than the endogenous IGF-1 produced following serum deprivation in the DM cells. Alternatively, the concentrations of IGF-1 used in this study (30ng/ml) may not have been sufficient or indeed too high to augment differentiation in human primary myoblasts. However, at least one previous study has demonstrated increased differentiation in primary human myoblasts with 30ng/ml R3IGF-1 (Foulstone et al. 2003). Comparisons between IGF-1 stimulated differentiation and differentiation (induced by serum withdrawal) in this study were only examined at 7 days post differentiation. Thus the possibility remains that the type points selected in the present study were too short to see any marked increase in IGF-1 stimulated differentiation.

B. Cell cycle regulator and MRF expression during differentiation

Despite the equivocal effects of the IGF-1 treatment, serum withdrawal alone was sufficient to promote a marked differentiation response in our human skeletal muscle cell cultures. The expression patterns of the cell cycle regulators and MRF were initially examined to establish the temporal pattern of myogenesis exhibited in the human primary skeletal muscle cultures. It was anticipated that this would allow the association of observed changes in the expression of novel genes with specific stages of the differentiation program.

The decision of myoblasts to proliferate or exit the cell cycle and progress towards skeletal muscle differentiation is determined in late G1 of the cell cycle and is governed by the activity of G1 cyclins/Cdk complexes (Pardee 1989). The cyclin / Cdk complexes cyclin D/Cdk4 and cyclin E / Cdk2, cooperate to control the G1 to S transition through the phosphorylation and inactivation of retinoblastoma protein (Rb). The commitment of myogenic cells into skeletal muscle differentiation requires prior irreversible cell cycle arrest and inactivation of cell cycle activators by cell cycle inhibitors (such as Rb p21, p27 and p57). Central to this process are the MRFs which are involved not only in the expression of muscle specific genes, but also cooperate with cell cycle regulators in mediating the transition from the proliferative stage. Previous studies have indicated the MyoD is capable of inducing the expression of p21 (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995) as well as other Cdk inhibitors , p27, p57 (Reynaud et al. 2000) and Rb (Martelli et al. 1994) during myogenesis. The time window for cells to exit the cell cycle and into differentiation is in
G1, when MyoD expression is maximal and Myf5 expression is lowest (Kitzmann et al. 1998; Kitzmann and Fernandez 2001). In the present study, MyoD expression was maximal at 0 h and 12 h post differentiation, followed by a small increase in p21 expression. p21 works redundantly with p57 (Zhang et al. 1999) by blocking progression through the cell cycle by binding to PCNA (to inhibit DNA synthesis) as well as the G₁ phase cyclin/Cdk complexes to prevent their enzymatic activity (Boulaire et al. 2000). As expected, PCNA expression was markedly reduced by 24 h post differentiation, despite only minor changes in the expression of p21. Although p57 expression was not measured in this study, it is possible that increased p57 expression might account for the attenuated expression of p21. The expression of myogenin, considered one of the earliest markers of differentiation, was increased at 12 h with expression peaking at 48 h and 72 h. Myogenin expression preceded the expression and activity of markers of terminal myoblast differentiation MHC and CKM. Myf6 expression, consistent with its suggested role at the latest stages of myogenesis, possibly during myobirillogenesis (Rudnicki and Jaenisch 1995) was down-regulated throughout the 72 h differentiation time-course.

Collectively, these observations suggested that our model exhibited the molecular markers of muscle differentiation in a chronologically appropriate manner. Importantly, the establishment of the temporal pattern of myogenesis in this model would allow the association of observed changes in the expression of novel genes with specific stages of the differentiation program. As discussed in chapter 5, the genes examined in this study were all observed to be up-regulated in the vastus lateralis during the 24 h recovery from a single bout of resistance exercise. The genes investigated were muscle LIM protein (MLP), thioredoxin interacting protein (TXNIP), ankyrin repeat and SOCS box-containing 5 (ASB5), and hypothetical protein FLJ38973.

C. Examination of the Expression Patterns of Novel Myogenic Genes

MLP was originally discovered in denervated skeletal muscle and has shown an enriched pattern of expression in striated muscle tissue (Arber et al. 1994). Several previous studies have identified important roles for MLP in myogenesis and in the promotion of myogenic differentiation (Arber et al. 1994; Arber et al. 1997; Louis et al. 1997; Shen et al. 2003). More specifically, the actions of MLP have been described late in differentiation potentially facilitating the assembly or stabilization of the myofibrillar apparatus by interacting with the structural proteins β1-spectrin, telethonin and α-actinin (Louis et al. 1997; Stronach et al. 1999; Flick and Konieczny 2000)
results of the present study show MLP slightly increased at 72 h, however, as described previously, MLP expression is likely to peak later in differentiation (i.e. after 6 days (Shen et al. 2003)), outside the time-frame of this study.

TXNIP, is a ubiquitous protein that binds with high affinity to thioredoxin and has been implicated the maintenance of cellular redox state and insulin secretion (Hui et al. 2004), development of hepatic steatosis (Donnelly et al. 2004; Van Greevenbroek et al. 2004), among others (Ikarashi et al. 2002; Yoshioka et al. 2004). In chapter 5, TXNIP expression was shown to be rapidly and transiently increased in skeletal muscle following a resistance exercise bout; providing the first evidence of a role for TXNIP in skeletal muscle. Interestingly, in the present study following the differentiation signal, TXNIP expression was rapidly increased and remained elevated throughout the differentiation time-course. The expression of TXNIP was the earliest induction of any of our markers of the myogenic program, which might suggest a very early role in the transduction of the differentiation stimulus. Similarly, TXNIP expression was markedly expressed very early (30 min) following the resistance exercise bout, supporting a potential role as an immediate responsive gene. Previous research has identified elevated levels of TNXIP expression in terminally differentiated epithelial cells of the human gastrointestinal tract (Takahashi et al. 2003) as well as reduced TXNIP expression in gastrointestinal cancers, characterized increases in their proliferative potential (Ikarashi et al. 2002). Collectively, these results suggest a potentially vital role for TXNIP in cellular differentiation. Whilst a role in differentiation remains speculative at this stage, it would be interesting to investigate the forced inhibition of TXNIP on the capacity of myobasts to attain the differentiated phenotype.

Conversely, the expression of ASB5 and hypothetical protein FLJ38973 demonstrated no altered expression patterns during primary myoblast differentiation. Very little is known about the functions of ASB5, one study has identified increased expression of this gene in growing collateral arteries following femoral ligation (Boengler et al. 2003). The authors noted an increased expression of ASB5 in both endothelial and smooth muscle cells which proliferate rapidly during collateral artery growth. Thus the possibility remains that ASB5 might exert its function on actively proliferating cells, despite no reduction in its expression post differentiation. Alternatively, it is intriguing to speculate that the expression of ASB5 might respond exclusively to the damage, contraction, or stretch imposed by resistance exercise. In support of this, the expression of ASB5 has been localized in some satellite cells whilst mature myocytes themselves do not appear to express this protein (Boengler et al. 2003). Thus the possibility still remains that ASB5 might constitute a novel molecule involved in proliferative response of satellite cells in response resistance exercise. Equally,
nothing is known of the functions of the unknown protein FLJ38973 and the results of this study provide little answers as to its role during skeletal myogenesis.

