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A Gene Therapy Approach for Treating Muscle Degeneration in Neuromuscular Disorders Using IGF-I Splice Variants

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A thesis submitted for the degree of Doctor of Philosophy to the University of London – May 2007

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PhD Thesis

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Title: A Gene Therapy Approach for Treating Muscle Degeneration in Neuromuscular Disorders Using IGF-I Splice Variants

Laboratory / Institution: Molecular Tissue Repair Unit, Department of Anatomy and Developmental Biology, University Department of Surgery, Royal Free and University College Medical School, University College London

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Abstract

Neuromuscular disorders, which associate with muscle degeneration, are not rare and affect millions of people worldwide. The impacts of such disorders vary from gradual loss of mobility and independence to severe disability and death, and therefore millions of patients suffer from them at every stage of their life. Because, there is currently no treatment of any form of such disorders, this study was aimed to develop a novel treatment for such disorders.

For a long time it has been known that Insulin-like Growth Factor (IGF-I) influences several cellular processes, including proliferation, differentiation, repair and maintenance. Like many genes, the IGF-I gene can be spliced to produce several isoforms, and in human muscle, it expresses at least two main isoforms which are a liver type, systemic form (IGF-I Ea) and an autocrine / paracrine form (IGF-I Ec). This second isoform has been named the Mechano Growth Factor (MGF) because of its mechanosensitivity.

The in vitro and in vivo effects of these two splice variants of the IGF-I gene were investigated in this study. In vitro roles of two splice variants of the gene were studied by the proliferation / differentiation effects of two alternative splice isoforms of the gene in animal muscle cell lines (mouse C2C12 and rat L6 E9). Proliferation /

differentiation assays were carried out using human primary cell cultures from biopsied muscles from congenital muscular dystrophy (CMD), fascioscapulohumeral muscular dystrophy (FSHD) and amyotrophic lateral sclerosis (ALS) patients as well as from healthy volunteers. Human primary muscle cells were treated with IGF-I Ea (long r¹IGF-I) and MGF E domain peptides, and immunocytochemistry techniques with Desmin, DAPI and FITC markers were used to detect proliferation state of myogenic commitment. The CPK and BCA protein assays were also used to determine the differentiation state following such peptide treatments. The results showed that MGF significantly increased muscle stem (satellite) cell proliferation in both animal and human muscles, both in healthy and in severe muscle wasting disorders. E domain of MGF dramatically increased proliferation in progenitor cell in CMD (68%), FSHD (74%) and ALS (49%) primary cultures. The results also confirmed that the MGF had no effect on myotube formation but that it increases myoblast progenitor cell proliferation, whilst systemic IGF-I peptide (IGF-I Ea) increased cell differentiation and facilitated myotube formation.

The effects of two IGF-I splice variants in muscle fibre growth were also studied in relation to Duchenne Muscular Dystrophy (DMD) by in vivo gene transfer method using the mdx mouse model. Such effects were investigated in both young and old mdx mice TA muscles by intramuscular injection of cDNAs in plasmid vectors pcDNA3.1NT/GFP. Maximum muscle tetanic contractile force was measured to determine the changes of muscle strength at 21 days after gene injection. The results showed that cDNA of MGF dramatically increased muscle fibre strength in young mdx mice (37 %) in only 3 weeks time. The MGF also increased muscle strength and

mass in the older mdx mice but to a moderate level (11%). In mdx mice, the changes in gene expressions of satellite cell markers (MyoD and myogenin) were determined by quantitative real-time reverse transcriptase PCR, 21 days after injection of the gene constructs into TA muscle comparing with TA muscle of untreated leg. The results showed that MGF had an effect in satellite cell activation, and it activated quiescent satellite cells in mdx mice.

This study showed that MGF has a significant effect in both in vitro and in vivo models, which were used in relation to treatment of muscle degeneration in DMD, CMD, FSHD and ALS. The study also showed that MGF has considerable potential to use as a therapeutic agent to treat muscle degeneration in such neuromuscular disorders.

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Abbreviations

aa: Amino acid
AAV: Adeno-associated virus or adeno-associated viral
Ab-I: IGF-I Receptor Blocking Antibody
ACh: Acetylcholine
AChE: Acetylcholinesterase
Ad: Adenovirus or adenoviral
AD: autosomal dominant
ADA: Adenosine deaminase
ADP: Adenosine diphosphate
ADS: Antibody Diluting Solution
ALS: Acid Labile Subunit
ALS: Amyotrophic Lateral Sclerosis
AOs: Antisense Oligonucleotides
AR: Autosomal recessive
ATP: Adenosine 5'-triphosphate
bp: Base pair
BCA: Biochroninic Acid
BMD: Becker Muscular Dystrophy
BrdU: 5-Bromo-2-deoxy-uridine
BSA: Bovine Serum Albumin
C2C12: Mouse myoblast cell line
CD34: Hematopoietic Progenitor Cell Antigen
c-met: Hepatocyte Growth Factor (HGF) receptor
c-terminal: Carboxy Terminal
CMD: Congenital Muscular Dystrophy
CMV: Cytomegalovirus
CNS: Central Nerve System
CPK: Creatine Phosphokinase
c- terminal: Carboxy-terminal
des-N-(1-3): N-terminal truncated IGF-I isoform
DAPC: Dystrophin Associated Protein Complex
DAPI: 4,6-diaminido-2-phenylindole
DD: Distal Muscular Dystrophy
DEPC: Diethyl pyrocarbonate
DMEM: Dulbecco's Modified Eagle's Medium
DMD: Duchenne Muscular Dystrophy
DMSO: Dimethyl Sulfoxide
DNA: Deoxyribonucleic acid
cDNA: Complementary DNA
dNTPs: Deoxynucleotide Triphosphates
ds : Double stranded
dsDNA: Double Stranded DNA
dUTP: 2'-Deoxyuridine 5'-Triphosphate
dys: animal dystrophin
EDMD: Emery-Dreifuss Muscular Dystrophy

EDTA: Ethylenediaminetetraacetic acid
EGF: Epidermal Growth Factor
Epo: Erythropoietin
EtOH: Ethanol
FBS: Foetal Bovine Serum also called FCS: Foetal Calf Serum
FGF: Fibroblast Growth Factor
bFGF: Basic Fibroblast Growth Factor
FITC: Fluorescein isothiocyanate
FSHD: Facioscapulohumeral Muscular Dystrophy or Facioscapulohumeral Dystrophy (FSH)
GAM: gene-activated matrices
GF: Growth Factor
GFP: Green Fluorescent Protein
GH: Growth Hormone
GHD: Growth Hormone Deficiency
GHIS: Growth Hormone Insensitivity Syndrom
GRMD: Golden Retriever Muscular Dystrophy
HFMD: Hypertrophic Feline Muscular Dystrophy
HGF: Hepatocyte Growth Factor
HIV: Human Immunodeficiency Virus
HSH: Hereditary Spastic Hemiplegia
HSV: Herpes Simplex Virus
IGF: Insulin-like Growth Factor
IGF-I: Insulin-like Growth Factor I
IGF-II: Insulin-like Growth Factor II
IGF1R: Type I Insulin-like Growth Factor Receptor
IGF2R: Type II Insulin-like Growth Factor Receptor
IGF-IR IR: Hybrid Receptor (Type I IGF and Insulin receptor)
IGFBP: Insulin - like growth factor binding protein
IGFBP-rP: Insulin - like growth factor binding protein related protein
IR: Insulin Receptor (IR-A: Insulin Receptor exon 11 -ve / IR-B: Insulin Receptor exon 11 +ve)
ISS: Idiopathic Short Stature
kDa: Kilo Daltons
kb: Kilo base
L6 E9: Rat myoblast cell line
LB: Luria Bertani
LGMD: Limb-Girdle Muscular Dystrophy
M: Molar
Mac25: IGFBP-related protein 1
M-cadherin:
MCGM: Muscle Cell Growth Medium
MD: Muscular Dystrophy
mdx: murine dystrophy x-linked mouse
mdx utm: dystrophin and utrophin deficient mouse
MGF: Mechano Growth Factor
MHC: Myosin Heavy Chain
MI: Myocard Infarctus
MLV: Murine Leukaemia Virus
mM: Mili-Molar
MMD: Myotonic Muscular Dystrophy
MND: Motor Neuron Disease
MRC: Medical Research Council
M-6-P: Mannose - 6 - Phosphate
M-6-PR: Mannose - 6 – Phosphate Receptor
Mrf4: Myogenic Regulation Factor 4
MS: Multiple Sclerosis
Myf3: Myogenic Factor 3, MyoD
Myf4: Myogenic Facotr 4, Myogenin

Myf5: Myogenic Factor 5
MyoD: Myogenic-determining Factors, Myf3
NGF: Nerve Growth Factor
NGM: Normal Growth Medium
Notch: A single pass transmembrane receptor protein / Notch signalling: A gene regulatory pathway
NT: N-Terminal (Amino-terminal)
NT GFP: N-Terminal / Green Fluorescent Protein
NSILA: Nonsuppressible Insulin-like Activity
Numb: a Notch signalling inhibitor
OPMD: Oculopharyngeal Muscular Dystrophy
Pax- (3 or 7): Pax molecule family members
PBP: Progressive Bulbar Palsy
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
qPCR: Quantitative Polymerase Chain Reaction
PDGF: Platelet Derived Growth Factor
pDNA: Plasmid DNA
pcDNA: Plasmid cDNA
pcDNA3.1 NT-GFP: Plasmid cDNA/N-Terminal- Green Fluorescent Protein
PLS: Primary Lateral Sclerosis
PMA: Progressive Muscular Atrophy
P S: Penicillin / Streptomycin
PSA: Prostate-Specific Antigen
rhIGF: Recombinant Human Insulin-like Growth Factor
RLB: Reporter Lysis Buffer
RNA: Ribonucleic acid
dsRNA: Double Stranded RNA
mRNA: Messenger RNA
rRNA: Ribosomal RNA
RNAi: RNA Interference
siRNA: Small Interfering RNA
RNase: Ribonuclease
rpm: rotor per minute
RT: Real Time
RT: Reverse Transcription
RT-PCR: Real Time Polymerase Chain Reaction
RT-PCR: Reverse Transcription Polymerase Chain Reaction
SCID: Severe Combined Immunodeficiency
SDS: Sodium Dodecyl Sulphate
SFHR: Small Fragment Homologous Replacement
SMA: Spinal Muscular Atrophy
ss: Single Stranded
SV40: Simian Virus 40
SYBR Green: a fluorogenic minor groove binding dye
Syndecan- (1, 3 or 4): Plasma cell-surface glycoproteins
TA: Tibialis Anterior
TCA: Trichloroacetic acid
TGF: Transforming Growth Factor
TNF: Tumor Necrosis Factors
TRI Reagent: Total RNA Isolation Reagent
UTR: Untranslated Regions
VEGF: Vascular Endothelial Growth Factor
VSG-G: G protein of Vesicular Stomatitis Virus
XR: X- linked recessive
WR: Working Reagent

Chapter 1:

Introduction

1.1 Introduction

1.1 Introduction

The impacts of musculoskeletal / neuromuscular diseases and the conditions, which associate with muscle wasting / weakness / loss, worldwide are enormous. The musculoskeletal / neuromuscular diseases and conditions vary from gradual loss of mobility and independence to severe disability and death; and loss of work hours. The treatment of such diseases and conditions cost in the order of 250 billion US dollars per year (Goldspink and Noble, 2006). All muscular / neuromuscular disorders characterised by muscle wasting and weakness, and they affect human beings (both sexes, males and females) at every stage of life: in their infancy, childhood, adolescence, adulthood, and also in their later years. In addition, during the aging, mammals lose up to a third of their skeletal muscle mass and strength (Barton-Davis et al, 1998). Furthermore, the aged population in the developed world is continuing to expand.

The neuromuscular disorders including muscular dystrophies are not rare. The incidence of neuromuscular disorders in general is 1 in every 1,000 births, (those of muscular dystrophies are almost 1 in every 2000) (<http://www.distrofia-mexico.org/diadm/ingles.htm>). According to this rate, currently, there might be more

than 6 millions patients with neuromuscular disorders worldwide. These problems are seen worldwide, with no country or region being an exception. World Health Organisation and others predict that some of these diseases and conditions will increase dramatically in the next two decades (Goldspink and Noble, 2006). However, now, there is no effective treatment of any form of such disorders and conditions. Therefore, it seems that the treatment for such muscle conditions associated with neuromuscular / musculoskeletal disorders / diseases will be more important in the near future, and it will thus be given high priority. Thus, the aim of this study was to attempt a novel gene therapy application for treating muscle degeneration in such disorders.

On the other hand, it is known that that IGF-I has very important roles in cell and tissue development and growth, including proliferation, differentiation, repair and maintenance, in many tissues, especially in skeletal muscle in many species. Like many genes, the IGF-I gene can be spliced to produce several isoforms, and in human skeletal muscle, it expresses at least two main isoforms which are a liver type, systemic form (IGF-I Ea) and an autocrine / paracrine form (IGF-I Ec / MGF). The recent publications strongly suggest that one of IGF-I isoforms, MGF, has a crucial role in tissue growth and repair, particularly in skeletal muscle in mice and rabbits. Such studies show that MGF acts as a general repair factor. These splicing variants of the IGF-I gene were therefore used in this study as gene therapy agents.

The experiments in this study were designed to two main parts: in vitro cell culture model and in vivo gene transfer method using the mdx mouse model. In in vitro cell

culture experiments, the roles of the isoforms of IGF-I gene in cell proliferation and different were investigated in different mammalian skeletal muscle cells (mouse, rat and human) with and without different neuromuscular disorders such as CMD, FSHD and ALS.

In in vivo animal experiments of the study were separated into two parts after injecting cDNAs constructs of IGF-I splice variants into TA muscles of mdx mouse, which is a mouse model of human Duchenne Muscular Dystrophy (DMD). In the first part, the effects of two IGF-I splice variants in muscle fibre growth were investigated in order to study the roles of such variants in DMD as gene therapy agents.

The damaged or degenerated muscles need extra nuclei of the muscle satellite (stem) cells for regeneration and repair. All muscular / neuromuscular disorders are characterised by muscle wasting and weakness. It is apparent that activation of the quiescent satellite cells and providing extra nuclei is crucial for treating such disorders. The second part of in vivo animal model experiments was therefore designed for investigating the roles of such variants in satellite cell activation. The changes in gene expression and mRNA transcripts of satellite cell markers (MyoD and myogenin) were determined by quantitative real-time R-T PCR.

The in vitro data of this study clearly showed that MGF increased cell proliferation dramatically both in healthy and degenerative muscle cells. The in vivo data showed that MGF increased muscle fibre growth in DMD by increasing muscle force. The study also showed that MGF activated satellite cells. Thrawout this study, in vitro and

in vivo data strongly suggested that MGF might be suitable as a therapeutic agent to treat muscle degeneration in neuromuscular disorders, such as DMD, CMD, FSHD and ALS as well as other wasting conditions. Thus, gene therapy applications using MGF transfer into skeletal muscle could therefore be an effective technique.

1.2 Background Information and Literature Review

1.2.1 Muscle

In nature, motion is one of the basic phenomena of life. In many animals muscle tissue has a crucial role in this motion and is involved in life sustaining processes such as respiration. In human and most animals, muscle accounts for about 40% of the body mass and although there are relatively few diseases of muscle, those, which affect muscle mass and muscle function are usually life threatening (Goldspink and Hansen, 1993, Emery and Muntoni, 2003).

The human body has three types of muscle tissues, which differ in function and form: skeletal muscle, cardiac muscle and smooth muscle. The skeletal muscle cells are long, cylindrical, cross-striated and multinucleate. They are organised into muscles that are responsible for the gross and fine movements of limbs and the maintenance of the body position and posture. The cardiac muscle cells are short, branched, cross-striated and usually with a single nucleus. They are confined to the heart and are rhythmically contractile. The smooth muscle cells are spindle-shaped, non-striated

and with a single, central nucleus. They are mainly found in vascular system, digestive tract and in uterus. (Martini, 2001; Tortora and Grabowski, 2003).

1.2.1.1 The skeletal muscle

1.2.1.1.1 The structure of skeletal muscle

The skeletal muscle tissue is composed of bundles of multinucleated muscle cells, called muscle fibers or myofibres, which are surrounded by a plasma membrane (sarcolemma). They are very different from that of mononucleated cells. They are 10 to 100 μm in diameter and from a few millimetres to several centimetres long. They contain up to several thousand nuclei derived from the fusing of myoblasts in fetal and postnatal life. Each muscle cell (myofibre) is packed with bundles of thick (myosin) and thin (actin) filaments, and organised into myofibrils.

As can be seen from Figure 1.1, myofibrils are subdivided into A bands (dark bands) and I bands (light bands). A bands are bisected by H zone, and M line, a dark line, and I bands are bisected by Z disk or Z line, a different dark line. The segment between two Z disks is termed a sarcomere, and consists of two halves of an I band and an A band. The sarcomere is the structural and functional unit of skeletal muscle. Each sarcomere contains two types of filaments, thick filaments (myosin II) and thin filaments (α -actin). (Lodish et al, 2001).

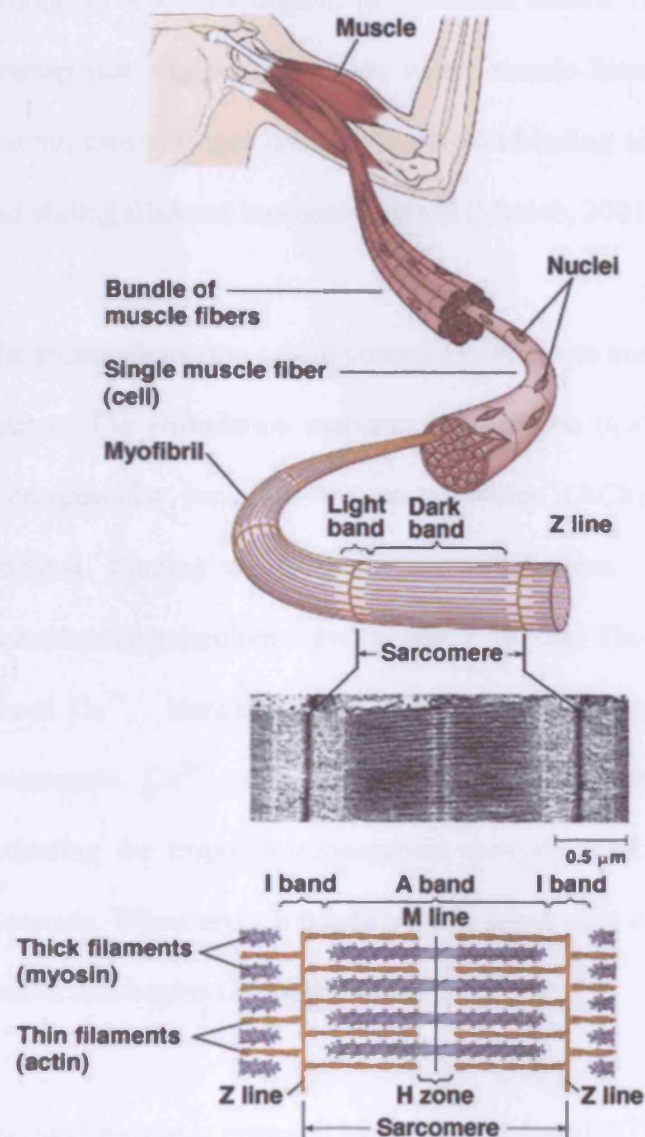


Figure 1.1: Skeletal muscle structure. (Taken from the web page at http://fajercpc.magnet.fsu.edu/Education/2010/Lectures/37_Muscle_System_files/image010.jpg)

1.2.1.1.2 Contraction mechanism of skeletal muscle

Skeletal muscles contract according to the sliding-filament theory, proposed by Hugh Huxley in 1954 (Huxley and Hanson, 1954). According to this theory, the thin (actin) filaments slide on the thick (myosin) filaments so that the actin and myosin filaments

overlap to a greater degree. In a relaxed muscle fiber, the thick and thin filaments overlap just slightly. However, when muscle fibers are stimulated by the nervous system, cross-bridges latch on to myosin binding sites on actin in the thin filaments, and sliding filament mechanism starts (Marieb, 2001).

The stimulations (the action potential) reaches to muscle fibers by the axon of a motor neuron. The stimulation activates calcium ion (Ca^{2+}) channels on the axon. At the neuromuscular junction, the acetylcholine (ACh) was released by the synaptic terminal, binding to receptors on sarcolemma. The action potential reaches to sarcoplasmic reticulum through the T tubule. The sarcoplasmic reticulum releases stored Ca^{2+} , increases Ca^{2+} concentrations of the sarcoplasm in and around the sarcomeres. Ca^{2+} binds to troponin C on the thin filaments of the myofibrils, activating the troponin-tropomyosin complex, and exposes active sites on the thin filaments. When myosin heads bind to active sites myosin cross bridges form and the contraction begins (Martini, 2001).

The binding site is powered by the hydrolysis of ATP. The myosin, which is bound to ATP, binds to the newly binding sites on the thin filaments, and then hydrolyses ATP to release ADP and an inorganic phosphate (Pi), providing energy to deliver a power (working) stroke. The releasing of ADP and Pi causes the myosin head to turn. Myosin is now bound in the strong binding sites. This event produces filament sliding, pulling the Z bands towards each other, and also shortens the sarcomere and the I band, which are shown in Figure 1.1 (Martini, 2001).

When the action potential ceases Ach is removed by acetylcholinesterase (AChE). The sarcoplasmic reticulum reabsorbs Ca^{2+} , and the concentrations of Ca^{2+} in the sarcoplasm decreases. When Ca^{2+} concentration comes to normal resting levels, the troponin-tropomyosin complex returns to its normal position. This event re-covers the active sites and prevents further cross-bridge interaction. Without cross-bridge interactions, no more sliding can occur, and then the contraction ceases. Finally, muscle relaxation takes place, and muscle returns passively to resting length position (Martini, 2001).

1.2.1.1.3 Embryologic origin of muscle tissue

Muscle is a derivation of mesodermal cells. All vertebrate skeletal muscles (apart from head and neck muscles) are derived from mesodermal precursor cells originating from the embryonic somites (epithelial spheres of paraxial mesoderm) (Buckingham, 2001; Buckingham et al, 2003; Parker et al, 2003; Zhao and Hoffman, 2004; Chargé and Rudnicki, 2004). The limb muscles, which are among the cells used in in vitro part of this study, arise from myogenic precursors located in the somites (lateral half / dorsal part of the somites), particularly leg muscles originate from somites 26-33 (Valasek et al, 2005). The limb muscles are also innervated by spinal nerves (Yu et al, 2002). On the other hand, the muscles of the head and cervical region, including cranial, neck and face muscles, are originated in the somitomeres, which are from the branchial arch region of the early embryo (Larsen, 1998; Carlson, 1999; Buckingham et al, 2003). For instance, the craniofacial masseter muscles, which again are amongst the cells used in this study, are derived from the sixth cranial somitomere and innervated by cranial nerves (Noden et al, 1999; Buckingham, 2001;

Yu et al, 2002). In brief, while the somites are differentiated into lower body muscles, the somitomeres are differentiated into the head and neck muscles.

1.2.1.1.4 The differences among skeletal muscles

Along with the differences of the embryologic origins, there are also some anatomical, physiological, biochemical, functional, compositional differences between muscles, muscle groups and muscle regions. A big diversity in fiber-types, and motor tasks among such muscles / groups / regions is seen (Korfage et al, 2005a; Korfage et al, 2005b). Large differences in fibre-type composition were observed among individuals, individual muscles, muscle groups and muscle regions (e.g., jaw muscles and limb muscles). For instance, the jaw muscles are quite active during a large variety of motor task, including mastication, biting, speech, and swallowing. (Korfage et al 2000; Korfage et al, 2005a; Korfage et al, 2005b).

1.2.1.1.5 The Myogenesis of Skeletal Muscle

The mature skeletal muscle fiber is a complex multinucleated cell. Up to this stage, the mammalian skeletal myogenesis proceeds through three stages: (a) the determination of muscle progenitor cells, (b) proliferation, and then (c) differentiation into mature muscle.

Initially, somites, which are collections of embryonic mesodermal cells, transform into myoblasts. These myogenic progenitor cells undergo mitotic divisions (proliferation), become postmitotic myoblasts due to the action of a set of transcription factors such as myogenin and MyoD (Davis et al, 1987; Goldspink and

Hansen, 1993; Hawke and Garry, 2001; Dhawan and Rando, 2005) and growth factors such as fibroblast growth factor (FGF) and transforming growth factor (TGF) (Carlson, 1999; Carlson, 2003; Dhawan and Rando, 2005). Further details about other transcription factors and growth factors, which have roles in myogenesis, can be found in other related sections of this thesis.

Postmitotic myoblasts differentiate into multinucleated myotubes by cellular fusion. The myotubes contain many nuclei but share a common cytoplasm. Subsequent to the fusion into myotubes, there is no further mitotic division of the muscle cells. The myotubes then develop into myofibres after innervation and attachment to the skeleton. This is the final stage of skeletal muscle differentiation.

In the course of adult muscle fiber growth, the extra nuclei are derived from satellite cells. There are residual mononucleated myoblasts, which take up positions between the muscle fibers and basal lamina. The myoblasts fuse to multinucleated myotubes and then to the muscle fibers.

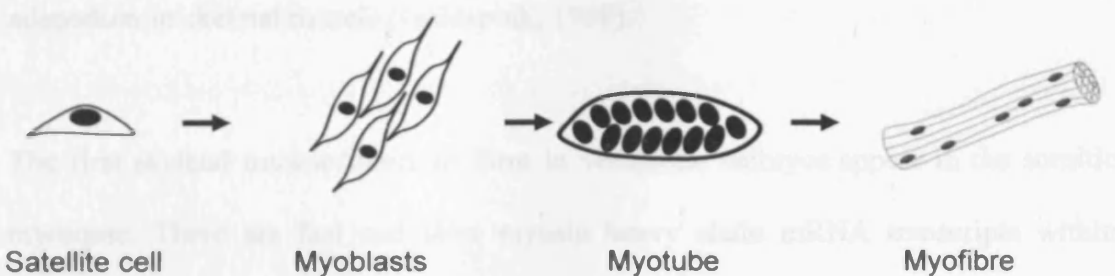


Figure 1.2: Myogenesis of Skeletal Muscle

1.2.1.1.6 Gene Expression in the skeletal muscle

Different gene families are involved in skeletal muscle development and functions: these include the myogenic regulatory factors (MRF), growth factors and the structural genes, e.g.: myosin heavy chain genes (MHC) and myogenic genes. For instance, MRFs initiates the terminal myogenic differentiation program by upregulating the genes such as those encoding myosin heavy chain, creatine kinase and sarcomeric actin proteins (Dhawan and Rando, 2005).

MHC isoforms expressed in skeletal muscles are encoded by a multigene family and are expressed in different developmental stages and fiber types. In humans and mice, skeletal myosin heavy chain (MHC) genes are clustered on chromosome 17p and 11 (Weiss et al, 1999).

The different molecular motors are encoded by different myosin heavy chain isogenes. These isogenes comprise a family of separate genes. The regulation and switching of different myosin heavy chain isogenes play a role during growth and adaptation in skeletal muscle (Goldspink, 1998).

The first skeletal muscle fibers to form in vertebrate embryos appear in the somitic myotome. There are fast and slow myosin heavy chain mRNA transcripts within myotomal fibers. Firstly, the embryonic fast myosin heavy chain isoform is expressed, and then the slow myosin heavy chains 1 and 3, and slow myosin heavy chain 2 (Sacks et al, 2003). The myogenic regulator factor genes play an important part in muscle development. One of these is MyoD. The MyoD gene plays a key role in

muscle development. Other genes such as Myf5, myogenin and Mrf4 are also involved in the development (Lodish et al, 2001).

1.2.1.2 Satellite cells

Investigations of adult mammalian organs have revealed tissue specific progenitor cells in almost every tissue (Sun et al, 2003). These progenitor cells are involved in normal tissue turnover and repair while some others undergo self-renewal. They are mostly multipotent progenitor cells, that is, cells capable of different lineages under certain conditions (Lakshmipathy and Verfaillie, 2005). Therefore, most of them can also be considered as stem cells (Weismann, 2000). In muscle tissue, such stem cells are the satellite cells (Partridge, 2004); in other words, the satellite cells are the primary stem cells in adult skeletal muscle (Collins and Partridge, 2005). The satellite cells provide a reserve capacity to add to or to replace the nuclei in differentiated muscle fibres.

The satellite cells are characterized by their location, lineage markers such as Pax-7, myogenin, MyoD and others, myogenic potential and ability to change in response to extrinsic and intrinsic signals including exercise, muscle damage, increased workload, electrical stimuli and degenerative muscle diseases (Dhawan and Rando, 2005; Goldspink, 2005).

Satellite cells have long been considered a distinct myogenic lineage responsible for postnatal growth, hypertrophy, regeneration, repair, and maintenance of the skeletal muscle (Dhawan and Rando, 2005; Seale et al, 2000). The skeletal muscle satellite

cells were first described in frog muscles in 1961 (Mauro, 1961; Katz, 1961) and then identified in adult avian and mammalian muscle (Schultz, 1976; Armand et al, 1983). They are thought to be the main source of new myonuclei in postnatal muscle (Moss and Leblond, 1971).

The numbers of satellite cells are very low in vivo (Dhawan and Rando, 2005). In the first few weeks after birth, the number of satellite cells in a rodent declines from about 30% to less than 5%. However, in adults, the number remains almost constant although the satellite cells numbers per fiber differ in fast-twitch and slow-twitch fibres (Schultz and McCormick, 1994).

For a while now, a debate about the reason of the low number of satellite cells has been going on. The debate concerns the question whether there is a considerable decline in satellite cell numbers with age, or a functional decline in their activation rather than a true loss of such cell number until very old age (Conboy et al, 2003; Dhawan and Rando, 2005).

1.2.1.2.1 The molecular mechanism of satellite cell quiescence, activation and self-renewal / replenishment

Every myofibre is associated with a number of satellite cells. In normal (healthy) adult muscle, satellite cells are mitotically quiescent, and become activated to divide in response to signals from exercise, muscle damage and/or increased workload and degenerative muscle diseases. Subsequent to the division, activated satellite cells become fusion competent myoblasts, they then differentiate into multinucleated

myotubes (Dhawan and Rando, 2005), and finally incorporate into mature muscle fibres as post-mitotic myonuclei (Bischoff, 1994).

1.2.1.2.2 Satellite cell quiescence

Some evidence suggests that the satellite cell quiescent state is under active transcriptional control (Yusuf and Fruman, 2003). The quiescent satellite cells express various proteins, which are also used for the identification and purification of such cells. Some of these markers are CD34 (Beauchamp et al, 2000), M-cadherin (Beauchamp et al, 2000), Pax-7 (Seale et al, 2000), syndecan-3, syndecan-4 and c-met (Cornelison et al, 2001). Myostatin is also expressed in quiescent satellite cells and McCroskery et al suggest that myostatin might actively promote the quiescence (McCroskery et al, 2003). Epigenetic mechanism and cytoskeleton might also play a role in the satellite cell quiescence (Dhawan and Rando, 2005). Grigoryev et al suggest that epigenetic mechanism induce and maintain quiescence in other lineages (Grigoryev et al, 2004; Dhawan and Rando, 2005). Grigoryev et al showed that major changes in epigenetic chromatin markers in centomeric heterochromatin and non-centomeric chromatin go with the reactivation of quiescent T-lymphocytes from mouse spleen. They examined the levels and intranuclear localization of major histone modifications and non-histone heterochromatin proteins in quiescent and reactivated mouse spleen lymphocytes. According to authors, reciprocal changes in the locations of these two proteins were observed in activated lymphocytes and cultured mouse fibroblasts induced into quiescence (Grigoryev et al, 2004). On the other hand, Dhawan and Helfman suggest that the cytoskeleton is a key regulator of proliferation: perturbation of microfilament-dependent signalling system in culture leads to

reversible quiescence (Dhawan and Helfman, 2004, Dhawan and Rando, 2005). Dhawan and Rando also suggest that the core program of quiescence is probably conserved, and might be important in the maintenance of satellite cell quiescence (Dhawan and Rando, 2005).

1.2.1.2.3 Satellite cell activation

The satellite cell activation is a multi-step process. The localisation of the satellite cells and the communication between myofibres and satellite cells are very important for the maintenance of quiescence. Therefore, any disturbance of the satellite cell environment can lead to its activation (Dhawan and Rando, 2005). When quiescent satellite cells receive an activating signal, they exit from the quiescent state and go to the transition from G_0 to G_1 phase of the cell cycle. After entering into their first cycle, they proceed through a highly proliferative intermediate progenitor stage and thus become intermediate progenitors. The intermediate progenitor stage is strongly regulated by the Notch signalling pathway (Conboy and Rando, 2002). These progenitors can be characterized by declining CD34 and high levels of Pax3 and Pax7. The progenitors then exit from this progenitor stage and progress to the fusion – competent myoblast stage and express Desmin (Dhawan and Rando, 2005). The myoblasts then progress to the fusion for myogenic differentiation stage, and become multinucleated myotubes. Finally myotubes differentiate into mature muscle fibres.

To sum up, the following factors and pathways have been identified as playing significant roles in satellite cell activation: signal transduction pathways, including expressing of c-met, syndecan-3, syndecan-4 and CXCR4; hepatocyte growth factor

(HGF) – c-met pathway; fibroblast growth factor (FGF) family pathway; and also, as mentioned above, the Notch signalling pathway (Dhawan and Rando, 2005).

1.2.1.2.4 Satellite cell replenishment and return to quiescence

Some intermediate progenitors do not progress into myoblast stage, and myogenic lineage. They remain in this progenitor stage until they return to quiescence as renewed satellite cells. Although the molecular mechanism of satellite cell replenishment remains unclear, there are some scenarios, such as the Numb scenario. The Numb is a Notch signalling inhibitor. For the Numb scenario, the daughter that inherits Numb, the signalling inhibitor, progresses along myogenic lineage and become myoblast; on the other hand, a daughter that does not inherit Numb returns to quiescence (Dhawan and Rando, 2005).

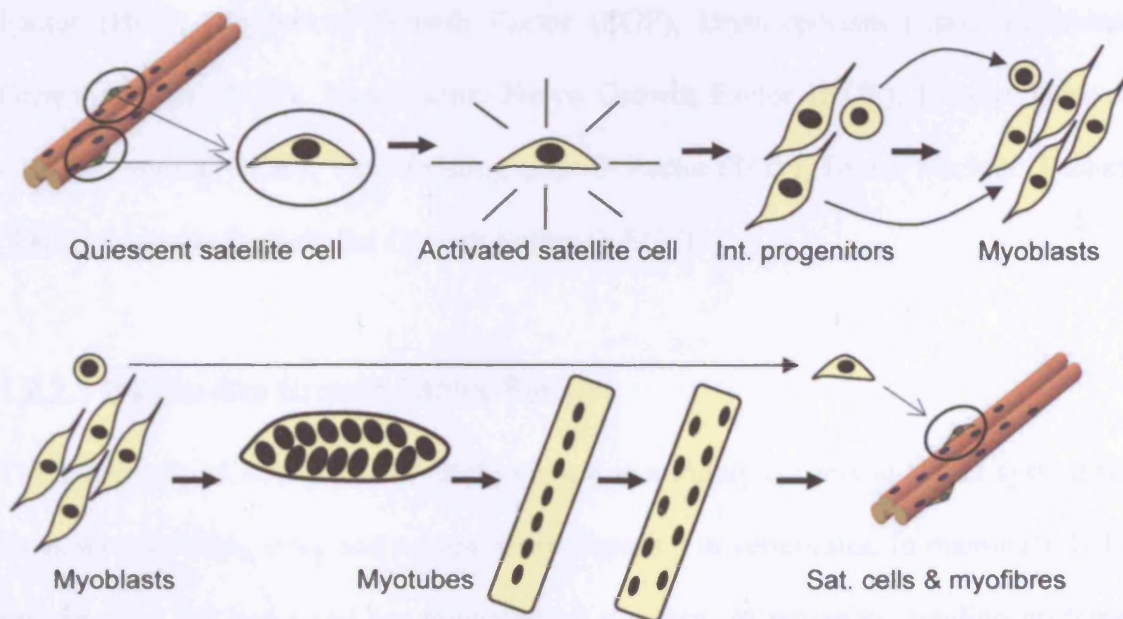


Figure 1.3: Satellite cell quiescence, activation, proliferation, differentiation and selfrenewal.

1.2.2 Growth Factors

Growth factors (GFs) are one of the major regulators of postnatal body growth. Some growth factors and hormones, such as Insulin-like Growth Factors (IGFs), Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF), and Thyroid Hormone, are involved in muscle cell proliferation and/or differentiation (Goldspink et al, 1993). Growth Factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation, and are essential to growth and repair. Some growth factors are quite versatile, stimulating cellular division in numerous different cell types, while others are specific to a particular cell-type depending upon the dose.

There is a range of these growth factors in serum, including Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), Erythropoietin (Epo), Fibroblast Growth Factor (FGF), Interleukins, Nerve Growth Factor (NGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor (TGF), Tumor Necrosis Factors (TNF), Vascular Endothelial Growth Factor (VEGF).

1.2.2.1 Insulin-like Growth Factor System

The Insulin-like Growth Factor (IGF) system is a highly conserved signal system for the control of embryonic and postnatal development in vertebrates. In mammals, IGFs act throughout the body and are regulated by a system of receptors, binding proteins, proteases and binding protein related proteins. The IGF system is a large family. It has two ligands (IGF-I and IGF-II), two high - affinity cell surface receptors (IGF1R and IGF2R), at least six high – affinity IGF binding proteins (IGFBP-1, IGFBP-2, IGFBP-

3, IGFBP-4, IGFBP-5, IGFBP-6), at least nine low – affinity binding protein related proteins (IGFBP – rP1 to IGFBP – rP9) and multiple binding protein proteases. Figure 1.4 shows the components of IGF system.