Skeletal muscle differentiation is a highly complex and coordinated process requiring the prior irreversible cell cycle arrest, before terminal differentiation, the expression of muscle specific genes and ultimately the formation of multinucleated myotubes. Here, the expression of novel myogenic genes in the context of the expression of known regulators and markers of skeletal myogenesis during the forced differentiation of human primary skeletal muscle cell cultures was investigated. Whilst the exact functions of these genes in skeletal muscle remains to be determined, these data provide additional insights into their regulation during myogenesis, and go some way to potentially identifying their mechanisms of action.
CHAPTER SEVEN

EXPRESSION OF THE NOVEL GENES FOLLOWING AN ACUTE BOUT OF ENDURANCE EXERCISE

I. INTRODUCTION

Skeletal muscle is an extremely dynamic tissue, which possesses the ability to adapt to a variety of physiological stimuli resulting in improved muscular capacity, specifically adapted to its functional demands. The plasticity of muscle is illustrated by the diverse phenotypic profiles of muscle evident following different exercise training regimes. For example, endurance training improves the metabolic properties of muscles, including an increase in number and size of mitochondria, switches in muscle fibre types from fast to slow isoforms, as well as the activity of enzymes related to fat and carbohydrate oxidation. Conversely, resistance training results in greater accumulation of contractile proteins, increased myofibre cross-sectional area, increased numbers of myonuclei and a shift in fibre type from slow to fast isoforms. Despite a great understanding of the specific phenotypic adaptations to endurance and resistance exercise. The knowledge surrounding the regulatory mechanisms governing the highly specified cellular level changes is limited. Through the use of molecular biology tools, we are starting to identify specific molecules and signalling pathways, which together result in the appropriate adaptation of skeletal muscle. For example, increases in the expression of genes related to fat and carbohydrate oxidation have been observed following both acute and chronic endurance exercise (i.e. GLUT4, PDK4) (Pilegaard et al. 2000; Hildebrandt et al. 2003; Pilegaard and Neuf 2004). Whereas resistance exercise has been shown to promote the expression of genes associated with muscle regeneration and development (i.e. MyoD, myogenin) (Willoughby and Nelson 2002; Bickel et al. 2003; Psilander et al. 2003).

In chapter 5, the identification of a number of genes that show increased expression in skeletal muscle following an acute bout of resistance exercise was described. Three of these genes: thioredoxin-interacting protein (TXNIP), ankyrin repeat and SOCS box-containing 5 (ASB5), and unknown protein FLJ38973, have not previously investigated
in skeletal muscle and as such provide unique targets for further investigation into the molecular regulation of hypertrophy. The fourth gene, muscle LIM protein (MLP), has previously been implicated in skeletal muscle hypertrophy however its mechanisms of action remain equivocal.

In chapter 6, the expression of these genes was investigated during the differentiation of human primary myoblasts. The results of this study showed that ASB5 and FLJ38973 mRNA was not altered at any time during proliferation arrest or differentiation. Conversely, the expression of MLP tended to increase and TXNIP mRNA increased rapidly and markedly following the initiation of differentiation. Whilst, these results suggested potential roles for TXNIP and MLP in the differentiation of skeletal muscle satellite cells during hypertrophy, it provided no clues as to the potential importance of the increased expression of ASB5 and FLJ38973 in skeletal muscle. Furthermore, we were no closer to determining if the responses of these genes were a unique response to resistance exercise or if they were responsive to skeletal muscle contraction or exercise in general. Therefore, in the present study the expression of resistance exercise responsive genes following a single bout of endurance exercise was investigated.

Additionally, the expression of the muscle regulatory factors (MRFs) was examined. Whilst the majority of studies to date have examined the expression of the MRFs in the context of resistance exercise, there is some suggestion that the MRFs might have the capacity to mediate muscle metabolic and contractile properties, not only towards phenotypes suited to resistance exercise, but also towards a more oxidative phenotype such as that observed following endurance training (Hughes et al. 1993; Bottinelli et al. 1994; Hughes et al. 1997; Hughes et al. 1999; Kadi et al. 2004a; Siu et al. 2004). Since these studies had focused on MyoD and myogenin, the expression of all four MRFs was investigated following an acute bout of endurance exercise. It was hypothesised that the expression of ASB5, TXNIP, MLP, FLJ38973 and the MRFs would not be altered following a single bout of endurance exercise.
II. METHODS

A. Subjects

Eight healthy, habitually active but non-endurance trained male subjects volunteered to participate in this study. Age, height and weight were on average 26.1 ± 1.6 years, 183.2 ± 2.5 cm, and 77.1 ± 2.9 kg (mean ± SEM). Peak oxygen uptake (VO\textsubscript{2} peak) determined by an incremental bicycle test was 47.5 ± 2 ml.kg\textsuperscript{-1}.min\textsuperscript{-1}. Exclusion criteria included endurance training status as evidence by a VO\textsubscript{2} peak greater than 55 ml.kg\textsuperscript{-1}.min\textsuperscript{-1} or previous history of a diagnosed condition or illness that would endanger the subjects during strenuous endurance exercise. Informed written consent was obtained from each subject before participation in the study, after the nature, purpose and risks of the study were explained. All experimental procedures involved in this study were formally approved by the Deakin University Ethics Committee.

B. VO\textsubscript{2} Peak

All subjects completed a VO\textsubscript{2} peak test on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands) with an incremental protocol and subjects pedalling at a self selected rate. Respiratory gasses were collected and analysed using Vista TurboFit software (Vacumetrics Inc. Ventura, CA) connected to Applied Electrochemistry Technologies oxygen (Model S-34) and carbon dioxide (Model CD-3A) analysers (Pittsburg, Pennsylvania). The gas analysers were calibrated prior to each test against commercially prepared (BOC, Australia) alpha rated gases of known concentrations.

C. Experimental Design

Subjects arrived at the laboratory in the fasted state for a resting muscle biopsy. For the 24 h preceding, and the days of the trial, subjects were provided with and consumed a standard diet of 20% fat, 14% protein, 66% carbohydrate and abstained from alcohol, caffeine, tobacco and additional exercise. Macronutrient intake was assessed using FoodWorks 3.0). Following the resting biopsy, subjects began cycling at a power output requiring approximately 70% peak oxygen consumption (average across all subjects throughout the exercise was 71.2 ± 2.1%). The exercise bout
lasted 60 min and subjects were permitted to consume water *ad libitum*. Further muscle samples were obtained 30 min after the exercise bout, 4 hours after the exercise bout and again 24 hours after the exercise bout.

**D. Muscle Biopsy Procedure**

The *vastus lateralis* muscle of the non-dominant leg was sampled by percutaneous needle biopsy technique as described previously (Chapter 4, section II, part D).

**E. Real-Time PCR Analysis**

1. **RNA Extraction**

The RNA extraction was performed as described previously (Chapter 4, section II, part E.1).

2. **Reverse Transcription**

The cDNA synthesis reaction was performed as described previously (Chapter 3, section II, part H.2)

3. **Primer Design**

PCR primers were designed as described previously (Chapter 3, section II, part H.3). PCR primer sequences are presented in Table 7.1.

4. **Real-time PCR reaction**

The real-time PCR reaction was performed as previously described (Chapter 3, section II, part H.4).

5. **PCR Efficiency**

Linearity of PCR primers was performed as described previously (Chapter 3, section II, part H.5), see Table 7.1 for details.

6. **PCR Analysis and Endogenous Control Selection**

Data was analysed using the comparative critical threshold (Ct) method where the amount of target normalised to the amount of the endogenous control is given by $2^{-\Delta\text{Ct}}$. β-actin has previously been identified as an appropriate endogenous control for endurance exercise studies (Mahoney et al. 2004) and as such was selected for the endogenous control for this study. The appropriateness of this gene as an
endogenous control was examined by using the $2^{\Delta CT}$ normalised to control values (Figure 7.1A). However, this analysis revealed significant changes in the expression of this gene and as such was deemed to be an inappropriate endogenous control to use for this study. Subsequently, cyclophilin was investigated as an endogenous control (Figure 7.1B). No significant change in its expression following endurance exercise was observed and therefore was selected as the endogenous control for this study.

*Figure 7.1.* Examination of the mRNA expression of the endogenous control β-actin (A) and cyclophilin (B) following an acute bout of resistance exercise. The expression of the endogenous control in a given sample was calculated by subtracting the $C_T$ of each sample from the $C_T$ of the corresponding pre or control value. The relative expression of the gene of interest normalised to control was calculated using the expression $2^{\Delta CT}$. Statistical analysis revealed no change in the expression of this gene at any time point following the exercise intervention. Therefore, this was considered an appropriate endogenous control for this study.
F. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA). Means were compared using a one-way ANOVA and any significant differences analysed using a Dunnet's multiple comparison test. Data is presented as mean ± standard error of the mean (SEM). A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.
Table 7.1. Real-Time PCR Primer Details.

<table>
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<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Forward Primer (5' → 3')</th>
<th>Conc, µM</th>
<th>Reverse Primer (5' → 3')</th>
<th>Conc, µM</th>
<th>Exon Divide</th>
<th>Amplicon Length</th>
<th>PCR Efficiency</th>
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<td>ASB5</td>
<td>AY057053</td>
<td>AAATGCAGTAACCTTAGACCATGTCA</td>
<td>2</td>
<td>ACATGCCACGTGATCTCCAA</td>
<td>2</td>
<td>---</td>
<td>67</td>
<td>0.8</td>
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<td>β-Actin</td>
<td>NM_001101</td>
<td>AAGCCACCCCACTTCTCTTAA</td>
<td>2</td>
<td>AATGCTATACCTCCCCTGTGT</td>
<td>2</td>
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<td>141</td>
<td>1.1</td>
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<td>Cyclophilin</td>
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<td>CATCTGCACCTGCCAGACTGA</td>
<td>4</td>
<td>TTCATGCCTTCTTTCCTTTGC</td>
<td>4</td>
<td>exon 4 &amp; 5</td>
<td>80</td>
<td>0.9</td>
</tr>
<tr>
<td>FLJ38973</td>
<td>NM_153689</td>
<td>GAATGGTTGCAAGGGAGAAA</td>
<td>4</td>
<td>GCGTCATTGTTAGTGTTGATTA</td>
<td>4</td>
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<td>87</td>
<td>1.1</td>
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<td>MLP</td>
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<td>TCAGTCTATGCTGCTGAGAAGGT</td>
<td>2</td>
<td>GATGGACACACGGAAACAG</td>
<td>2</td>
<td>exon 4 &amp; 5</td>
<td>72</td>
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<td>CCACTCGCGACAACT</td>
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<td>Myf6</td>
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<td>2</td>
<td>CACAGTGGCAGGCTTCAAT</td>
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<td>myogenin</td>
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<td>TGATGCTGCCACCAGTTG</td>
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<td>exon 2 &amp; 3</td>
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<td>4</td>
<td>---</td>
<td>73</td>
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</table>

Primer sequences were designed using Primer Express Version 2 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search (http://www.ncbi.nlm.gov/BLAST/). Exon information was obtained from the Ensembl Genome Database (http://www.ensembl.org/). The concentrations of forward and reverse primers are indicated along with the intron-exon divide the primers were designed around (if possible) and amplicon size in base pairs. ASB5, ankyrin repeat and SOCs box-containing 5; MLP, muscle LIM protein; PDK4, pyruvate dehydrogenase kinase 4; thioredoxin interacting protein.
III. RESULTS

The expressions of the resistance exercise responsive genes were examined following a single bout of endurance exercise (Figure 7.2). Interestingly, the expressions of TXNIP, MLP and FLJ38973 were not altered at any time-point post exercise. Conversely, the expression of ASB5 was rapidly increased \((p<0.05)\) by approximately 2-fold, 30 min following the cessation of exercise before returning to resting levels by 4 h.

Additionally, the expression of the muscle regulatory factors was investigated (Figure 7.3). Myf5 expression appeared to increase by approximately 2-fold at 30 min however considerable between-subject variability was evident and this increase failed to reach significance. MyoD and Myf6 expression was not altered at any point following endurance exercise. Myogenin expression appeared to increase at 24 h, although again this failed to reach significance.

Finally, the expression of PDK4, previously shown to be up-regulated following endurance exercise (Pilegaard et al. 2000; Hildebrandt et al. 2003; Pilegaard and Neufer 2004), was examined to ensure the exercise bout in conducted this study was sufficient to elicit expected increases in gene expression (Figure 7.4). As expected, PDK expression significantly increased by approximately 10-fold 4 h following the endurance exercise bout.
Figure 7.2. The effects of a single bout of endurance exercise on the expression of resistance exercise sensitive genes. Real-time PCR analysis was performed on TXNIP, MLP, ASB5 and FLJ38973. Values are means ± SEM of 8 subjects.

*Significantly different from control (p<0.05)
Figure 7.3. The effects of a single bout of endurance exercise on the expression of the Muscle Regulatory Factors. Real-time PCR analysis was performed on Myf5, MyoD, myogenin, and Myf6. Values are means ± SEM of 8 subjects. No significantly different changes in gene expression were observed (p>0.05).
Figure 7.4. The effects of a single bout of endurance exercise on the expression of PDK4. Values are means ± SEM of 8 subjects.

**Significantly different from control, p<0.01
IV. DISCUSSION

Following a single bout of resistance exercise, microarray technology was used to identify genes whose expression changed markedly, and therefore had the potential to play important roles in the repair and adaptation of skeletal muscle. Since very little is known about the functions of these genes (TXNIP, ASB5, MLP and FLJ 39873) in muscle, this study aimed to determine whether these changes were specific to resistance type exercise, or a common response to muscle contraction.

Previously in chapter 5 it was demonstrated that ASB5 gene expression increased markedly 4 and 24 h following a single bout of resistance exercise. Interestingly, ASB5 expression also increased in the present study, 30 min following a single bout of endurance exercise. The expression of ASB5 had been shown to be significantly increased in endothelial and smooth muscle cells during arteriogenesis. Since increased capillarisation is an adaptive response to both endurance and resistance type exercise. It seems that the likely explanation for the increased expression of ASB5 mRNA following both of these types of exercise is as an adaptive response to an increased need for capillaries within the muscle. In support of this, increased VEGF, a very early marker of arteriogenesis, is evident following both endurance and resistance exercise well before capillarisation itself is likely to have occurred (Gustafsson et al. 1999; Takahashi et al. 2002; Degens et al. 2003b). Thus, it appears likely that ASB5 is a novel molecule implicated in the initiation of arteriogenesis, although its mechanisms of action remain to be determined.

The lack of increased expression of TXNIP, MLP and FLJ38973 following endurance exercise seem to suggest a reliance on conditions specifically imposed on the muscle during resistance type exercise for their transcriptional activation. Numerous divergent signals unique to each type of exercise might be responsible for the different molecular responses of, and ultimately, phenotypic endpoint of resistance versus endurance type training. Potential regulators of early gene expression in muscle include, but are not limited to: exercise induced myotrauma, energy demands, intracellular calcium, growth factors, and mechanical stretch. Different exercise paradigms have the obvious potential to alter many different signalling pathways within muscle ultimately resulting in the unique patterns of gene expression and muscular adaptation. Thus TXNIP, MLP and FLJ38973 might constitute critical early molecular adaptations within skeletal muscle with the potential, following training, to promote muscle hypertrophy.
Several studies have suggested that MRFs may be involved in determining structural and metabolic characteristics of postmitotic skeletal muscles (Hughes et al. 1993; Bottinelli et al. 1994; Hughes et al. 1997; Adams et al. 1999; Allen et al. 2001). Specifically, these studies have suggested that myogenin expression is associated with the slow myosin heavy chain expression and increased oxidative capacity, whereas MyoD is related to the expression of the fast isoforms and a shift towards greater glycolytic activity. This is the first study to investigate the gene expression of all members of the MRF family following a single bout of endurance exercise. Whilst no significant change was evident in any of the MRFs, Myf5 expression tended to increase however considerable variability was evident between subjects likely contributing to the lack of statistical power. Conversely, in a previous study (see chapter 4) utilizing identical time-points post exercise, marked increases in both MyoD and myogenin were evident following a single bout of resistance exercise. The results of this study seem to suggest that immediate changes in the gene expressions of the MRFs are not critical adaptations to endurance exercise.