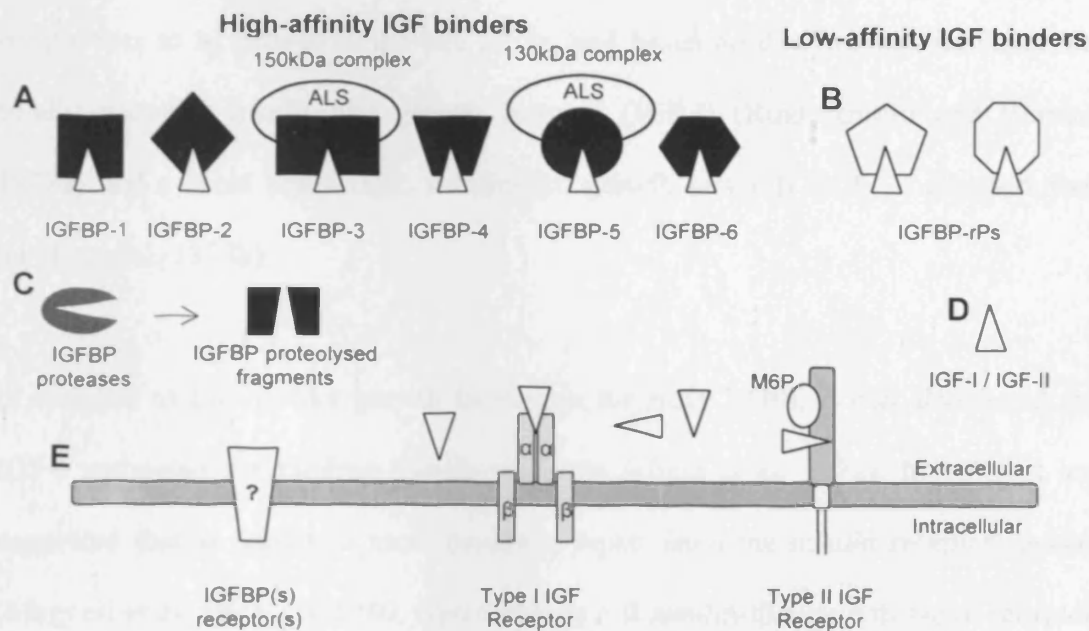


Figure 1.4: IGF System. Abbr: IGF-I and IGF-II: IGF I and II; IGFBP: IGF binding protein, IGFBP-rP: IGF binding protein related protein and M6P: Mannose - 6 - Phosphate. α : α subunit of IGF-IR; β : β subunit of IGF-IR. A: IGF binding proteins, B: IGFBP related proteins, C: Proteases, D: IGF-I and/or IGF-II, E: IGF receptors (Adapted from Hwa et al, 1999).

1.2.2.1.1 The historical background of IGF system

The Insulin-like Growth Factors (IGF) were first identified in 1957, when W. Salmon and W. Daughaday reported a serum factor(s) that mediated cartilage sulfation and the bone growth activity of growth hormone (GH). This factor was therefore termed the "sulfation factor" and was produced by hepatic cells after exposure to GH (Salmon and Daughaday, 1957). Nearly a decade later, in 1966, the factors were renamed as nonsuppressible insulin-like activity I / II (NSILA I/II)- (Froesch et al, 1966). Then, in

the early 1970s, Dulak and Temin renamed them as multiplication-stimulating activity (MSA) (Dulak and Temin, 1973). During this period, Daughaday renamed them again as somatomedins (Daughaday, et al, 1972). In the 1970's, it was discovered that both factors, NSILA and Somatomedin, might be very similar to insulin. Similarity to insulin was to be proven in the late 1970s, and based on this finding, the factor was finally renamed insulin-like growth factor I (IGF-I) (Rinderknecht and Humbel, 1978a) and a short while later, insulin-like growth factor II (IGF-II) (Rinderknecht and Humbel, 1978b).

In addition to Insulin-like growth factors, in the early 1970s, it was also found that IGF-I competed for binding insulin receptors (Hintz et al, 1972). In 1975, it was suggested that at least two more receptors (apart from the insulin receptor) existed (Megyesi et al, 1975). In 1980, type I and type II insulin-like growth factor receptors (IGF-IR and IGF-IIR) were discovered (Rechler et al, 1980), and then the molecular sizes of all three receptors (IR, IGFIR and IGFIIR) were determined in the early 1980s (Massague and Czech, 1982). More recently, the binding proteins of IGFs (IGFBPs) were found (Jones and Clemmons, 1995).

1.2.2.1.2 IGF Receptors

IGF-I and IGF-II ligands interact with various cell surface receptors that particularly recognise and bind the IGFs. There are two known receptors in IGF system: IGF-I receptor -IGF-IR- (also known type 1 IGF receptor), and IGF-II receptor -IGF-IIR- (also known type 2 IGF receptor or cation – independent mannose-6-phosphate receptor -M-6-PR-). IGF-IR has IGF mediated signalling functions, whereas IGF-IIR

has currently no known IGF signalling function (Jones et al, 1995). Each receptor is the product of a unique gene (Jones and Clemmons, 1995).

1.2.2.1.2.1 Type 1 IGF receptor (IGF-IR)

IGF-IR binds both IGF-I and IGF-II with high affinity. This receptor has been identified in essentially all tissues except liver, and virtually all of the biological activities of the IGFs result from binding to the type 1 receptor. IGF-IR (and also IR) is a transmembrane tyrosine kinase receptor. It has two extracellular α subunits and two membrane spanning β subunits which form a β - α - α - β arrangement (Figure 1.5). As can be seen from Figure 1.5, the major ligand binding determinants are located within the α subunits and the intrinsic tyrosine kinase domain is located in the cytoplasmic part of the β subunits (Denley et al, 2005, Adams et al, 2000).

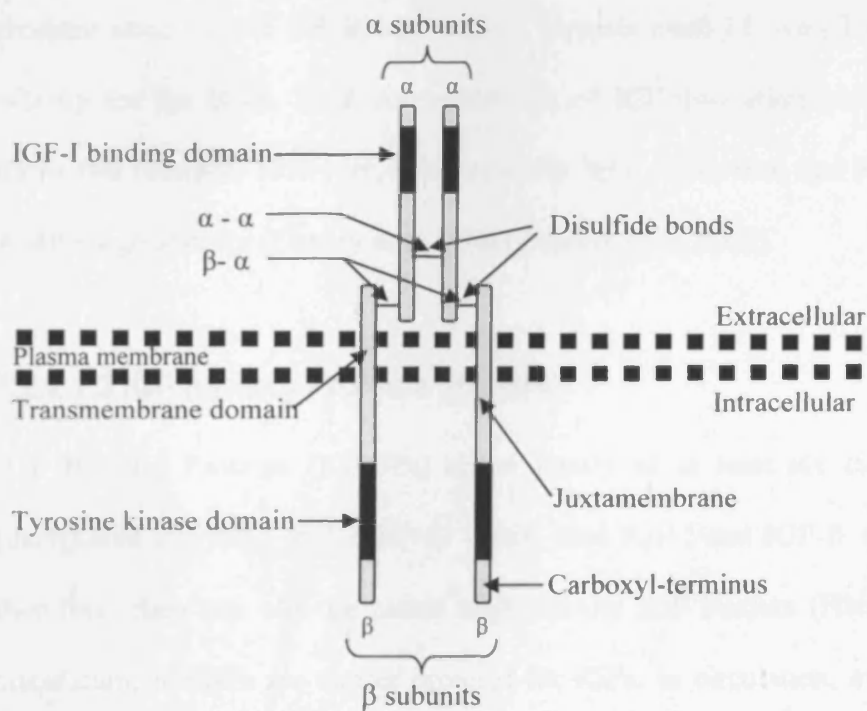


Figure 1.5: IGF-I receptor

1.2.2.1.2.2 Type 2 IGF receptor (IGF-IIR)

IGF-IIR binds IGF-II with high affinity but binds IGF-I with very low affinity and does not bind insulin (Denley et al, 2005). IGF-IIR has no intrinsic signalling transduction capability. It appears primarily to be involved in clearance and degradation of IGF-II. This is also the cation-independent mannose-6-phosphate receptor used for targeting mannosylated enzymes to lysosomes (Denley et al, 2005).

1.2.2.1.2.3 Insulin (IR) and hybrid (IGF-IR/IR) receptors:

The insulin receptors –IR- (IR-A and IR-B) and hybrid IGF and insulin receptors (IGF-IR/IR) can also interact with IGFs, but insulin receptors (IRs) bind IGF-I with 100-fold lower affinity than insulin. The insulin receptor has two isoforms: insulin receptor exon 11 +ve (IR-B) and insulin receptor exon 11 –ve (IR-A). IR-B has low affinity for the IGFs. High concentrations of IGF may stimulate insulin signalling through this receptor. However, it has recently been discovered that IGF-II binds to IR-A with high affinity (Denley et al, 2005; Pandini et al, 2002).

1.2.2.1.3 IGF Binding Proteins (IGFBPs)

IGF Binding Proteins (IGFBPs) are a family of at least six circulating proteins (designated IGFBP-1 to IGFBP-6) which bind IGF-I and IGF-II with high affinity, therefore, they can also be called high affinity IGF binders (Hwa et al, 1999). In circulation, IGFBPs are carrier proteins for IGFs. In circulation, over 99 % of IGFs are in complex with a member of the IGFBP family, particularly with IGFBP-3; and less than 1% of IGFs circulate in an unassociated form (Fürstenberger and Senn,

2002). In plasma, more than 90 % of circulating IGFs are bound to IGFBP-3 and IGFBP-3 / ALS complex (Fürstenberger and Senn, 2002).

IGFBPs control distribution, function and activity of the IGFs in various cells, tissues and body fluids by binding them. For high affinity IGF binding, both N- and C-terminal regions of IGFBPs are required; whereas in bindings IGFs with low affinity, they require just one of them, either N- or C- terminals. IGFBP-3 and IGFBP-5 can form a ternary complex with IGFs and acid labile subunit (ALS) (Denley et al, 2005, Hwa et al. 1999).

IGFBPs coordinate and regulate the biologic activity of IGFs in several ways, such as,

- a) To transport IGFs in plasma to deliver them to tissues; and to control their diffusions and effluxes from the vascular space; and therefore they determine physiological concentrations of IGFs;
- b) To increase the half life of IGFs in circulation (less than 10 minutes to 12-15 hours) and to regulate clearance of the IGFs;
- c) To modulate, inhibit, potentiate or facilitate actions of IGFs and interactions of IGFs with their receptors;
- d) To provide specific binding sites for the IGFs in extracellular and pericellular space (Russo et al, 2005; Jones and Clemmons, 1995).

IGFBPs functions are site-specific. Until recently, it was thought that they act just by the sequestration of the IGF peptides, by preventing receptor interactions. However, there is increasing evidence that IGFBPs, such as IGFBP-3 and IGFBP-5, have also

independent functions, even in intracellular actions (Davies et al, 2006; Duan and Xu, 2005; Clemmons, 1998).

The mature peptides of IGFBPs are between 216 and 289 amino acids, and 24 and 43-45 kDa. Their concentrations are various. The concentrations of IGFBP-2, 4, 5 and 6 in serum are around 2-15 nM. But, that of IGFBP-3 in serum is quite high (about 100 nM) and that of IGFBP-1 in serum is very low (relatively constant).

Although the liver is the major source of circulating IGFs, IGFBPs and ALS, most cell types produce one or more members of the IGFBP family. Hepatocytes synthesize IGFBP-1, IGFBP-2, IGFBP-4 and ALS; and hepatic Kupffer cells synthesize IGFBP-2 and IGFBP-3. Again, IGFBP-3, IGFBP-4 and IGFBP-6 are expressed in vascular smooth muscle cells, while IGFBP-4 and IGFBP-5 are produced by a wide range of osteoblast cells.

1.2.2.1.4 IGFBP related Proteins (IGFBP-rPs)

There are also 9 other binding proteins (designated IGFBP-rP-1 to IGFBP-rP-9) which bind IGFs with low affinity because they bind IGFs by just their N- terminal. The biologic activities of the IGFBP-rPs are currently unclear.

Although there are six binding proteins, some scientists have been suggesting seven binding proteins, including IGFBP-7. IGFBP-7 has been also called Mac25. Today, it is quite clear that IGFBP-7 is one of the IGFBP related proteins (IGFBP-rP1), and not that of IGFBPs.

The IGFBP-rP1 (previously termed as Mac25 and IGFBP-7) has been independently cloned in various cellular systems, and thus has been previously identified as IGFBP-7 (Haugk et al, 2000; Oh et al, 1996), Mac25 (Swisshelm et al, 1995; Murphy et al, 1993) and is a tumour-derived adhesion factor (Akaogi et al, 1996), and also a prostacyclin-stimulation factor (Yamauchi et al, 1994).

1.2.2.1.4 IGFBP Specific Proteases

In IGF system, there are also a range of IGFBP specific proteases including prostate-specific antigen (PSA), matrix metalloproteases, cathepsin, thrombin and serine proteases. Today, there is increasing evidence that IGFBP specific proteases are involved in the regulation of IGFBP functions and turnover. IGFBPs are cleaved at specific sites by those proteases. Following limited proteolysis, IGFBPs exhibit a reduced affinity for IGFs and some IGFBP fragments appear to have IGF independent activity (Baxter, 2000).

1.2.2.1.5 IGF Ligands

The IGF system has two ligands: IGF-I and IGF-II (Figure 1.6). Those two Insulin-like Growth Factors, IGF-I and IGF-II, are both growth-promoting peptides. IGF-I and IGF-II, are single chain polypeptides, respectively 70 and 67 amino acids in length, with a highly conserved amino acid sequence, and complicated and conserved gene structures (Woelfle et al, 2005; Stewart and Rotwein, 1996; Daughaday and Rotwein, 1989; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). The mature peptides of IGF-I and IGF-II (70 and 67 amino acids) are very similar

among mammals, birds and amphibians (Russo et al, 2005; Perfetti et al, 1994; Koval et al, 1994; Kajimoto and Rotwein, 1991; Chan et al, 1990; Shimatsu and Rotwein, 1987; Zapf and Froesch, 1986). Each of these IGFs has a number of variant forms, resulting from the use of alternative gene promoters and alternative splicing. IGFs are structurally related to insulin and are in a class of peptides that have a structure similar to proinsulin (insulin-like effects). The mature IGFs consist of B, C, A and D domains. Structurally, both IGFs resemble insulin in having two chains connected by disulfide bonds. Large parts of the sequences within the A and B domains are homologous to the A (α) and B (β) chain of human proinsulin. This sequence homology with insulin is 43 % for IGF-I and 41 % for IGF-II (Duan, 2005). Although IGFs and insulin are closely related in primary sequences and biologic activity, the IGFs are main growth factors for nearly all tissues in the body, whereas insulin predominantly regulates glucose uptake and cellular metabolism.

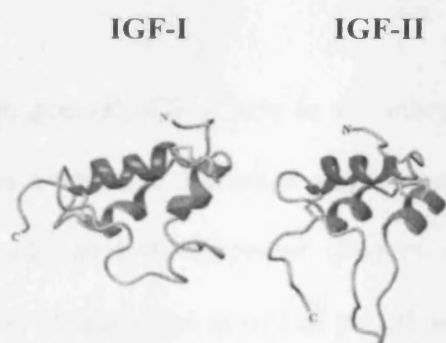


Figure 1.6: IGF ligands, IGF-I and IGF-II.

IGF-I is one of the main growth factors that stimulates protein synthesis in almost all tissues including muscle tissue. It is also one of the main regulators of tissue mass during early life (McKoy et al, 1999; Daughaday et al, 1972). IGF-I is the primary

mediator involved in the response of cells to growth hormone (GH). IGF-I plays a critical role in normal body growth. It induces cellular activities, including cell growth, differentiation, migration, and mitosis (Stewart and Rotwein, 1996); and has autocrine and paracrine activities in addition to the endocrine activities.

1.2.2.1.5.1 IGF-II

Human IGF-II gene has been localised on the short arm of chromosome 11 (11p15). IGF-II consists of 67 amino acids residue with a molecular mass of 7.47 kDa. It is also a single chain polypeptide. The amino acid sequence of IGF-II molecule is 62 % identical to IGF-I (Dupont and Holzenberger, 2003; Fürstenberger and Senn, 2002; Rinderknecht and Humbel, 1978b). The IGF-II gene has 10 exons, including 4 leader exons, 1 alternative exon and 3 common (mature) protein-coding exons. The IGF-II gene has also 4 promoters (p1-4) and these precede the common coding exons 7, 8 and 9 exons (Rinderknecht and Humbel, 1978b).

In general, IGF-II acts as an embryonic and foetal growth factor, whereas IGF-I acts as a postnatal and adult growth factor. IGF-II plays a fundamental role in embryonic and foetal development (Davies et al, 2006). Although, in humans, IGF-II also expresses in the postnatal period and is produced in various tissues thrawout life, its role in the postnatal period seems to be less important. The serum concentration of IGF-II is considerably higher than IGF-I (IGF-II: 400-600 ng/ml, IGF-I: 100-200 ng/ml) (Dupont and Holzenberger, 2003).

1.2.2.1.5.2 IGF-I

1.2.2.1.5.2.1 The Biochemical Structure and Expression of IGF-I gene

The human IGF-I gene has been localised on the long arm of chromosome 12 (12q22-12q24) (Brissenden et al, 1984) and human IGF-I cDNA was first characterised by Jansen et al. in 1983 (Jansen et al, 1983). Although human mature IGF-I peptide is a 70 amino acid single-chained polypeptide and its size is just 7.65 kDa, its gene has over 100 kb of genomic DNA (Tricoli et al, 1984). In humans, the expression of the IGF-I gene leads to the production of three different sizes of mRNA at 1.1, 1.3 and 7.6 kilo bases (Rotwein, 1984). As it was mentioned above, the IGF-I is organised into four peptide domains: B, C, A, and D.

As can be seen from Figure 1.7, the IGF-I gene has 6 exons, four of which are alternatively spliced (Smith et al, 2002). Although they do not alter the structure of the mature peptide, the alternative splices generate different precursor peptides. Different tissues produce IGF-I for autocrine and paracrine actions (Stewart and Rotwein, 1996). Exons 1 and 2 are promoter exons derived from different transcription start sites. Exons 5 and 6 are subject to a complex alternative splicing pattern. Exon 4 usually splices to exon 6 but sometimes it splices to exon 5, representing 10% of IGF-I transcripts. A minor isoform results from splicing of exons 4 and 5, and from an alternative 5' splice site in exon 5 to exon 6. (Smith et al, 2002)



Figure 1.7: IGF-I gene.

IGF-I is synthesised in the liver systemically. In addition, both IGF-I and IGF-II are expressed in many non-hepatic tissues. Particularly during exercise, the major source of circulating IGF-I is skeletal muscle (Goldspink and Yang, 2004). Thus, IGFs are expressed both systemically and locally and therefore, they have endocrine and autocrine / paracrine actions (LeRoith et al, 2001).

IGF-I expression is affected at many levels including gene transcription, splicing, translation and secretion. IGF-I expression is also affected by hormonal (GH), (Bichell et al, 1992; Meton et al, 1999), nutritional (Thissen et al, 1994), tissue-specific and developmental factors (Russo et al, 2005; Dupont and Holzenberger, 2003; Sara and Carlsson-Skwirut, 1994). The IGF-I has multiple isoforms that have specific functions and at least six main isoforms expressed by human muscle: Classes I and II IGF-I Ea, and IGF-I Eb and IGF-I Ec (MGF).

1.2.2.1.5.2.2 The Biological Actions of IGF-I

Insulin-like growth factor - I is an anabolic and mitogenic peptide. For a long time, a substantial amount of research showed that IGF-I plays a key role in growth and development (Jones and Clemmons, 1995; Stewart and Rotwein 1996; Dupont and Holzenberger, 2003), and has tissue - specific multifunction, involving several cellular processes including tissue proliferation (Jones and Clemmons, 1995; Stewart and Rotwein 1996), differentiation (Florini and Magri, 1989; Jones and Clemmons, 1995; Stewart and Rotwein 1996), maintenance (Yang et al, 1997) and repair (Adams, 1998; Goldspink and Yang, 2004) in almost all tissues including muscle, brain, nerve, bone, mammary glands, cartilage, pancreas, ovary, skin and fat tissues (Jones and

Clemmons, 1995; Stewart and Rotwein, 1996; Dupont and Holzenberger, 2003); and has autocrine and paracrine activities in addition to the endocrine activities.

IGF-I is not just produced by different tissues, but it also has different functions in different organ systems of the body, including the muscular, the digestive and gastrointestinal, the reproductive, the cardiovascular, and also peripheral and central nervous systems. IGF-I has some anabolic effects such as the stimulation of the synthesis of glycogen, lipids and proteins. It also increases glucose metabolism in adipose tissue and inhibits lipolysis (Reece et al, 1994).

It is currently quite clear that IGF-I and some other IGF system components have also key roles in the cell cycle (Furlanetto et al, 1994), aging (Dupont and Holzenberger, 2003; Goodman-Gruen and Connor, 1997), lifespan (Dupont and Holzenberger, 2003), migration, mitosis (Stewart et al, 1996), apoptosis (Fürstenberger and Senn, 2002; Baxter, 2001) and cancer development (Davies et al, 2006; Fürstenberger and Senn, 2002).

The mitogenic effects of IGF-I arise as a result of increased DNA synthesis. IGF-I upregulates the expression of cyclin D1 (G₁ cyclin), cyclin B1 (G₂ cyclin) and cyclin-dependent kinases such as cdc2 and cdck2 (Furlanetto et al, 1994).

Serum IGF-I levels decrease with age (Goodman-Gruen and Connor, 1997). In postnatal and childhood period, until puberty, IGF-I levels increase slowly. During puberty, the increase dramatically rises and after puberty, the levels of IGF-I begins to

decrease slowly until old age. In elderly men and women, this level is very low (Goodman-Gruen and Connor, 1997).

Several studies have also suggested that the insulin / IGF signalling pathway may control body size and longevity in invertebrates (Gems and Partridge, 2001), and the growth hormone/IGF-I signalling axis may perform the same function in mammals (Bonkowski et al, 2006). Because, reduced calorie restriction extends longevity in organisms ranging from yeast to mammals. Mutations affecting somatotropic, insulin, or homologous signaling pathways can increase life span in worms, flies, and mice, and there is considerable evidence that reduced secretion of insulin-like growth factor I and insulin are among the mechanisms that mediate the effects of calorie restriction on aging and longevity in mammals. (Bonkowski et al, 2006). Those studies have shown that insulin/IGF signalling is a key regulator of lifespan in both nematodes and flies (Dupont and Holzenberger, 2003).

1.2.2.1.5.2.3 IGF system and Cancer

For a while, numerous studies have shown that there is a link between serum concentrations of IGF-I and different IGFbps, particularly IGFbp-3, (with an increased risk of breast, prostate, colorectal and lung cancers). Many studies have shown that high levels of IGF-I and low levels of IGFbp-3 predict increased cancer risk (Fürstenberger and Senn, 2002). The action of IGF-I is predominantly mediated through the type I IGF receptor (IGF1R). IGF1R is also involved in several oncogenic transformation processes (Davies et al, 2006; Fürstenberger and Senn, 2002).

1.2.2.1.5.2.4 IGF system and Apoptosis

The IGFs also have a cellular action to inhibit cell death in certain situations (Haunstetter and Izumo, 1998). This action has been shown particularly in haematopoietic cells (Williams et al, 1990; Jones and Clemmons, 1995). An increasing number of studies have shown the anti-apoptotic effect of IGF-I and IGF system. In brief, IGF-I inhibits apoptosis by increased expression of the bcl-xL (Parrizas and LeRoith, 1997).

1.2.2.1.5.2.5 IGF-I gene and its alternative splicing

1.2.2.1.5.2.5.1 Alternative Splicing

After the discovery of exons and introns in the Adenovirus hexon gene in 1977 (Sambrook, 1977), it was proposed that different combination of exons could be spliced together to produce different mRNA isoforms of a gene. This was therefore termed as alternative splicing (Gilbert, 1978).

In the beginning of the 1980s, alternative splicing was shown in several genes, and in the 1990s, it was estimated that 5% of genes might have alternative splicing (Sharp, 1994). Since then, the estimates of the percentage of alternative splicing in human genes have increased dramatically. In recent years, different research groups estimated the percentage of alternative splicing in human genes as follows: 22 % (Croft et al, 2000), 35 % (Miranov et al, 1999), 38 % (Brett et al, 2000), 42 % (Modrek et al, 2001), 55 % (Kan et al, 2001), at least 59 % (Int Hum Gen Seq Co. 2001), up to 60% (Modrek and Lee, 2002), more than 75 % (Lynch, 2004; Johnson et al, 2003) and finally, 80 % (Lee and Wang, 2005).

Although some genes have limited numbers of alternative splicing, some genes have a huge number of alternative splicing. For instance, alternative splicing of dystrophin gene generates at least 16 different alternative transcripts (Sironi et al, 2002). On the other hand, the combinatorial alternative splicing of different exons of the neurexin gene generate up to 40,000 different isoforms (Modrek and Lee, 2002; Graveley, 2001). At present, current studies indicate that primary transcripts from more than 80 % of all human genes undergo alternative splicing, generating a great number of protein isoforms (Lee and Wang, 2005). As can be seen below, similar to most of the genes, IGF-I gene also has different alternative splice variants in different conditions and tissues.

1.2.2.1.5.2.5.2 Alternative splicing of IGF-I

As explained before, the IGF-I gene has 6 exons, four of which are alternatively spliced (Gilmour, 1994; Smith et al, 2002), while the other two are common exons (exons 3 and 4) which encode mature IGF-I. The structure of human IGF-I gene and its alternative ways of splicing are shown in Figure 1.8.

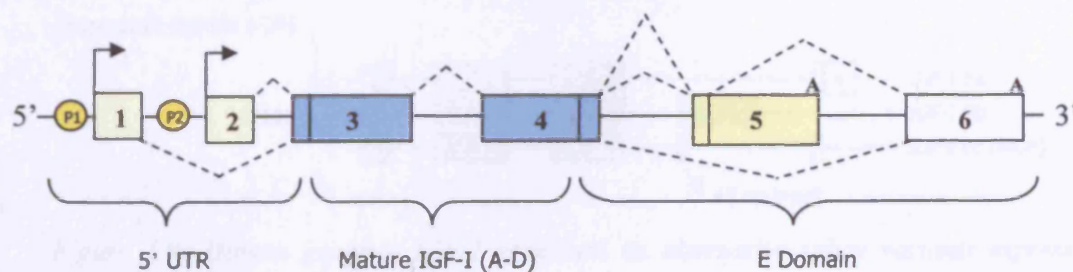


Figure 1.8: Human genomic IGF-I gene and its alternative splice variants ways

Exons 1 and 2 are alternative leader exons (Gilmour, 1994; Goldspink and Yang, 2004) with transcription start sites. Exons 1 and 2 are differently spliced to the common exon 3 and produce class 1 and class 2 IGF-I mRNA transcripts (Goldspink and Yang, 2004; Welle et al, 1993). Exons 3 and 4 code the mature IGF-I (B, C, A, and D domains) as well as the first 16 amino acids of the E domain. Exons 5 and 6 code the alternative parts of the E domain and because of a reading frame shifts in the splicing of IGF-I Ec (MGF) (Goldspink and Yang, 2004). There is also a change in the coding.

Alternative splicing and / or inclusion of exons lead to a family of IGF-I mRNAs which all encode the same mature IGF-I; but they differ in sequences 3'. Exons 1 and 2, which are 5' leader exons, lead to two different sets of transcripts: Class 1 and Class 2. Classes 1 and 2 alternative splicing of IGF-I gene is shown in Fig. 1.9.

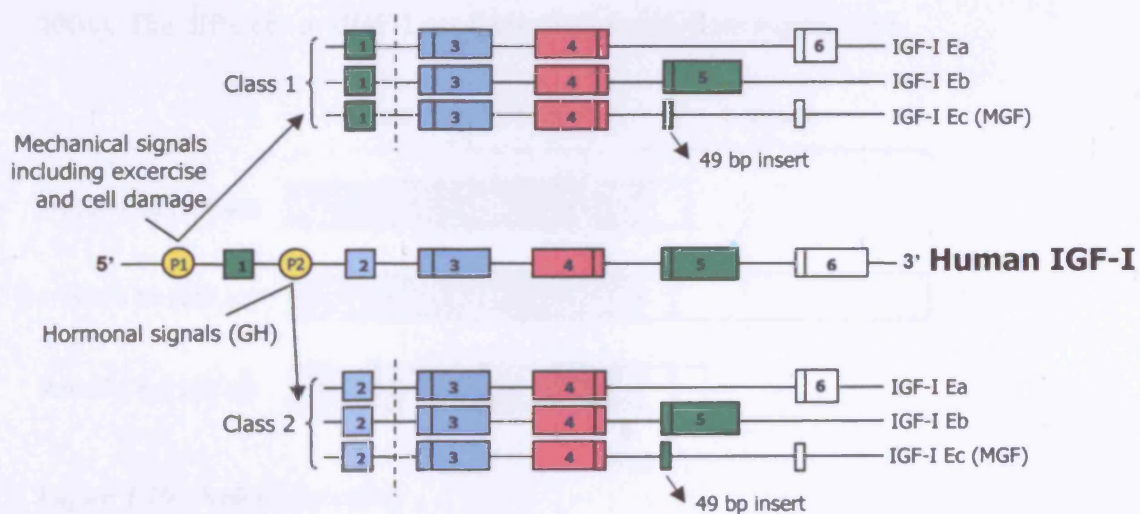


Figure 1.9: Human genomic IGF-I gene and its alternative splice variants expressed in skeletal muscle.

As can be seen from Figure 1.9, Classes 1 and 2 alternative splicing of the primary IGF-I transcripts result in at least six different IGF-I mRNAs. These are: Class 1 IGF-I Ea (exons 1, 3, 4 and 6), class 1 Eb (exons 1, 3, 4 and 5), class 1 Ec (exons 1, 3, 4, 5 and 6); class 2 Ea (exons 1, 3, 4 and 6), class 2 Eb (exons 1, 3, 4 and 5) and class 2 Ec (exons 1, 3, 4, 5 and 6) (Goldspink and Yang, 2004).

1.2.2.1.5.2.5.3 ProIGF-I proteins

The 3' alternative splicing of IGF-I gene leads to three different proIGF-I proteins, which are, proIGF-IEa, proIGF-IEb and proIGF-IEc. ProIGF-IEb is the longest peptide which has 147 amino acid residues. ProIGF-IEa is the shortest peptide and has 105 amino acid residues. IGF-IEc has 110 amino acid residues. All three proteins share a common mature IGF-I peptide, which has 70 amino acid residues, as well as the 16 amino acids sequence at N-terminal part of the E domain (Goldspink and Yang, 2004). The different proIGF-I peptides can be seen from Figure 1.10.

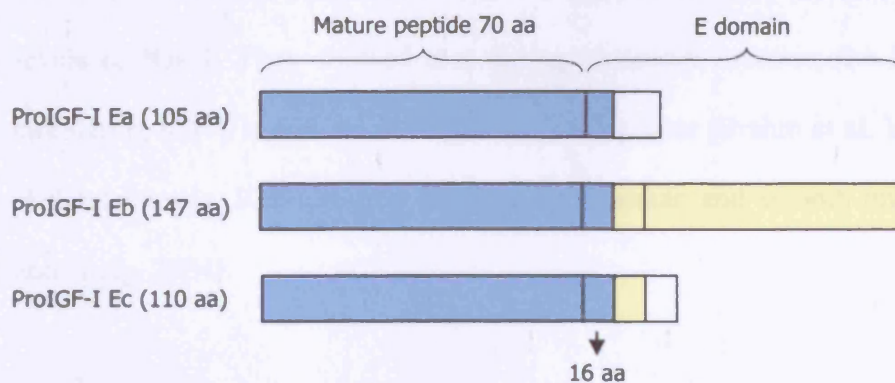


Figure 1.10: ProIGF-I proteins

1.2.2.1.5.2.5.4 The Alternative Splicing of IGF-I in Muscle Tissue

For a long time, it had been known that IGF-I was produced by the liver under the influence of growth hormone (GH). It is now realised that IGF-I is not just expressed by liver tissue, but also by many other tissues in the body, including muscle tissue. It is now known that IGF-I exists as different forms of alternative splice variants, each of which has a different physiological action (Goldspink and Yang, 2004).

The skeletal muscle is a highly complex and heterogeneous plastic tissue which is capable of regenerating itself and of remarkable adaptation in response to various stimuli including exercise. For a long time, it has been known that when a muscle is exercised, that particular muscle undergoes an increase in mass, but not all muscles of the body. On the other hand, for sometime, it has been noted that IGF-I is up-regulated by skeletal muscle during exercise, and therefore, the expression of the mRNA of IGF-I increases as a result of exercise (DeVol et al, 1990; Czerwinski et al, 1994). Furthermore, Brahm et al showed that the exercise increased the circulating levels of IGF-I. They showed that during extensive exercise, the large part of the circulating IGF-I is derived from muscle, not by liver (Brahm et al, 1997). As well as skeletal muscle, IGF-I is also expressed by cardiac and smooth muscle (Goldspink and Yang, 2004).

Some recent studies have shown that at least two splice variants of the IGF-I gene are expressed by skeletal muscle including human and animal muscles. The first isoform of IGF-I is IGF-I Ea. This is expressed by muscles, even at rest. The IGF-I Ea is similar to the liver or the systemic / the hepatic endocrine type of IGF-I and has a

general anabolic action. Although the IGF-I Ea splice variant is expressed even in resting muscle, it is also expressed by active muscle, in response to mechanical stimuli including exercise. The first splice variant of IGF-I has several abbreviations such as IGF-I Ea, L.IGF-I, m.IGF-I (Yang et al, 1996; McKoy et al, 1999; Hameed et al, 2003; Goldspink and Yang 2004).

The second isoform is expressed in muscle, only in response to mechanical activity. It was cloned by Yang et al (1996) in rabbit tibialis anterior muscles when subjected to an electrical stimulation, stretch and immobilisation model (Yang et al, 1996). This isoform was classified as IGF-IEb in rats and IGF-IEc in humans. This was termed as Mechano Growth Factor (MGF) in the literature because of the mechanosensitivity of its expression. The MGF is generated in muscle subjected to stretch and overload (Yang et al, 1996; McKoy et al, 1999; Hameed et al, 2003; Goldspink and Yang 2004).

According to Aperghis and Goldspink, muscle does not express only class 1 splice variants of IGF-I mainly in response to mechanical activity, but it also expresses class 2 variants (Goldspink and Yang, 2004). Adams' group showed that those two splice variants' expression kinetics are different with each other. Their research shows that the MGF is expressed earlier than IGF-I Ea (Haddad and Adams, 2002). Following muscle damage, firstly the MGF is produced. It lasts a day or so, and then MGF expression starts declining as IGF-I Ea increases. IGF-I Ea stays for much longer (Hill and Goldspink, 2003).

1.2.2.1.5.2.5.5 The Alternative Splicing of the IGF-I Gene in Neuronal Tissue

The alternative splicing of the IGF-I in neuronal tissue is similar to muscle tissue. Both isoforms, IGF-I Ea, and MGF, are expressed in brain and spinal cord. Aperghis et al showed that MGF has neuroprotective effects (Aperghis et al, 2004). CNS also express N-terminal truncated IGF-I isoform, des-N-(1-3) (Ballard et al, 1987; Szabo et al, 1988; Carlsson-Skwirut et al, 1989).

1.2.2.1.5.2.5.6 The Alternative Splicing of the IGF-I Gene in Other Tissues

The liver is the main source of the circulating IGF-I. Different alternative splice variants of IGF-I gene (IGF-I Ea, Eb and Ec) were first detected in the liver tissue. The cDNA of liver IGF-I (L.IGF-I or IGF-I Ea) was first reported in 1983 (Jansen et al, 1983). The second splice variant was expressed in human liver is IGF-I Eb, and it was detected in 1986 by Rotwein (Rotwein, 1984). The third variant, IGF-I Ec, was reported by Chew et al in liver cells in 1995 (Chew et al, 1995).

1.2.2.1.5.2.5.7 Mechano Growth Factor (MGF)

15 years ago, Professor Goldspink's group showed that when the mature rabbit tibialis anterior (TA) muscle was electrically stimulated while held in the stretched position by plaster cast immobilization, muscle mass was increased by 35% just in 7 days (Goldspink et al, 1992). The group found considerably increased RNA in the stretched / stimulated muscle, not in the resting control one. They then detected a RNA transcript from stretched / stimulated muscle using different oligonucleotides primers and RT-PCR. When the group analysed the sequence, they saw that it was derived from the IGF-I gene by alternative splicing (Yang et al, 1996). The newly discovered

alternative splice variant had a different carboxyl peptide sequence and E-domain to liver type of IGF-I and therefore it was differentiated from the systemic / liver type of IGF-I (IGF-I Ea). The new splice variant has been classified as IGF-IEb in rats and IGF-IEc in humans. Because of the confusion in terminology and its mechanosensitivity it was called Mechano Growth Factor (MGF).

As can be seen from Figure 1.9, alongside the common exons 3 and 4, the MGF differs by the inclusion of 49 bp insert in humans, and (52 bp insert in rats) from the first part of the exon 5 of the E domain of IGF-I gene. Neither 49 nor 52 bp are multiples of 3. Therefore, the 3' RNA sequence encodes for a different C-terminal peptide sequence than that the liver type of IGF-I (Goldspink and Yang, 2004).