Previous studies have demonstrated increased nuclear localization of myogenin following a single bout of exercise (Kadi et al. 2004a) and increased myogenin mRNA and protein levels following 8 weeks of endurance exercise (Siu et al. 2004). Despite the lack of increase in myogenin mRNA expression in the present study it is possible that the cellular localisation of myogenin protein might be adequate in mediating the short term adaptation of muscle following endurance exercise. Whereas repeated bouts of exercise might be necessary for increased myogenin gene and protein expression. Importantly, current understanding of the genes whose trans-activation is directly regulated by members of the MRF family is limited. Further examinations into specific gene targets of each MRF will further clarify the divergent functions of these critical regulators of muscle development.

Resistance and endurance exercise results in the activation of numerous divergent signalling pathways that ultimately result in the unique expression of muscle specific proteins and development of a phenotypic profile highly suited to its functional demands. In this study, the expressions of resistance exercise sensitive genes was further characterised by examining their expression following endurance exercise. Furthermore, we have examined the primary regulators of the muscle phenotype, the MRFs following endurance exercise. These data provide further evidence of the unique molecular mechanisms that regulate the exercise-induced adaptations of skeletal muscle.
CHAPTER EIGHT
GENERAL DISCUSSION & FUTURE DIRECTIONS

I. INTRODUCTION

Skeletal muscle confers numerous vital functions including locomotion and postural behaviour, strength, breathing and metabolism. Unfortunately, diseases of skeletal muscle such as age related sarcopenia, muscular dystrophies, cancer cachexia, disuse atrophy and other wasting disorders (e.g. AIDS) can lead to considerable morbidity, loss of functional capacity and in the worst cases, lethality. Understanding the mechanisms regulating skeletal muscle mass is therefore of great importance. Both in the development of therapeutic strategies that may alleviate some of the pathological conditions associated with diseases of muscle. But also in the development of preventative measures that might assist in the maintenance of muscle mass to arrest the currently inevitable muscle atrophy associated with ageing.

The specific aims of this thesis were:

• To develop and test a human skeletal muscle specific cDNA microarray for global transcriptional profiling and the identification of novel gene transcripts.

• To further enhance current understanding of the process of myogenesis by examining the expression profile of rhabdomyosarcoma cells undergoing forced differentiation using the human skeletal muscle specific microarray.

• To further test the sensitivity and reliability of the microarray in identifying changes in gene expression in a biological system. Thus ensuring reliable and reproducible would be attainable from the microarray in subsequent studies using less readily available human skeletal muscle samples.

• To investigate the expression of myogenic and atrogenic genes in human skeletal muscle following an acute bout of resistance exercise.
• To use the human skeletal muscle specific microarray to study the time-course of transcriptional changes and identify novel genes expressed following an acute bout of resistance exercise.

• To identify whether the expression of the novel genes identified in chapter 5 were (1) activated at any time during proliferation, cell cycle arrest or differentiation or (2) activated by the additional mitogenic and myogenic stimulation provided by exogenous IGF-1 peptide supplementation.

• To examine whether the novel genes identified in chapter 5 were a unique response to resistance exercise or if they were responsive to skeletal muscle contraction or exercise in general.

II. SUMMARY OF MAJOR FINDINGS

The broad aim of this thesis was to further understand the mechanisms of hypertrophy by identifying novel genes involved in resistance exercise induced increases in skeletal muscle mass. In chapter 2, the development of a human skeletal muscle specific microarray was discussed. Approximately 11,000 cDNA clones derived from a normalised human skeletal muscle cDNA library were arrayed onto the microarray along with 270 genes with known functional roles in skeletal muscle. It was demonstrated that the expression profiling data derived from the microarray was accurate, reliable and would allow expression analysis and identification of novel genes in a range of different experimental systems. The results presented demonstrate the robustness of the microarray in producing accurate and reliable data. In chapter 3, the human muscle specific microarray was used to profile the temporal expression of genes during the differentiation of a model of skeletal muscle myogenesis, rhabdomyosarcoma cells. It was demonstrated that, despite aberrant differentiation in vivo, during forced differentiation, rhabdomyosarcoma cells exhibit a pattern of gene expression consistent with that observed during normal myogenesis. Furthermore, two genes (PTMA and Tim10) were identified as being markedly down-regulated during rhabdomyosarcoma differentiation. This was the first study to investigate the expression of these genes in rhabdomyosarcoma.
The activation and proliferation of satellite cells has been identified as a critical component of adaptive skeletal muscle hypertrophy. In chapter 4, the expression of several cell cycle regulatory factors was examined following a single bout of resistance exercise. It was identified that, whilst markers of cell proliferation were not altered within the 24 h following an acute exercise bout, a marker of cell cycle withdrawal, p21 was markedly and rapidly induced by 4 h post exercise. Similarly, the expression of two members of the MRF family, MyoD and myogenin were also rapidly induced by 4 h. The time-course of these changes however, likely preclude the involvement of satellite cells in these gene responses. It was proposed that MyoD and p21 act within the myonuclei of mature myofibres to reduce apoptotic cell death. In addition, the expression of several negative regulators of skeletal muscle hypertrophy, myostatin and the atrophy related genes, MURF-1 and atrogin-1 was investigated. To date, this is the first study to examine the expression of these genes following a single bout of resistance exercise in human subjects.

In chapter 5, the human skeletal muscle microarray was used to identify novel genes involved in the early recovery from resistance exercise. Four genes (hypothetical protein FLJ38973, ASB5, MLP, and TXNIP) demonstrating marked increases in expression following resistance exercise were identified and had not previously been investigated in this context. To further understand the functions of these genes in skeletal muscle, a human primary skeletal muscle cell culture model was employed to examine the expression of the genes during proliferation, cell cycle arrest and differentiation. No change in expression was observed for MLP, ASB5 and FLJ38973 throughout the time-course of differentiation. Conversely, TXNIP expression was rapidly increased and remained elevated throughout the 72 h of differentiation. Finally, the expression of these novel genes was examined following a single bout of endurance exercise to determine if the responses observed in chapter 5 were a unique response to resistance exercise or if they were responsive to skeletal muscle contraction or exercise in general. The expressions of TXNIP, MLP and FLJ38973 were not altered by the endurance exercise protocol, whilst ASB5 expression increased at 30 min post exercise.

The results of this thesis describe the identification of four genes whose expression had not previously been examined in skeletal muscle in response to an acute bout of resistance exercise; consequently increasing the overall body of knowledge regarding the transcriptional activity following a single bout of resistance exercise. Additionally, the potential functions of these genes in the early adaptive response to resistance exercise was further characterised by examining their expressions during myogenic differentiation as well as during the recovery from a single bout of endurance exercise.
Collectively, the results described in this thesis, in part, describe the potential functional roles of these genes in skeletal muscle.

A. ASB5

ASB5 was originally detected in endothelial and smooth muscle cells proliferating rapidly during collateral artery growth following femoral ligation (Boengler et al. 2003). In this thesis, ASB5 was observed to be up-regulated following both resistance and endurance exercise bouts, however no change in ASB5 expression was demonstrated during the differentiation of human primary skeletal muscle cells in culture. From these and previous studies, it seems that the likely functions of the observed increases in ASB5 mRNA following endurance and resistance exercise is as an adaptive response to an increased need for capillaries within the muscle. The exact functional role of the ASB5 protein in these processes however remains unknown.

B. TXNIP

TXNIP, is a ubiquitous protein that binds with high affinity to thioredoxin and has been implicated the maintenance of cellular redox state and insulin secretion (Hui et al. 2004), development of hepatic steatosis (Donnelly et al. 2004; Van Greevenbroek et al. 2004), among others (Ikarashi et al. 2002; Yoshioka et al. 2004). In this thesis, TXNIP expression was shown to be markedly, and transiently up-regulated 30 min following a single bout of resistance exercise, whilst no change was observed following endurance exercise. Interestingly, it was also demonstrated that TXNIP mRNA was markedly induced during differentiation. Several possible functions for TXNIP in skeletal muscle can be hypothesized from the results presented here. Firstly, The lack of induction following endurance exercise seems to suggest a reliance on conditions specifically imposed on the muscle during resistance type exercise for its transcriptional activation. Secondly, increased TXNIP expression during differentiation in vitro might suggest a potential role in the differentiation of satellite cells in vivo. However, the very early (30 min) and transient nature (return to resting levels by 4 h) of TXNIP expression following resistance exercise would likely preclude a role for TXNIP in satellite cell differentiation within this time frame. It is possible therefore, that TXNIP might be acting within the myonuclei to exert its functions on the adapting/repairing myofibre. Importantly, considerable work is necessary to further delineate its functions within skeletal muscle.
C. MLP

MLP was originally discovered in denervated skeletal muscle and identified as a positive regulator of the myogenic program in myogenic cells (Arber et al. 1994). In this thesis, it was demonstrated for the first time that MLP transcriptional expression is increased by resistance exercise whilst not being altered following endurance exercise. Numerous functions have been attributed to MLP in skeletal muscle including facilitating the assembly or stabilization of the myofibril apparatus (Flick and Konieczny 2000) contributing to the mechanical stretch sensor machinery (Knoll et al. 2002), and regulating myogenesis through the interaction with the myogenic transcription factors, MyoD, Myf6 and myogenin (Kong et al. 1997). Collectively these data seem to suggest multiple complementary roles for MLP in sensing mechanical stretch, regulating the subsequent transcriptional response and ultimately facilitating the assembly or stabilization of the contractile apparatus. Thus it appears that in response to a stimulus like resistance exercise, MLP plays a critical and coordinated role in the repair and adaptation of skeletal muscle.

D. Hypothetical Protein FLJ38973

The hypothetical protein FLJ38973 was originally identified during a large-scale screen of ESTs for the purpose of identifying the full-open reading frame cDNA sequence for each human and mouse gene (Mammalian Gene Collection Program Team et al. 2002). Whilst no subsequent study has investigated FLJ38973, bioinformatics analysis using revealed that it is located on chromosome 2 and produces, by alternative splicing, 2 different transcripts (aDec03, bDec03) together encoding 2 different protein isoforms. In the thesis, FLJ38973 mRNA was demonstrated to be up-regulated following an acute bout of resistance exercise, whilst no changes in expression were observed during differentiation or following a single bout of endurance exercise. This is the first collection of studies to investigate this gene and provides valuable data on its possible functions in vivo. Considerable work is necessary to further characterise the tissue distribution, cellular localisation, and most important, biological functions of the protein(s) encoded by this gene.
III. FUTURE DIRECTIONS

This thesis describes the identification and characterisation of four novel genes whose expression was increased during the early recovery from a single bout of resistance exercise in human vastus lateralis muscle. Subsequently, the expression patterns of these genes was investigated during the differentiation of primary human skeletal muscle cells in culture as well as following a single bout of endurance exercise. Whilst these studies go some way towards characterising the functions of these genes, considerable work is necessary to delineate their biological functions in skeletal muscle. In this section, details of future studies designed to advance current understanding of functions of these gene products will be discussed.

Throughout this thesis, each study focused not only on the identification of novel genes, but also on understanding further the importance of the myogenic pathways in the development of skeletal muscle hypertrophy. Despite the recent advances in the understanding of the molecular regulation of skeletal muscle hypertrophy, considerable gaps remain. Further research required to advance knowledge in this area will be discussed.

A. Functional analysis of novel gene targets using gene knockdown and over-expression techniques

1. Background

In chapter 5, the identification of several novel resistance exercise responsive genes was discussed. Subsequently, the potential functions of these genes were examined during myogenic differentiation in culture (chapter 6) and following a single bout of endurance exercise (chapter 7). Whilst these studies provided considerable information on the transcriptional regulation of these genes, very little is currently known about their functions within skeletal muscle. Targeted gene knockdown and over-expression techniques are effective ways of analysing the functions of genes in vitro.

2. Aims

(i) To investigate the functions of selected genes by examining the phenotypic changes induced by targeted knockdown in myogenic cells in culture.
(ii) To investigate the functions of selected genes by examining the phenotypic changes induced by targeted over-expression in myogenic cells in culture.

3. Key Methods

3.1 RNA Interference

RNAi has been widely adopted as the simplest and most effective method of suppressing a gene’s product resulting in null or hypomorphic phenotypes. Subsequently, the biological effects of knocking down a target gene can be examined and functions of a gene inferred. In mammalian cells, RNAi is typically induced by the introduction of small inhibitory RNAs (siRNA) (specifically designed to target the gene of interest whilst minimizing the effects on non-target genes) into the cell culture system (Sago et al. 2004).

Following siRNA synthesis, delivery of the siRNA into the cell system is usually accomplished via transfection with a lipid- or amine-based reagent. Delivery into primary cells, however, can be problematic, if not impossible, using standard transfection methodologies. In these cases, electroporation using a specialized, gentle-on-cells buffer and optimized pulsing conditions generally results in very efficient siRNA delivery without compromising cell viability. Following transfection, the effectiveness of the siRNA in inducing knockdown of its intended target needs to be assessed. siRNAs exert their effects at the mRNA level, therefore, quantitative real-time PCR analysis would be required for siRNA validation and transfection optimization purposes. Subsequently, the effect of the targeted knock-down of the gene can be assayed using standard functional, morphological and phenotypical measures. For example, targeted gene knockdown of IL-6 in muscle cells has been used previously to examine its effect on myogenic differentiation (Baeza-Raja and Munoz-Canoves 2004).

3.2 Over-expression studies

Over-expression techniques involve the forced expression of a specific gene and subsequent examination of biological changes induced by the gene product. Standard techniques are available for cloning of the selected gene into an expression plasmid and the transient transfection of the target gene into the cell line of interest. Classical
transfection technologies that are widely used include the DEAE-dextran method, the calcium phosphate method, electroporation, and liposome mediated transfection.

Following verification and optimisation of the transfection techniques, standard functional, morphological and phenotypical measures would be employed to examine the effect of the forced over-expression of the gene. For example, over-expression studies have been effectively employed to examine the effect of integrin binding proteins on muscle differentiation (Li et al. 1999) and the effect of Chemokine-like factors on myoblast proliferation (Xia et al. 2002).

4. Significance

The genes identified in this thesis are all likely to be involved in the repair or adaptation of muscle. Therefore measures of proliferation (e.g. PCNA mRNA, DNA content), differentiation (e.g. creatine kinase activity, MHC protein content) and myofibre size (hypertrophy) would all be appropriate end-point measures to assess possible functions of each target gene in vitro. For example, the knock-down of a gene exhibiting markedly reduced creatine kinase activity and MHC expression would likely play a critical role in myogenic differentiation. Similarly, dramatic increases in the rate of proliferation following the over-expression of a particular gene would likely indicate a role in the cellular proliferation. Therefore these studies would be valuable in further examining the functions of these genes in skeletal muscle.

B. Examination of cellular localisation and tissue distribution of selected novel genes

1. Background

Microarray analysis and gene expression analysis in general are valuable measures of the regulation of biological processes. However, mRNA expression patterns are by themselves insufficient for the quantitative description of a biologically active product. Further examination of the functions of the novel resistance exercise sensitive genes would be advanced by the examination of the cellular localisation and tissue distribution of the proteins encoded by each gene. Whilst commercially produced antibodies may be available for some of the proteins of interest, others may need to be custom produced. The development of a polyclonal antibody to target the protein encoded by the gene of interest would provide numerous avenues for further investigation into its functions.
2. Aims

(i) To investigate the cellular localisation of selected novel proteins in skeletal muscle using confocal microscopy.