It has been shown in a recent C2C12 cell culture study that the systemic or liver type IGF-I (IGF-I Ea) activates differentiation into myotubes, whereas the MGF activates proliferation, and prevent differentiation by remaining in the mononucleated state. It has been also suggested that MGF has a role in the activation of satellite cells (Yang and Golspink, 2002).

Furthermore, a very recent human muscle exercise study has shown that high resistance exercise results in MGF mRNA in the young, but not in the elderly (Hameed et al, 2003). The molecular weight of MGF is 5 kDa and is expected to have a shorter half – life than the liver IGF-I's which are stabilized by binding proteins. Therefore the half-life of MGF is less than 10 minutes. The MGF is likely to act in an

autocrine/paracrine mode of action rather than a systemic endocrine one (Hameed et al, 2002).

1.2.2.1.5.2.6 Therapeutic implications of IGF-I and IGF system

Our knowledge of IGF system has increased substantially in recent years due to the developments in molecular and cellular biology. We can now easily measure circulating levels of the members of the IGF system in order to understand the possibly pathophysiological changes in certain clinical situations. We also now have an opportunity to identify the genetic alterations as the underlying cause of such situations. The human beings with a variety of illnesses and different disorders have altered circulating IGF-I and IGFBPs levels (Pozo et al, 2005). Numerous studies have shown that these levels change in many diseases and disorders. The data provided from the circulating levels of IGFs bring about a new approach for treatment of such clinical situations. So far, many therapeutic implications of the components of the IGF system have been applied to many clinical situations. Particularly using recombinant human IGF-I (rhIGF-I) or recombinant human IGF-I/IGFBP-3 complex has opened the potential for therapy for a wide range of pathological conditions (Savage et al, 2004). Therefore, the potential therapeutic applications of IGF system are very broad. Studies have shown that IGF system does have some therapeutic effects, in one way or another, on several human diseases, ranging from neuromuscular or neurodegenerative diseases to growth disorders; from malignancy to diabetes (Monzavi and Cohen, 2002; Savage et al, 2004, Russo et al 2005). The following disorders have either been already treated in some way with the products of IGF systems attempt this has been carried out mainly using animal models:

- Traumatic / hypoxic or ischemic muscle and neuronal tissue damages including skeletal and cardiac muscle, peripheral and central nerve damages, MI, stroke, neuropathies, spinal cord injuries, brain ischemia. (Gluckman et al, 1992; Guan et al 1993; Sharma et al, 1998; Guan et al, 2003; Goldspink and Yang, 2004; Cortes et al, 2005; Dluzniewska et al, 2005; Russo et al, 2005);
- Neuromuscular disorders, including myopathies, muscular dystrophies and ALS (Singleton and Feldman, 2001; Mitchell et al, 2002; Kaspar et al, 2005; Russo et al, 2005; Wilczak and Keyser, 2005);
- Neurodegenerative diseases, including ALS, Alzheimer, Multiple Sclerosis and Cerebellar ataxia (Dore et al, 2000; Gasparini and Xu, 2003; Watson and Craft, 2003; Carro and Torres-Aleman, 2004; Russo et al, 2005; Kaspar et al, 2005; Wilczak and Keyser, 2005);
- Growth disorders, including IGF-I gene defects, Growth Hormone Deficiency (GHD), Growth Hormone Insensitivity Syndrome (GHIS or Laron syndrome), Acromegaly, Idiopathic short stature (ISS) (Monzavi and Cohen, 2002; Savage et al, 2004);
- Endocrinal diseases, including type I and II diabetes, insulin resistance syndrome (Zenobi et al, 1992; Cheetham et al 1995; Thraikill, 2000; Monzavi and Cohen 2002; Savage et al, 2004);
- Bone diseases, including osteoporosis (Rosen and Pollak 1999);
- Chronic inflammatory and nutritional disorders, including chronic inflammatory bowel diseases, Crohn's Disease and Chronic Juvenile Arthritis (Savage et al, 2004);
- Different types of cancer, including breast, prostate, colorectal, lung cancers (Monzavi and Cohen 2002).

1.2.3 Degenerative Muscle Disorders

1.2.3.1 Muscular Dystrophies

Muscular dystrophies (MDs) are a group of neuromuscular disorders which vary in their pathology and inheritance patterns. At least 30 different muscular dystrophy forms have been identified to date (Vainzof and Zatz, 2003). They all are inherited myogenic disorders characterised by progressive muscle wasting and weaknesses of variable distribution and severity. They share a set of clinical and pathological characteristics but vary in severity and inheritance pattern, and molecular defect.

Muscular dystrophies are caused by loss of the linkage between the extracellular matrix and the actin cytoskeleton (Laval and Bushby, 2004). The most common form of MD's is Duchenne muscular dystrophy (DMD). DMD affects 1 in 3500 live male births (O'Brien and Kunkel, 2001).

Nine major forms of MDs are as follows: Myotonic muscular dystrophy (MMD, also known as Steinert's Disease), Congenital muscular dystrophy (CMD), Duchenne Muscular Dystrophy (DMD, also known as Pseudohypertrophic), Becker muscular dystrophy (BMD), Emery-Dreifuss muscular dystrophy (EDMD), Distal muscular dystrophy (DD – Miyoshi MD), Facioscapulohumeral dystrophy (FSHD or FSH, also known as Landouzy-Dejerine), Oculopharyngeal muscular dystrophy (OPMD), Limb-girdle muscular dystrophy (LGMD) (Emery, 2002). Figure 1.11 shows the affected muscles in six different types of muscular dystrophy.

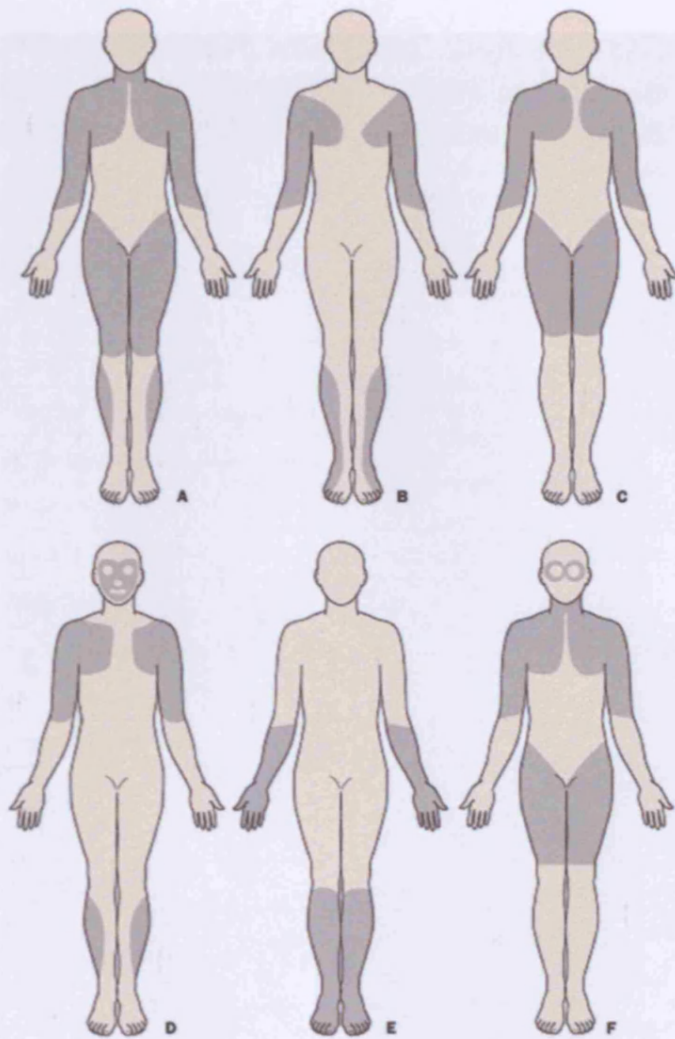


Figure 1.11: Muscle weakness in different types of dystrophies. Shaded areas are the affected muscles. A: Duchenne and Becker, B: Emery-Dreifuss, C: Limb-Girdle, D: Fascioscapulohumeral, E: Distal and F: Oculopharyngeal. Taken from Emery, 2002)

The genetics of many types of muscular dystrophies have now been known in details. Different forms of MDs have different pathology and inheritance patterns. Therefore, the gene loci and protein defects in the commonest forms of muscular dystrophies vary. These are shown in Table 1.1.

The gene loci and protein defects in the commonest forms of muscular dystrophies		
DISORDERS	GENE LOCUS	PROTEIN DEFECT
Congenital MD (AR)	6q	α_2 -Laminin (merosin)
Congenital MD (AR)	12q	Laminin receptor (α -integrin)
Congenital MD (AR)	9q	Fekutin (Fukuyama dystrophy)
Congenital MD (AR)	1p	Selenoprotein N1 (rigid spine syndrome)
Congenital MD (AR)	1p	Glycosyltransferase (muscle – eye – brain disease)
Duchenne and Becker MD (XR)	Xp21	Dystrophin
Emery – Dreifuss MD (XR)	Xq28	Emerin
Emery – Dreifuss MD (AD/AR)	1q	Lamin A / C
Distal MD (AD)	14q, 2q	Not known
Distal MD (AR)	2p	Dysferlin
Facioscapulohumeral MD (AD)	4q	Not known
Oculopharyngeal MD (AD)	14q	Poly (A) – binding protein 2 (PAB 2)
Limb – girdle MD (AD)		
1 A	5q	Myotilin
1 B	1 q	Lamin A / C
1 C	3p	Caveolin 3
1 D	6q	Not known
1 E	7q	Not known
1 F	2q	Not known
Limb – girdle MD (AR)		
2 A	15q	Calpain - 3
2 B	2p	Dysferlin
2 C	13q	γ - sarcoglycan
2 D	17q	α -sarcoglycan (adhalin)
2 E	4q	β - sarcoglycan
2 F	5q	δ -sarcoglycan
2 G	17q	Telethonin
2 H	9q	Not known
2 I	19q	Fekutin-related protein
2 J	2q	Titin

Table 1.1: Gene loci and protein defects in the commonest forms of muscular dystrophies. Abbr: AR: Autosomal recessive, AD: autosomal dominant, XR: X- linked recessive. (Adapted from Emery, A. E. H., 2002; and Bogdanovich et al, 2004)

The different forms of muscular dystrophy vary in their pathology and inheritance patterns. For instance, congenital muscular dystrophy is an autosomal recessively inherited disorder that presents hypotonia and weakness of muscles. It has several different forms. Some, such as Fukuyama muscular dystrophy, occur with mental retardation and some transpire without it. The congenital muscular dystrophies are caused by the absence of certain proteins such as merosin, laminin and fektin in birth or within the first few months of life (Emery, 2002). It is seen in severe and early contractures (shortening or shrinking of muscles that cause joint problems). On the other hand, Duchenne Muscular Dystrophy (DMD) and its milder allelic variant, Becker muscular dystrophy (BMD) are X linked recessive disorders. DMD is caused by the absence of dystrophin protein that is encoded by the dystrophin gene which is the largest mammalian gene known. When some mutations, such as several types of deletions and other rearrangements, occur in the gene, the dystrophin protein cannot be translated, except in the BMD where dystrophin protein is truncated. Figure 1.12 shows the molecular pathologies of different forms of MDs.

1.2.3.1.1 Dystrophin and Dystrophin Associated Protein Complexes

The dystrophin gene (*dys*) within Xp21 is the largest identified mammalian gene, which spans approximately 2.6 Mb of genomic sequence and occupies almost 2% of X chromosome, and nearly 0.05 % of entire human genome (Bogdanovich et al, 2004). The gene consists of 79 exons and the corresponding 14 kb dystrophin transcript is expressed in skeletal, cardiac and smooth muscle, with lower levels in brain. In normal muscle, dystrophin gene encodes a 427 kDa dystrophin protein composed of 3685 amino acid residues. The dystrophin protein localises to the

cytoplasmic surface of sarcolemma and is enriched membrane of neuromuscular junction (Kapsa et al, 2003; Bogdanovich et al, 2004).

The dystrophin protein is organised into four structural domains: (a) The amino-terminal acting binding domain, (b) a central rod domain, (c) cystein-rich domain, and (d) a carboxy-terminal domain. The N-terminal of dystrophin binds actin filaments of cytoskeleton. The rod domain is the majority of the protein. The cystein-rich domain binds intracellular calcium and ZZ domain. Finally, the C-terminal of dystrophin binds some dystrophin associated proteins such as dystrobrevin, syntrophin, and β -dystroglycan.

The dystrophin is located beneath the muscle sarcolemma associated with a membrane-spanning protein complex that connects the cytoskeleton to the basal lamina. The protein complex associated with dystrophin is therefore of key importance in the pathogenesis of the muscular dystrophies. Mutations in different components of the dystrophin associated protein complex (DAPC) cause different forms of MDs (see Table 1.1 and the Figure 1.12). The α -Dystroglycan is an extracellular component of DAPC and is linked to the sarcolemma by interaction with a trans-membrane complex consists muscle specific β -dystroglycan and sarcoglycan complex (α , β , δ , and γ sarcoglycans). The cytoplasmic tail of the β -dystroglycan binds the dystrophin. The α -Dystroglycan also binds components of extracellular matrix, including laminin, biglycan, agrin, and perlecan.

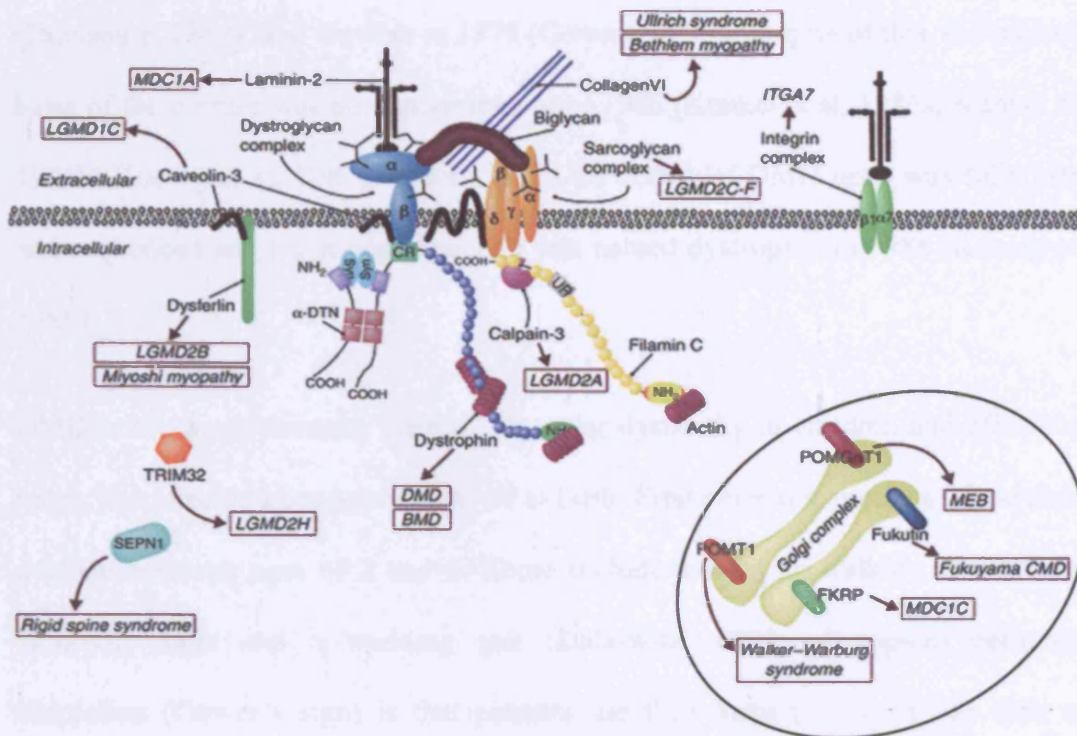


Figure 1.12: Muscular dystrophies and the membrane associated proteins. The figure shows the location of different membrane and enzymatic proteins associated with muscular dystrophies. Diseases are shown in boxes. The figure in a circle shows Golgi complex and related proteins and diseases. Abbr: MDC and CMD: Congenital muscular dystrophy, LGMD: Limb-Girdle muscular dystrophy, DMD: Duchenne muscular dystrophy, BMD: Becker muscular dystrophy. (The figure is taken from Dalkilic and Kunkel, 2003, by kind permission of Dr Isin Dalkilic from Neromuscular Disease Project in MGH of Harvard University).

1.2.3.1.2 Duchenne / Becker / Muscular Dystrophy (DMD / BMD)

Duchenne muscular dystrophy (DMD) is a X-linked fatal progressive muscle wasting disorder that affects only male children (females are the carrier). With an incidence of ~ 1 in 3,500 male new born, it is the most common muscular dystrophy and one of the most common fatal hereditary disorders.

DMD was first described by Edward Meryon in 1851 (Meryon, 1851), and then its clinical and muscle histology was detailed by Duchenne a decade later in 1861

(Duchenne, 1861), and Gowers in 1879 (Gowers, 1879). In spite of this, the molecular basis of the disease was not determined until 1986 (Kunkel et al, 1986a; Kunkel et al, 1986b; Koenig et al, 1987). The entire 14-kb cDNA of DMD gene was fully cloned and sequenced and the encoded protein was named dystrophin in 1988 (Koenig et al, 1988).

DMD is the most common form of muscular dystrophy in children and affects only boys. The affected boys appear normal at birth. First clinical symptoms of the disease develop between ages of 2 and 6. These include a delay in walking, difficulties in climbing stairs and a waddling gait (Dubowitz, 1978). A typical behavioural adaptation (Gower's sign) is that patients use their arms to "climb" up their own thighs, when changing from a lying to a standing position. The muscles decrease in size and grow weaker over time though they may appear larger. Calf muscles show a marked pseudo hypertrophy while all proximal muscles become progressively weaker. Disease progression varies, but many sufferers need a wheelchair by the age of 12. In most cases, the arms, legs, and spine become progressively deformed. Some sufferers are mildly retarded. Severe breathing and heart problems mark the later stages of the disease. The most DMD patients die from respiratory or cardiac failure in the early 20s (in some rare cases in the early 30s).

Becker muscular dystrophy (BMD) is quite similar to Duchenne muscular dystrophy, but the difference is that the disease is much milder than DMD. The symptoms appear later and progress more slowly. It affects 1 in every 20,000 new born boys (van Deutekom and van Ommen, 2003). BMD usually appears between the ages of 2 and 16 but can also appear as late as age of 25. Like DMD, it affects only males and

causes heart problems. Even though the clinical symptoms are similar to DMD, they are significantly delayed. Disease severity varies. Sufferers can usually walk into their 30s and live further into adulthood. Some BMD patients even remain ambulant until old age (England et al, 1990).

The main pathological problem in DMD and/or BMD is the mutations in the gene encoding dystrophin protein. The mutations in DMD and/or BMD result in the absence or the severe reduction of the dystrophin protein. The dystrophin protein is completely absent from the sarcolemma in muscles from DMD patients and some animal models, including the mdx mouse and GRMD dog. On the other hand, in BMD patients, dystrophin protein still exists but it is internally truncated. Therefore, the dystrophin protein has semi function (and milder phenotype).

Dystrophin also plays a role in the brain. This role is not sufficiently understood currently, but the absence of dystrophin in DMD may result in a mild cognitive impairment (Blake and Kroger, 2000; Mehler, 2000).

The progressive nature of the disease results from the inability of the affected muscle to efficiently replace damaged muscle fibres. This is mainly due to an exhaustion of the potential to regenerate fibres from muscle progenitor cells, in concert with the gradual replacement of muscle fibres by connective tissue (Reimann et al, 2000). An useful, although unspecific diagnostic marker, even before the onset of a clinical phenotype, is the strongly increased serum creatine phosphokinase activity. The histological hallmarks of the disease in skeletal muscle are: fibre size variation, cluster of necrotic fibres, central nuclei in regenerating fibres, fibrosis and infiltration of

macrophages and CD4+ lymphocytes (Gorospe et al, 1990; McDouall et al, 1990; Blake et al, 2002).

A definite diagnosis for DMD is based either on the absence of dystrophin protein in a muscle biopsy or on the molecular identification of a mutation in the dystrophin gene. About 65% of dystrophin mutations are large deletions which occur preferentially at two mutation “hot spots” and 5% of these are large duplications (Koenig et al, 1989). The remaining 30% which is distributed over the whole gene are point mutations or small-scale deletions/duplications, including 18% of nonsense mutations, 8% small deletions or insertions, and the remaining are splice site mutations, and missense mutations (Roberts et al, 1994). According to the “reading frame theory” (Monaco et al, 1988) a deletion or duplication causing a frame shift leads to a premature termination of translation.

The resulting dystrophin protein is truncated, not functional, and due to low mRNA and protein stability only present at very low levels. Patients with such mutations develop DMD. Surprisingly, even these patients show a small subset of dystrophin positive fibres. The occurrence of so-called revertant fibres is directly associated with the nature of mutation in individual patients. While some mutations allow revertant fibres, others do not. A natural exon skipping mechanism was proposed to be the underlying molecular mechanism. Overall, revertant fibres are detected in approximately 50% of DMD patients and account for 0.2 – 4 % of the total number of muscle fibres (Burrow et al, 1991; Nicholson et al, 1993).

In BMD, the mutations usually cause the loss of a small, non-essential part of the molecule while the reading frame stays intact. In BMD, the deletions mainly occur from exon 13 (Sitnik et al, 1997) or 17 (England et al, 1990) to 48 (Bogdanovich et al, 2004). This deletion allows the expression of smaller dystrophin protein. The resulting dystrophin could have varying degrees of functionality, depending on the size and the position of the deleted sequence. This is reflected by the wide spectrum of the clinical severity of BMD patients. Female carriers are not or only mildly affected because they are normally able to compensate the defect via the functional dystrophin copy on their second X chromosome.

Current treatment options for DMD and BMD are very limited. No effective pharmacological treatment is available so far, while supportive therapies like artificial ventilation have moderately prolonged patient's survival but offer no permanent cure (Scheuerbrandt, 1998). The recent therapeutic applications for Duchenne / Becker Muscular dystrophies can be seen in 1.5.1.8.

1.2.3.1.3 Congenital Muscular Dystrophy (CMD)

Congenital muscular dystrophies (CMD) are a heterogeneous group of autosomal recessively inherited disorders, which present with hypotonia, muscle weakness and contractures, associated with dystrophic changes on skeletal muscle biopsy (Sellick et al, 2005). CMDs have several different forms with different gene defects such as Ulrich CMD (21q22.3 and 2q37). Walker-Warburg Syndrome (9q34.1), Muscle-eye-brain disease (1q32-q34) and Fukuyama muscular dystrophy (19q13.3) with and without mental retardation and with geographic high-incidence foci (Tubridy et al,

2001; Emery, 2002; Kirchner and Bonnemann, 2004). Most recently, another CMD locus was identified on 4p16.3 by using a high-density SNP array (Sellick et al, 2005). CMDs are caused by the absence of some extracellular matrix proteins such as α 2-laminin (merosin), α integrin, fekutin, glycosyltransferase and selenoprotein N1 in birth or within the first few months of life (Mukherjee and Mittal, 2004). Therefore a proportion of children with CMD show an absence or a marked deficiency in a merosin which divides CMD into 2 groups, merosin-negative and merosin-positive (Tome et al, 1994). In CMD, it is associated with severe and early contractures (shortening or shrinking of muscle that causes joint problems). Unfortunately, there is no effective treatment for any form of CMD (Emery, 2002; Kirchner and Bonnemann, 2004).

1.2.3.1.4 Facioscapulohumeral Dystrophy (FSHD)

Facioscapulohumeral dystrophy (FSHD) is an autosomal dominant muscle disorder characterised by progressive weakness and wasting of facial, scapulohumeral, anterior tibial, pelvic and pectoral-girdle and upper arm muscles including retinal vascular disease, sensory hearing loss and in severe cases some with detectable abnormalities of the CNS (Mukherjee and Mittal, 2004). FSHD is the third most common inherited muscular disorder after Duchenne muscular dystrophy and myotonic dystrophy (Fitzsimons, 1999; Upadhyaya and Cooper, 2002; Topler and Gabellini, 2004; Yamanaka et al, 2004); and the estimated prevalence is 1 in 20,000 (Fitzsimons, 1999; Kissel, 1999). A deletion has been found in the subtelomeric region of chromosome 4q35 (Fitzsimons, 1999; Upadhyaya and Cooper, 2002; Topler and Gabellini, 2004; Yamanaka et al, 2004) and at least 95% of families with FSHD show linkage to such

deletion (Pradhan, 2003). FSHD currently has no effective treatment (Fitzsimons, 1999).

1.2.3.1.5 Emery-Dreifuss Muscular Dystrophy (EDMD)

Emery-Dreifuss Muscular Dystrophy (EDMD) appears from childhood to early teens. The symptoms are weakness and wasting of shoulder, upper arm and shin muscles. Joint deformities and frequent cardiac complications are also common. The disease usually progresses slowly. EDMD is X-linked recessive (females are carriers) and defective proteins are emerin and lamin A/C.

1.2.3.1.6 Limb-Girdle Muscular Dystrophy (LGMD)

Limb-Girdle Muscular Dystrophies (LGMD) are also a diverse group of muscle-wasting disorders, and they characteristically affect the large muscles of the pelvic and shoulder girdles (Laval and Bushby, 2004). LGMDs are autosomal recessive, or X-linked recessive disorders, and appears from childhood to middle age. Defective proteins of LGMDs vary. Some of these are myotilin, lamin A/C, laminin-alpha2, collagen VI, caveolin 3, calpain-3, dysferlin, α , β , γ and δ sarcoglycans, titin, telethonin or myotilin, fekutin-related protein and E3-ubiquitin ligase.

To date, five autosomal dominant and ten autosomal recessive forms of limb-girdle muscular dystrophies have been identified: the first four forms of AR LGMDs are the sarcoglycanopathies (LGMD2C - γ - sarcoglycan, 2D - α -sarcoglycan / adhalin, 2E - β -sarcoglycan and 2F - δ -sarcoglycan). The other forms are LGMD2A (calpain 3), LGMD2B and Miyoshi myopathy (dysferlin - 2p31), LGMD2G (telethonin),

LGMD2H (9q31-11), and LGMD2I (Fekutin-related protein - 19q13.3) and LGMD2J (Titin) (Vainzof et al, 2001; Emery, 2002; and Bogdanovich et al, 2004). On the other hand, Myotonic Dystrophy (MMD), Oculopharyngeal Muscular Dystrophy (OPMD) and Distal Muscular Dystrophy (DD), all are autosomal dominant disorders.

1.2.3.1.7 Animal models for Muscular Dystrophies

Animal models have provided valuable clues to understand of DMD pathogenesis and offer a good opportunity for the development and testing of therapeutic approaches (Allamand and Campbell, 2000). Several species of animals have been identified in which mutations in the dystrophin gene (in murine, canine and feline) lead to the absence of dystrophin and to development of dystrophy with varying degrees of severity. Some of these are as follows:

1.2.3.1.7.1 The mdx mouse

The most commonly used animal model of DMD is C57Bl/10ScSn mdx (murine dystrophy x-linked) mouse (Bulfield et al, 1984). A point mutation in the dystrophin gene (nonsense mutation in exon 23, in position 3185) results in a premature stop codon (CAA - > TAA) (Sicinski et al, 1989). The mice have a small number of revertant fibres (about 1%). Although the mdx is a genetic and biochemical homologue of the DMD, it has a milder phenotype. The clinical phenotype of the mdx mice is much more benign than in human DMD patients (Gillis, 1999). Therefore, these animals have a nearly normal life expectancy and move normally at old ages.

Typical features of regenerated muscles of mdx mice are centrally located nuclei. Under normal conditions nuclei return to the periphery of the muscle fibre after a

regeneration process is completed. In mdx muscles very high levels of central nuclei were observed over the entire lifespan. Central nucleation is an easily measurable and well accepted indicator of muscle pathology in mdx mice.

There are also some other murine models of DMD, such as dy/dy mouse (C57Bl/6 J – dy2J) and mdx/utm (dystrophin and utrophin deficient) mouse and several different immunodeficient strains of mouse (Collins and Morgan, 2003). On the other hand, some other murine models of different muscular dystrophies are also available, such as, dysferlin deficient C57BL/10.SJL-Dysf mouse (von der Hagen et al, 2005).

1.2.3.1.7.2 The GRMD dog

There are several dog models of DMD such as Golden Retriever (Cooper et al, 1988), Rotweiller (Partridge, 1991) and the German short-haired pointer (Schatzberg et al, 1999). Of these, the Golden Retriever Muscular Dystrophy (GRMD) dog has been the most extensively studied and best characterised animal model of DMD. GRMD is caused by a point mutation in consensus splice acceptor in the introns 6 of canine dystrophin gene. The mutation results in the deletion of exon from the mRNA thereby introducing a frame shift which leads to a premature stop codon. The mutation is 3'splice-site point mutation in introns 6 (in position 739-2a). Affected pups gain weight slower than unaffected siblings. The affected dogs generally die within days or months (Cooper et al, 1988). The GRMD dogs are a good model for DMD because of their severe clinical phenotypes, their large size and the accompanying histopathological progression (Howell et al, 1997; Cozzi et al, 2001).

1.2.3.1.7.3 The HFMD cat

There is also a dystrophin deficient cat: Hypertrophic feline muscular dystrophy (HFMD) cat. (Carpenter et al, 1989; Gaschen and Burgunder, 2001; Collins and Morgan, 2003). Hypertrophy is the most notable effect in affected animal. An abnormal gait and some necrosis are present but the overall phenotype is benign. Affected cats also suffer from cardiomyopathy (Gaschen et al, 1999). The mutations of HFMD cat is deletion of Dp427m and Dp427p promoter regions.

Although there are currently no primate models of dystrophin deficiency, some non-mammalian and invertebrae models of disease are available for research, such as zebrafish (Chambers et al, 2001; Rubinstein 2003), the nematode *C. elegans* (Baumeister and Ge, 2002) and the sea urchin (Neuman et al, 2001).

1.2.3.1.8 Therapeutic Approaches for Muscular Dystrophies

Muscular dystrophies, particularly DMD, affect hundreds of thousands patients worldwide. Because no effective treatment is available, great efforts are underway to develop a cure. Once an effective approach is established to one of these, it might be applicable with modification in large numbers of other heritable diseases. Because of the complexity of muscular dystrophies, an optimal therapeutic approach can be targeted all genetic (mutations in genes), biochemical (absence of proteins), cellular (myofibre necrosis), tissue (reduction of myofibre size and number), functional (loss of muscular function) and clinical levels of the physiopathological changes including paresis and paralysis, column deformities, and cardio-respiratory insufficiencies.

Until now, many different strategies have been studied and developed to treat muscular dystrophies. The majority of these various therapeutic strategies have been for DMD over the years. Therefore, it will mainly concentrate on the potential therapies for DMD. Such therapeutic strategies have been studied and developed on mainly dystrophin deficient animal models of DMD, particularly on mdx mice. However, many DMD human clinical trials are currently ongoing.

Different levels of progress for these different therapeutic strategies have been achieved in three main approaches. Therefore, in general, therapeutic strategies for DMD can be classified into the following four major groups based on their approaches:

- Gene therapy approaches (viral, plasmid and oligonucleotide-based),
- Cell therapy approaches (myogenic, stem cell transplantation),
- Pharmacological therapy approaches (anabolic agents and gentamicin),
- Alternative therapy approaches (upregulation of some other alternative therapeutics, such as utrophin or IGF-I).

1.2.3.1.8.1 Genetic approaches

The main goal of gene therapy strategies for DMD is either to replace the defective dystrophin gene with a functional copy or to “repair” the mutated dystrophin gene. Up to now, for vector based supplementary gene therapy (for trying to replace the defective gene with a functional copy), both viral and plasmid vectors have been used to deliver genetic materials to treat DMD. On the other hand, as non-vector based

corrective gene therapy, small double stranded fragments (>~100bp) and synthetic single stranded oligos (<100 bp) have been used.

For viral vector based gene therapy, a number of viral vector systems for gene therapy of muscle disorders are currently under development, such as adenoviral (full-length, mini- or micro-dystrophin cDNA transfer), adenoassociated viral (mini-, or micro-dystrophin cDNA transfer), retroviral (mini-dystrophin cDNA transfer), lentiviral (mini-dystrophin cDNA transfer) and herpes simplex viral (full-length dystrophin cDNA transfer). The advantages of their high capacity and of being able to accommodate the full-length dystrophin cDNA and promoter, adenoviral vectors have been used widely to treat DMD. Different variants of adenoviral (Ad) vectors have been used in many mdx mice and human clinical trials (Vincent et al, 1993; Acsadi et al, 1996; Guibinga et al, 2001; Gilbert et al, 2001; Jiang et al, 2001; Maione et al, 2001; O'Hara et al, 2001). Particularly in recent years, the adenoassociated viral (AAV) vector based gene therapy have been used to deliver mini- or micro-dystrophin cDNA and have yielded comparatively better results (Xiao et al, 1996; Wang et al, 2000; Stedman et al, 2000; Duan et al, 2001 ; Yuasa et al 2002 ; Harper et al, 2002 ; Fabb et al, 2002 ; Watchko et al, 2002 ; Skuk et al, 2002 ; Kapsa et al, 2003 ; van Deutekom and van Ommen, 2004 ; Gregorevic et al, 2004). The serotype of AAVs has been recently improved and most recent variants of these, such as pseudotyped AAV types 5 and 6 are now much more effective (more than 200 folds) for in vivo (Duan et al, 2001, Gregorevic et al, 2004). Alongside Adenoviral and AAV vector system, retroviral (Dunckley et al, 1993; Fassati et al, 1997; Skuk et al, 2002), lentiviral (Kafri et al, 1997) vectors have been also used to treat DMD. Although viral

gene therapy for DMD mainly focuses on adenoviral, adenoassociated, and retroviral vectors, several additional viral systems have also been applied to DMD. These include herpes simplex virus (Akkaraju et al, 1999), Epstein-Barr virus (Tsukamoto et al, 1999) and chimeric adeno-retrovirus (Tsukamoto et al, 1999).

As non-viral vector based gene therapy for DMD, mainly naked DNA (plasmid cDNA) has been used (Liu et al, 2001; Romero et al, 2004). High level and long-lasting gene expression has been reached by the development of improved plasmid DNA transfer techniques, such as intravenous injection with high pressure (Budker et al, 1998), optimised electroporation conditions (Vicat et al, 2000; Vilquin et al, 2001) and use of ultrasound (Danialou et al, 2002).

As mentioned above, for corrective gene therapy, depending on the nature of the mutations, small fragment homologous replacement (SFHR), chimeraplasty and oligonucleotide-mediated exon skipping strategies have been hitherto used. The repair of point mutations in the dystrophin gene has recently been demonstrated in mdx mice (in cultured cells and in muscles) (Bertoni and Rando, 2002). This single base pair repair is called chimeraplasts, which are single stranded RNA/DNA oligonucleotides. The chimeraplasts bind in a first step to the targeted sequence and then in a second step, trigger the repair of the mutation through the endogenous DNA repair machinery (Barlett et al, 2000). In theory, chimeraplasty may correct permanently almost all kinds of point mutations and therefore is a very general and promising approach; however, in application, it has a very limited effect, such as 1 – 2% of all fibres per muscle were repaired so far (Rando et al, 2000).

Another possibility of DMD gene therapy is to skip a stop codon mutation or to restore an open reading frame after shift mutation. To specifically induce exon skipping of mutations in DMD patients, antisense oligonucleotides (AOs) were used. Successful skipping of the mdx stop codon mutation (in exon 23 of the mouse dystrophin) has already been demonstrated in vitro and in vivo, when AOs specific for the junction of introns 22 and exon 23 were applied (Duncley et al, 1998; Rando et al, 2000; Mann et al, 2001; Lu et al, 2003; Goyenvalle et al, 2004). Currently, three large clinical trials are being planned to take place in the Netherlands, the UK and Australia (Wilton and Fletcher, 2005). Among them, two European consortia (in the Netherlands/Belgium and in the UK) are preparing for a clinical trial on antisense oligonucleotides (AON) in DMD (Muntoni et al, 2005). According to the report about the meeting held in Naarden, the Netherlands on 22-24 October 2004, the UK consortium project funded by the Department of Health is testing novel AON targeted against exon-intron boundaries of exon 51 and 53 in DMD patients. The Dutch / Belgian consortium is testing exon 51 in DMD patients (Muntoni et al, 2005).

According to Prof Muntoni, UK consortium participants are Prof Francesco Muntoni, Prof Terry Partridge and Prof Dominic Wells of Imperial College London, Prof Kate Bushby of Newcastle University, Dr Matthew Woods of Oxford University, and Prof George Dickson of Royal Holloway College – London. (Prof Muntoni's talk in GTAC - UK Gene Therapy Advisory Committee - public meeting in July 2004; <http://www.advisorybodies.doh.gov.uk/genetics/gtac/meetings.htm> -*Delegate Pack for Open Meeting 2004*).

1.2.3.1.8.2 Cellular approaches

For cellular approaches, myoblast and stem cell transplantations have been used. For myoblast transplantation, Terry Partridge's group transferred cultured normal myoblasts into dystrophin-deficient mdx/nude neonatal mice (Partridge et al, 1989). However, the clinical trials of myoblast transplantations were unsuccessful (Karpati et al, 1993; Miller et al, 1997; Neumeyer et al, 1998; Skuk, 2004; Dubowitz, 2004). On the other hand, a few systemically injected bone-marrow cells and liver-derived stem cells were also introduced to dystrophin-deficient muscle (Bittner et al, 1999; Malouf et al, 2001; Zuk et al, 2001). However, the efficiency levels were low (Gussoni et al, 2002).

1.2.3.1.8.3 Pharmacological approaches

One of novel strategies for DMD therapy is to introduce gentamicin, an aminoglycoside antibiotic, into dystrophin-deficient muscle. Gentamicin-treated mdx mice have shown increase of dystrophin expression and functional improvement (Barton-Davis et al, 1999). However, human gentamicin based clinical trials were not effective (Wagner et al, 2001). In addition, calcium homeostasis, using calcium channel blockers such as diltiazam/nifedipine, was also used to treat DMD (Johnson and Bhattacharya, 1993).