(ii) To investigate the tissue distribution of selected novel genes using western blot analysis.

3. Key Methods

3.1 Fluorescence Microscopy

Specific primary antibodies could be used to examine the cellular localisation (if not already known) of the novel skeletal muscle sensitive proteins. Furthermore, immunohistochemical techniques could be used to track the cellular localisation of these proteins in mounted muscle tissue following, for example, a single bout of resistance exercise. Similarly, immunocytochemical techniques might be used to examine the possible changes in cellular localisation during proliferation and differentiation of satellite cells in culture.

3.2 Western Blot Analysis

The development of specific primary antibodies or the use of commercially available antibodies specific to the protein of interest would provide an opportunity to compare the expression of the selected protein across many different tissues. Analysis of the protein expression in many tissues would be carried out using standard western analysis techniques.

4. Significance

These analysis strategies would further advance current understanding of the potential functions and mechanisms of actions of the proteins encoded by these novel genes. For example tissue distribution analysis might reveal that a particular protein is expressed exclusively in skeletal muscle or alternatively is enriched in skeletal and cardiac muscles tissues whilst demonstrating minimal expression in other tissues. Such a protein would be of particular interest as a potentially critical mediator of the skeletal muscle phenotype.
C. Effects of resistance exercise training on the expression of selected novel resistance exercise responsive genes

1. Background

The novel resistance exercise sensitive genes discussed in this thesis were identified following a single bout of resistance exercise. However a single bout of resistance exercise is unlikely to confer marked phenotypic or functional changes within the skeletal musculature. Therefore it would be of interest to examine the effects of a resistance exercise training protocol, of sufficient intensity and duration to induce marked increases in muscle hypertrophy and strength, on the expression of these previously identified genes.

2. Aims

(i) To examine the mRNA expression of novel resistance exercise sensitive genes following 12 weeks of high intensity resistance exercise training.

(ii) To examine the protein expression of novel resistance exercise sensitive genes following 12 weeks of high intensity resistance exercise training.

3. Key Methods

Approximately 10 young male subjects would be recruited to undergo a 12 week heavy resistance exercise training protocol. Vastus lateralis muscle biopsies would be collected before exercise and following a single bout of resistance exercise. Following the 12 weeks of training, subsequent biopsies would be collected at rest and again following a single bout of exercise.

RNA and protein would be extracted from the muscle biopsies and analysed to measure RNA and protein expression for each of the novel resistance exercise sensitive genes.

4. Significance

The study would provide further valuable data on both the transcriptional regulation of these genes as well as the translational regulation of the proteins following a single bout of resistance exercise. The great advantage of this study design would however, be to examine the effect of training on the expression of these genes and proteins. If these genes are indeed important regulatory components of the hypertrophic response, a marked increase in their expression would be likely following the intensive
training regime. Thus valuable data collected from this study would further current understanding of the molecular regulation of skeletal muscle hypertrophy.

D. Identification of novel genes demonstrating decreased expression following acute resistance exercise

1. Background

In chapter 5, a human skeletal muscle specific microarray was used to examine the global transcriptional response of skeletal muscle to a single bout of resistance exercise. 7,229 elements out of the 11,000 spotted onto the microarray showed adequate expression levels, with 3112 elements demonstrating increased expression following resistance exercise whilst 1436 elements decreased in expression. Considerable time and cost involved in sequencing gene elements prevented the analysis of all differentially expressed elements so a subset of these genes were selected for sequencing and further analysis. Only genes demonstrating increased expression were selected for this analysis. Genes showing reduced expression however are likely to constitute and equally important biological adaptation following resistance exercise.

2. Aims

(i) To examine the collected microarray data to identify novel genes down-regulated following a single bout of resistance exercise in human subjects.

3. Key Methods

3.1 Microarray data analysis

Further detailed analysis of the collected microarray data would be required to isolate elements demonstrating marked and consistently reduced expression following a single bout of resistance exercise. A subset of these elements will be selected for further analysis.

3.2 Sequencing

Sequencing analysis would be performed on the selected clones as previously described.
3.3 Bioinformatics analysis

Extensive bioinformatical analysis will be performed on the selected genes to identify the gene sequence, transcript structure, amino acid profile in addition to known or predicted functions of the protein.

3.4 Real-Time PCR

Real-Time PCR analysis would be performed to validate the results of the microarray.

4. Significance

Several proteins (most notably myostatin) have recently been identified as powerful negative regulators of skeletal muscle mass. Further analysis of the microarray data collected for this thesis has the potential to isolate further novel genes with critical biological roles in skeletal muscle.

E. Examination of alternate pathways potentially involved in skeletal muscle hypertrophy

1. Background

Considerable recent research into the molecular mechanisms regulating skeletal muscle mass has greatly enhanced the current understanding of this complex process. In this thesis, several novel resistance exercise responsive genes have been identified that might constitute important components of early adaptive response to resistance exercise. Furthermore, a number of genes with identified roles in myogenesis were examined to investigate potential roles in the early adaptive response to resistance exercise. Despite these recent breakthroughs, a substantial amount of work is required to further unlock the complex regulatory mechanisms mediating skeletal muscle hypertrophy. For example, the mechanism(s) by which skeletal muscle senses skeletal muscle contraction and transduces this information into downstream cellular level responses remains unknown. Similarly, it is unclear how satellite cells and mature myofibres act cooperatively to mediate myofibre adaptation and hypertrophy. It is likely that there are numerous as yet uncharacterised pathways that might contribute to skeletal muscle hypertrophy however further research is necessary to investigate these questions further.

2. Suggested targets for further investigation

Recent research has demonstrated that the Notch signalling pathway is a key determinant of muscle regeneration. Importantly, components of the Notch signalling
mechanism are impaired with age, contributing to the reduced regenerative potential evident in elderly muscle (Conboy et al. 2003). It is unclear whether this pathway plays an important role in the regeneration of human skeletal muscle nor whether it might contribute to skeletal muscle adaptation and hypertrophy. Therefore, this pathway constitutes a potentially interesting target for further investigation.

Much of our understanding of the biology of skeletal muscle has come from the study of embryonic muscle development. The processes of muscle regeneration and adaptation has been described as a recapitulation of the stages of embryonic muscle development and subsequently may share parallel morphological steps (Grounds 1991; Chambers and McDermott 1996). These initial stages of myogenesis are regulated by positive and negative signals, including Wnt, bone morphogenetic protein and sonic hedgehog family members. Therefore, these myogenic regulators would provide interesting candidates as potential mediators skeletal muscle adaptation.
IV. CONCLUSIONS

In summary, the findings of this thesis are as follows:

1. The mRNA expression of two genes, PTMA and Tim10 were identified as being down-regulated following forced myogenic differentiation of rhabdomyosarcoma cells.

2. The temporal pattern of the MRF expression over the time-course rhabdomyosarcoma differentiation was identified as exhibiting a remarkably similar pattern of expression to that exhibited in normal myogenesis.

3. A single bout of resistance exercise was shown to be sufficient to increase the expression of the MRF member’s myogenin and MyoD but not Myf5 and Myf6. The cell cycle regulator p21 was demonstrated to increase following resistance exercise whilst no change was observed for PTMA, cyclin D1 and p27. Myostatin mRNA expression was shown to be significantly reduced following acute resistance exercise.

4. Four novel genes (ASB5, TXNIP, MLP, FLJ38973) were identified as being increased following an acute bout of resistance exercise. These genes have not previously been implicated in the early molecular response of skeletal muscle to resistance exercise in humans.

5. The expression of TXNIP was demonstrated to be increased during the differentiation of human primary skeletal muscle cell culture. ASB5, MLP and FLJ38973 expression was not altered during differentiation.