1.2.3.1.8.4 Alternative approaches

Among other approaches to treat DMD, a few other therapeutic strategies can be mentioned. One of these is utrophin upregulation. Utrophin is an alternative protein to

repair the mutated dystrophin, because utrophin is dystrophin paralog, and it is already moderately upregulated in dystrophin-deficient muscle (Tinsley and Davies, 1993). The utrophin functionally replaced dystrophin in some transgenic and viral-gene transfer studies (Tinsley et al, 1996; Wakefield et al, 2000).

1.2.3.1.8.5 Non – molecular approaches / specific management implications

Although some promising developments in the preclinical field and various therapeutic approaches, there are yet no fundamental treatments aimed at the underlying pathology for any kind of muscular dystrophies. Nevertheless, some evidence based specific managements are available for many years. Many of the life threatening complications of these diseases such as cardiac and respiratory system complications can be prevented and / or treated by these managements in order to increase life expectancy and to improve quality of life (Bushby and Straub, 2005).

According to Bushby and Straub (2005), some of these specific management implications are as follows:

- Drug treatments (the use of corticosteroids [prednisone/prednisolone and deflazacort] are useful in maintaining or improving strength);
- Physiotherapy and orthopaedic surgery (MD patients with joint contractures may benefit);
- Cardiac surveillance and treatment (treatments of arrhythmias and disrhythmias);
- Respiratory management (prevention and treatment of increasing respiratory insufficiency thraw the prophylaxis; and treatment of chest infections with immunization, cough augmentation techniques and prompt treatment by antibiotics);

- Pain management;
- Anaesthesia..

1.2.3.2 Motor Neurone Diseases / Amyotrophic Lateral Sclerosis

Motor neurone diseases (MND) are a group of disorders, which are characterised by progressive degeneration of the motor neurones of the brain, brain stem or spinal cord. It can affect both upper and lower motor neurones. Upper motor neurones (cortico-spinal tract cells) originate in the brain. The lesions in upper motor neurones cause characteristic signs such as spasticity, muscle stiffness, brisk reflexes, abnormal reflexes (e.g. Babinski reflex) and spastic weakness (Shaw, 1999, Strong, 2003).

The lower motor neurones originate in the brain stem or the anterior horn cells of the spinal cord and innervate muscle. The lesions of lower motor neurones cause characteristic signs such as muscle wasting, muscle fasciculation, flaccid weakness, and hypertonia and diminished reflexes (Talbot, 2002).

Motor neurone disease is rare, with an overall prevalence of around 6 in 100,000. Incidence rises with age and this is estimated at approximately 1-2 per 100,000 per annum overall, increasing to 10 in 100,000 in people aged 65-85. Only 10 % of cases are familial (inherited), and the remaining 90 % sporadic (Shaw, 1999).

The aetiology of MND is unknown yet. Degeneration of the motor neurones leads to weakness and wasting of muscles. This generally occurs in arms or legs initially, some groups of muscles being affected more than others. Some individuals may

develop weakness and wasting in the muscles supplying the face and throat, causing problems with speech and difficulty with chewing and swallowing. Therefore, the symptoms of disease are spasticity, weakness, paralysis and impairment of speech, swallowing and breathing problems. There is no effective treatment for MND that substantially halts or slows disease progression. The average survival is only about three years after the starting of symptoms (Shaw, 1999, Strong, 2003).

Some authors term the Motor Neurone Disease as Amyotrophic Lateral Sclerosis (ALS). MND is the generic term used more in Europe whilst ALS is sometimes used more generically in the USA. These diseases are also known as “Maladie de Charcot”, and are often referred to in America as Lou Gehrig’s disease, after the famous baseball player who died of the disease. The most famous person who has MND is Professor Stephen Hawking, the Cambridge University physicist, mathematician and cosmologist.

Motor Neurone Diseases can be classified as follows:

1) Idiopathic Motor Neurone Diseases:

- Amyotrophic Lateral Sclerosis (ALS),
- Progressive Bulbar Palsy (PBP),
- Progressive Muscular Atrophy (PMA),
- Hereditary Spastic Hemiplegia (HSH),
- Spinal Muscular Atrophy (SMA),
- Spinobulbar Atrophy (SBMA or Kennedy’s disease),
- Primary Lateral Sclerosis (PLS),

- Familial ALS,
- Juvenile ALS,
- Madras Motor Neurone Disease,
- Monomelic Motor Neurone Disease.

2) Toxin-related Motor Neurone Diseases:

- Lathyrism,
- Konzo and Guamanian ALS.

On the other hand, Figlewicz and Orrell suggest another classification. They suggest that MNDs may be divided into three categories according to their motor neuron involvements (Figlewicz and Orrell, 2003):

i) Motor Neurone Diseases with lower motor neuron involvement:

- Spinal Muscular Atrophy (SMA),
- Spinobulbar Muscular Atrophy (SBMA or Kennedy's disease),
- Progressive Muscular Atrophy (PMA)

ii) Motor Neurone Diseases with upper motor neuron involvement:

- Primary Lateral Sclerosis (PLS)1,
- Primary Lateral Sclerosis (PLS) 2,
- Spastic Paraplegias;

iii) Motor Neurone Diseases with combined upper and lower motor neuron involvement:

- Amyotrophic lateral sclerosis (ALS),
- Progressive Bulbar Palsy (PBP).

Amyotrophic lateral sclerosis (ALS), the commonest form (over 80 percent) of motor neuron disease (MND), is an age dependent fatal neurodegenerative disorder due to motor neurons' degeneration. The overall prevalence is around 5 in 100 000. The aetiology of MND is largely unknown at present. Degeneration of the motor neurones leads to weakness and wasting of muscles which generally occurs in arms, legs or bulbar region (Strong, 2003). There is no effective treatment for ALS that substantially halts or slows disease progression (Strong, 2003). The majority of individuals affected with ALS live for less than 5 years, usually about three years (Shaw, 1999). Animal model for ALS is transgenic mouse expressing mutant superoxide dismutase 1 (SOD1).

1.2.4 Gene Therapy

Although there are many definitions, one of the simplest and concise definitions of gene therapy can be the introduction of DNA-based genetic material into humans for treating genetic disorders. According to GTAC (UK Gene Therapy Advisory Committee), gene therapy is “The deliberate introduction of genetic material into human somatic cells for therapeutic, prophylactic or diagnostic purposes.” (12th Annual Report of the UK Health Minister’s Gene Therapy Advisory Committee – GTAC- , 2005).

More detailed definition of GTAC about gene therapy is as follow:

“... [a] gene therapy medicinal products mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either in vivo or ex vivo, of a prophylactic, diagnostic or therapeutic gene (i.e. a piece of nucleic acid), to

human / animal cells and its subsequent expression in vivo. The gene transfer involves an expression system contained in a delivery system known as a vector, which can be of viral, as well as non-viral origin. The vector can also be included in a human or animal cell.” (The definition was given in Part IV of Directive 2003/63/EC [amending Directive 2001/83/EC]) (GTAC - 12th Annual Report, 2005). Thus, the main goal of gene therapy is to treat genetic diseases by using genes as a therapeutic drug (Lasic, 1997), and its main effort is to deliver normal copies of missing or defective genes into the individual.

Gene therapy can be classified into two broad categories:

- **Germ-line gene therapy**
- **Somatic gene therapy**

In germ-line gene therapy, reproductive germ cells are altered. Therefore, the modifications of germ cells can affect the patient’s offspring because the genetic changes will be passed on to next generations. On the other hand, somatic gene therapy involves only the alteration of somatic cells, and therefore, the genetic changes only affect the individual, not his / her offspring. Somatic cell gene therapy is now the only gene therapy technique in use in humans.

There are also two principal ways in which vectors (gene transporters) can be administered to carry new genes into target cells:

- **Ex vivo gene therapy**
- **In vivo gene therapy**

In ex vivo somatic gene therapy, the target cells are removed from the body; vectors with new gene are inserted into the cells in the laboratory and cultured, and then the cells with vectors are reinserted into the body. In contrast, in vivo somatic gene therapy, the vectors with the gene are directly injected into the body, either into the target cells of the tissue of interest (in situ), or the bloodstream for systemic delivery.

1.2.4.1 The historical background of gene therapy

Although the first approved gene therapy clinical trial started 15 years ago, in 1990 (Blease et al. 1995), gene therapy discussions and experiments began much earlier. The 'gene therapy' term was coined to distinguish it from the Orwellian connotations of human genetic engineering, which was derived from the term genetic engineering (Wolf and Lederberg, 1994). The 'genetic engineering' term was first used at the 6th International Congress of Genetics held in New York in 1932 (Crow, 1992; Wolf and Lederberg, 1994). The experiments of gene transfer into animals and humans increased after the beginning of gene transfer in bacteria in 1960s (Wolf and Lederberg, 1994). The earliest discussions of possibility for gene therapy was made by Edward Tatum in a letter dated October 1962. 4 years later, E. Tatum, Joshua Lederberg and others discussed the possibility of gene therapy at a symposium in New York, in May 1966 (Tatum, 1966, Lyght, 1967). In 1970, Stanfield Rogers tried to treat two girls with a genetic disease, argininemia by argininase gene in a papilloma virus; but the attempt was unsuccessful (Rogers, 1976). Discussions of gene therapy maintained their place in scientific agenda, especially in publications (Davis, 1970; Freese, 1971; Anderson, 1972; Friedmann and Roblin, 1972; Morrow, 1976). Then, in 1977, Michael Wigler and Richard Axel transferred a gene into mammalian cells

(Wigler et al, 1977). In 1979, W. F. Anderson succeeded in the first transfer of a single copy of a functional Thymidine Kinase gene using microinjection into mouse cells and corrected the genetic deficiency of the cells (Anderson et al, 1980). In Early 1980s, viral vectors were developed and used for transferring genes into cells. The first retroviral, (Joyner et al, 1983; Miller et al, 1983; Watanabe and Temin, 1983, Mann et al, 1983 and Willis et al, 1984), then adenoviral (Van Doren et al, 1984) and adeno-associated viral vectors (Tratschin et al, 1984 and Hermonat and Muzyczka, 1984) were developed. The first in vivo gene transfer was obtained in 1985 (Eglitis et al, 1985). In 1989, first human gene transfer clinical trial took place in 1989 (Rosenberg et al, 1990) and next year, the first gene therapy journal, Human Gene Therapy, began to be publishing.

On 14th September, 1990, the first approved successful gene therapy clinical trial took place and an US team transferred the T cells to 4 years old girl Ashanthi DeSilva with ADA – deficient Severe Combined Immunodeficiency (SCID), by retroviral vector carrying normal ADA gene (Blease et al. 1995). The attempt was successful. After ADA-deficient SCID trial, the first non-viral gene therapy clinical trial began in 1992 and Gary Nabel et al injected a DNA/liposome complex into HLA-B7 tumours (Nabel et al, 1992). Gene therapy clinical trials had increased rapidly and in 1993, the 100th gene therapy clinical trial was submitted to US authorities for approval. And then, in September 1999, the first gene therapy victim occurred in the Institute of Human Gene Therapy of the University of Pennsylvania. Jesse Gelsinger, an 18 year-old male from Arizona, died following infusion of an adenoviral vector. He was the only patient to have ever died as a direct result of a gene therapy application.

1.2.4.2 Gene delivery vectors

The most important issue in gene therapy is the delivery of the genetic material into the target cells in the body. The vehicles to deliver the gene of interest to the target cell are defined as vectors. Therefore, the choice of vector is crucial in any gene transfer protocol because it can result in either success or failure.

Currently there is no ideal or a “good universal vector” in gene therapy (Fry and Wood, 1999). All of the vectors that are currently available have both advantages and disadvantages. Different vectors have different disadvantages as follows: Low efficiency (non-viral vectors), high labour and high cost production (viral vectors); inability to infect nondividing cells (retroviral vectors); safety concerns including unwanted immunological effects (adenoviral vectors) and cytotoxic effects (Herpes simplex virus vectors); the capacity of carrying limited genetic material (adeno-associated viral vectors) and so forth. Therefore, some authors suggest that an ideal gene delivery vector should fulfil at least the following requirements: (Somia and Verma, 2001; Pfeifer and Verma, 2001)

- Safety: The vector should be non-toxic, non-inflammatory, non-immunogenic and should be stable for safe storage;
- High transfection efficiency;
- The capacity of easy design and production in high concentration at low cost;
- Sustained and regulated transgenic expression: The gene delivered by the vector should be expressed in a desired way. The gene or the product of the gene should be biodegradable; its expression should be under the control of a reversible manner;

- Long-term expression: The gene delivered by the vector should be expressed and be stable for a long-term.
- Specific targeting of expression: The vector should be specifically targeting the cells or tissues of interest.
- Infection of dividing and nondividing cells: The vector should be able to efficiently transduce all kind of cells, both dividing and nondividing alike.
- Site-specific integration: The vector should integrate the delivered gene into the host genome at specific site(s) to repair specific genetic defect(s).

Current gene therapy (somatic) vectors can be divided into two categories and thus classified as viral and non-viral vectors. The viruses used as viral vectors invade host cells, and transport their genetic information into the nucleus either to become part of the host's genome by integrating the transgene into the host's genome, or to constitute an autonomous genetic unit. On the other hand, the non-viral vectors are synthetic or are physical gene delivery system and they do not integrate the transgene into the host's genome, they stay as episomal.

1.2.4.2.1 Viral Vectors

Viral vectors can be classified in different ways, usually as follows:

1) Based on their genetic materials:

- a) RNA virus vectors (Retroviral and lentiviral vectors),
- b) DNA virus vectors (Adenoviral, adeno-associated viral, herpes simplex virus – HSV- vectors, and others),

2) Based on their integration into the host's genome:

- a) Integrating vectors (Retroviral, lentiviral and adenoassociated viral vectors),

b) Unintegrating vectors (Adenoviral vectors and HSV),

3) Based on their envelope: (Table 1.2)

a) Enveloped vectors (Retroviral and lentiviral vectors, HSV),

b) Non-enveloped vectors (Adenoviral and adeno-associated viral vectors)

VIRAL VECTORS							
Vector	Genetic material	Packaging capacity	Tropism	Vector genome forms	Inflammatory potential	Main limitations	Main advantages
<i>Enveloped</i>							
Retroviruses	RNA	8 kb	Dividing cells only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis	Persistent gene transfer in dividing cells
Lentiviruses	RNA	8 kb	Broad (dividing and non-dividing)	Low	Integrated	Integration might induce oncogenesis	Persistent gene transfer in most cells
HSV	dsDNA	>30 kb	Broad (neurons, muscles and stem cells)	High	Episomal	Inflammatory	Large packaging capacity
<i>Non-enveloped</i>							
AAV	ssDNA	4.7 kb	Broad	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-inflammatory; non-pathogenic
Adenoviruses	dsDNA	7.5 kb	Broad	High	Episomal	Inflammatory	Extremely efficient transduction of most tissues

Table 1.2: The main groups of viral vectors (adapted from Thomas et al, 2003)

1.2.4.2.1.1 Retroviral vectors:

Retroviruses are RNA viruses and thus, their genomes contain two identical single-stranded RNA molecules in the size range of 3.5-10 kb (Fry and Wood, 1999). They also have their own reverse transcriptase enzymes. Therefore, they insert their RNA genome into the host cell DNA. Once integrated, these vectors become a part of the host DNA and express the transgene throughout the life of the cell. Therefore,

retroviruses give good gene expression over a long term. Retroviral vectors were subjected to the first clinical trial on human gene therapy for the correction of ADA deficiency (Blease et al. 1995) as mentioned before. The large part of the retrovirus vectors currently used in gene therapy models are derived from the simple Maloney murine leukaemia virus (MLV), a C-type oncoretrovirus. Therefore, the retroviruses are also called as oncoretroviruses.

The titres of retroviruses are in low range (up to 1×10^7 colony-forming units per ml) (Fry and Wood, 1999). But newly developed pseudotyped retroviruses have higher titres and a much broader host range than the wild type retroviruses (Lundstrom, 2003). For pseudotyping, the MLV envelope glycoprotein is generally pseudotyped with the G protein of vesicular stomatis virus (VSG-G) (Thomas et al, 2003).

Retroviruses can only transduce dividing cells; therefore they cannot infect non-dividing cells. However, most of the cells in an adult body do not divide, including neurons and muscle cells. The inability of infecting non-dividing cells is the main disadvantage of the retroviruses.

1.2.4.2.1.2 Lentiviral vectors

The lentiviruses are also RNA viruses. Lentiviruses are often pseudotyped with VSG-G. Although lentiviruses are members of the retrovirus family, and belong to a subclass of retroviral vectors, their special features necessitate a separate description for them. The majority of the lentiviral vectors used in gene therapy are derived from human immunodeficiency virus (HIV). Therefore, lentiviral vectors can accommodate

larger gene inserts and then provide long-term transgene expression (Vigna and Naldini, 2000; Lundstrom, 2003).

Since lentiviruses are a subclass of retroviruses, they have the advantage of possessing the ability to integrate into the host genome. Contrary to retroviral vectors, the lentiviral vectors can also infect efficiently the non-dividing cells, and can thus be applied for non-dividing, terminally differentiated mammalian cells, including lymphocytes, macrophages, neuronal and muscle cells. This feature makes lentiviral vectors very attractive among gene therapy delivery systems (Trono, 2000; Pfeifer and Verma, 2001). Recently, some lentiviral vectors were improved and developed for transduction of hepatocytes in vivo (Follenzi et al, 2002). Like other retroviruses, lentiviruses have also medium size packaging capacity, around 8 kb.

1.2.4.2.1.3 Adenoviral vectors (Ad)

Adenoviruses are non-enveloped, medium sized, double stranded DNA viruses with 36 kb genome. They do not integrate into the genome of the target cell. They remain as an extra chromosomal entity in the nucleus of the target host cell. Adenoviral vectors were introduced to the field of gene therapy in the early 1990s. Adenoviral vectors were first described in the early 1950's (Ginsberg, 1999; Warren et al, 2003). Since then they have undergone three stages of their evolution, namely the first, second and third generations of Adenoviral vectors (Warren et al, 2003).

Adenoviral vectors are very efficient at transducing a large number of cell types, even nondividing cells, and have a high level transgene expression. However, the target

cells for adenoviral vectors are liver cells, and almost 100% of liver cells are transduced following injection of an adenoviral vector into a mouse's tail (Morral et al, 1999; Fry and Wood, 1999).

The key features of adenoviruses, such as the ability of generating high-titer virus stocks and the high level transgene gene expression, have made them very popular gene therapy vectors. Early versions (E) of adenoviruses had very toxic side effects and strong immunological reactions. But the many viral genes of the earlier versions of adenovirus vectors were deleted in their newer, late generation vectors (L), such as second and third-generations, and their immunogenicities have been decreased by significant improvements. Despite the recent improvements, a major disadvantage of adenoviral vectors in vivo is still immune responses. Because most people have been exposed to natural adenovirus' infections, such as common cold viruses, the antibodies of the host's body recognise the adenoviral vectors and produce a response against them. Consequently, this response can cause the destruction of vector-transduced cells, and leads to local tissue damage and inflammation (Fry and Wood, 1999).

1.2.4.2.1.4 Adenoassociated viral vectors (AAV):

The adeno-associated viruses (AAV) are nonpathogenic parvoviruses, and contain single stranded DNA genome of 4.7 kb (Pfeifer and Verma, 2001). In spite of this, eight serotypes of AAV have been identified in humans and primates, the most AAV vectors derived from AAV2 (Pfeifer and Verma, 2001; Thomas et al, 2003).

Currently, AAV vectors are among the most popular gene delivery system, because they have long-term and efficient transgene expression in a large number of cell types such as liver, muscle, retina, and CNS (Rabinowitz and Samulski, 1999). Nevertheless, they still have some disadvantages, such as small packaging capacity. However, unlike the adenoviruses, which can cause a high degree of cell damage (cytopathogenicity), the adeno-associated viral vectors cause only a little damage to the host cells (Fry and Wood, 1999) and they do not appear to be associated with toxicity.

1.2.4.2.1.5 Other viral vectors

Other than above vectors, there remain several other different viruses used in gene therapy delivery systems. Some of these are herpes simplex viruses (HSV), baculoviruses, alpha-viruses, pox viruses, Epstein-Barr viruses and vaccine viruses. They all also have several limitations along with their advantages.

1.2.4.2.2 Nonviral Vectors

Along with viral vectors, there are also many nonviral vectors that deliver transgenes into the target cells by physical and/or chemical techniques. The use of nonviral vectors involve mainly two ways, which are a direct injection of naked / plasmid DNA (pDNA), or mixing naked plasmid DNA with physical and/or chemical compounds that allow it to cross the cell membrane and protect DNA from degradation. Therefore, nonviral gene delivery systems can be classified into two main categories: synthetic vectors including cationic lipids and polymers, and naked plasmid DNA (Huang, et al, 1999). Some synthetic vectors for delivery DNA are as

follow: cationic carriers such as cationic lipids or liposomes, cationic and also non-ionic polymers, cationic amphiphiles; electroporation and nucleofection; in vivo DNA electro transfer, biolistic gene gun and ballistomagnetic gene transfer, jet injection, ultrasound and microbubble-enhanced ultrasound, hydrodynamic pressure, gene-activated matrices (GAM) and so forth (Schmidt-Wolf and Schmidt-Wolf, 2003).

Main groups of non-viral vectors can be seen from Table 1.3.

There are also some other types of gene delivery vectors, such as hybrid vectors (viral and nonviral components), peptide based gene delivery systems, nanoparticles, and physical and chemical combinations (magnetofection) (Schmidt-Wolf and Schmidt-Wolf, 2003).

NONVIRAL VECTORS							
Method	Size of DNA	Target cells	Transfection efficiency	Gene expression	Preparation	Application	
Naked plasmid DNA	No limit	myocytes	10-30%	Until death of cell/myocyte	Easy and cheap	In vivo	
Electroporation	150 kb	Mitosis / resting	Stable <0.1-1%	-	Easy	In vitro	
Microinjection	No limit	Mitosis / resting	Stable <0.1-1%	-	200/400 inject/h	In vitro	
Particle bombardment	10 000 copies	Mitosis / resting	Stable <0.1-1% transient <20%	2-12 months	Easy	In vitro and in vivo	
Lipofection	No limit	Mitosis / resting	Stable <0.1-1% transient 80%	-	Easy	In vitro and in vivo	
Ligand mediated	> 48 kb	Mitosis / resting	Up to 80% in vitro	High, transient	Labour intensive	In vitro and in vivo	

Table 1.3: Main groups of non-viral vectors. (Adapted from Schmidt-Wolf, 2003)

1.2.4.2.2.1 Naked / Plasmid DNA

Naked DNA is an attractive nonviral vector because of its simplicity. For a long time it has been known that muscle cells are capable of taking up and expressing plasmid DNA. Gene transfer by naked plasmid DNA to the skeletal muscle was first demonstrated by intramuscular injection 15 years ago. Wolff et al showed that the plasmid DNA could be delivered into mouse muscle cells in vivo and result in an expression (Wolff et al, 1990). Plasmid DNA can stably express in muscle for at least 2 years (Wolff et al, 1992).

Contrary to common belief, long-term transgene gene expression from naked plasmid DNA is possible even without chromosome integration if the target cell is postmitotic (as in muscle) or slowly mitotic (as in hepatocytes) (Wolff *et al*, 1992; Miao *et al*, 2000; Herweijer *et al*, 2001; Zhang *et al*, 2004; Wolff and Budker, 2005).

The administration of naked nucleic acids including naked plasmid DNAs and siRNAs into animals has increasingly been used as a valuable research tool (Herweijer and Wolff, 2003; Hodges and Scheule, 2003; Wolff and Budker, 2005).

They have also been used in several human clinical trials, such as Duchenne muscular dystrophy, peripheral limb ischemia, and cardiac ischemia (Davis *et al*, 1996; Tsurumi *et al*, 1997; Romero *et al*, 2002; Wolff and Budker, 2005).

Nonviral vectors including plasmid DNA have no viral genes and, unlike viral vectors, they cannot cause diseases. For instance, plasmid or naked DNA, unlike viruses, does not contain a protein coat (hence the term “naked”). Because of not

having a protein coat, the naked DNAs cannot move from a cell to another cell freely, and integrate into the chromosome. As a result of not having a protein coat, the naked DNA does not cause harmful reactions as viruses. Therefore, it seems today that the simplest and safest nonviral gene delivery system among current gene therapy systems is the direct injection of naked / plasmid DNA, although it is too early to say which gene transfer method and gene therapy approach is safest and most efficacious (Wolff, 1997; Schmidt-Wolf and Schmidt-Wolf, 2003). In spite of its safety, simplicity and reliability, the plasmid DNA has also its limitations such as low efficiency. Because the transfection efficiency of plasmid DNA by intramuscular injection is relatively low, it only establishes transgene expression at the injection site or the injected muscle (Liu et al, 2001). In contrast, many muscle disorders, such as muscular dystrophies, require therapeutic gene expression in most muscles in the body. Therefore, plasmid DNA needs to be administered systemically in order to achieve effective gene therapy for such disorders (Liu et al, 2001).

1.2.4.2.3 Comparison of Gene Transfer Vectors

As mentioned before, the ideal gene delivery system should be specifically targeting, non-toxic, non-immunogenic, non-inflammatory, biodegradable; carrying large genetic material, having high efficiency, being produced at low cost and less labour (Schmidt-Wolf and Schmidt-Wolf, 2003). Nevertheless, none of the gene therapy delivery systems currently in use meets all the above criteria.

Viral vectors have problems with safety, toxicity, immunologic reactions, and the lack of cell specific targeting. Furthermore, viral vectors are rapidly cleared from the blood

circulation by limiting transfection (Schmidt-Wolf and Schmidt-Wolf, 2003). On the other hand, nonviral delivery systems, particularly naked plasmid DNA, have several advantages over viral vectors:

- Nonviral vectors are generally easy to prepare and scale up. The design and production of naked plasmid DNA is cheaper with less labour; whereas virus and viral vector production are quite expensive and labour intensive.
- Nonviral vectors generally have no limitations for the size of delivered gene, whereas viral vectors have limitations by the size of their viral capsid; therefore nonviral vectors do not have such problems in carrying larger genetic materials;
- Nonviral vectors are generally much safer in vivo. Some viral vectors may still retain viral genes and promoters that could express in human cells under certain conditions, and they can cause some dreadful diseases or severe immune responses. Such dreadful diseases and severe reactions did occur in the near past. For instance, in 2000, 18 years old male patient, Jesse Gelsinger, died during a viral vector based gene therapy clinical trial by Institute of Human Gene Therapy of University of Pennsylvania of USA (Nature news, 2000). And again, in 2002, two boys of ten children who have severe combined immune deficiency (SCID) had developed leukaemia –like conditions during a French retroviral based gene therapy clinical trial for SCID led by Drs Alan Fisher and Marina Cavazzano-Calvo of the Necker Hospital for Sick Children in Paris (Nature news, 2002). On the contrary, the naked plasmid DNA does not elicit a specific immune reaction because of not having a protein coat unlike viruses, and therefore it does not induce an antibody response against itself, such as anti-DNA antibodies. Therefore naked plasmid DNA administrations are reasonably safe and can be administered repeatedly (Jiao et al, 1992).

The efficiency of gene transfer of the currently available nonviral delivery systems in delivering transgene into skeletal muscle and other tissues by direct injection is relatively low and variable in contrast to viral vectors *in vivo*, especially in larger animals such as nonhuman primates (Jiao et al, 1992). However, in recent years efficiency of naked plasmid DNA transfer has been increased. The recent developments demonstrate that naked plasmid DNA can be delivered efficiently to cells *in vivo*, either via electroporation, or by intravascular delivery (Herweijer and Wolff, 2003; Wolff and Budker, 2005).

Herweijer and Wolff suggest that electroporation increases uptake of injected naked plasmid DNA into muscle tissue and skin (Herweijer and Wolff, 2003). The technical improvements in electroporation have increased both gene expression efficiency and safety (Somari et al, 2000). Many recent publications have demonstrated that electroporation techniques have increased gene expression in different cell types *in vivo*. For instance, they have increased the gene expression levels in muscle 10-fold higher than injection of plasmid DNA without electroporation (Hartikka et al, 2001).

Intravascular injection of pDNA also results in a very effective gene transfer in liver and muscle. Particularly tail vein plasmid DNA delivery has made naked DNA transfer a very effective, viable gene therapy method. Plasmid DNA delivery via the tail vein in rodents provides a simple and effective *in vivo* gene transfer technique.

In this method, plasmid DNAs were delivered by rapid injection in large volumes (around 10% of the body weight) via the portal vein, the hepatic vein, and the bile

duct in mice and rats. The rapid delivery of large volume into a vessel leads to the liver by the vena cava. High levels of gene expression were first achieved by the rapid injection of naked DNA in large volumes via an artery route with both blood inflow and outflow blocked surgically (Budker *et al*, 1998; Zhang *et al*, 2001; Wolff and Budker, 2005). Intravenous delivery has also been shown to be effective (Liu *et al*, 2001; Hagstrom *et al*, 2004; Liang *et al*, 2004; Wolff and Budker, 2005). The tail vein delivery results in very high level of gene transfer. For example, 10-15% of the hepatocytes are transfected in mice following injection of 10 µg pDNA, whereas the expression level in liver rose up to 40% by tail vein injection method (Herweijer *et al*, 2001; Maruyama *et al*, 2002; Zhang 2002; Herweijer and Wolff, 2003). The procedure also proved effective in larger animals such as dogs and nonhuman primates (Eastman *et al*, 2002; Zhang *et al*, 1997).

The Wolff group has also demonstrated high level gene expression *in vivo* in different skeletal muscle groups in mice, rats, dogs and monkeys via intravascular plasmid DNA delivery (Herweijer and Wolff, 2003). By this method, small interfering RNA (siRNA) can also be delivered very efficiently and results in a knock out of the target gene expression (Lewis *et al*, 2002; McCaffrey *et al*, 2002; Wolff and Budker, 2005). On the basis, it seems accurate to suggest that nonviral gene transfer techniques, particularly naked plasmid DNA delivery, will become much more important.

1.2.4.3 Skeletal Muscle and Gene Therapy

Skeletal muscle is an attractive target tissues for gene therapy applications of both muscle and non-muscle disorders due to its anatomical, cellular and physiological

properties. Gene transfer into muscle tissue can produce a variety of physiologically active proteins and may ultimately be applied to the treatment of many diseases.

Several factors make skeletal muscle an attractive route for gene transfer: i) The tissue is abundant, making up about 40% of the body weight of an average adult; ii) skeletal muscle is accessible to most of the delivery systems currently used for gene transfer; and iii) there is no significant cell replacement in skeletal muscle tissue, so that introduced genes are not rapidly lost following mitosis, and transgene expression can thus persist for relatively long periods (Liu et al, 2001). Since the first report in 1990, using skeletal muscle as a direct target for transgene via naked plasmid DNA delivery (Wolff, 1990), many publications have identified that different proteins can be synthesized and correctly processed by skeletal muscle.

So far, almost all gene delivery systems, both viral and nonviral, have been used for delivering transgenes to muscle tissue, particularly skeletal muscle. As well as nonviral systems, many genetically modified viruses, such as retroviruses, lentiviruses, herpes simplex viruses, Epstein-Barr virus, adenoviruses and adeno-associated viruses have tested for gene delivery vehicles into muscle (Lu et al, 2003). Among above, adenoviral and adeno-associated viral vectors have been found to be the most efficient vehicles so far for transducing muscle fibers (Lu et al, 2003).

1.2.4.4 Clinical Trials of Gene Therapy (Worldwide)

A clinical trial is a study in human volunteers to answer specific questions about new drugs or therapies to treat diseases. Therefore, clinical trials are used to determine

whether new drugs or treatments are safe and effective in human subjects. Clinical trials have the following four phases: Phase I, II, III and IV.

Until January 2007 (between 1989 and July 2006), a total of 1272 gene therapy clinical trials have taken place worldwide. (The majority of these trials (67%) were conducted to treat different cancers (The website of the Journal of Gene Medicine, Updated Jan 2007 - <http://www.wiley.co.uk/genetherapy/clinical>). The classification of the trials can be seen in Figure 1.13.

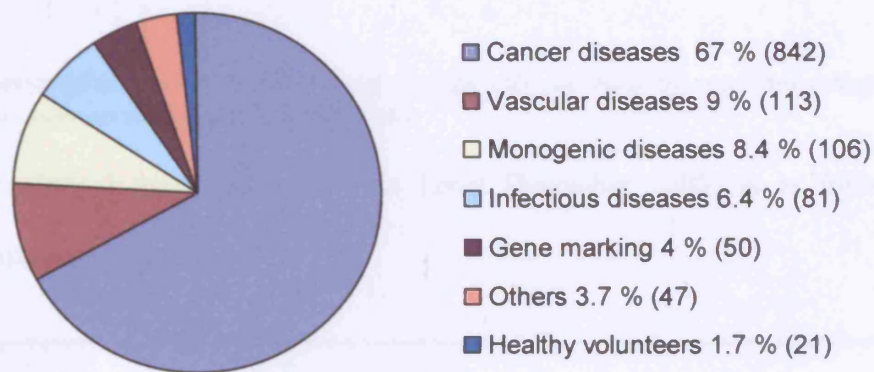


Figure 1.13: Indications addressed by gene therapy clinical trails (adapted from <http://www.wiley.co.uk/genetherapy/clinical/>)

On the other hand, as can be seen from Figure 1.14, in the same period (between 1989 and January 2007), the majority of these gene therapy clinical trials (65 %) had been conducted in the USA. Only 28 % of these were in European countries (Asia 2.9 %, Australia 1.5 % and Africa 0.1 %) (The website of the Journal of Gene Medicine, Updated Jan 2007 - <http://www.wiley.co.uk/genetherapy/clinical>). 12 % of the trials had been conducted in the UK; followed then by Germany (5.9 %) and Switzerland (3.3 %). The other European countries such as France, Belgium, Italy and the

Netherlands were in a 1-2 % in range (1.6%, 1.5%, 1.2%, and 1% respectively) (The website of the Journal of Gene Medicine, updated Jan 2007).

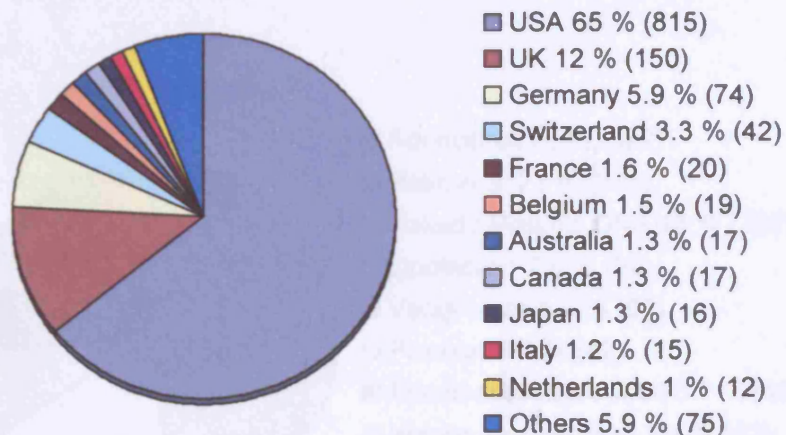


Figure 1.14: Geographical distribution of gene therapy clinical trials by countries (adapted from <http://www.wiley.co.uk/genetherapy/clinical/>)

The complete clinical trials list worldwide (until December 2005) is as follows (GTAC 12th Annual Report, 2005):

<u>COUNTRIES</u>	<u>%</u>	<u>COUNTRIES</u>	<u>%</u>	<u>COUNTRIES</u>	<u>%</u>
Australia	1.5	Germany	6.5	Singapore	0.2
Austria	0.2	Israel	0.5	South Korea	0.3
Belgium	1.6	Italy	1.1	Spain	0.3
Canada	1.1	Japan	1.3	Sweden	0.2
China	0.3	Mexico	0.1	Switzerland	3.5
Czech Republic	0.1	Netherlands	0.6	UK	11.7
Denmark	0.2	New Zealand	0.2	USA	64.8
Egypt	0.1	Norway	0.3	Multi-country	1.0
Finland	0.3	Poland	0.3		
France	1.7	Russia	0.1		

Table 1.4: Gene therapy clinical trials in worldwide by December 2005. (Source: Journal of Gene Medicine – taken from GTAC 12th Annual Report, 2005)

A various range of gene delivery systems was used for these clinical trials in the same period. More than 70% of the vectors used in the trials were viral vectors, and about

26 % were non-viral vectors (18 % of these were naked/plasmid DNA, and 7.9 % of them were lipofection). Complete vector distributions worldwide can be seen in Figure 1.15.

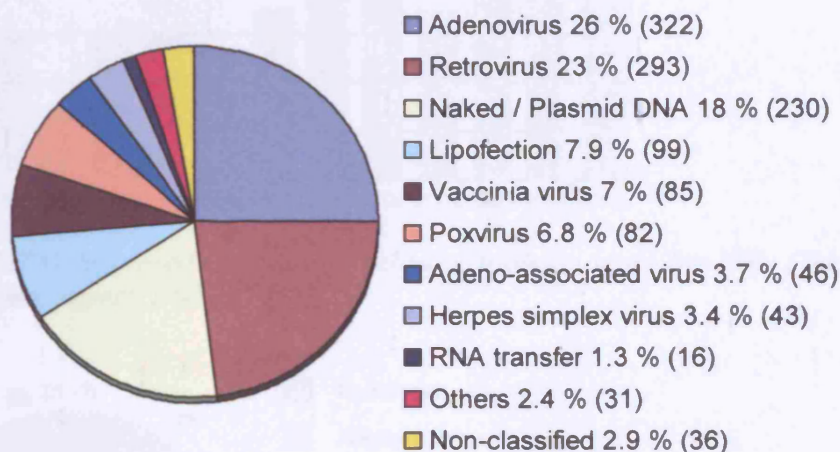


Figure 1.15: Vector used in gene therapy clinical trials (adapted from <http://www.wiley.co.uk/genetherapy/clinical/>)

1.2.4.5 UK Gene Therapy Trials

First gene therapy study was conducted in the UK in 1993. Since then, until December 2005, (1993-2005) UK Gene Therapy Advisory Committee (GTAC) processed 126 clinical trials applications and approved 113 of them. Five approved trials were then withdrawn. Remaining 108 trials were analysed as follows (GTAC 12th Annual Report [January 2005 to December 2005]):

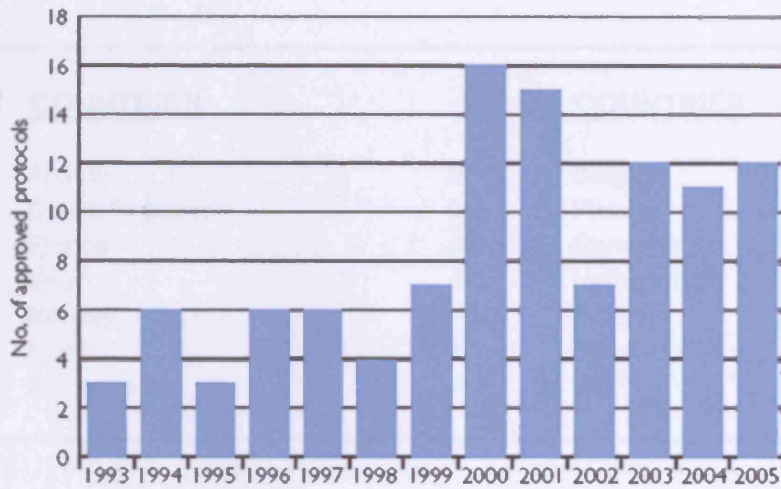


Figure 1.16: GTAC approved gene therapy clinical trials by years (n=108). (Taken from GTAC 12th Annual Report, 2005)

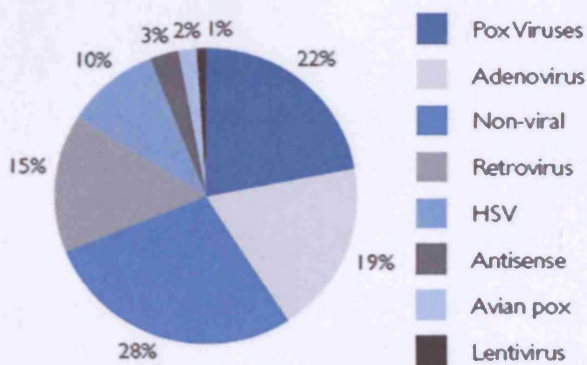


Figure 1.17: GTAC approved gene therapy clinical trials by vector system (n=108). (Taken from GTAC 12th Annual Report, 2005)

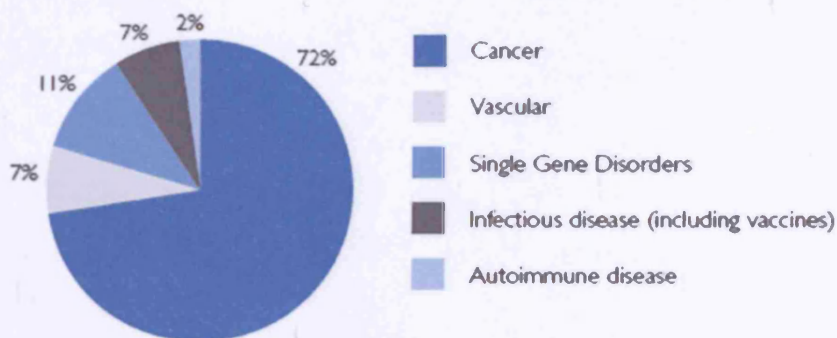


Figure 1.18: GTAC approved gene therapy clinical trials by diseases (n=108). (Taken from GTAC 12th Annual Report, 2005)

<u>COUNTRIES</u>	<u>%</u>	<u>COUNTRIES</u>	<u>%</u>
Austria	0.6	Belgium	5.6
Czech Republic	0.3	Finland	0.9
France	5.9	Germany	22.8
Italy	4.0	Netherlands	2.2
Norway	1.2	Poland	0.9
Spain	1.2	Sweden	0.6
Switzerland	12.3	UK	41.4

Table 1.5: Gene therapy clinical trials by countries in Europe by December 2005. (Source: Journal of Gene Medicine – taken from GTAC 12th Annual Report, 2005).

1.3 The Aim and Hypothesis of the Study

1.3.1 The purpose of the study

As can be seen from the introduction / background and the literature review of this study, neuromuscular / musculoskeletal disorders affect millions worldwide. The current expansion of the aged population in the developed world is predicted, by the World Health Organisation, to accelerate some of these diseases to epidemic proportions in the next two decades (Goldspink and Noble, 2006). Nevertheless, there is currently no effective treatment of any form of these disorders. Therefore, the treatment for such disorders is vital. Thus, the aim of this study was to attempt to develop a novel gene therapy application for treating muscle degeneration in such disorders.

The IGF-I has a very important role in cell and tissue development and growth, including proliferation, differentiation, repair and maintenance, and has several alternative splicing variants. One of these variants, MGF, also has a crucial role in tissue growth and repair, particularly in muscle; and it is now accepted as a general tissue growth/repair factor. There are some therapeutic implications of IGF-I for different pathological conditions including neuromuscular disorders. However, so far,

the mature peptide of IGF-I was only used for such therapeutic implications. Neither the therapeutic effects of IGF-I splice variants in such neuromuscular disorders, nor the roles of them were studied in primary cell culture models in human muscle from the patients with such disorders has never been investigated yet. Therefore, the main purpose of this study was to investigate such effects of IGF-I splice variants on degenerative muscle conditions in human and animals in vitro and in vivo models in comparison with normal conditions.

1.3.2 The purpose of in vitro part of the study

So far, the effects of IGF-I splice variants on human primary cell culture models have not been investigated. Therefore the aim of in vitro part of the study was to investigate such effects in in vitro cell culture models, comparing with rodent muscle cell lines with primary cultured human healthy and degenerative muscle cells such as with different muscular dystrophies and ALS.

1.3.3 The purposes of in vivo parts of the study

When cDNAs of different IGF-I splice variants in plasmid vectors are introduced into wild-type mouse skeletal muscle tissue, the MGF increased the muscle fibre size 25 % within 3 weeks (Goldspink and Yang, 2001; Goldspink and Yang, 2004). Similar experiments were carried out by Lee Sweeney's group (Barton-Davis et al, 1998) using cDNA of systemic liver-type of IGF-I in viral vectors. These experiments also resulted in a 15% increase in muscle mass (muscle force), but within over 4 months instead of 3 weeks.

Nevertheless, the effects of IGF-I splice variants were never investigated in a murine model of DMD, which is the mdx mouse. Therefore, the purpose of in vivo part of the study was to investigate in vivo effects of IGF-I splice variants in mdx mouse muscles. Thus, the aim of the first part of in vivo model of the study was to investigate the effect of such variants in muscle tissue growth by measuring maximum muscle force after introducing cDNAs of the variants to TA muscles of mdx mice by comparing with untreated – control TA muscles. On the other hand, Hill et al suggest that MGF has a significant effect in satellite cell activation after tissue damage in wild-type rats (Hill and Goldspink, 2003a, and Hill et al, 2003b). However, so far, no study has shown the roles of IGF-I splice variants in satellite cell activation mechanism in degenerative muscle tissue. The second part of in vivo model of the study concentrated on the effects of IGF-I splice variants with particular reference to MGF in satellite cell activation, which shows tissue growth and regeneration in skeletal muscles of mdx mice.

1.3.4 Hypothesis of the study

Following two hypothesis were established based on above background:

- 1- E domain of MGF, in comparison with IGF-I mature peptide, influences cell proliferation and prevents myogenic differentiation in primary cultured muscle cells from patients with CMD, FSHD and ALS, and rodent muscle cell lines.
- 2- The coding sequence of MGF, in comparison to the coding sequence of IGF-I Ea, improves the maximum tetanic contractile force of muscles and activates satellite cells in the mdx mouse.

Chapter 2:

Materials and Methods

2.1 Design of Experiments

2.1 The Design of Experiments

Emerging on the hypothesis of this thesis, the objective of this study was to investigate the roles of the IGF-I splice variants in muscle cell proliferation and differentiation. Therefore, the work focused on the roles of such variants in muscle cell proliferation and differentiation in *in vitro* and *in vivo* models. The work was designed as (i) *in vitro* cell culture model, (ii) *in vivo* animal model of mdx mouse.

- (i) *In vitro* - cell culture model experimentation was designed for investigating the effects of IGF-I splice variants in myoblast proliferation and differentiation in different vertebrates' species including human primary muscle cell culture from the patients with different neuromuscular disorders comparing with primary muscle cells from healthy individuals.
- (ii-a) *In vivo* - gene transfer experimentation was designed for investigating *in vivo* effects of IGF-I splice variants in muscle contractile force after introducing cDNAs of such variants into the skeletal muscles of the murine model of DMD;
- (ii-b) *In vivo* - satellite cell activation experimentation was designed for investigating the roles of such variants in skeletal muscle regeneration and

repair by determining gene and mRNA expression of the activated satellite cell markers after introducing cDNAs of such variants into TA skeletal muscles of mdx mouse.

2.1.1 In vitro - cell culture model experimentation

2.1.1.1 Cells

In in vitro part of the study, it was aimed to investigate the effect of IGF-I isoforms on different types of myoblasts from different vertebrates' species. Therefore, animals (rat and mouse) and human myoblasts were selected as different type of the myoblasts, which are muscle derived cells that contain a population with myogenic potential that can vary enormously.

The skeletal muscles differ according to their anatomy, functions, fibre – type composition, and as well as according to their embryologic origins among individual muscles, muscle groups and muscle regions. (Larsen, 1998; Carlson, 1999; Noden et al, 1999; Korfege et al, 2000; Buckingham, 2001; Buckingham et al, 2003; Korfege et al, 2005a; Korfege et al, 2005b). Therefore, as human primary muscle cells, different muscles from different parts of human body, such as craniofacial masseter muscle as a head-neck and cervical region, and quadriceps – vastus lateralis- muscle as a limb muscle were chosen for investigating the effects of IGF-I isoforms in different muscle groups of the human body. Along with different muscles from healthy individuals, the effects of IGF-I isoforms were also investigated in three different severe degenerative muscles from patients with CMD, FSHD and ALS.

To investigate the effects of IGF-I isoform on proliferation in different rodent myoblast cell lines, rat myoblast cell line (L6 E9) and mouse myoblast cell line (C2C12) were used.

For the proliferation assays, for human primary muscle cells, the following muscle cells were used:

- (i) Healthy craniofacial masseter muscles (obtained from biopsies of three healthy individuals). The assays were repeated twice.
- (ii) Healthy limb muscles, (obtained from biopsies of the vastus lateralis of two healthy individuals). The assays were repeated twice.
- (iii) Degenerative craniofacial masseter muscle with CMD (obtained from biopsy of a patient with CMD). The assays were repeated three times.
- (iv) Degenerative lower limb muscle with FSHD (obtained from biopsies of the vastus lateralis of two different patients with FSHD). The assays were repeated twice.
- (v) Degenerative lower limb muscle with ALS (obtained from biopsies of the vastus lateralis of three different patients with ALS). The assays were repeated twice.

For the differentiation assays with human primary muscle cells, healthy craniofacial masseter muscle cells were only used, because differentiation assays with muscle cells of masseter muscle, and those of limb muscles from the patients with different neuromuscular disorders failed.

2.1.1.2 Proliferation Assays

2.1.1.2.1 Proliferation assays for animal cell lines

For both animal cells, rat myoblast cell line (L6 E9) and mouse myoblast cell line (C2C12) were treated with E domain of IGF-I Ec [MGF]. Different concentration conditions of E domain of MGF peptide were used with both animal cell lines. The concentrations for E domain of MGF peptide were as follows: 0, 1, 2, 5, 50 and 100 ng/ml. The similar concentrations, which Yang and Goldspink were used their experiment before, were chosen (Yang and Goldspink, 2002). A set of 8 wells of a 96 well plate was used for each condition.

2.1.1.2.2 BrdU Proliferation Assay

BrdU (5-bromo-2-deoxyuridine) proliferation assay is a non-radioactive method for determination of cellular proliferation in living cells. BrdU works by substituting for thymidine during DNA replication and incorporating into the newly synthesised DNA. The antibodies, which are specific for BrdU, are used to detect the incorporating BrdU into the replicating DNA. Therefore, the indicating cells by a monoclonal antibody against BrdU show the proliferating cells. BrdU cell proliferation assays based on a 96 well microtiter plate are used as a parameter for routine cell proliferation applications. Thus, BrdU assays were used to investigate the role of MGF in myoblast proliferation in rat and mouse myoblast cell lines.

2.1.1.2.3 Proliferation assays for human primary cultured cells

For human myoblast cell, the cells were not sorted, therefore non-sorted / mixed cells were used in order to obtain maximum physiological condition, and also to reduce the cost of experiments. Because the cells were not separated, fibroblasts and myoblasts were together in the culture. Then again, because there were not only one type of cell in the culture, immunocytochemistry technique was chosen instead of BrdU proliferation assay in order to investigate the effect of IGF-I isoforms on myoblast proliferation in primary cultured human muscle cells, both in healthy and pathologic conditions.

Fluorescently labelled antibodies (Desmin and FITC) and a DNA binding fluorescent stain (DAPI) were used to determine different cell types in cultures. Thus, the ratios of progenitor myoblasts to other cells were measured after following treatment with different IGF-I isoforms. An indirect method of detection was used with a monoclonal primary antibody detected by a FITC conjugated secondary antibody and viewed using fluorescent microscopy.

2.1.1.2.3.1 Optimisation of proliferation assays

Initially, three time-points were used as 24, 48 and 72 hours in order to determine optimal culture time. The normal (healthy) human craniofacial primary cells were treated with MGF (500 ng/ml), long r³ IGF-I (10 ng/ml) and both MGF and r³ IGF-I together (500 and 10 ng/ml, respectively) for 24, 48 and 72 hours. For positive control, some cells were not treated with any of such peptides for the same time points. As can be seen from Figure 2.1, MGF had not increased myoblast proliferation

at either 24 or 72 hours. However, it increased myoblast proliferation at 48 hours (Figure 2.1). Therefore, 48 hours had been determined as the time point for the human primary cell culture experiments. After determination of the optimal time point, which was 48 hours, all cells in the experiment process were treated for 48 hours.

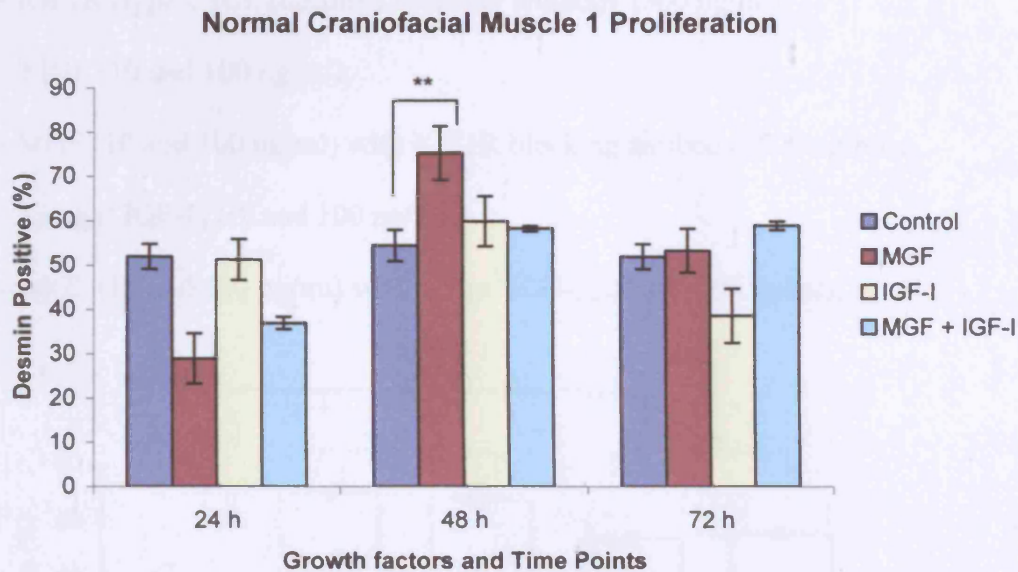


Figure 2.1: Investigating time points for myoblast proliferation on healthy human craniofacial primary cells. The concentration for MGF was 500 ng/ml; for long r3 IGF-I was 10 ng/ml and for MGF + long r³ IGF-I is 500 ng/ml (for MGF) and 10 ng/ml (for long r³ IGF-I). Data were presented as mean \pm s.d. $p < 0.05$ was considered significant.

As with the BrdU assays, two different IGF-I peptides were used as different IGF-I isoforms. For mature IGF-I receptor domain, recombinant human IGF-I (long r³ IGF-I) (Sigma) was used as IGF-I Ea isoform, and for IGF-I Ec, an E peptide of MGF was used that was synthesised with Department of Biochemistry in UCL. Also the IGF-I receptor blocking antibody (Ab-I) (Oncogene) was used in order to investigate whether or not MGF had a different signalling pathway from systemic IGF-I. The healthy and pathological human primary cells were also treated with Ab-I with and

without MGF. To determine optimal concentrations, different concentrations and repeats were used for MGF and long r³ IGF-I. Conditions were as follows:

- 1 – Only defined medium, (for positive control);
- 2 - IGF1R (type 1 IGF receptor) blocking antibody (500 ng/ml);
- 3 – MGF (10 and 100 ng/ml);
- 4 - MGF (10 and 100 ng/ml) with IGF1R blocking antibody (500 ng/ml);
- 5 – Long r³ IGF-I (10, and 100 ng/ml);
- 6 – MGF (10 and 100 ng/ml) with long r³ IGF-I (10 and 100 ng/ml);

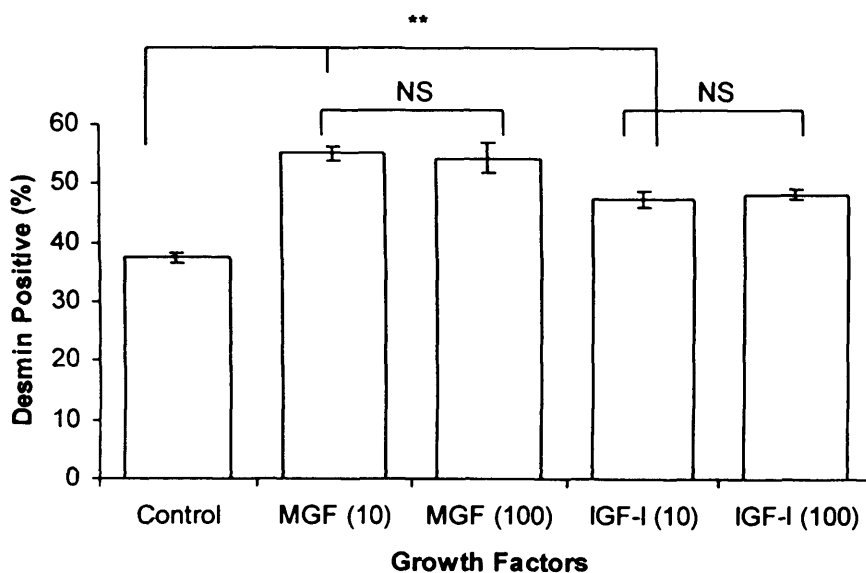


Figure 2.2: Investigating optimal concentrations for myoblast proliferation on healthy human limb muscle primary cells. The concentrations for MGF were 10 and 100 ng/ml; for long r³ IGF-I was 10 and 100 ng/ml. Data were presented as mean \pm s.e.m. $p < 0.05$ was considered significant. NS non-significant.:

As can be seen from Figure 2.2, which shows optimum dose concentration result, effects of E domain of MGF with 10 and 100 ng/ml were very similar. Similar effects were seen for long r³ IGF-I doses. Effects of long r³ IGF-I peptide with 10 and 100

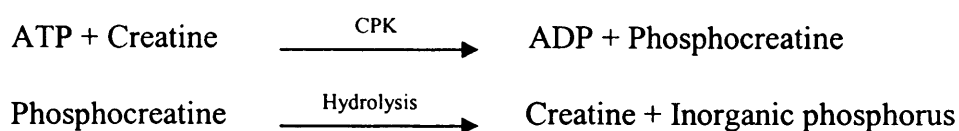
ng/ml were similar again (Figure 2.1). Therefore, optimum concentrations for both MGF and long r³ IGF-I were chosen as 10 ng/ml for all human primary cultured cell proliferation assays.

2.1.1.3 Differentiation assays

The role of IGF-I isoforms in differentiation was also investigated in the part of in vitro cell culture model of the study. As detailed to a certain extent in section 1.2.1.1.5, myoblasts differentiate to form multinucleated myotubes by cellular fusion. Therefore, the myotube formation was investigated in order to determine the roles of IGF-I isoforms in differentiation process in healthy and pathological human primary muscles. The creatine phosphokinase (CPK) activity on myotubes was measured for these differentiation assays because myoblasts does not have any CPK activity where as the myotubes do. Therefore, the CPK/Protein ratio was used in order to analyse the role of IGF-I isoforms in myoblast differentiation in human muscle cells. Hence, both CPK assays and BCA protein assays were used in order to investigate the role of IGF-I isoforms during myoblast differentiation / myotube formation.

2.1.1.3.1 Creatine phosphokinase (CPK) assay

The enzyme creatine phosphokinase (CPK) catalyses the reversible phosphorylation of ADP by phosphocreatine to form ATP and free creatine. The schematic reaction is as follows:



The reaction may be followed in either direction by measuring the formation of one or the other of the end products. CPK activity is measured using the colorimetric method based on the generation of phosphorus according to the above reaction. The inorganic phosphorus produced is then measured colorimetrically by Fiske and SubbaRow procedure and is proportional to CPK activity. The kit used for CPK assay in this study is the Sigma Diagnostic CPK kit. It is intended for use in the qualitative, colorimetric determination of creatine phosphokinase in serum at 620-800 nm.

2.1.1.3.2 BCA protein assay

BCA Protein Assay kit (Pierce) is detergent-compatible formulation based on bioinchroninic acid (BCA) for the colorimetric detection and quantitation of total protein. The method combines reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the sensitive and selective colorimetric detection of cuprous cation (Cu^{+1}) using a unique reagent containing BCA. The complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20 – 2 000 $\mu\text{g/ml}$).

2.1.1.3.3 The conditions for differentiation assays

For these assays 96 well – plates were used for both BCA protein assay and CPK assays (One plate was for CPK assay, and another plate was protein assay). For 96 well plates, 10×10^4 cells were put into each well. For 96 well plates, 16 conditions with 5 repeats (16 X 5) were used. The time point was the same as proliferations assays, 48 hours, for both assays.

The cells were treated again with and without MGF and long r³ IGF-I separately or together. Conditions for differentiation experiments were the same in both CPK and BCA protein assays. These are as follows: (1) - Only fusion medium, no growth factor, (for positive control); (2) - MGF (10 and 100 ng/ml); (3) - r³ IGF-I (10 ng/ml); and (4) - MGF (10 and 100 ng/ml) with long r³ IGF-I (10 ng/ml);

2.1.2 In vivo - gene transfer experimentation

2.1.2.1 Introduction

Up to the present time, various therapeutic strategies to treat DMD have been proposed. Many different viral and non-viral plasmid vectors have been used to deliver genetic materials to treat DMD. The main goal of gene therapy strategies for DMD so far was either to add a functional gene copy or to “repair” the mutated dystrophin gene. The main approach of this study was to regenerate degenerative muscles, instead of replacing or repairing the mutated dystrophin gene. For this aim, cDNA of IGF-I splice variants (IGF-I Ea and MGF) in plasmid/naked DNA vectors were transferred into limb skeletal muscles (TA muscles) of old and young mdx mice, and then 3 weeks after injection maximum muscle forces and weights of TA muscles were measured. Because the muscles of mdx dystrophic mouse are unable to express MGF even during mechanical stimuli (Goldspink and Yang, 2001; Goldspink and Yang, 2004; Goldspink, 2005), dystrophin deficient mdx mice was chosen as in vivo animal models and cDNAs of IGF-I variants were injected into TA muscles of mdx mice.

On the other hand, the skeletal muscle provides a good biological example of a structure-function relationship. Since the 1600s, anatomical, physiological as well as biomechanical properties of skeletal muscle have been studied in some detail (Blix, 1895; Lieber, 1999). Since then, it has been identified that the primary purpose of skeletal muscle is to generate force and movement. The anatomical structure of skeletal muscle, specifically the number and arrangement of muscle fibers, has a profound effect on muscle force and body movement (Lieber, 1999). Muscle mass in size and length and also the numbers of muscle fibre affect the muscle's contractile force. Therefore, the greater numbers of muscle fibers and the larger muscle fibres in size are all contribute for the increase in the contractile force of muscle (Marieb, 2001). Therefore, it is possible to investigate the changes of numbers and size of muscle fibers by measuring the maximum muscle force.

2.1.2.2 Effects of IGF-I splice variants based gene therapy

A considerable body of research suggest that muscle mass in size, particularly the total cross sectional area of muscle fibres affect muscle's contractile force. Therefore, large number of large muscle fibres increase the contractile force of muscle (Marieb, 2001). Thus, 21 days after injecting cDNAs of MGF and IGF-I Ea, and also a pcDNA3.1NT/GFP vector not containing any cDNA of IGF-I splice variants into one of TA muscles, the differences of maximum muscle force between injected and uninjected control muscles presented the effects of IGF-I variants as gene therapy agents in animal model of Duchenne Muscular Dystrophy (DMD).

2.1.2.3 Animals

The mdx mouse, an animal model of human DMD, was chosen for in vivo experimentation. Therefore, the mdx mouse, which is a genetic and biochemical homologue of the DMD, has been broadly used for a long time although it has a milder phenotype of human DMD. Because of this background, mdx mice were used for in vivo investigation of effects of IGF-I splice variants as gene therapy agents on Duchenne Muscular Dystrophy.

Before starting the animal experimentation, a Home Office laboratory animal management, welfare and surgery training (module 1, 2, 3 and 4) was undertaken. The training was successfully completed, and a personal licence was acquired for working with mouse, rat, rabbit, guinea pig and hamsters (from administrative responsibilities to major surgical procedures). After that, all procedures for animal experimentation were performed in accordance with the institutional guidelines for the care and use of laboratory animals, and were approved by the Home Office in the UK.

For animal experimentation, young and old mdx mice (C57Bl/10ScSn mdx) were used. The mdx mice were obtained from the cages in Prof Goldspink's mdx mice breeding store in Royal Free CBU unit of Royal Free and University College Medical School (Hampstead Campus) in UCL. For in vivo gene transfer experiments, two sets of animal experiments were carried out, as young and old mdx mice.

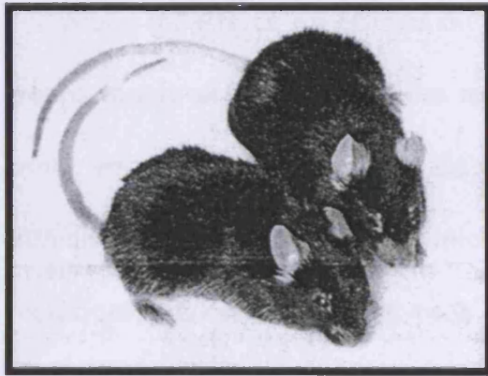


Figure 2.3: Mdx mice

23 young mdx mice (12 females and 11 males) were used for young mdx mice gene transfer experiments. Their ages were between 4 and 8 weeks old, and their bodyweights ranged from 22.86 g to 30.97 g (26.18 ± 4.78 g).

For old mdx mice experimentation, 23 old mdx mice between 12 and 26 months old (11 females and 12 males) were used. The bodyweights of the old mdx mice used ranged from 22.40 g to 36.50 g (32.14 ± 9.74 g). The bodyweights of old animals were very broad, because some of the animals were very old (over 2 years old).

Two different cDNAs of IGF-I splice variants, IGF-I Ea and IGF-I Ec (MGF), were inserted into a plasmid vector and used for investigating in vivo effects of these IGF-I splice variants on young and old mdx mice. Therefore, before injecting cDNAs of two different IGF-I alternative splice variants to the animal muscles, both young and old mdx mice were divided into three groups, as

- (i) IGF-I Ea,
- (ii) IGF-I Ec (MGF)
- (iii) Vector-only (control).

For young mdx mice experimentations, animals were randomly separated into three groups in two sets, such as seven mice for vector-only group, eight mice for MGF group, and eight mice for IGF-I Ea group. For the older mdx mice experimentation, animals were similarly separated into three different groups as vector: Seven mice for vector-only and eight mice for each of MGF and IGF-I Ea groups. However, in the course of experiments, one older mouse from each group of vector-only and MGF died. The final numbers of groups were six mice for vector-only, seven mice for MGF, and eight mice for IGF-I Ea group. It is known that when the mdx mice, particularly female mdx mice, get older, they become weak.

2.1.2.4 Gene delivery system

The naked/plasmid DNA gene delivery system has some advantages such as its simplicity and safety in contrast to other types of gene transfer. The plasmid / naked DNA delivery also provides more opportunities for local intramuscular gene injection. Emerging on such advantages it has, plasmid cDNA gene delivery system (pcDNA3.1NT/GFP of Invitrogen) was selected as the delivery system of this study.

2.1.2.5 Determining the time course for pcDNA gene transfer

A while ago, some groups introduced different IGF-I variants into skeletal muscle in order to regenerate muscle fibers, instead of replacing or repairing the mutated dystrophin gene. These include Lee Sweeney and Nadia Rosenthal's groups in University of Pennsylvania and Harvard University. They carried out similar strategy and technique (Barton-Davis et al, 1998). They transferred cDNA of liver / systemic type of IGF-I (IGF-I Ea) in adeno-associated virus (AAV) vector into mdx mouse

muscles. They measured muscle force mechanically 4-9 month after injection. They suggested that they found the IGF-I Ea expression promotes an average increase of 15% in muscle mass and strength in healthy young adult mice (Barton-Davis et al, 1998) comparing with uninjected control muscles.

When Goldspink and Yang introduced cDNAs of IGF-I splice variants including MGF into TA muscles of wild type C57BL/10 mouse by the naked DNA/plasmid vector delivery system, they found that MGF increased average of 25% in muscle fibre size in injected muscle by comparing uninjected control muscle, within just 3 weeks (Goldspink and Yang, 2001; Goldspink and Yang, 2004).

On the other hand, according to Wolff's group, CMV promoter-driven plasmid DNA expression is very high on day 1, after injection, but it starts diminishing strongly on day 2. It gets relatively stable on day 4 until the end of week 3. In this period, between the 4th and 21st day, expression levels remain relatively stable or decline very slowly. After the end of 3rd week, expression level starts dropping very sharply again (Herweijer et al, 2001; Herweijer and Wolff, 2003). However, plasmid DNA can be detected for at least 12 weeks after injection. According to above background, the time interval was chosen as 3 weeks (21 days) in this study.

2.1.3 In vivo - satellite cell activation experimentation

2.1.3.1 The selection of the markers for satellite cell activation

In general, the satellite cells are identified and characterized in terms of molecular markers, which are expressed by them. Along with other proteins, the activated satellite cells express the following myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin and MRF4. MyoD (also called Myogenic Factor 3 -MYF-3 / Myf-3-, or Myoblast determination protein 1 in human), and myf5 expressions are involved in early stage of satellite cell activation (proliferation stage), whereas myogenin and MRF4 expressions are associated with terminal differentiation (Hawke and Garry, 2001). Upon satellite cell activation, an increase in MyoD transcript and protein occur within 12 hours (Dhawan and Rando, 2005). In the late stage of activation, an increase occurs in myogenin (also called myogenic factor-4 – Myf4- in human) and other MRFs expressions. The increase of myogenin and other MRFs initiates the terminal myogenic differentiation program by upregulating the genes such as those encoding myosin heavy chain, creatine kinase and sarcomeric actin proteins (Dhawan and Rando, 2005). Myogenin appears at the earliest stage in differentiating myoblasts or muscle progenitor cell phase and it is not expressed in proliferating myoblasts. Similar findings were proposed by Zammit et al (2006). They suggest in most recent paper that although there are many satellite cell markers, the most important markers are CD34, Pax7, Myf5/ β -gal, PCNA, MyoD, Myogenin and MLC3F-tg (Zammit et al, 2006). CD34 and Pax7 are markers for mainly quiescent stage of the satellite cells, MyoD is for proliferation stage, and Myogenin is for differentiation stage of those cells (Zammit et al, 2006). Therefore, MyoD and Myogenin were selected for

investigating satellite cell activation. Schematic of satellite cell myogenesis and some typical markers of each stage can be seen from Figure 2.4.

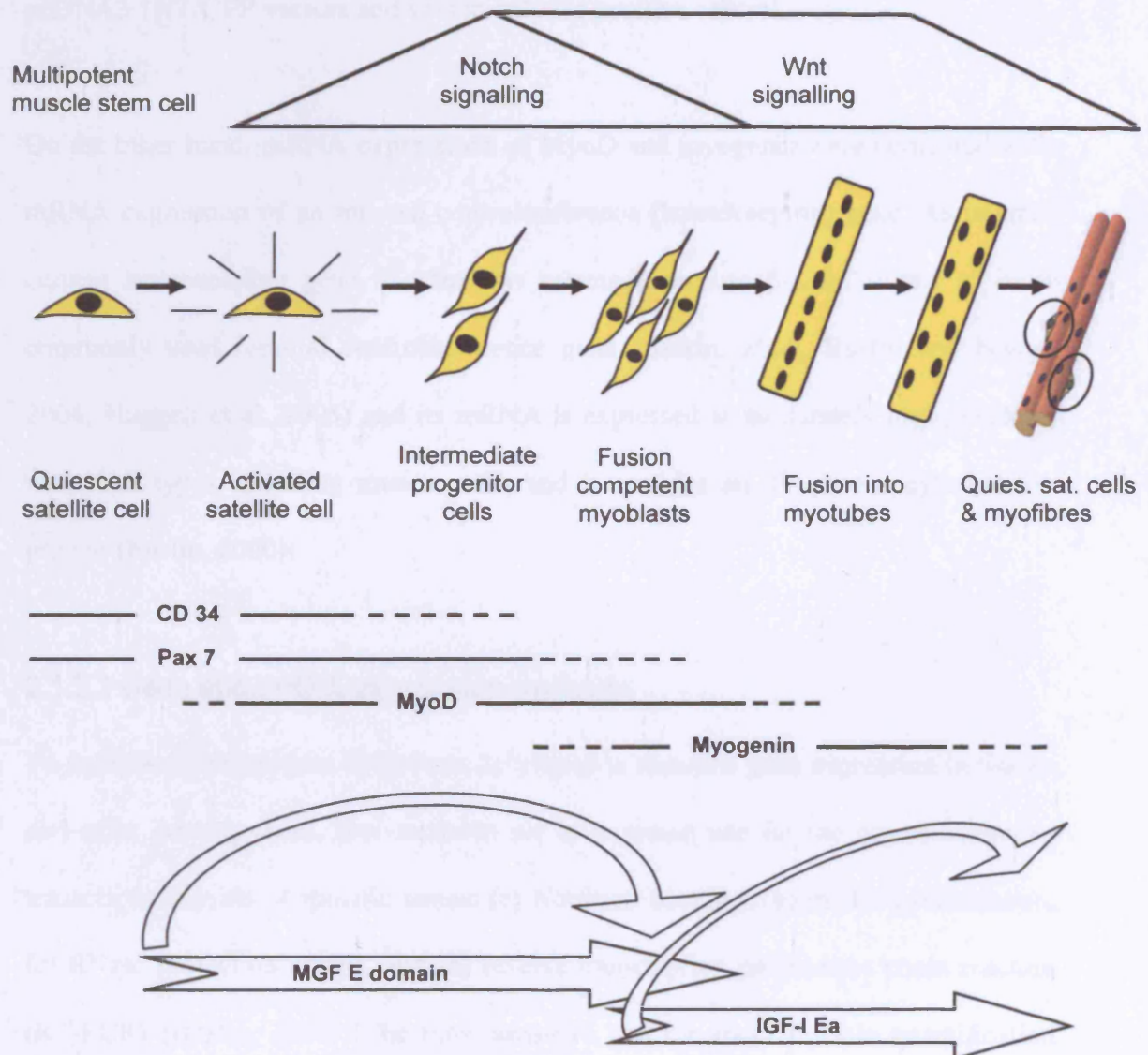


Figure 2.4: Schematic of satellite cell myogenesis and markers typical of each stage.

As a result, MyoD and myogenin was selected as satellite cell activation markers, because both markers play an important role in skeletal muscle myogenesis, particularly in satellite cell activation. The MyoD was selected as the marker for the early stage of satellite cell activation, and myogenin was selected as the marker for the late stage of such activation. The mRNA expression levels of these two satellite cell

activation markers were investigated in TA muscle tissues of young mdx mice, 21 days after injecting cDNAs of IGF-I splice variants (IGF-I Ea and MGF) in pcDNA3.1NT/GFP vectors and vector-only for positive control.

On the other hand, mRNA expressions of MyoD and myogenin were compared with mRNA expression of an internal control/reference (housekeeping) gene. As internal control housekeeping gene, β -actin was selected, because β -actin is one of most commonly used internal control/reference gene (Bustin, 2000; Bustin and Nolan, 2004; Huggett et al, 2005) and its mRNA is expressed at moderately high levels in most cell types including muscle cells and it encodes an ubiquitous cytoskeleton protein (Bustin, 2000).