6. The expression of ASB5 was increased following a single bout of endurance exercise. The expression of MLP, TXNIP and FLJ38973 was not altered by this exercise protocol. Similarly, the expression of the MRFs was not altered by a single bout of endurance exercise.
REFERENCES


APPENDIX A

ANKYRIN REPEAT AND SOCS BOX-CONTAINING PROTEIN5

Official Gene Name: Ankyrin repeat and SOCS box-containing protein 5
Official Gene Symbol: ASB5
Alternative symbols: None
Chromosomal Location: 4q34.2
Gene Size: 55.4 kb
Number of Exons: 7
mRNA Length: 8613 bases
Open Reading Frame Length: 987 bases (329 amino acids)
Protein pl/Mw: 6.34 / 36340.85

Transcript Structure:
cDNA and amino acid sequence:

```
GAAGCAGCTGATATTTTCTTTCCTCAGGACCAGCTGTTCAGGAGCATCCCGGACGAGAGCCA
GGCACATTTGAGACTTGGATCCAACTAAAGACCGCCGCAGATTCTTCTGCAGCAATGGTC
M  S
GTGTTAGAAGAAAATCGGCCGTTTGCTCAACAATTCTCCATGCTACTTTTACACATTTACTTT
V  L  E  E  N  R  P  F  A  Q  Q  L  S  N  V  Y  F  T  I  L
TCGCTGTTCGTTTATAGGTGAAAAATCAGGCTTGGCACCATCTCAGCTATTCTAC
S  L  F  C  F  K  L  F  V  K  I  S  L  A  I  L  S  H  F  Y
ATACGAAAAAGCCAGGAAAGCGGAAAGGATAGCTGAAATTTAATGGGAATACCC
I  V  K  G  N  R  K  E  A  A  R  I  A  A  E  F  Y  G  V  T
CAAGGACACAGGTCTGGCGAGATGATCATCACCACCTATCGAGCAGCAGAAGCTGGCC
Q  Q  S  G  S  W  A  D  R  S  P  L  H  E  A  A  S  Q  G  R
CTTCTTGCTCTTGAGAAACATTATTCATCAAGGTTTATATGTAATACGATACCTTACAGAC
L  L  R  T  L  L  S  Q  G  Y  N  V  N  A  V  T  L  D
CATGTACCCGGTTGGACAGGACGACGCTCCTGGAGATCAATGGCAGATCCGGAAGACTCTG
H  V  T  P  L  H  E  A  C  L  G  D  H  V  A  C  A  R  T  L
CTGGAGACCAAGATCTTAAATGGATACGATATCGCTATCCCTTTATCAACAC
L  E  A  G  N  V  N  A  I  T  I  D  G  V  T  P  L  F  N
GCCATGCTCCAGGAGCCCAGTGGGTGAGCTGCTCTTGAGATATGGTGCAAAAGCC
A  C  S  Q  G  S  P  S  C  A  E  L  L  L  E  Y  G  A  K
CACGCTGAGCTCTGTCCTTACCTACCCAAGGAGAGGCCCCAGTAGAGCATCCATGAGAA
Q  Q  S  E  I  V  N  L  L  L  E  F  G  A  D  I  N  A  K
ATTACAGAGCTTTGCCGCCATTTAGATGGACGATCTGCATGACTGATGCTGGAAAGGTA
N  T  E  L  L  R  P  I  D  V  A  T  S  S  S  M  V  E  R  I
TTGCTCTTACAGTGAAGCTACCCCAAGCTCTTACCTACGCTATGGATGCTATCCGA
L  L  Q  H  E  A  T  P  S  S  L  Y  Q  L  C  R  L  C  I  R
AGCTACATAGAAAACCAAGATGTCCTATCTCCGCAAGCAGGATGGTTACTG
S  Y  I  G  K  P  R  L  H  L  I  P  Q  L  Q  L  P  T  L  L
AAGAATTTTCTPAGCTATGAAAACAGTAAGAGGATATCTTCAATCTTGAGAAATCAG
K  N  F  L  Q  Y  R  *
```
The coding sequence of the ASB5 cDNA and its deduced amino acid translation (in black). Exons are represented in alternating blue and red text, whilst non-coding regions are highlighted in yellow. The stop codon (TGA) is shown as an asterisk (*).
APPENDIX B

THIOREDOXIN INTERACTING PROTEIN

Official Gene Name: Thioredoxin interacting protein
Official Gene Symbol: TXNIP
Alternative Gene Names: THIF, VDUP1, HHCPA78, EST01027
Chromosomal Location: 1q21.2
Gene Size: 3.9 kb
Number of Exons: 8
mRNA Length: 8820 bases
Open Reading Frame Length: 1173 bases (391 amino acids)
Protein pI/Mw: 7.46 / 43689.39

Transcript Structure:
cDNA and amino acid sequence:

```
GCTTAGTGTAACCAAGCGGCGTATATTTTTTAGGGCGCCTTTTCGAAAACCTAGTAGTTAAT
ATTCATTTTGAATTCAACTCATTATTTTTAAGCTCAAACTGCTTAAGAATACCTTAATT
CCTTAAAGCGGTAGGATCTGCTGGATGAGGCCTTATGAGGTTGATGAGTTGAACTGCTGCT
VKA VRL TACG VAKV LWMQGS

CAGCAGTCCCAACACGACTCTGGAGTACCTGCTGCTATAGAAGACCGCCCTCTCTCGAGAC
Q CCK QTSE YLRYEDTLLEDCAGCCCAACAGTGTGAGATGCTGATGACCCAGGAAACAAATAGAGTACAAG
QPTGENSEMVIMRPNGKYEYTTCGGCTTTGAGCTCTTCCAGGCCCTTGGAGAATCTTCTTGAAGAGAC
FGFELPQGPLGTSTSFKGYGC

GTAGACTAAGGGAAGCTTCTTCTTGAGCGCAGCGCGCCGCCAGGGCAACACTACAGAGCAAG
VDYWVWKAFLDRPSQPTQETKAAAAACTTGGAGATGCTGATGCTGATGCAATACCCCTGTATTATGGCCACCTTGG
KNFVEVDLVVDVNTPDMAPVTCTGGCTAAAGAAAGAAAGAATTTCCTGCTATCTCTGATATGTCGGGTCTGTCC
SACKEKKVSFCMFIPDRSVV

TCTGGCTGATTGAGAAGAACAGATCTTGAGATCCTTTCTCCATAGCTTCTGAGAC
SARIDRKGFCGCDEISIHADTTTGGAATACATGTTCCCGATGTGGGCTCCCAAAAGTGGCACTTGTTGGGCGCTATCACT
FE NTCSRI IVVP KAIAV RHTTACCTGGACATGGCCGACCAAAGTGCTGACCTGAGAGGTTGATCAGTCCATAGGGCAAT
YLAGOQTKVLTQKLSSVRGNCATATTACTGCGGACTGTGGCATTGGGCCAGGCTTCCGGCTCTGAGAATC
HIISGCTASWRGKSLRVQKIAGGCCCTACTGCTGGCTGAAACACCTTCTGAGTGAATTCTTCTTACTGACTTGATGCTTG
RPSIIGNEDCNILRVEYLISIYVAGGTCTGCTGAAATCCAGAAGATTCCCTTCTTGAAGAGGAGAC
SVPGSKVKILDLPVLVIGRS

GGTCTAAGCGACAGAACTCCAGCATGGCCAGCCACAGGCTCAGATGATGTTGGGTA
GLSSRSTSMASTRSSSSSEM SWVATCTGAACATATCCCTCTATACCCAAGAAGCTCCTCCCTGCTATATGGATGCTATCCTGGAA
DLNIDTPPEAPCP YMVDIPEATCGGACCTTGAGCAGGCCAACACTTCTCTGCTCTAGATGACAGGTAGGGCTCTCAAGAC
DHRLESPPTPLDDMDGSDQ
AGGCCCTATCTTTATGTATGCTCCCTTCTGAGTCTTACATGCGCACCACCCGACTTATACCTGAG
SPIFMAYPFEKFMPPTYTEGTTGACCTCGTACATCCACACACATAGTGCACTGAGCATGTGGAAGAAAAGAAGACAGCT
VDPCILNNNQ*
```