2.1.3.2 Gene and mRNA expression methods

To date, many techniques have been developed to measure gene expression in tissues and cells. Among them, four methods are in common use for the quantification of transcription levels of specific genes: (a) Northern Blotting, (b) in situ hybridisation, (c) RNase protection assays, and (d) reverse transcription polymerase chain reaction (RT-PCR) (Bustin, 2000). The most sensitive and the most flexible quantification method among above four methods is RT-PCR (Bustin, 2000). Therefore, this method can be used to determine the presence or absence of a transcript, to estimate and compare mRNA levels in different samples, and to characterised patterns of mRNA expressions. Therefore, the quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) technique was used to determine gene and mRNA expression of myogenin and MyoD.

2.2 Materials

Animals

C57BL/10ScSn- mice (mdx mice)

Cells:

- L6 E9 rat myoblasts cell line

The cells were taken from the liquid NO₂ store of the Molecular Tissue Repair Unit of Department of Anatomy and Developmental Biology / Department of Surgery in Royal Free Campus of UCL, which were bought from European Cell Lines Bank)

- C2C12 mouse myoblasts cell line

The cells were taken from the liquid NO₂ store of the Molecular Tissue Repair Unit of Department of Anatomy and Developmental Biology / Department of Surgery in Royal Free Campus of UCL, which were bought from European Cell Lines Bank)

- Primary Human Muscle Cells:

- Craniofacial Masseter Muscle non-sorted Cells:

- The Healthy Cells:

The cells were taken from the liquid NO₂ store of Department of Biomaterials and Tissue Engineering in Eastman Dental Institute of UCL obtained from consented healthy children at the Eastman Dental Hospital.

The Cells with Congenital Muscular Dystrophy (CMD):

The cells were taken from the liquid NO₂ store of Department of Biomaterials and Tissue Engineering in Eastman Dental Institute of UCL obtained from the children with CMD disorder, patients undergoing dental surgery and the removal of third molars under general anaesthesia at the Eastman Dental Hospital

Limb muscle non-sorted cells of the vastus lateralis:

The Healthy Cells:

The cells from healthy individuals were obtained from Dr Richard W. Orrell of University Department of Clinical Neuroscience of Royal Free Hospital.

The Cells with ALS

The cells from patients with ALS were obtained from Dr Richard W. Orrell of University Department of Clinical Neuroscience of Royal Free Hospital.

The Cells with FSHD

The cells with FSHD were prepared from the biopsies (percutaneous needle biopsy), which were carried out with Richard W. Orrell of University Department of Clinical Neuroscience of Royal Free Hospital on patients with Fascioscapulohumeral dystrophy (FSHD).

IGF-I peptides

MGF peptide:

The 24 amino acid peptide of MGF was synthesized by Department of Biochemistry of UCL according to sequence for C-terminal of MGF transcript. The sequence of the synthetic peptide was: NH₂-Y(Tyr)-Q(Gln)-P(Pro)-P(Pro)-S(Ser)-T(Thr)-N(Asn)-K(Lys)-N(Asn)-T(Thr)-K(Lys)-S(Ser)-Q(Gln)-R(Arg)-R(Arg)-K(Lys)-G(Gly)-S(Ser)-T(Thr)-F(Phe)-E(Glu)-E(Glu)-H(His)-K(Lys)-COOH. The N terminus was modified by a PEG derivative (O'-O-bis(2-aminopropyl)polyethylene glycol 1900) (Jeffamine) via a succinic acid bridge. The crude peptide was precipitated, and the product was separated by centrifugation. After being washed and vacuum-dried, the peptide was purified by HPLC and then analysed.

Human Recombinant IGF-I peptide:

Human Recombinant IGF-I (long r³ IGF-I) (from Sigma-Aldrich)

Antibodies

IGF1R blocking antibody - Ab-I – (Oncogene)

Monoclonal mouse anti-human Desmin antibody (DAKO)

DAPI

Anti mouse FITC

Anti-BrdU

Kits

BrdU labelling and detection kit III (Roche)

Creatine Phosphokinase (CPK) kit (Sigma)

BCA Protein Assay Reagent Kit (Pierce)

QIAGEN EndoFree Plasmid Maxi Kit (Qiagen)

Omniscript Reverse Transcriptase Kit (QIAGEN)

QIAquick Gel Extraction Kit (QIAGEN)

Hoods:

Culture Hoods (All cells were treated in class II biological safety cabinets with double fan)

Fume Hoods

CO₂ Incubators:

All cells were grown via incubation in a humidified environment at 37 °C and 5% CO₂.

Devices and machines

Light microscopy

Hemocytometer (Sigma)

Plate reader

pH meter

Electronic balance

Different centrifuges (Sarval GS3 rotor, Sarvall SS-34 rotor, and micro centrifuges)

Shaker

Autoclave

Oven

Microwave

Ultraviolet camera
GeneSpec I device (Naka Instruments)
Force transducer (Harvard Instruments Co. Ltd)
Lightcycler machine (Roche Diagnostics Inc, Germany)
Homogeniser
Minigel tanks
Homoeothermic blanket
Electrical shaver
Surgical devices (Scalpels etc)
Silk thread
27 gauge needles
PC Computer

Softwares

Spike 2 – Version 4- software (CED- Cambridge Electronic Design - UK).
GeneSpec I software (Naka Instruments)
Omiga 2.0 software (Oxford Molecular)
Microsoft Office 2003 software package

Tissue Culture Plastics and Glasses

Flasks with vented caps (25 cm², 75 cm² and 150 cm²) (Orange)
Multi-well plates (flat bottom and U-shaped bottom) (6 and 96 wells) (Orange, Nunclon and Corning)
Centrifuges Tubes (20 ml and 50 ml) (Orange)
Petri dishes (glass and plastic, 35 mm, 60 mm, 100 mm and 150 mm) (Orange and Nunclon)
Pipettes (1, 2, 5, 10 and 25 ml) (Orange)
Plastic tubes (0.5 ml and 2 ml) (Nunclon)
Cryotubes (2 ml) (Nunclon)
Glass cover slips (13 mm)
Tube racks

Erlenmeyer Flasks (Orange)
Beakers (250 ml and 1000 ml)
Glass and plastic bottles (different sizes)

Solutions and chemicals

DMEM (Gibco)
PBS (Gibco)
Gelatin (20 %, Sigma)
FBS / FCS (Sigma)
Trypsin / EDTA (Sigma)
Trypan blue
DMSO
BSA (30% IgG – free) (Sigma)
EGF
bFGF
Insulin
Dexamethasone
Feutin
Vitamin E (Trolox)
Vitamin H (Biotin)
Vitamin C
Transferrin
Sodium Selenite
Albumax-1
Trypton X-100
Citiflour
Lysine
Sodium Azide - NaN_3 (Sigma)
Paraformaldehyde
Methanol (neat)
Acetone (neat)
Absolute Ethanol – EtOH (neat)

0.5 mM gly gly buffer
1 X Reporter Lysis Buffer (RLB)
Fiske and SubbaRow solution
Agarose
Tryptone (Sigma)
Yeast extract (Sigma)
NaCl (Sigma)
Bacteriologic agar (Sigma)
Urethane
Ethidium bromide
TAE
TBE
PCR dye
DNA size marker ladders (50, 100 and 1000 Kb)
OCT (optimum cutting temperature)
Liquid Nitrogen
RNA Later solution (QIAGEN)
2X Quantitect SYBR Green PCR Master Mix (QIAGEN)
TRI reagent (Sigma)
RNase-free water (Promega)
DEPC - treated water
Chloroform (Sigma)
Sodium Acetate
Isopropanol
RQ1 RNase-free DNase (Promega)
10 X RT Buffer (QIAGEN)
Omniscript Reverse Transcriptase enzyme (QIAGEN)
dNTPs (QIAGEN)
RNase inhibitor (Promega)

Antibiotics

Penicillin (Sigma)

Streptomycin (Sigma)

Ampicillin (Sigma)

Tetracycline (Sigma)

Vectors

pcDNA3.1/NT-GFP vector (Invitrogen)

Restriction enzymes

Apa I restriction enzyme

Kpa I restriction enzyme

Reaction Buffer (Buffer 4)

Primers

MyoD (Sigma) (NCBI NM 010866) 123 bp Mw: 38.139 g/mol KD ssDNA

Upperstream (forward) –TTTCTTCACCACACCTCT-

Downstream (reverse) –TTAACTTTCTGCCACTCC-

Myogenin (Sigma) (NCBI NM 031189) 102 bp Mw: 31.547 g/mol KD ssDNA

Upperstream (forward) –AGACGAAACCATGCCCAA-

Downstream (reverse) –TAAAAGCCCCCTGCTACAGA-

β -actin (Sigma) (GenBank AB117093) 186 bp mw 41.695 g/mol KD ssDNA

Upperstream (forward) -GACGATGATATTGCCGCACT-

Downstream (reverse) -GATACCACGCTTGCTCTGAG-

Random hexamers primers (QIAGEN)

2.3 Methods

2.3.1 In vitro - cell culture experimentation

2.3.1.1 Introduction

To investigate the role of IGF-I splice variants in vitro on skeletal muscle cells; different cell types from different species, such as mouse, rat and human, were used. For animal cells, rat (L6 E9) and mouse myoblasts (C2C12) cell lines; and for human cells, primary healthy and degenerative muscle cells, both from craniofacial and lower limb muscles were investigated because of their different embryologic origins,. The characteristics of these cells were determined via light, phase and fluorescent microscopy.

2.3.1.2 Cells

The healthy primary craniofacial masseter muscle non-sorted cells and the craniofacial masseter muscle primary cells with congenital muscular dystrophy were acquired from the liquid nitrogen store of Department of Biomaterials and Tissue Engineering in Eastman Dental Institute of UCL. The human craniofacial masseter muscle biopsies were previously obtained from healthy children and the children with CMD disorder, patients undergoing dental surgery and the removal of third molars under general anaesthesia at the Eastman Dental Hospital. For having primary

cultured human craniofacial muscle cells, the biopsies were finely diced and the fragments plated into 0.2% gelatin coated T80cm² culture flasks, incubated in standard growth medium (SGM: 20% FCS and 1% P/S in DMEM) and maintained at 37°C in humidified air/CO₂. The expanded cells were then stored under cryogenic conditions.

The human primary limb muscle cells of the vastus lateralis, from healthy individuals and from patients with ALS, were obtained from Dr Richard W. Orrell of University Department of Clinical Neuroscience of Royal Free Hospital. A number of biopsies (percutaneous needle biopsy) were also carried out with Dr Orrell on patients with Fascioscapulohumeral dystrophy (FSHD). Biopsy procedure was made as follows: Following local anaesthesia with 1 % lignocaine (lidocaine; Phoenix Pharma Ltd, UK), muscle biopsies were taken from the vastus lateralis muscle using the percutaneous needle biopsy technique (Bergström, 1962) with applied suction. 0.5-3 cm of human vastus lateralis muscle tissue samples were then treated via explant primary culture technique.

2.3.1.3 Primary Cell Culture

Initially, a 6-well plate was coated before addition of cut-tissue samples for incubation. For coating, 1 ml of 0.2 % gelatin in PBS was added to each well. The plate was shaken on a plate shaker for 2 hours for coagulation. After coagulation, gelatin was removed from wells. 0.2% gelatin coated plate contains 1 ml of DMEM with 1% of P/S/well was carried to an operation room in sterile conditions on ice. 0.5 cm of quadriceps (vastus lateralis) muscle tissue sample taken from a patient using the

percutaneous needle biopsy technique with applied suction was put in a well containing DMEM with P/S. The plates were immediately carried into a hood in the tissue culture laboratory.

The tissue was washed by DMEM and chopped very finely by microsurgical scissor and scalpels. The pieces in DMEM were transferred to a 50 ml centrifuge tube by 10 ml wetted pipette. The pieces were washed by resuspending in DMEM and the pieces were left to settle for a while, and then the supernatant fluid was removed. The pieces were washed again in DMEM, settled; and the supernatant was removed one more time; and finally some DMEM was added onto the washed pieces.

The pieces in DMEM were transferred to the wells of the plate by pipetting. Each wells contained 10-15 pieces. DMEM was removed and 200 µl of growth medium with 40% of FBS and 1% of P/S was added onto each well to be adhered. The pieces were then incubated at 37 °C and 5% CO₂ overnight.

Following day, 200 µl of growth medium was gently added again and the medium volume was increased everyday until each well had 2 ml of 40% of growth medium. The medium was changed weekly until a substantial outgrowth of cells was observed. When the cells were confluent, which took about 5-8 weeks, the explant was picked off from the centre of the outgrowth with a sterile scalpel and transferred to another coated 6 well plate for further culturing. The cells of the first plate were then passaged by routine passage technique and seeded into a 0.2% of gelatin pre-coated 75 cm² tissue culture flask. See 2.2.3.

2.3.1.4 Routine Cell Culture

The cells were grown in a medium with 10 % FBS and medium changed every 3 – 4 days. The cells were grown until they formed a monolayer sheet on the plastic (70-80 % confluent) at 37 ° C and 5% CO₂, which took between 3 and 21 days.

2.3.1.5 Routine Passage

The growth medium was aspirated off and the monolayer cell culture content was washed 3 times with Ca²⁺ / Mg²⁺ free PBS. The cells were harvested from the plastic flask using 1 X Trypsin / EDTA at 5 ml 1 X Trypsin / EDTA per 75 cm². The trypsin digests the ECM links and EDTA Ca²⁺ on which cell-cell adhesion is dependent. The flasks were incubated until all of the cells had rounded up and detached from the plastic, which took approximately 5-10 minutes. Trypsin action was terminated by addition of 5 ml of growth medium with 10 % FBS. Trypsined cell / medium mixture was transferred to a 50 ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes to pellet the cells. The supernatant was discarded gently and the cells re-suspended in 5 ml of medium by titrating with 10 ml pipette. Re-suspended cells were counted using haemocytometer. The cells were seeded again at 2 X 10⁵ cells per 75 cm² flask at 37 ° C and 5% CO₂ for continued growth.

2.3.1.6 Cell Density Determination

The cell density was determined using a haemocytometer viewed under a light microscope at a magnification of 10 times. 7µl of the sample of the trypsinised and resuspended cells were collected and put into a 0.5 ml plastic tube. The cell sample was mixed with 7µl of Trypan blue, and the mixture was allowed to stand for 10

minutes. 7 μ l of trypan blue/cell suspension were transferred to both chambers of the haemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action (Figure 2.5). At a 10 X magnification, the grid was located and the cells were counted within the four primary squares, which are circled in Figure 2.6. The value was averaged for the four primary squares and by using the calculation formula ($c = n \times 10^4$).

c = cell concentration (cells / ml), n = average number of cell counted.

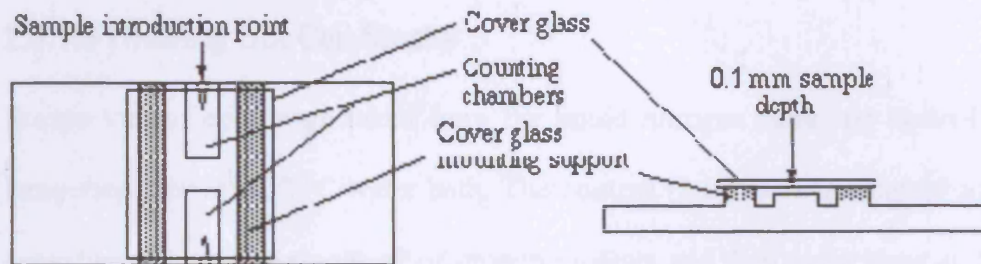


Figure 2.5: Mounting a cover glass onto a haemocytometer slide.

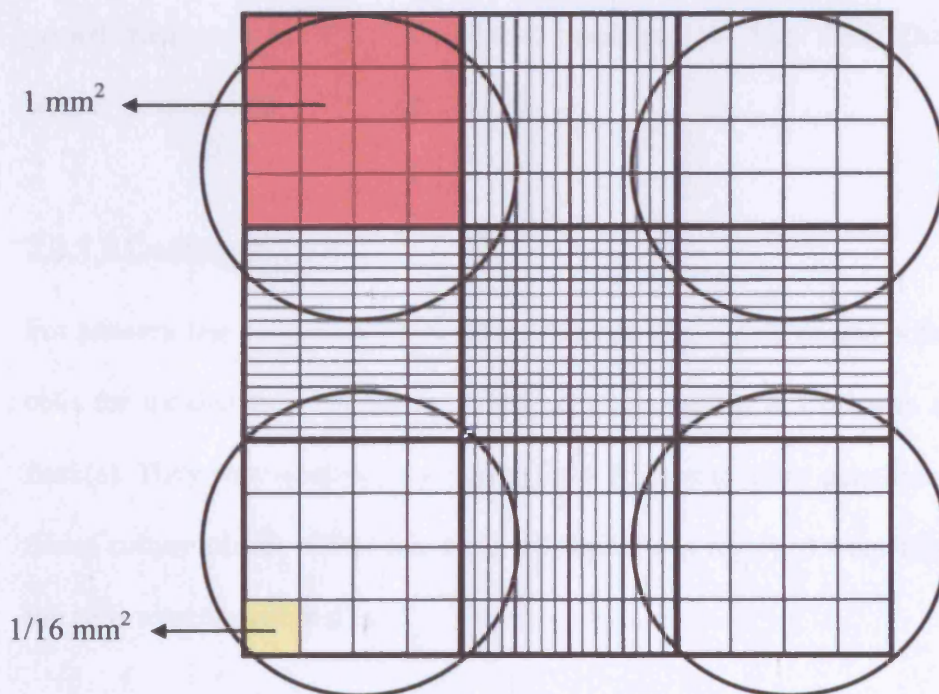


Figure 2.6: The grids and squares in a haemocytometer slide.

2.3.1.7 Freezing down Cell Stocks

After trypsinisation and centrifugation, cells were counted and 1×10^6 of cells were re-suspended in 10 % DMSO in FBS. The 1 ml of cell solution was transferred to a cryotube, labelled and placed in a plastic container with isopropanol, overnight at -80°C . Next day, the cryotubes were transferred to a liquid nitrogen tank for long term storage.

2.3.1.8 Thawing Out Cell Stocks

Frozen vial of cells were taken from the liquid nitrogen store and thawed in room temperature or in a 37°C water bath. The content (1 ml) was transferred to a 50 ml centrifuge tube containing 9 ml of growth medium and then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 15 ml of growth medium with 10 % FBS and then transferred to 75cm^2 flask. The flask(s) were left for incubation at 37°C and 5% CO_2 .

2.3.1.9 Coating Flasks

For primary non-sorted human muscle cells, the flasks were coated before addition of cells for incubation. For coating, 5 ml of 0.2 % gelatin in PBS was added into the flask(s). They were shaken on a shaker for 1-2 hours to allow gelatin to adhere to the tissue culture plastic. After this time, all liquid was removed from flask(s) and then the cells were transferred in.

2.3.1.10 Proliferation Assays

To investigate the effects of IGF-I splice variants cell proliferation in muscle cells of different species, different proliferation assay methods were used for different cell types as explained before. For rat L6E9 and mouse C2C12 cell lines, BrdU (5-Bromo-2-deoxy-uridine) labelling and detection kit III (Roche – Cat. no. 1 444 611), and for non-sorted human primary cells immunocytochemistry assays were used.

2.3.1.10.1 BrdU Proliferation Assay

2.3.1.10.1.1 Plating Cells

Frozen vials of rat L6E9 and mouse C2C12 cells were taken out from the liquid nitrogen store and thawed in room temperature. The cells were transferred into T75 flasks with growth medium and grown until becoming 70-80 % confluent and then harvested and counted by using a haemocytometer. Both rat L6E9 and mouse C2C12 cell lines were plated into 96 well cell culture plates at a density of 5×10^3 per well in 200 μ l of growth medium (for L6E9, in 20 % FBS with 0.5 % Ampicillin and 0.5 % Gentamicin in DMEM, and for C2C12 cells, 10 % FBS with 0.5 % Ampicillin and 0.5 % Gentamicin in DMEM) and incubated at 37 ° C and 5% CO₂ for 3 days. Growth mediums was removed from wells and the cells then washed 3 times with 100 μ l of serum free medium with antibiotics in above concentrations. The washed cells were added 200 μ l of serum free medium with antibiotics and incubated 37 ° C and 5% CO₂ for 24 hours. Next day, serum free medium was removed and different dozes of MGF and systemic type of IGF-I (R³IGF-I) in 200 μ l of DMEM with 100 μ g/ml of BSA were added into wells. Then, the cells treated with growth factors were incubated at 37 ° C and 5% CO₂ for 24 hours again.

2.3.1.10.1.2 Labelling Cells with BrdU

10 µl of 10 µM BrdU labelling solution were added into each well for all 4 plates for 3 hours. The wells were washed 3 times with 250 µl of washing medium containing 10 % FBS in PBS. After the last wash, the cells were fixed with 200 µl of pre-cooled fixative per well for 30 minutes at room temperature. The fixative was removed and the cells were washed 3 times again with 250 µl of washing medium again. The washing solution was removed. The plates were left without buffer at 4 °C overnight.

2.3.1.10.1.3 Detecting Cells with BrdU

Next day, wells were washed once with 250 µl of washing medium containing 10 % FBS in PBS and then the solution was removed. Then 100 µl of nuclease working solution was added into per well and the plate was incubated at 37 °C and 5% CO₂ for 30 minutes. The nuclease working solution was removed and the cells were washed again 3 times with the washing medium. The washing solution was removed and the cells were incubated with 100 µl of anti BrdU – POD at 37 °C and 5% CO₂ for 45 minutes and then washed again 3 times with 250 µl of the washing solution. The solution was removed and 100 µl of peroxidase substrate was added and then incubated at room temperature for about 30 minutes until positive samples show a green colour. After occurring green, the colour the plates were mixed with shaker and measured in microtiter plate reader at 405 nm with a reference wavelength at 490 nm.

2.3.1.10.2 Immunocytochemistry Proliferation Assay

2.3.1.10.2.1 Preparing Coverslips

A small jar with an airtight lid was filled to 2/3 of its volume with 70 % absolute ethanol (EtOH) in tissue grade water. A moderate number of 13 mm glass coverslips (from BDH) were put in the jar containing 70% EtOH. The jar was closed tightly and sealed with parafilm and then left overnight. The coverslips were removed from the jar and placed into a clean Pyrex Petrie dish, using a forceps and a needle. Excess EtOH was pipetted off and a small volume of tissue grade water was added to prevent coverslips from aggregation. The glass Petrie dish was wrapped in aluminium foil and labelled with autoclave tape and then baked at 200 °C for 2 hours. The baked Petrie dish was cooled. The cooled coverslips were transferred to a new plastic sterile Petrie in a hood. The plastic Petrie was then sealed with parafilm and stored in a sealed sandwich box to store.

2.3.1.10.2.2 Preparing the Cells for Proliferation Assay

Three pre-prepared 13 mm coverslips were placed into each well of a 6 well cell culture plate. 1 ml of 0.2 % gelatin in PBS was added into each well of the plate(s). The plates were shaken on a shaker for 1-2 hours. And then the gelatin was removed.

Human primary non-sorted cells were plated out onto the coverslips in wells of 6 well plate(s) at a density of 4.5×10^4 in 2 ml of the defined medium (Muscle Cell Growth Medium – MCGM) per well and left to grow at 37 °C and 5% CO₂ overnight. For details of MCGM, see appendix. After incubating the cells on the coated coverslips overnight, next day, MGF, long r³ IGF-I, and IGFIR blocking antibody (Ab-I) were

added in different concentrations for different conditions (Figure 2.7). The coverslips on which the cells were placed were incubated at 37 °C and 5% CO₂ for 24, 48 and 72 hours for different time points, and then fixed.

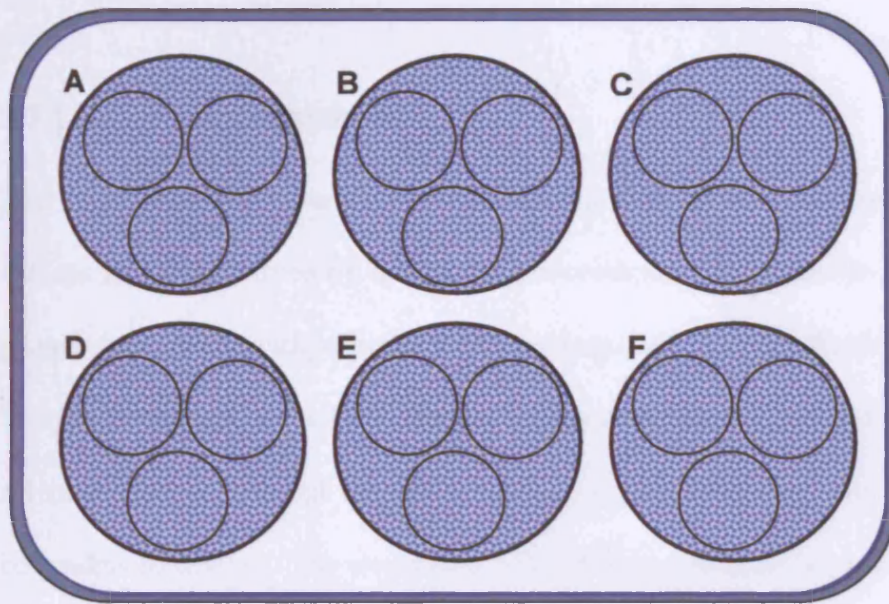


Figure 2.7: The plated out human primary muscle cells onto the coverslips in 6 well plate, and the main conditions for each well. A, B, C, D, E, and F are the wells of a 6 well plate which are in a shape of big circles. The small circles are coverslips. All wells had non-sorted human primary muscle cells at a density of 4.5×10^4 in 2 ml of Muscle Cell Growth Medium – MCGM. A had only the cells in MCGM for control; B had the cells in MCGM + Ab-I; C had the cells in MCGM + MGF; D had the cells in MCGM + MGF + Ab-I; E had the cells in MCGM + long r^3 IGF-I; and F had the cells in MCGM + MGF + long r^3 IGF-I.

2.3.1.10.2.3 Fixing and Storing the Treated Cells on Coverslips

50 % of the culture volume (1 ml) was removed from the wells. An equal volume (1ml) of ice cold methanol was added onto cells to ensure cells were exposed to a minimum volume of 1:1 culture medium / methanol. The cells were fixed on ice for 5 minutes, and then 50 % the methanol / culture medium mixture were removed gently. The coverslips in wells were washed with addition of 2 ml of PBS for each well. 2 ml of ice-cold methanol was added to each well and the cells were fixed again on ice for

a further 10 minutes. The methanol was then removed and coverslips washed again with 2 ml of PBS again. PBS was removed and 2 ml of 0.1 % sodium azide (Na N₃) in PBS was added onto each well to store fixed coverslips. 6 well plates containing fixed coverslips were sealed with parafilm and stored at 4 °C prior to use.

2.3.1.10.2.4 Immunostaining

The fixed coverslips were removed from the wells with a microsurgical crossover forceps and were curved tip needle. The coverslips were drained by their edges being placed in contact with absorbent paper tissue so that the fluid was removed by “wicking” or capillary action. The coverslips were then washed x 6 with PBS in a 60 ml container and placed on pedestals by scooping PBS in order not to allow the coverslips to dry-out. The coverslips with PBS on pedestals were drained again with absorbent paper tissue and then 100 µl of 0.25 % Triton X-100 / PBS were added onto each coverslips to permeabilise cell membrane for 15 minutes at room temperature. The coverslips were drained and washed 6 times with PBS, and were drained again. For myoblast staining, the coverslips incubated with 50 µl of monoclonal mouse anti-human desmin antibody (DAKO clone D33) diluted (1: 200) in special Antibody Diluting Solution –ADS (ADS: 10% dentured FBS, 0.1M lysine and 0.1% Sodium Azide in PBS) for 1 hour at room temperature. For positive control, 50 µl of PBS was added onto one randomly chosen coverslip, instead of monoclonal mouse anti-human desmin antibody.

After one hours treated with monoclonal mouse anti-human desmin antibody, the treated coverslips were drained, washed and drained again as above. For nuclear

staining, all coverslips incubated with 50 µl of donkey anti-mouse FITC / DAPI antibody in ADS for 30 minutes at room temp. The dilutions were 1 in 200 for FITC (Jackson Lab ltd – USA), and 1 in 1000 for DAPI. The coverslips were drained, washed 6 X PBS, drained and mounted onto labelled glass slide with Citifluor antifade media and then sealed edges with clear nail varnish. 3 coverslips were mounted for each slide. The mounted slides were stored at 4-6 °C for counting.

2.3.1.10.2.5 Epi-fluorescence and light contrast microscopy:

Cell - associated fluorescence and morphology were visualised with epi-fluorescence and Leica Modulation Contrast microscopy, using an inverted Leica DMIRB microscope equipped with a COHU CCD camera and FW4000TZ imaging software in order to capture and save images. The cells were counted under a fluorescent microscope at a magnification of 63 X. Fluorescents were blue (for DAPI nuclear stained – for all cells), and green (for Desmin stained – for myoblast). All cells were counted in a field, in blue and green fluorescent.

2.3.1.11 Differentiation Assays

BCA protein assay reagent kit (Pierce), and creatine phosphokinase (CPK) kit (Sigma), and immunocytochemistry techniques were used to investigate cell differentiation (fusing mononuclear cells to multinuclear myotubes).

2.3.1.11.1 Differentiation Experiments by CPK Assays

2.3.1.11.1.1 Preparing the Cells

Cells were grown in normal muscle growth medium – NGM- (20% FBS and 1% penicillin /streptomycin in DMEM) in T75 flasks until having enough number of cells. 96 well – plates were used for both the BCA protein assay and CPK assays. For 96 well plates, 200 μ l of NGM containing 10×10^4 cells were put into each well. For 96 well plates 16 conditions (16 X 5) were used. The time point was 48 hours for both assays. For each experiment, two pre-gelatinised plates were prepared (1 for protein assay, 1 for CPK assay).

After plating, cells in NGM were incubated at 37 °C for 3-5 days until 70 – 80 % confluent. The cells were washed with DMEM 3 times and then growth factors in 200 μ l of muscle cells defined medium were added. 2 different defined mediums were used. They were either 2 % FBS and 1 % P/S in DMEM, or 0.5 % BSA, with low insulin (5 ng/ml) with 1 in 100 P/S in DMEM. As presented in chapter 2, the conditions containing different concentrations of growth factors were different such as positive control, different conc. of MGF, different conc. IGF-I, different conc. MGF and r³ IGF-I combinations, control with IGF-I receptor blocking antibody, MGF with IGFIR blocking antibody. The cells with and without growth factors (for positive control) were incubated at 37 °C and 5% CO₂ for 48 hours.

After 48 hours, the cells were washed with 3 X PBS and then added 150 μ l of 0.5 mM gly gly buffer for 96 well plates experiments to fix the cells, and then filmed, foiled

and stored at -70°C without adding any buffer. For immediate using, the cells were not put into -70°C . Experiment was carried on adding 1 X Reporter Lysis Buffer (RLB).

2.3.1.11.1.2 Lysition cells with Reporter Lysis Buffer

A sufficient amount of 1 X RLB was prepared with dH_2O from 5 X RLB stock. The defined medium containing growth factors was removed from wells and then was washed with 3 X PBS. 150 μl of 1 X RLB was added into each well. For frozen cells, they were thawed and 150 μl of 1 X RLB was added. The cells were shaken on a shaker at room temperature for 5-7 minutes. After that, the cells were incubated for 15 minutes at room temp. The cells in 1 X RLB solutions were crashed carefully using a tip. The crashed cells were taken into pre-labelled tubes and centrifuged at 10 000 rpm for 2 mins. Then, the supernatant was carefully transferred into another labelled tube. (Only the supernatants were transferred, not pallets). The tubes were foiled and stored at -70°C for later protein and CPK assays.

2.3.1.11.1.3 Preparation of standards and working reagent

First of all, the standards and working reagent (WR) were prepared. For WR, 50 parts of BCA reagent A were mixed with 1 part of BCA reagent B (50:1). For preparation of standards, different amount of albumin (BSA) were mixed with either 0.5 mM gly gly buffer (for 96 well plates) or 1 X RLB (for 24 well plates). Final BSA concentrations were 0, 0.05, 0.125, 0.250, 0.5, 1, 1.5 and 2 mg/ml.

2.3.1.11.1.4 Procedure of Protein Assay

3 X 25 μ l of each standard and samples were pipetted into a labelled 96 well plate. 200 μ l of WR was added into each well (to both, to standards and samples). The plate content was mixed on a plate shaker for 30 seconds. It was incubated at 37 $^{\circ}$ C for 30 minutes and then cooled at room temperature for 10-15 minutes. Finally, the absorbance was measured at 562 nm on a plate reader.

2.3.1.11.1.5 Procedure of CPK Assay

A set of labelled 1.5 ml centrifuge tubes were prepared (3 tubes for each standard and sample).

	TEST	BLANK 1	BLANK 2
Trizma buffer sol.	-	100 μ l	-
Creatine sol.	100 μ l	-	100 μ l
Sample	30 μ l	-	-
0.5 mM gly gly buffer	-	30 μ l	30 μ l
Water	100 μ l	100 μ l	100 μ l

Above reagents were added into centrifuge tubes as shown above in the table and were then mixed. The tubes were placed in 37 $^{\circ}$ C water bath to warm up for 1-2 minutes. 10 μ l of ATP – Glutathione solution was added to each tube and mixed and then incubated at 37 $^{\circ}$ C water bath for 30 minutes to initiate the reaction.

160 μ l of ice cold 20 % Trichloroacetic acid (TCA) was added to stop the reaction and were mixed and was left at room temperature for 5 minutes. The tubes were centrifuged at 2000 rpm for 5 minutes.

100 μ l of supernatants of tubes were transferred to a new set of 1.5 ml centrifuge labelled tubes, which were prepared earlier. 400 μ l of water, 100 μ l of Acid Molybdate solution and 25 μ l of Fiske and SubbaRow solutions were added to each tube including blanks. The tubes were mixed well and left for 30 minutes at room temperature for colour development. Finally, 150 μ l of the solution of each tube was transferred to 96 well plate and immediately measured at 620 nm on a plate reader.

2.3.1.11.1.6 Standard Calibration for CPK Assay

The phosphorus standard solution was mixed with distilled water in different concentrations to prepare 0, 0.5, 1, 1.5, 2 and 2.5 g/tube for final standard. The standard solutions were transferred to 1.5 ml centrifuge tubes. 100 μ l of Acid Molybdate solution was added to each standard tube and mixed well. Then, 25 μ l of Fiske and SubbaRow solutions was added to tubes and were mixed again. The standard tubes were incubated at room temperature for 10 minutes and then 150 μ l of the tube content was transferred to the same sample 96 well plate. The plates measured at 620 nm on a plate reader in 10 minutes. After measuring, a standard calibration curve was prepared.

2.3.1.11.2 Differentiation Experiments by Immunocytochemistry

The cells were grown in normal muscle growth medium – NGM- (20% FBS and 1% penicillin /streptomycin in DMEM) in T75 flasks until having sufficient number of cells. After preparing the baked coverslips (see 2.2.10.2.1), 3 prepared 13 mm coverslips were placed into each well of a 6 well cell culture plate and were coated by

0.2% gelatin as mentioned in 2.2.10.2.1.2. Different primary human degenerative muscle cells were plated out onto the coverslips in wells of 6 well plate(s) at a density of 2×10^5 in 2 ml of the Normal Growth Medium - NGM (20% FBS and 1% P/S in DMEM), and were left to grow at 37°C and 5% CO_2 until approximately 80% confluent. When the cells on coverslips were more than 80% confluent, the NGM was changed with the MCDM (Muscle Cells Differentiation Medium). The MCDMs were either 0.5 % BSA, with low insulin (5 ng/ml) with 1 in 100 P/S in DMEM or 2 % FBS and 1 % P/S in DMEM. Before adding the MCDM, the NGM was removed and the cells on coverslips were washed with DMEM three times.

The cells were daily checked under light microscopy for observation of fusing myoblasts to myotubes. As soon as fusing starts different growth factors were added onto the wells for different conditions. The conditions containing different concentrations of growth factors were as follows: no growth factors for positive control, MGF (10 and 100 ng/ml), long r^3 IGF-I (10 and 100 ng/ml) for IGF-I Ea, and MGF plus long r^3 IGF-I (10+10, 10+100, 100+10 and 100+100 ng/ml). The cells with and without growth factors (for positive control) were incubated at 37°C and 5% CO_2 for 48 hours, and then fixed and stored as mentioned in 2.2.10.2.1.3. The immunostaining and counting protocols were the same as mentioned in 2.2.10.2.1.4 and 2.2.10.2.1.5. Differentiation status would be determined by counting both Desmin positive myoblast and Desmin positive myotubes. The percentages of the cells would be then calculated. After that, the percentages of myotubes would be compared to those of myoblasts. As can be seen from 3.2.3.3.2, the differentiation by

immunocytochemistry assay was not successful. Therefore differentiation states for degenerative muscle cells were not determined.

2.3.2 In vivo – gene transfer experimentation

2.3.2.1 Plasmid design and gene construction

The pcDNA3.1/NT-GFP vector (Invitrogen), which is driven by CMV (cytomegalovirus) promoter, was used in this part of the study to produce IGF-I isoforms in muscle fibres of mdx mice. The CMV (cytomegalovirus) was used as a promoter. The pcDNA3.1/NT-GFP contains 6160 nucleotides (Figure 2.8). The cDNAs of rabbit MGF and IGF-I Ea were previously cloned in Prof. Goldspink's group. These were subcloned into Kpn I (on 1650th bp, GGTAC/C) / Apa I (on 1730th bp, GGGCC/C) sites of the pcDNA3.1/NT-GFP. The multiple cloning site of pcDNA3.1/NT-GFP was between 1645-1738 bases. The constructs were checked by sequencing from T7 promoter upstream of GFP (Green Fluorescent Protein) to ensure that MGF and IGF-I Ea cDNA insertions had been cloned into the multiple cloning site of the vector. As well as MGF and IGF-I Ea constructed plasmids, a non constructed plasmid (for positive control) were stored in -80 °C.

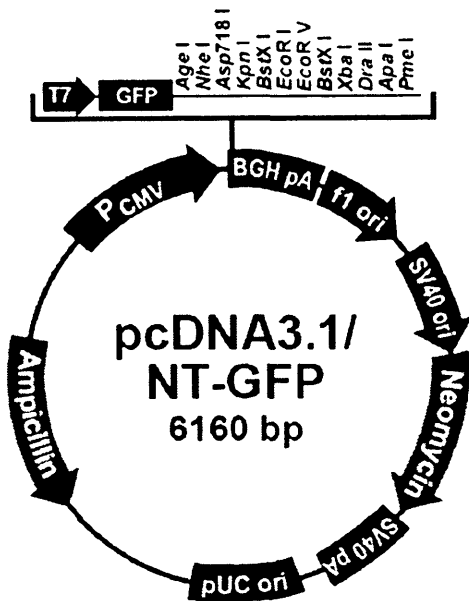


Figure 2.8: *pcDNA3.1/NT-GFP* vector map and its multiple cloning site.

2.3.2.2 Preparation of LB Agar Plates for Plasmid Purification

Firstly, 1 litre of Luria Bertani (LB) Agar was prepared. For preparation of LB Agar, 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g bacteriologic agar were put in a 1 litre clean bottle, and dissolved in 1 litre of distilled water by mixing well. The suspension was autoclaved at 121 °C for 15 minutes. LB Agar was then allowed to cool about 60 °C, and was then added an appropriate amount of ampicillin (the final concentration of ampicillin was 100 µg/ml).

The LB Agar was poured into 60 mm of Petri plates at room temperature. It was waited for until the gel in the plates to set. After setting, the plates were put in 37 °C of oven to dry off for about 2 hours. The LB Agar plates were separated into three groups and they were labelled as “MGF”, “IGF-I Ea”, and “Vector only”. The *pcDNA* 3.1 GFP/NT plasmid vectors containing cDNAs of IGF-I Ea and MGF, and only

vector (not containing any cDNA) were taken from -70°C store and were then placed on dry ice. The pcDNA 3.1 GFP/NT plasmid vectors were cultured onto labelled LB Agar plates. The plates were placed in a 37°C oven for overnight to enable the growth of colonies. The next day, the plates were checked for whether or not they had colonies. The colonised plates were filmed and stored at 4°C for future experimentations.

2.3.2.3 Preparation of LB medium:

Three 100 ml of LB medium was prepared. For preparation of the medium, three 500 ml of clean flasks were labelled as “MGF”, “IGF-I Ea” and “Vector only”. 1 g tryptone, 0.5 g yeast extract and 1 g NaCl were put into each flask. Then 100 ml of distilled water was added into each flask and they were mixed well for dissolving. The pH was adjusted to 7.0. The medium in flasks was autoclaved at 121°C for 15 minutes and were then cooled to about 60°C and then added ampicillin ($100\ \mu\text{g/ml}$).

2.3.2.4 Plasmid DNA purification (Maxi prep)

A QIAGEN EndoFree Plasmid Maxi Kit (Qiagen) was used for plasmid DNA purification. MGF, IGF-I Ea, and vector only plates were picked out from cold store. A single colony was picked out from each plate and inoculated into labelled autoclaved LB mediums. LB mediums were put in a shaking incubator. The colonies in LB mediums were grown at 37°C for 16 hours with vigorous shaking at 300 rpm.

Next day, the bacterial cells were poured into 250 ml of previously autoclaved and labelled centrifuge bottles and were harvested by centrifugation at $6000 \times g$ (6500 rpm

in Sarval GS3 rotor) for 15 mins at 4 °C. All traces of supernatant were removed from the bottles by not disturbing the pallets. 10 ml of chilled resuspension buffer (buffer P1) containing 50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A was added onto the bacterial pallet in bottle. The bacteria were resuspended completely by vortexing until no cell clumps remained. 10 ml of Buffer 2 (Lysis buffer) containing 200 mM NaOH, 1% SDS (w/v), was added to lysate and mixed gently by inverting 6 times and was incubated at room temperature for 5 minutes. The QIAfilter Maxi Cartridge was prepared during the incubation period. The cap was screwed onto the outlet nozzle of the QIAfilter Maxi Cartridge and it was placed into a labelled 50 ml of sterile centrifuge tube.

10 ml of chilled Buffer P3 (Neutralisation buffer) containing 3.0 M potassium acetate, pH 5.5 was added onto the lysate and mixed gently immediately by inverting 6 times. The lysate was poured into the barrel of the QIAfilter Maxi Cartridge, and it was then incubated at room temperature for 10 minutes. The cap was removed from the cartridge's outlet nozzle. The plunger was inserted gently into the cartridge and the cell lysate was filtered into a sterile 50 ml tube. 2.5 ml of Buffer ER was added to the filtered lysate and mixed well gently by inverting the tube 10 times and then incubated on ice for 30 minutes.

During the incubation period, QIAGEN – tip –500 was prepared for equilibration. The tip was placed into another sterile 50 ml tube and was applied 10 ml of Buffer QBT (Equilibration buffer) containing 750 mM NaCl; 50 mM MOPS, pH 7.0 and 15 % isopropanol (v/v), 0.15 % Triton X – 100 (v/v) and was waited for until the column to

be emptied by gravity flow and to be drained completely. After incubation, the filtered lysate was poured into the tip and was allowed to enter the resin by gravity flow.

The tip was washed twice by 30 ml of the washing buffer [1.0 M NaCl; 50 mM MOPS, pH 7.0 and 15 % isopropanol (v/v)]. The washed tip was placed into an autoclaved 30 ml endotoxin-free glass tube and was applied 15 ml of the elution buffer containing 1.6 M NaCl; 50 mM MOPS, pH 7.0 and 15 % isopropanol (v/v) to collect the elute. The DNA was precipitated by 10.5 ml (0.7 volume) of room-temperature isopropanol in order to elute the DNA. It was mixed well and immediately centrifuged at 15,000 x g (11,000 rpm in Sarvall SS-34 rotor) for 30 minutes at 4 °C. The supernatant was decanted very carefully.

The DNA pellet was washed by 5 ml of room-temperature endotoxin-free 70 % ethanol (100% ethanol was diluted in the endotoxin-free water); and then centrifuged at 15,000 x g for 10 mins. The supernatant was again decanted carefully without disturbing the pellet. Finally, the pellet was air-dried for 10 minutes, and then the DNA was redissolved in 100 µl of sterile PBS. 10 µl of purified plasmid DNA was separated for DNA cutting to assess the quality of the purified DNAs, and then remaining purified DNAs was stored in -20 °C.

2.3.2.5 The cutting and checking of DNAs using restriction enzymes

In order to assess the quality of the purified DNAs, purified DNAs were cut by restriction enzymes Apa I and Kpa I in a master mix and then visually inspected after staining by ethidium bromide under ultraviolet light.

Reagents	For each tube (ml)	For master mix tube (4X) (ml)
Apa I	1	4
Kpn I	1	4
Reaction buffer (Buffer 4)	2	8
BSA	0.2	0.8
dd H ₂ O	5.8	23.2
Total volume	10	40

Table 2.1: A master mix for DNA cutting to investigate DNA quality

All reagents were aliquoted into a labelled sterile tube on an ice, and were then mixed by vortexing. Then, 10 μ l of master mix solution was put into new labelled sterile tubes. 10 μ l of purified plasmid DNA were added into the tubes containing 10 μ l of master mix solution. The solutions were mixed again by vortexing and were then placed into a water bath at 37 °C for 2 hours in order to cut the DNAs.

While the DNAs were being cut, a 1.5 % of agarose minigel was prepared. For gel, 0.75 g of agarose was put in a 200 ml of flask and 50 ml of 1 X TAE was added into it. The suspension was microwaved for 80 seconds. The flask containing gel was cooled under cold tap water. Then, 2.5 μ l of Ethidium Bromide was added into 50 ml of cooled (warmed) gel for inspecting visually under ultraviolet light. A minigel tank was set and then the gel was poured into the minigel tank and was kept until being set. After the gel was set, a sufficient volume of 1 X TAE was added into the tank. As soon as the purified plasmid DNAs were cut, 3 labelled 1.5 ml sterile tubes were prepared. 20 μ l of cut DNAs were put into each labelled tube. 4 μ l of dye (1 in 6 volumes) were added into the tubes and mixed. 24 μ l of the DNA and dye mixture (20 μ l of DNA and 4 μ l of dye) were loaded into wells of the gel, and also 10 μ l of 50 Kb

ladder marker. The gel was electrophoresed at 90 V for 40 minutes. Finally the electrophoresed gel was imaged by ultraviolet camera. The image was then printed out. The image of cut DNAs can be seen from Figure 2.7 below.

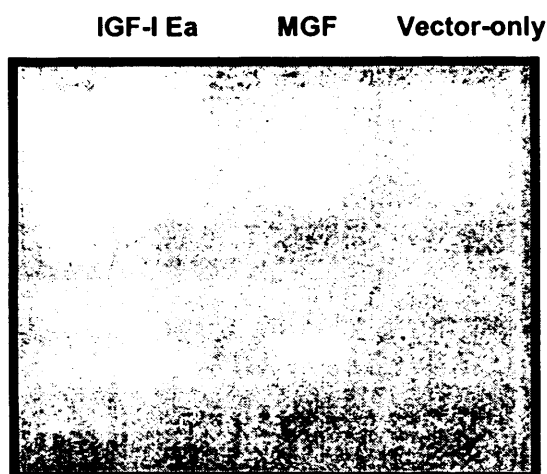


Figure 2.9: 1.5 % agarose gel picture showing cut purified DNAs in pcDNA3.1/NT-GFP vectors. There is only one product shown for each group as IGF-I Ea, MGF and Vector-only. Lane 1: 50 kb DNA size markers; lane 2: IGF-I Ea; lane 4: MGF; and lane 6: vector-only.

As can be seen from Figure 2.7, while IGF-I Ea and MGF were clearly visualised as having good quality DNAs, on the other hand, no DNA product was seen in vector-only group because it does not contain any cDNA insert.

2.3.2.6 Measuring of DNA concentration

DNA concentrations were determined by spectroscopy at 260 nm using the GeneSpec I device and software (Naka Instruments, Japan). Firstly, the GeneSpec I parameters were set as follows: the calculation mode was dsDNA, the calculation item was pure calculation and the dilution fact was 1.

A cuvette was washed with 5 μ l of DEPC-treated water and the water was then removed. The cuvette was re-washed with 5 μ l of absolute ethanol which was then

removed. Then the possible remaining ethanol was vaporised by waiting for 1-2 mins. 1.5 μ l of sterile PBS was put into the 'room' of the washed cuvette and then the cuvette was placed into the device. Then the "baseline" item on the screen was clicked. The PBS was removed from the 'room' and then the room of the cuvette was washed again with 5 μ l of DEPC water and absolute ethanol as explained above.

1.5 μ l of the sample DNA was put into the room and the 'measure' item on the screen was clicked. The value was recorded and also written on the label of the sample tube. The DNA sample in the room was then removed and washed again with 5 μ l of DEPC water and ethanol. The second sample was measured as mentioned before. All samples were measured in the same way, and the values were recorded and written on the sample tubes. Finally, the values were printed out and recorded in the lab book. The concentrations were adjusted to 1 μ g/ μ l through ethanol precipitation, vaporising or diluting with PBS.

2.3.2.7 Direct Intramuscular Injection of Plasmid DNAs into Animal Muscles

1 μ g/g (of the bodyweight of mice) of plasmid DNA in PBS (1 μ g / μ l) was injected through a 27 gauge needle into the middle of the right Tibialis Anterior (TA) muscle of the mice. The injections were applied directly into TA muscles by not making any skin incision. (Figure 2.10) No injection was made to the left TA muscles of animals for control. The same injections were applied into right TA muscles of all animals.

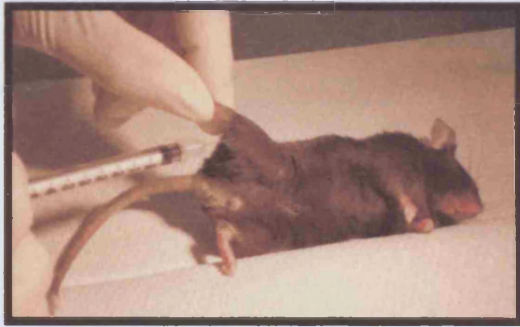


Figure 2.10: Direct injection of plasmid DNAs into TA muscle of a mouse

2.3.2.8 The Surgery Procedure

After injections, all animals were carefully monitored on a daily basis until the 21st day. In this period (on the 15th and 17th day after the injection), as was mentioned above, it was observed that two animals were in acute distress. After discussing with the vets in the CBU, these two animals were killed by cervical dislocation.

On the 21st day, animals were taken to the operation room. Surgery procedure was undertaken under terminal anaesthesia by 25% urethane diluted in double distilled H₂O (2.5 g urethane diluted in 10 ml of dd H₂O). Initially, a mouse was taken out of the cage with care and was then anaesthetised intraperitoneally using 0.3 ml of 25% urethane.

The anaesthetised animal was placed on a platform maintained at 37 °C with a temperature-controlled homoeothermic blanket. Both right and left legs were carefully shaved using an electrical shaver. A superficial longitudinal incision was made carefully onto right leg of animal on Tibialis Anterior (TA) muscle area and skin, and the fascia of the muscle was opened. The muscle and its tendon were carefully

dissected from their distal insertions, taking care to avoid damage to neurovascular pedicle (Figure 2.11). The TA muscle was separated from the other leg muscles.



Figure 2.11: Dissecting of TA muscle

The distal tendon of the right TA muscle was cut and then fixed by a silk thread to an isometric transducer that was linked to a computer. Additionally, a deep horizontal incision was made to the right side of gluteal area of the animal, and the sciatic nerve was found. A silk thread was passed underneath the nerve, and then both electrodes of a bipolar silver electrode were attached to the nerve by being passed underneath the nerve. A cotton bud was placed in between the electrodes to not touch each other.

The muscles were then activated indirectly by delivering maximal electric stimuli (square pulses, 0.2 sec pulse duration, 8V and 50 Hz) to the sciatic nerve proximal to nerve graft. The stimuli were generated by dual impedance research stimuli (Harvard Instruments Co. Ltd) and were delivered with a shielded bipolar silver electrode (Figure 2.12).

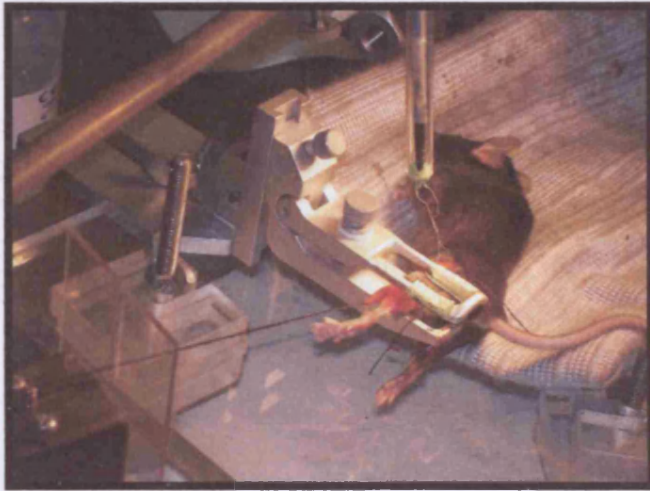


Figure 2.12: Measuring muscle force of mouse TA muscle

2.3.2.9 The Maximum Muscle Force Measurement

Before maximum muscle force measurement, a linear calibration of force transducer were figured out using different weight. Details of linear calibration of force transducer and the figure of it can be found from section 4.1.2.1.

After a linear calibration of force transducer were figured out, optimal muscle length (L_0) was determined by measuring the length of the TA muscle. The strongest contractions were generated following supramaximal stimuli by a device (Cambridge Electronic Device Ltd). The maximum muscle force was measured and recorded into a computer using a Spike 2 software program. The same surgical procedure was applied to the TA muscle of the untreated left control leg, and the maximum muscle force was measured and recorded by the same way. The same surgery and measurements were repeated for all animals.

Both the right and left TA muscles were removed from the animal (Figure 2.13) after physiological maximum muscle force were measured and then both TA muscles were weighed separately. Then TA muscles were cut into 3 pieces horizontally. The middle piece of the muscle was mounted in embedding medium (optimum cutting temperature compound – OCT) on a cork, and then was immediately frozen in isopentane cooled to the temperature of liquid nitrogen in order to prepare the future frozen sections for immunohistochemical analysis. The samples were then stored in -80°C . However, immunohistochemical analysis were not used for this study.

The distal and proximal parts of samples were put into labelled tubes containing $300\ \mu\text{l}$ of RNA Later solution (QIAGEN) and then the tubes were stored in -80°C for future quantitative real-time RT-PCR analysis.

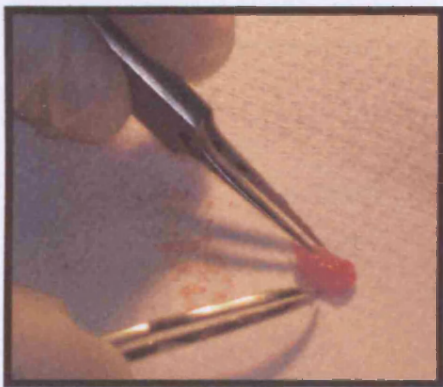


Figure 2.13: Mouse TA muscle

2.3.3 In vivo - satellite cell activation experimentation

2.3.3.1 The Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR has been developed by quantitative real-time RT-PCR technique in 1993 (Higuchi et al, 1993). Since then this technique has been increasingly used for the quantification of specific mRNA expression. Because quantitative real-time RT-PCR technique is highly sensitive and allows quantification of transcripts even in very low abundance, this technique was selected to determine satellite cell activation markers transcripts.

2.3.3.1.1 SYBR Green

SYBR Green I is a fluorescent dye which binds to the minor groove of dsDNA. Therefore, fluorescent signals of different amounts can be detected during various stages of PCR by the lightcycler machine and its software. As SYBR Green I fluorescent dye, 2X Quantitect SYBR Green PCR Master Mix (QIAGEN) was used.

2.3.3.1.2 Lightcycler

The cDNA amplifications and data collection was carried out using the Lightcycler machine (Roche Diagnostics Inc, Germany) with incorporation of fluorescent dye SYBR Green I. The lightcycler performs the real-time RT-PCR in small volume glass capillary tubes, contain within a rotor-like carousel. The rotor-like carousel are heated and cooled in an air stream (Bustin, 2000).

2.3.3.1.3 The Internal Control Reference Gene and Control Reactions

To facilitate optimisation, comparison and troubleshooting of the RT-reactions, different controls procedures including using internal control gene expression, an internal control / reference / housekeeping gene (β -actin) was used. All transcripts of mRNA levels for specific primers, including MyoD and myogenin, in different samples were compared with transcription levels of β -actin expression. Along with β -actin, other controls were used (two positive and three negative controls). For positive controls, (1) transcription levels of mRNA expressions from the TA tissues of injected and uninjected muscle samples from vector-only group, and (2) uninjected TA muscle samples from all three groups, and for negative controls, (i) No RNA, (ii) No RT, and (iii) only RNase-free water were used.

2.3.3.1.4 RNA Isolation

RNAs were isolated by TRI reagent (Sigma) according to manufacturer's instructions. First of all, the tissue samples which are in the tubes containing RNA Later were taken out from -70°C store and dissolved at room temperature, and then placed on ice. A couple of samples were randomly selected and their weights were measured. Having found the average weight, the volume of appropriate TRI reagent was then determined. The average tissue weight was about 30g, so the volume of TRI reagent was selected as 500 μl (500 μl TRI reagent per up to 50 mg of tissue).

In a fume hood, RNA Later was removed, and tissue samples were placed onto a clean tissue paper. The samples were dried and cut finely, and were then put into a sterile labelled 2 ml cryotube. 500 μl of TRI reagent was added into the cryotube. The

samples in the cryotube, containing TRI reagent, were homogenised well by sterile homogeniser until no pulp remained. After complete homogenisation, the probe of the homogeniser was washed in 5 ml of DEPC-treated water and 5 ml of absolute ethanol. The homogeniser was shaken well in order to dry. Another sample was taken and then dried and cut and homogenised in TRI reagent as explained above.

The homogenised samples in the cryotubes were transferred into a new labelled 1.5 ml sterile centrifuge tubes and then centrifuged at 12,000 x g for 10 min at 4 °C. The supernatants were again transferred into other new labelled 1.5 ml tubes, and the tubes were allowed to stand for 5 minutes at room temperature. 100 µl of chloroform was added into each tube. The tubes were covered tightly and vortexed for 15 seconds and then waited for 15 mins at room temperature. The mixture was centrifuged at 12,000 x g for 15 minutes at 4 °C. The centrifugation separated the mixture into three phases: a colourless upper aqueous phase for RNA, a white inter phase for DNA, and a red lower organic phase for protein. The colourless upper aqueous phase was carefully transferred into a fresh labelled tube without disturbing the white inter phase. 250 µl of isopropanol was added into the tube and mixed very well and then waited for 10 minutes at room temperature. The mixture was spanned down at 12,000 x g for 10 minutes at 4 °C and all supernatant was removed carefully without disturbing the pellet. The pellet was washed by adding 500 µl of 75% ethanol in RNase-free water and vortexed. The sample was centrifuged at 7500 x g for 5 minutes at 4 °C. All supernatant was decanted again. The RNA pellet was dried for 10 minutes for air – drying. Finally, 50 µl of diethylpyrocarbonate (DEPC)-treated water was applied onto

dried pellet and was mixed well in order to dissolve the pellet. It was waited for 20 minutes at room temperature and then the dissolved RNA was stored at -20°C .

2.3.3.1.5 Ethanol Precipitation

The concentrations of RNAs (or DNAs) were increased or decreased through ethanol precipitation. Therefore low concentrations of RNAs were treated by ethanol precipitation and the concentrations were increased. The following protocol was carried out for RNA ethanol precipitation. 50 μl of RNA was put into a sterile 1.5 ml centrifuge tube. 10% of total volume of RNA (5 μl) of Sodium Acetate (3M Sodium Acetate, pH 5.2) was added onto the RNA (5 μl of Sodium Acetate onto 50 μl of RNA, total mixture was 5+50=55 μl) and the mixture was mixed well. 2.5 volumes of total mixture of -20°C 100% absolute EtOH was added onto the mixture (EtOH volume was $55 \times 2.5 = 140 \mu\text{l}$). RNA, Sodium Acetate and ice-cold EtOH mixture was incubated at -80°C for 30 minutes, and then was centrifuged at 13,000 rpm for 10 minutes at 4°C . The supernatant was carefully removed, taking care for the pellet. 500 μl of 75% EtOH was added into the tube, which contains a pellet. After vortexing the tube to dissolve the pellet, the tube was centrifuged at 13,000 rpm for 5 minutes at 4°C . The supernatant was completely removed, taking extra care for the pellet again. The lid of the tube was left open and the pellet was air-dried by waiting at room temperature for about 10 minutes. Finally, 30 μl of DEPC-treated water was applied onto dried pellet and mixed well to dissolve it. The dissolved RNA was placed on ice and the concentration of the RNA was determined by spectroscopy at 260 nm, and then stored at -20°C .

2.3.3.1.6 The Determination of RNA concentrations

The concentrations of RNAs were also determined by measuring the absorbance at 260 nm (A_{260}) in the Gene Spec I spectrophotometer (Naka Instruments, Japan), with the same protocol, as explained in chapter 3 (3.2.3.4). However, the calculation mode of the parameters of the GeneSpecI software was set as RNA, instead of dsDNA. Other parameters and protocols were the same as those detailed 3.2.3.4 (measuring DNA concentrations).

2.3.3.1.7 The DNase Treatment of RNA Samples (DNase Digesting)

Successful reverse transcription is dependent on the purity of the mRNA used as the template. To remove any DNA contamination from RNA samples during RT-PCR is therefore crucial for having optimal results. The RNA templates should be DNA-free. Extra care should be taken to avoid the potential nucleic acids contamination. Thus, along with taking extra care in laboratory bench to minimize the potential cross-contamination, all RNA samples were also treated with 10 units of RQ1 RNase-free DNase (Promega) at 37 °C for 30 min followed by inactivation at 65 °C for 10 minutes in order to remove the possible amplification of contaminating genomic DNA. The samples were treated with DNase as explained in the following protocol:

Firstly, the RNA samples were thawed on ice. Then 1000 units of RQ1 RNase-free DNase (Promega), 10x Buffer (Promega) and RNase-free water were thawed at room temperature and then were stored on ice immediately after being thawed. Each solution was mixed by short-vortexing, and was then centrifuged briefly to collect

residual liquid from the sides of the tube. 1000 units of RQ1 RNase-free DNase was diluted as 50 units/ μ l. Then, a master mix was prepared for DNase digesting.

The master mix was prepared for 53 samples (46 for young mdx mice RNA samples, 2 for negative controls and 5 for the extras for unexpected pipetting losses). All samples were treated with DNase at the same time in order to have optimal results. The master mix for DNase digesting was prepared on ice according to the following table (Table 2.2):

Reagents	Stock solution	Final concentration	Volume for per reaction	Volume for master mix
RQ1 DNase	50 units	10 units	5 μ l	5 X 53 = 265 μ l
Buffer	10 X	1 X	2.5 μ l	2.5 X 53 = 132.5 μ l
Total			7.5 μl	397.5 μl

Table 2.2: A master mix for DNase digesting for RNA samples from young mdx mice TA muscles.

As shown in Table 2.2, to prepare a mister mix, 265 μ l of DNase (50 units/ μ l) were put into the master mix tube for 53 samples (46 RNA samples, plus 2 negative controls and 5 extras). 132.5 μ l of 10 X Buffer were added into the tube. Total volume of the master mix was 397.5 μ l. The tube was mixed by being vortexed shortly.

After preparing the master mix, 48 fresh autoclaved tubes were labelled (46 for RNA samples and 2 for negative controls). 7.5 μ l of master mix solution was distributed into individual labelled tubes. The tubes were kept on ice. The variable volumes of RNA samples were individually aliquoated to each labelled tube according to their concentrations to make up their concentrations as 5 μ g per reaction. No RNA sample was put into one of the negative control tubes (into the “no RNA” labelled negative

control tube). Appropriate RNA was added into second negative control tube (into the “no RT” labelled negative control tube). The variable volumes of nuclease-free water were also individually aliquoted into the tubes according to RNA aliquotations to make up a total volume of 25 μ l per reaction.

Following short-vortexing and centrifuging briefly to collect residual liquids from the walls of the tubes, the tubes were incubated at 37 $^{\circ}$ C for 30 minutes. 2.5 μ l of stop solution (Promega) were added into each tube, and the tubes were then short-vortexed and short-centrifuged again. Finally, the tubes were incubated at 65 $^{\circ}$ C for 10 minutes for inactivation the DNase. After the final incubations, the tubes, which contained DNase treated samples, were placed on ice and their concentrations were determined again by spectroscopy at 260 nm. All samples were then stored at -20 $^{\circ}$ C.

2.3.3.1.8 Reverse Transcriptase Reaction (cDNA synthesis)

Reverse transcriptase (RT) reactions were made for cDNA synthesis. Omniscript reverse transcriptase (QIAGEN) were used for the RT reactions. RT reactions for all RNase-free DNase treated-RNA samples from young mdx mice TA muscles were also carried out at the same time in order to yield optimal results.

Firstly, all DNase treated RNA samples including negative controls were thawed on ice. Then, random hexamers primers, 10x Buffer, dNTP mix, RNase inhibitor and RNase-free water were thawed at room temperature and were then stored on ice immediately after being thawed. Each solution was mixed by short-vortexing, and was then centrifuged briefly to collect residual liquid from the sides of the tube. The RT

reaction was carried out using the following concentrations of the following reagents which are shown in the table below (Tab 2.3):

Reagents	Stock solution	Final concentration	Wolume/reaction
Buffer RT	10 X	1 X	3
dNTPs	5 mM	0.5 mM	3
Random primers	50 pmol/ml	2.5 pmol/ml	1.5
RNAse inhibitor	10 u/μl	0.5 u/μl	1.5
RT enzyme	4 u/ μl	0.2 u/μl	1.5
Template RNA	-	0.75 μg/per reaction	variable
RNAse-free water	-	-	variable
Total volume			30 μl

Table 2.3: RT Reaction reagents and their concentrations

For the RT reaction, firstly, the master mix was prepared for 53 samples (46 DNase treated-RNA samples from young mdx mice TA muscles and plus 2 negative controls and plus 5 extras for unexpected pipetting losses). The mixture of the master mix was prepared according to the following table (Table 2.4).

Reagents	Stock solution	Final concentration	Volume for per reaction	Volume for master mix
Buffer RT	10 X	1 X	3 μl	3 X 53 = 159 μl
dNTPs	5 mM	0.5 mM	3 μl	3 X 53 = 159 μl
Random primers	50 pmol/ml	2.5 pmol/ml	1.5 μl	1.5 X 53 = 79.5 μl
RNAse inhibitor	10 u/μl	0.5 u/μl	1.5 μl	1.5 X 53 = 79.5 μl
RT enzyme	4 u/ μl	0.2 u/μl	1.5 μl	1.5 X 53 = 79.5 μl
Total			10.5 μl	556.5 μl

Table 2.4: A master mix for the RT reaction for RNase-free DNase treated-RNA samples from young mdx mice TA muscles.

As can be seen from Table 2.4, to prepare a master mix for 46 DNase treated-RNA samples from young mdx mice TA muscles and 2 negative controls, all aliquotations were prepared for 53 samples (46 for RNA samples, 2 for negative controls, and 5 for the extras). All the following reagents were put into a 1.5 ml of fresh autoclaved centrifuge tube to prepare a fresh master mix: 159 μl of 10 X Buffer RT (QIAGEN) (3 μl for each reaction), 159 μl of 5mM deoxynucleotide triphosphates (dNTPs)

(QIAGEN) (3 μl for each reaction), 79.5 μl of random hexamers primers (QIAGEN) (50 pmol/ml) (1.5 μl for each reaction), 79.5 μl of RNase inhibitor (Promega) (10 u/ μl) (1.5 μl for each reaction) and 79.5 μl of Omniscript Reverse Transcriptase enzyme (QIAGEN) (4 u/ μl) (1.5 μl for each reaction). The total volume of the master mix was 556.5 μl , and that of per reaction was 10.5 μl . After putting all above reagents into the same tube (the master mix tube), the tube was short-vortexed and then centrifuged briefly.

48 freshly autoclaved tubes were labelled (46 for the RNA templates and 2 for negative controls – one for noRT enzyme with RNA, and one for no RNA with RT). 10.5 μl of master mix solution was distributed into individual labelled tubes except the “no RT” labelled negative control tube. For the “no RT” labelled negative control tube, a separate master mix was prepared with no RT enzyme. Instead of RT enzyme, 1.5 μl of RNase-free water was added into the master mix of the “no RT negative control”. All tubes were kept on ice. The variable volumes of RNA templates were individually aliquoted to each labelled tube to make up their concentrations 0.75 μg per reaction, except to the “no RNA” labelled negative control tube. An appropriate volume of RNase-free water was put into the “no RNA” labelled negative control tube instead of RNA template. Then, variable volumes of nuclease-free water were individually aliquoted into the tubes according to RNA aliquotations to make up a total volume of 30 μl per reaction (Table 2.5). Individual tubes were then short-vortexed and centrifuged briefly to collect residual liquids from the walls of the tubes. Finally, all samples were incubated in a water bath tank at 37 $^{\circ}\text{C}$ for one hour, and

were heated to 93 °C for 5 minutes to inactivate the reverse transcriptase enzyme. The samples were then stored at -20 °C.

Reagents	Volume for each reaction
Master mix	10.5 µl
Template RNA	variable volume
RNAse-free water	variable volume
Total	30 µl

Table 2.5: RT reaction components for quantitative real-time RT-PCR.

2.3.3.1.9 Primer Design

The primer sequences were designed by Omega 2.0 software (Oxford Molecular). After finding the gene sequence of mouse MyoD, Myogenin and β-actin genes from NCBI web page, the sequences were transferred to Omega 2.0 designing software, and specific primers were designed for mouse MyoD, Myogenin and β-actin. The parameters of Omega 2.0 software were set as follows: The primer length: 18-24, G/C content, 40-60 percent; PCR product size: 100-300 bp; and annealing temperature: 50-65 °C. The Table 2.6 below shows the specific mouse primers for MyoD, Myogenin and β-actin.

Myogenin	Forward (5')	AGACGAAACCATGCCCAA	18 mer
	Reverse (3')	TAAAAGCCCCCTGCTACAGA	20 mer
MyoD	Forward (5')	TTTCTTCACCACACCTCT	18 mer
	Reverse (3')	TTAACTTTCTGCCACTCC	18 mer
β-actin	Forward (5')	GACGATGATATTGCCGCACT	20 mer
	Reverse (3')	GATACCACGCTTGCTCTGAG	20 mer

Table 2.6: Specific primers used to quantify MyoD and Myogenin expression by Real-time RT-PCR.

2.3.3.1.10 Optimising the annealing temperature of primers

Appropriate annealing temperature (T_m) of primers during PCR might be higher or lower than annealing temperature calculated from the sequence of the primers. Therefore, for optimising annealing temperatures, T_m was first calculated from nucleotide sequence of primers, according to the following formula.

$$T_m (^{\circ}\text{C}) = 2 (A+T) + 4 (G+C)$$

The annealing temperatures of each primer were then separately determined by real-time RT-PCR using SYBR Green I detection dye and lightcycler. For initial experiment, the target annealing temperature was started 5-6 $^{\circ}\text{C}$ below the calculated temperature. The target temperature in the annealing segment of the amplification programme was then increased 2 $^{\circ}\text{C}$ in each trial. Melting curves and points were analysed for each trail, and then, the annealing temperature for each primer was determined according to their melting peaks (whether or not having non-specific melting peaks). Annealing temperatures were determined for each primer as follows:

MyoD: 54 $^{\circ}\text{C}$, myogenin 57 $^{\circ}\text{C}$ and β -actin: 62 $^{\circ}\text{C}$.

2.3.3.1.11 The Preparation of the standards

For the quantification of different mRNA transcript levels for target genes and the control gene (β -actin), the appropriate homologues of the gene standards were used to confirm that the cDNA and the standard were amplified with the similar efficiency.

For preparing the standards, a sample, which has a high concentration, was selected and treated by RNase-free DNase according to the previously described protocol. The same sample was treated by RT-reaction and its cDNAs were synthesised. Then, a

real-time RT-PCR experiment was carried out by SYBR Green I master mix and lightcycler for 40 cycles and at 57 °C annealing temperatures. The 2% of agarose gel was then autoclaved at 121 °C for 15 minutes to remove possible RNase contamination, the PCR product was loaded into the gel and then electrophoresed at 100 mA for one hour by 2% agarose mini gel. The gel product was then visualised by ethium bromide-stained 18S and 28S rRNA under ultraviolet light.

In order to make the standards, the electrophoresed DNA products in the gel were extracted by Gel Extraction method, QIAquick Gel Extraction Kit (QIAGEN). The extraction protocol was as follows: The electrophoresed DNA fragments were excised from 2% of agarose gel with a clean and sharp scalpel under UV light in a dark room. Extra agarose was removed by cutting the slices as small as possible to minimize the size of the gel slices. The cut gel slices were weighed in a 15 ml colourless tube. 6 volumes of Buffer QG (guanidine thiocyanate) were added to 1 volume of the gel, because the gel concentration was 2%. The gel size was ~300 mg, therefore, 1800 µl (6X300) of Buffer QG was added onto the tube contains sample gel.

The tube containing gel-Buffer QG mixture was incubated in a hot bath at 50 °C until the gel slices had completely dissolved (it took about 15 minutes). During the incubation period, the tube was vortexed every 2-3 minutes for helping the gel to dissolve. The coloured of the dissolved gel Buffer QG mixture was yellow. 1 gel volume (300 µl) isopropanol was added to the sample mixture and mixed well. A spin column was placed in a 2 ml collection tube. 800 µl of sample was applied to the column and was centrifuged at 13,000 rpm (~17,900 x g) for 1 minute to bind the

DNA. The flow-thraw collected in the collection tube was discarded and the same spin column was placed in the same collection tube again. Another 800 μl of sample was applied to the column and centrifuged and the flow-thraw was discarded again until all sample solution was applied to the column. 750 μl of Buffer PE containing ethanol was added to the column and was centrifuged for 1 minute at 13,000 rpm to wash the DNA. The flow-thraw was discarded again, and the column was centrifuged for an additional 1 minute at 13,000 rpm. The column containing washed DNA was then placed into a sterile 1.5 ml microcentrifuge tube. Then, 50 μl of DEPC-treated water was carefully added onto the centre of the column membrane and the column was centrifuged for another 1 min. at 13,000 rpm to elute the DNA. After the column was removed, the concentration of the eluted DNA, which was collected in the tube, was measured.

The concentrations were arranged as 1×10^{-1} (0.1) $\mu\text{g}/\mu\text{l}$ and 1×10^{-2} (0.02) $\mu\text{g}/\mu\text{l}$ by diluting with DEPC-treated water. The samples were then stored at -20°C to be used as standards in future PCR experiments. During PCR experiments, the stored standards were diluted several times again with DEPC-treated water to have 10^{-1} $\mu\text{g}/\mu\text{l}$ to 10^{-8} $\mu\text{g}/\mu\text{l}$ of standards.