The coding sequence of the TXNIP cDNA and its deduced amino acid translation (in black). Exons are represented in alternating blue and red text, whilst non-coding regions are highlighted in yellow. The stop codon (TGA) is shown as an asterisk (*).
APPENDIX C

MUSCLE LIM PROTEIN

Official Gene Name: Cysteine and glycine rich protein 3
Official Gene Symbol: CRP3
Alternate Gene Names: Muscle LIM Protein (MLP), cardiac LIM protein (CLP), LMO4, CMD1M
Chromosomal Location: 11p15.1
Gene Size: 10.1 kb
Number of Exons: 5
mRNA Length: 5693 bases
Open Reading Frame Length: 582 bases (194 amino acids)
Protein pI/Mw: 8.89 / 20968.89

Transcript Structure:

```
Ex 1  Ex 2  Ex 3  Ex 4  Ex 5  Ex 6

Int 1 4032 bp
Int 2 9477 bp
Int 3 1787 bp
Int 4 1170 bp
Int 5 2205 bp

Ex 1 101 bp
Ex 2 140 bp
Ex 3 169 bp
Ex 4 133 bp
Ex 5 94 bp
Ex 6 470 bp
```
cDNA and amino acid sequence:

The coding sequence of the MLP cDNA and its deduced amino acid translation (in black). Exons are represented in alternating blue and red text, whilst non-coding regions are highlighted in yellow. The stop codon (TGA) is shown as an asterisk (*).
HYPOTHETICAL PROTEIN FLJ38973

Official Gene Name: Hypothetical Protein FLJ38973
Official gene symbol: FLJ38973
Alternative symbols: None
Gene Product: hypothetical protein FLJ38973
Chromosomal Location: 2q33.1
Gene Size: 17.0 kb
Number of Exons: 2
mRNA length: 8021 bases
Open Reading Frame Length: 807 bases (269 amino acids)
Protein pI/Mw: 8.65 / 30918.17
Transcript Structure:
cDNA and amino acid sequence:

CCCTCAAGGGCGGCTCCGCAGCCCGGGGGCCGCGCGCGCCGACCGTTGAGCCGCCGGCTGAGCC
GCCTGCTGAGTCCCTCCCTGCTGGGGCTCCAGAAGCCGCTCCACCTCGGAAAGCCGCTCTGC
GGCGCGGCGGCC
ATGTGGGGGTTCAGGCTCCTGCGGTCGCCGCCGTTGCTGCTCCTGCTGCCGCAGCTCGGA
M  W  G  F  R  L  L  R  S  P  P  L  L  L  L  P  Q  L  G
ATCGGAAACGCCTCGTCCTGCTCTCAGGCCAGAACCATGAACCCGGGCGGCAGCGGCGGC
I  G  N  A  S  C  S  Q  A  R  T  M  N  P  G  G  S  G  G
GCGCGATGCTCCCTCTCGGCCGAGGTGCGCCGCCGTCAGTGCCTGCAGCTTTCCACCGTG
A  R  C  S  L  S  A  E  V  R  R  R  Q  C  L  Q  L  S  T  V
CCTGGAGCCGGTCCACCGCGACGAAAGGATATTGCTCCCTGCTGTTGGCGGGCGGCCGGGGAGGGA
P  G  A  D  P  Q  R  S  N  E  L  L  L  A  A  A  G  E  G
CTGGAGCGGCGGACCTCCCGGGGACCACAGGAAAGGAGGGCAGCGGCGGCCGCGCCCGAG
L  R  Q  D  L  P  G  P  D  A  K  E  F  Q  P  P  Q
CATCGCCTCTCTATTCTCCTGGAGATGCGAGAATTACATGAAATTATGACTCGTCAT
H  H  V  L  Y  F  P  G  D  V  Q  N  Y  E  I  M  T  R  H
CTCGAGAAATAAATAATGGGAAAACCTGAGCTACTGAAATTGGCTACCATTTTAGCCCCAC
P  E  N  Y  Q  W  ENW SLEN VAT ILAH
CGGTTCCGCAATAGTTATATTGGTTGATAAAATATGTTCCCGGAATGCTATTGCAAAAATTC
R  F  P  N  S  Y  I  W  V  I  K  C  S  R  M  L  H  K  F
AGCTGATAGTACAATTTGGTAAAGTAACATGTTTGTGGTCGCCGCGAACACAATACATGC
SCYDNNFVKSNNFGAPEHNTD
TTTGAGCCTTTTAAACGACCTTTATATAGTTAATAGCTTTTTATATTATAAGTCGAGAT
FGAFKHLYMLLVNAFNLSQN
AGTTATCAAAAGAAAGAATTTGGAATTTGGAATAGACTCCTCCTACTGCTAACTGAGA
SLSKKSLNVWNKDISIASNCR
TCCAGTCTCCTCTACTACAGAATGGTGTCCGAGGAGAAAAATGAGGACCCTGTAAGAAA
SSPSHTTNGCQGEKVRTCEK
TCCTGATAGCTGCCACGAGTTTTTTATACCCACATCAATAAGACTCCATTTCATTTTACCTTG
SDESAFMSGYPPPSLNDASFTL
ATTTGAGTACGTAACAAAGGTTGTTGTTTGTGAATCACTGTGTGTTTTTGATATGGAAGAGGCC
IGFSKGCVVLNQLLFELKEA
AAAGAAACAAAGAACATAGATGCTTTTATCAAAGACATAGAACAAAGACATAGATTGCGGTGAAT
KKDKIIDAFIKSIRTMYWLD
GGTGATCCATTTCGAGGAAGCTATTTGTTACTATTCCGAAACATCTGTTGAGAGAATTT
GCHSSGGSNTWVTYPEVLKEF
GCAACAAACAGGAATTATTCGCTCAGACCTCAGATGAACTACCATACGTAATACTGCCCCAATTG
AQTVGIIVTHVTPYQQVRDPM
AGATCTTGGATTGGAAGGCAAAGAAATATTCTTGGCATACTTGUGGATCTTGTAATG
RSWIGKEHKKFQVQILDGLM
CAGGGTACGATCCAAAATCTTATTTTACAAGAAGGACTCTCCTCCCATAGAATACACTCTAGG
QTVTSQIHFTKEAPSIENHFRTTCCATGAAAGATTCTTATTAATAGCTTGAAGAAGACTATAV
VHVEF
AGCATTTTTGGATGTTATAAAATTCAGATAATGGGATGTAAATTCCATAGCTCAGTATTGCGATG
TTGGGTTAGTGCGGGAGAACACATCTTTAAATATTGGTATGTTAGTAATATATTTGAAAA
TCTAAAAAGATGTTTTGTTATTAATGTCGAGGATATAACACCGTTTTAAAATGAAAGGAC
AACATTGTCTCTTTAAACAAAGAAAGGTTACGAGCTTTTCATCAATTGGATATTG
AAGTATGTATTTAAACTATTGGTGAAATTAACTTTTTTGAATATGAGATTTTGGAGAAAA
ATTCTATTTCTCTCATAAAACAAAAGGAAAAGGTTACGAGGCTTTTCATCATTTGGAAAT
AAGCTCACCGTGCTCTTTTGGTTTTGTATTAAACTCCTTCCTTGACTCATTTTCAGTTGTCGAGGCGGCC
The coding sequence of the FLJ38973 cDNA and its deduced amino acid translation (in black). Exons are represented in alternating blue and red text, whilst non-coding regions are highlighted in yellow. The stop codon (TGA) is shown as an asterisk (*).