2.3.3.1.12 The Quantitative Real-Time RT-PCR

Firstly, great care was taken to minimise any contamination in this stage of the experiment. For instance, the laboratory bench was wiped thrawout by DEPC-treated water twice and then was cleaned again by 70% ethanol. Furthermore, the bench was wiped thrawout by nuclease-free aerosol. All materials, which were used in this stage

of the RT-PCR, including tubes, pipettes, were freshly autoclaved. The gloves used were frequently changed.

All template cDNA samples, including negative controls, 2X Quantitect SYBR Green PCR Master Mix, DNA primers and RNase-free water were thawed on ice. Each individual solution was mixed carefully. Then, a master mix was prepared according to 35 samples (24 DNA samples, plus 6 standards, plus 2 negative controls [1 for “no RT” sample, 1 for “no RNA” sample or 1 for “no RNA” sample, 1 for RNase-free water], and plus 3 extras for unexpected pipetting losses). The master mix volumes were aliquoted according to Table 2.7 below.

Reagents	Volume for each sample	Volume for master mix (for 35 samples)
2X SYBR Green PCR master mix	10 μ l	10 X 35 = 350 μ l
RNase-free water	6 μ l	6 X 35 = 210 μ l
Forward (5') primer (5mM)	1 μ l	1 X 35 = 35
Reverse (3') primer (5mM)	1 μ l	1 X 35 = 35
Total	18 μl	630 μl

Table 2.7: A master mix components for Real-time RT-PCR using LightCycler system.

The master mix tube was mixed thorawly. The lightcycler capillaries were prepared. As shown in Table 2.8, 18 μ l of master mix solution was distributed into each lightcycler PCR capillaries. Then, 2 μ l of DNA templates, including standards and negative controls were individually added to PCR capillaries, paying attention to their numbers.

Master mix	18 μ l
cDNA template	2 μ l
Total	20 μl

Table 2.8: Reaction components for Real-time RT-PCR using LightCycler system.

The covers of the capillaries were closed. The capillaries were then gently centrifuged very briefly and then put in the lightcycler machine and the program was set. The Lightcycler machine was programmed according to the program outlined in Table 2.9 below. After setting the programme, the PCR was run. 3 repeats were carried out for each condition.

STEP	TARGET TEMP. (°C)	HOLDING TIME (sec)	TEMP TRANSITION (°C/sec)
Initial activation step			
Initial activation step	95	900 (15 min)	20
Amplification step			
Denaturation	94	15	20
Annealing	57	25	20
Extension	72	25	20
	81	5	20
Melting step			
	95	0	20
	65	30	20
	95	0	0.1
Cooling step			
	40	30	20
Cycle number : 40			

Table 2.9: Real-time cycler conditions for the Lightcycler system.

2.3.3.1.13 Analysing quantitative real-time RT-PCR products

After the completion of PCR amplification, the data was analysed with the lightcycler software (Roche Diagnostic).

2.3.3.1.13.1 Melting Curve and Melting Peaks

All samples were compared by analysing melting curves and melting peaks for checking whether or not it had any unspecific PCR amplification. As can be seen from Figure 2.15, all PCR amplifications were melted at the same temperature (around 82 °C) and there was no unspecific amplification which melts in different temperature. Figure 2.15 also shows that, negative control samples did not have any melting peak,

which meant that there were no amplifications of the negative control sample. The straight linear at the bottom of both melting curves (Figure 2.14) and melting peaks (Figure 2.15) represent the negative control sample.

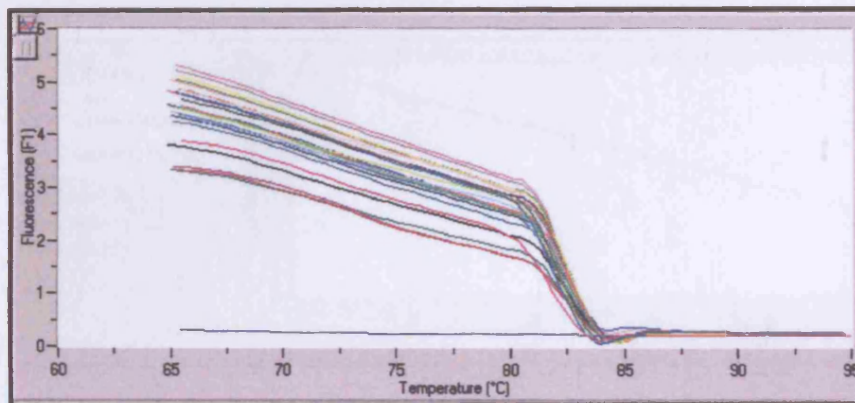


Figure 2.14: Melting curves. The straight linear at the bottom represents the negative control.

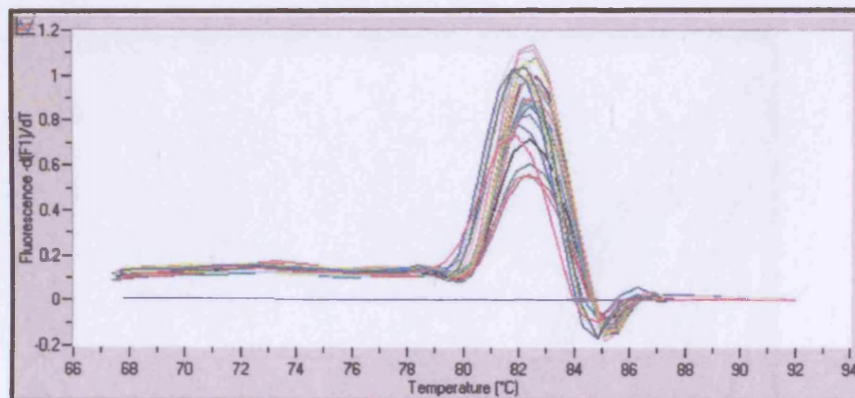


Figure 2.15: Melting peaks. The straight linear at the bottom represents the negative control.

2.3.3.1.13.2 Standard logs

For every single run, a serial standard dilution (6 standards for each assay) was prepared. The standard dilutions were as 1×10^{-1} , 10^{-3} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} . After the completion of the PCR amplification, a standard curve was made by the lightcycler program (Roche) according to their log concentrations. The standard curve was analysed according to their linear curve, and its slope, intercept, mean squared

error and r . Figure 2.16 shows a linear standard curve in six magnitudes of copies of MyoD. The slope of the standard curve was -3.938, and intercept was 3.222, mean squared error was 0.156 and the r was 1.00.

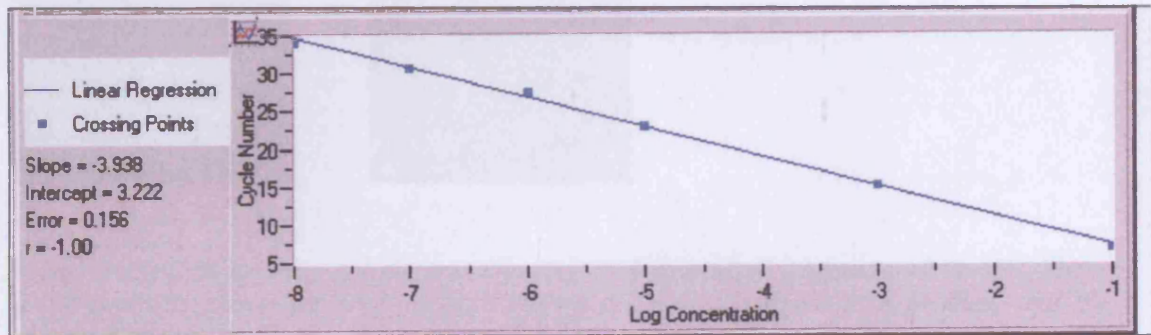


Figure 2.16: Standard curve. The panel shows a linear standard curve in six magnitudes of copies of MyoD. The standard curve generated by the Lightcycler software ranging from 1×10^{-1} to 1×10^{-8} ng of MyoD.

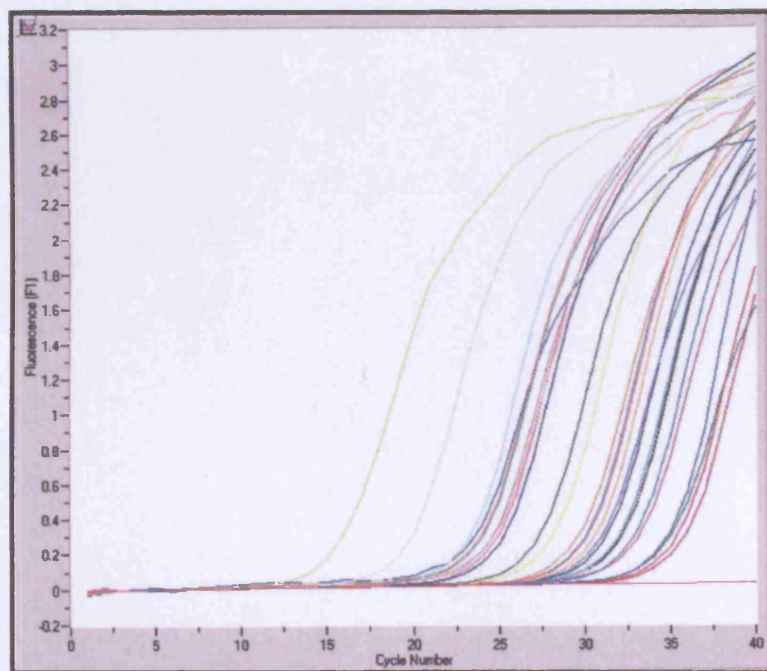


Figure 2.17: An amplification profile of standards and samples. The standards and samples show similar amplification efficiency.

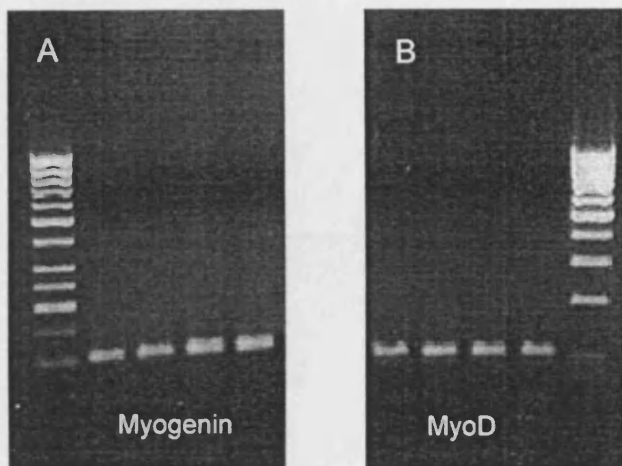


Figure 2.17: 2 % agarose gel pictures showing PCR products myogenin and MyoD. There are 4 products shown for each target. Picture A shows myogenin PCR product, and the picture B shows MyoD. Lanes 1 in A, and 5 in B are 100 bp DNA size markers; and lanes 2-5 in A are myogenin PCR product and, lanes 1-4 in B are MyoD PCR products.

2.4 Statistical analysis

Microsoft Excel software program of Microsoft Office 2003 software package were used for statistical analysis of this study. For in vitro cell culture experimentation data, 1-way ANOVA test was applied. The data were pooled for all runs for all observations on each condition, and the data were presented as mean \pm s.e.m. or \pm s.d. On the other hand, for in vivo – animal experimentations data of the study, paired t-test was applied to data. For the PCR part of the animal experimentation, the data were not pooled for all runs. Instead of pooling, paired t-test were applied using the mean values data (the mean values of all runs of the same samples were separately determined for every single sample); and then the data for injected muscle samples were compared with the data for uninjected muscle samples by paired t-test of Microsoft Excel statistics program. The data were presented as mean \pm s.e.m.

For significance test, $p < 0.05$ was considered significant (*). On the other hand, (**) was $p < 0.005$, and (***) was $p < 0.0005$.

**In Vitro Effects of IGF-I Isoforms
on Myoblast Proliferation and Differentiation
in Rodent Muscle Cell Lines and in
Degenerative and Healthy Human Primary
Muscle Cells**

3.1 The Effects of MGF on Myoblast Proliferation in Rodent Cell Lines

3.1.1 INTRODUCTION

For a very long time, it has been known that, cells can grow in cell culture conditions. Although the history of cell culture dates back to early twentieth century, even to nineteenth century, animal cell culture did not become a routine technique until the 1950s. Since then, it is a very common method to investigate normal physiological and biochemical functions of cells as well as pathological ones.

One of the main hypothesis of this study was that E domain of MGF influences muscle cell proliferation in in vitro cell culture models with muscle cells from different species, including human beings and rodents. Therefore, in this part of the study, in vitro - cell culture model experimentation with different rodent muscle cell lines (rat L6 and mouse C2C12) was designed for investigating the effects of IGF-I isoforms in animal myoblast proliferation.

3.1.2 METHODS

To study the effects of IGF-I isoform on muscle cells proliferation in different rodent myoblast cell lines, rat myoblast cell line (L6 E9) and mouse myoblast cell line

(C2C12), and BrdU proliferation assay technique were used. For both animal cells, rat myoblast cell line (L6 E9) and mouse myoblast cell line (C2C12), were treated with two different IGF-I isoforms (E domain of IGF-I Ec [MGF], and recombinant peptide of IGF-I [long r³ IGF-I]).

BrdU proliferation assays are used as a parameter for routine cell proliferation applications in living cells. BrdU works by incorporating into the newly synthesised DNA. The antibodies, which are specific for BrdU, are used to detect the incorporated BrdU into the replicating DNA. Therefore, the cells detected by a monoclonal antibody against BrdU indicates the proliferating cells. Thus, BrdU proliferation assays were used to investigate the roles of IGF-I isoforms in myoblast proliferation in rat and mouse myoblast cell lines.

3.1.3 RESULTS

3.1.3.1 The Effect of MGF in Myoblast Proliferation on Rat L6 Cells.

MGF did not show any effect on rat L6 cell lines. As can be seen from figure 3.1, any effect of MGF were not observed in the cell proliferation on rat L6 cell line even in different doses. The levels of cell numbers were similar between control cells and the cells which were treated with the different concentrations of MGF. In spite of three repeats, no significant changes on the cell proliferation on rat L6 cell line were observed. Because L6 cells were not responsive to MGF, no further work was done with these cells.

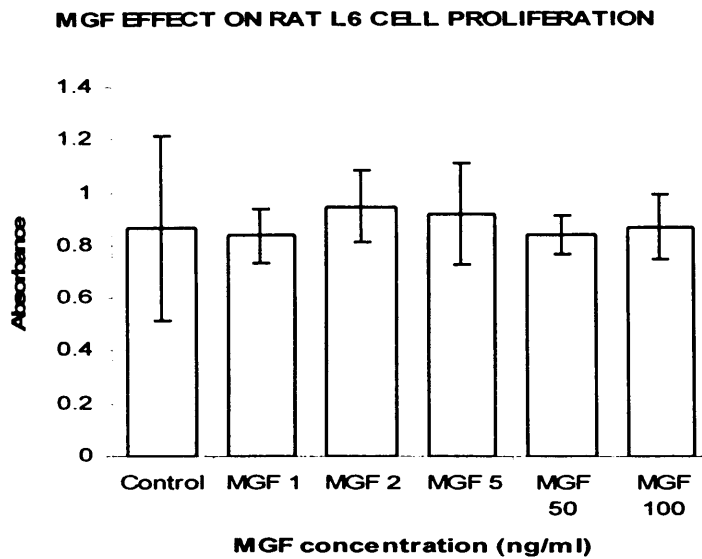


Figure 3.1: The effect of MGF on myoblast proliferation on rat L6 cells. MGF concentrations were various (0, 1, 2, 5, 50 and 100 ng/ml). $p > 0.05$ (non significant). Data were presented as mean \pm s.d.

3.1.3.2 The Effect of MGF in Myoblast Proliferation on Mouse C2C12 Cells

The same proliferation experiment was repeated three times on mouse C2C12 cell line by BrdU assay in the same conditions. Significant changes were observed in these experiments. The numbers of the dividing cells, which had been treated with MGF in different concentrations, were increased substantially. As can be seen from Figure 3.2, particularly in the numbers of the cells which were treated with 5, 50 and 100 ng/ml of MGF induced proliferation more than 2 fold. Figure 3.2 shows that, the effect of MGF was dose dependent. MGF had no effect in 1 ng/ml dose. The effect started in 2 ng/ml dose, and the doses between 5 and 100 ng/ml showed similar maximal results.

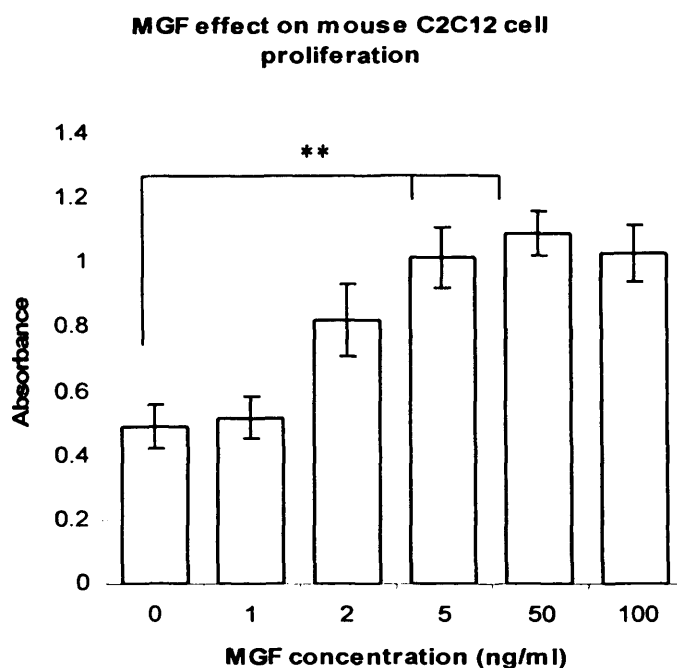


Figure 3.2: The effect of MGF on mouse C2C12 cells. MGF concentrations were various (0, 1, 2, 5, 50 and 100 ng/ml). $p < 0.005$ (** very significant). Data were presented as mean \pm s.d.

3.1.4 DISCUSSION

The study showed that MGF has a significant effect on mouse myoblast (C2C12) proliferation. The proliferation effect of MGF on C2C12 was dose dependent, and the effect was therefore not high in lower doses. In recent years, the physiological effects of two main splice variants of the IGF-I gene in muscle cells were studied and found that one of these variants (MGF) significantly increased myoblast proliferation in some vertebrates, including rabbits and mice (Yang et al, 1997; Yang and Goldspink, 2002). Yang and Goldspink showed that MGF increased mouse C2C12 myoblast proliferation, and its effect was higher than IGF-I Ea (Yang and Goldspink, 2002). This study demonstrated that the MGF had a significant effect on mouse myoblast proliferation; therefore, the study confirmed Yang and Goldspink's findings.

The study also showed that the MGF had no effect on myoblast proliferation in rat myoblast cell lines (L6 E9). Rosen et al suggests that IGF-I expression very low or nonexistent in the neonatal rat L6 cell line (Rosen et al, 1993). On the other hand, Braun et al suggests that L6 cells are restricted in their expression of myogenic factors (Braun et al, 1989), and MyoD is not expressed in L6 cells (Braun et al, 1989; Rhodes and Konieczny, 1989). MyoD is vital for myogenesis, and myoblast proliferation cannot start without MyoD expression. Therefore, MGF might be stimulating in L6 E9 cells, but the proliferation could not occur due to lack of MyoD expression. Thus, it seems that MGF could not affect myoblast proliferation in the lack of MyoD expression conditions.

3.2 The Effects of IGF-I Isoforms on Human Primary Muscle Cells

3.2.1 INTRODUCTION

In recent years, the physiological effects of two main splice variants of the IGF-I gene in muscle cells were studied and observed that one of these variants, which is the MGF, dramatically increased myoblast proliferation in some vertebrates, including rabbits and mice (Yang et al, 1997; Yang and Goldspink, 2002). As can be seen from first part of this chapter (3.1), similar effects was also observed in this study in mouse myoblasts – C2C12 cell lines . However, the physiological functions of the splice variants of the gene on human muscle in in vitro culture models have not been previously investigated. Therefore, this study, for the first time ever, investigated such peptides in humans muscles in in vitro primary cultured cell model.

3.2.2 METHODS

The effects of IGF-I isoforms on human primary myoblast proliferation (from healthy and degenerative muscles) were investigated by using immunocytochemistry assay; and on myotube formation by using CPK/BCA protein assay. As mentioned in section 1.1.1, because head and neck, and limb muscle tissues have a different embryologic origin, two different human muscle cells, namely the craniofacial masseter (head and

neck) and vastus lateralis (lower limb) muscle cells, were used in this study. Proliferation experiments have been completed with healthy craniofacial and limb muscle cells from healthy individuals; craniofacial muscle cell with congenital muscular dystrophy from a dystrophic (CMD) patient, limb muscles with motor neurone disease/ALS from different ALS patients and limb muscles with fascioscapulohumeral dystrophy from different FSHD patients. All five types of cells have been treated with IGF-I peptides with different doses, and fixed, stained with monoclonal primary and secondary antibodies, mounted on to slides and then stored at 4 °C.

3.2.2.1 Counting of the Immunostained Cells

30 fields were blindly counted in each coverslip by epi-fluorescence and phase contrast microscopy.

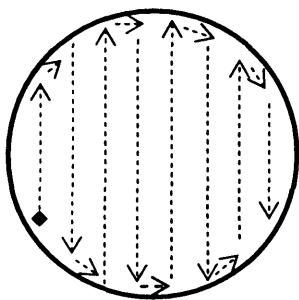


Figure 3.3: Methodology of counting of immunostained muscle cells on coverslips. Diamond shape (◆) shows starting point, and arrows show moving way.

For blind counting, the labels of the slides were closed, mixed and then relabelled as 1, 2, 3, 4, and so on. For counting, first of all, a field at left bottom corner of the coverslip was chosen and DAPI positive and Desmin positive cells in the field were

counted. Then another field next to first field was chosen by moving up vertically, and cells were counted. When reaching the top, it was moved to right horizontally. Then moved down vertically again. All fields were systematically counted by this way, which can be shown from Figure 3.3, until 30 fields were counted. Extra care was taken for not counting the same cells (or fields) twice.

The explant culture methodology generates cells of mixed lineage. These are daughter cells of satellite cells (muscle precursor cells) most of which are committed to myogenesis (desmin positive cells); and, the daughter cells of connective tissue cells (desmin negative cells). Therefore, some cells in this experiment were observed as desmin positive and some as desmin negative. On the other hand, the staining levels of desmin positive cells were differently stained, such very weak, weak, moderate or strong. Therefore, the staining levels were marked as + (very weak), ++ (weak), +++ (moderate), or ++++ (strong). Thus, very weak cells, which were marked as (+), did not consider as desmin positive and hence they were marked as desmin negative cells. Only the cells marked as (++) , (+++) , or (++++) were considered as desmin positive ones.

As can be seen from Figure 3.4, the nuclei of all living cells (DAPI positive cells) were seen in blue (A1, A2 and A3). On the other hand, in the same field, some cells were seen in green (desmin positive cells – myoblasts) (B1, B2 and B3), and some were not seen (desmin negative cells – fibroblasts) (B2 and B3). All living cells in the same field were also seen in the phase contrast microscope (C1, C2 or C3).

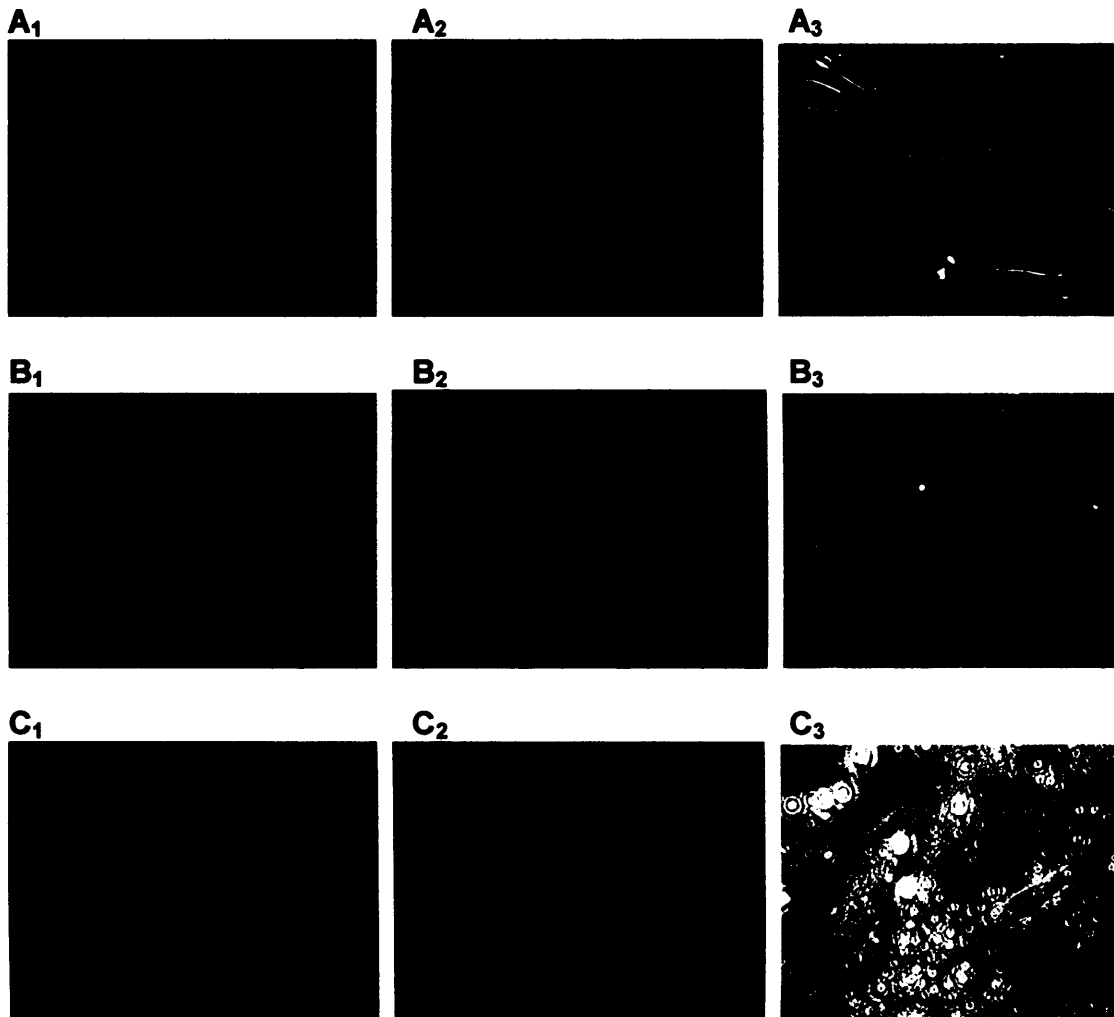


Figure 3.4: The non – sorted (mixed) cells under fluorescent and phase contrast microscopy. 1 for DAPI nuclear stained cells which indicates all living cells in a field, (nuclei are blue); 2s for desmin positive cells, which indicates myoblasts in the same field (myoblasts are green) and desmin negative cells; and 3s indicates all living cells in the same field under phase contrast microscopy. A, B and C show different fields. My: myoblast, Fb: Fibroblast.

After counting all coverslips on all slides and recording data in a lab book, the labels were re-opened, and recorded according to their original labels. Finally, the calculations of percentage were done. The ratios of the desmin positive myoblasts (green) to the DAPI positive living cells (blue) were used for interpretation.

3.2.3 RESULTS

3.2.3.1 Results for proliferation assays

3.2.3.1.1 Healthy Human Muscle

3.2.3.1.1.1 Healthy Human Craniofacial Muscle Cells

Figure 3.5 show that, MGF had increased myoblast proliferation significantly. The increase of MGF was 38 percent. On the other hand, long r³ IGF-I, and MGF and long r³ IGF-I together had not increased myoblast proliferation significantly. As can be seen from figure 3.5 and table 3.1, the long r³ IGF-I had increased myoblast ratio only 17 percent. When both IGF-I peptides were added together, the ratio had increased only 19 percent.

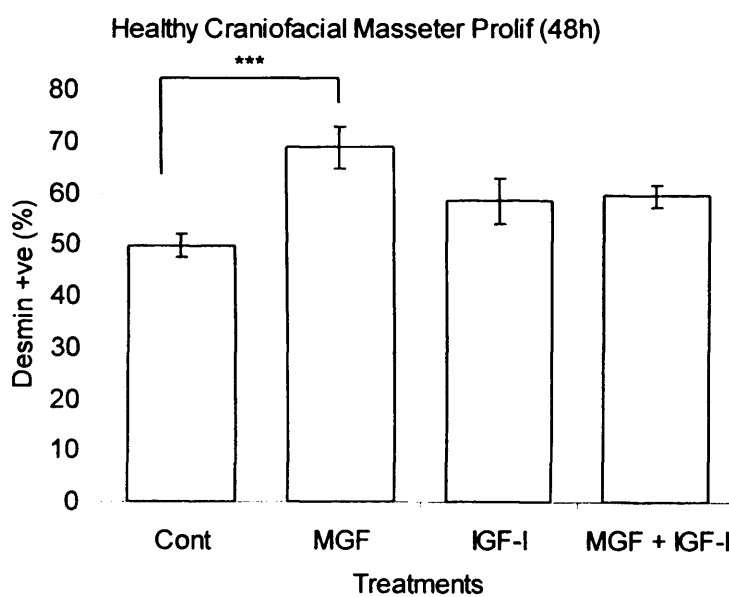


Figure 3.5: The effects of IGF-I isoforms on healthy craniofacial muscle cells. The concentration for MGF was 10 ng/ml; for recombinant IGF-I was 10 ng/ml and for MGF + long r³IGF-I was 10 ng/ml (for MGF) and 10 ng/ml (for long r³IGF-I). (***): p Value: <0.0005. Data were pooled for all runs and presented as mean \pm s.e.m.

Treatments (growth factors)	Desmin + ve mean (%)	Increase (%) (compare to control)
Control	49.8	-
MGF	68.8	38.2
Long r ³ IGF-I	58.4	17.3
MGF + long r ³ IGF-I	59.5	19.4

Table 3.1: Data for myoblast proliferation experiments on healthy craniofacial masseter muscle cells

3.2.3.1.1.2 Healthy Human Limb Muscle (Vastus Lateralis) Cells

The same effects of IGF-I isoforms were also observed on normal human limb muscles. Figure 3.6 and table 3.2 show that both, MGF and r³ IGF-I had increased myoblast proliferation significantly. The MGF raised it significantly (51 %). In contrast, systemic IGF-I increased the proliferation only to 23 percent.

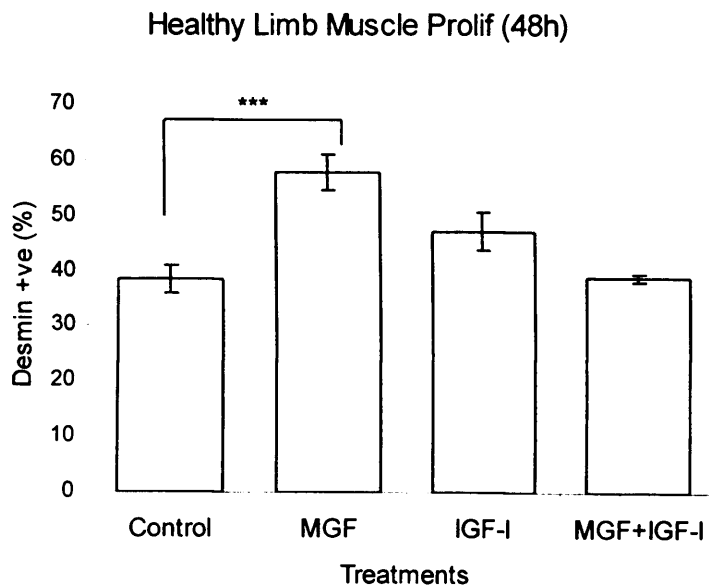


Figure 3.6 The effects of IGF-I isoforms on healthy human limb muscle cells. The concentrations for both MGF and long r³ IGF-I were 10 ng/ml. (***) : $p < 0.0005$ Data were pooled for all runs and presented as mean \pm s.e.m.

On the other hand, as can be seen from the figure 3.6 and table 3.2, when MGF and long r³IGF-I are used together, the effects decreased dramatically. When MGF and long r³IGF-I were used together, almost no change was observed (only 1% increase).

Treatments (growth factors)	Desmin + ve Mean (%)	Increase (%) (compare to control)
Control	38.4	-
MGF	57.9	50.6
Long r ³ IGF-I	47.1	22.6
MGF + long r ³ IGF-I	38.8	1

Table 3.2: Data for myoblast proliferation experiments on healthy limb muscle cells

3.2.3.1.2 Degenerative Human Muscle

3.2.3.1.2.1 Proliferation Experiments on the Muscle Cells with CMD

Figure 3.7 and Table 3.3 show again that IGF-I isoforms had similar effects on the muscle cells from the muscle of a patient with CMD. In this experiment, craniofacial muscle cells from a patient with CMD had been treated with MGF, long r³ IGF-I and Ab-I, together and separately.

Myoblast proliferation was increased significantly when CMD cells had been treated with MGF both with and without IGFIR blocking antibody. The MGF had increased myoblast proliferation as 68 percent. The long r³ IGF-I with and without MGF had also increased myoblast ratio, but only slightly (26%), not as dramatically as MGF. (Figure 3.7 and table 3.3). On the other hand, another significant fact was observed. Myoblast ratio was very low, around just 10 percent (10.4 percent). (Figure 3.7 and table 3.3) In contrast, in normal human primary muscle non-sorted cells, the rate of myoblasts in mixed cells was between 38 and 49 percent. In normal (healthy) limb

muscle cells, the ratio was 38 percent (Figure 3.7 and Table 3.2), while in normal craniofacial cells, the ratio was 49 percent (Figure 3.5 and table 3.1).

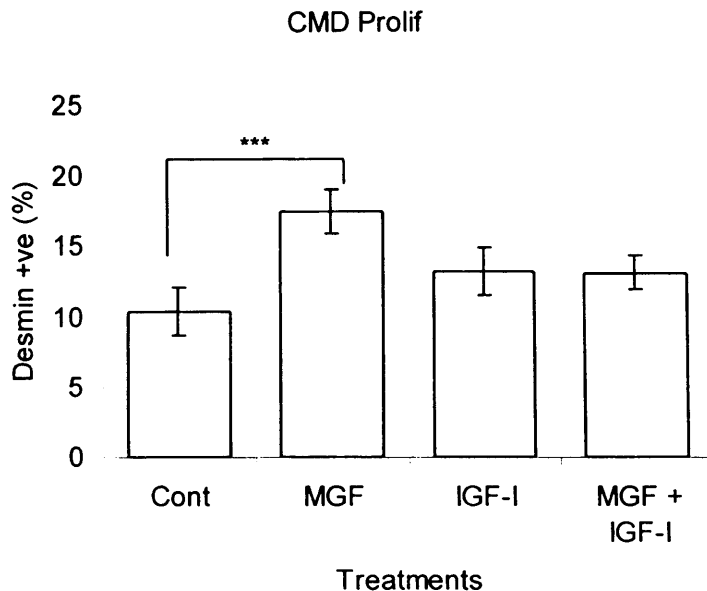


Figure 3.7: The effects of IGF-I isoforms on the muscle cells from the muscle of patients with CMD. The concentrations for both MGF and long r^3 IGF-I were 10 ng/ml (***) : $p < 0.0005$. Data were pooled for all runs and were presented as mean \pm s.d.

Treatments (growth factors)	Desmin + ve Mean (%)	Increase (%) (compare to control)
Control	10.4	-
MGF	17.5	68.1
Long r^3 IGF-I	13.2	26.6
MGF + r^3 IGF-I	13.1	26.2

Table 3.3: Data for proliferation experiment on craniofacial muscle cells with CMD.

3.2.3.1.2.2 Proliferation Experiment on the Muscle Cells with ALS

As can be seen from Figure 3.8, quite similar effects have been observed on myoblasts from muscle of patients with ALS. MGF increases myoblast proliferation dramatically again (49%). The long r^3 IGF-I did not increase the proliferation with and without MGF.

A low ratio of myoblast was observed again in ALS, like in congenital muscular dystrophy. In ALS, the ratio was much lower (just 4.8 percent). Data can be seen from Table 3.4.

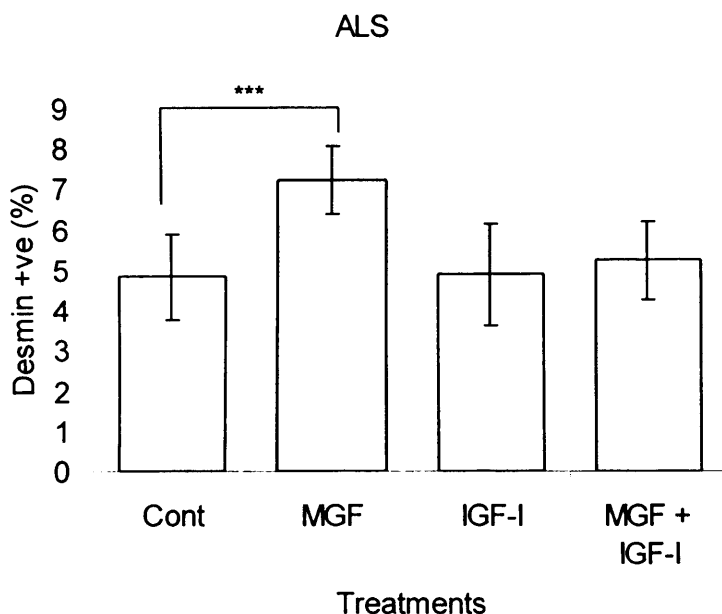


Figure 3.8: The effects of IGF-I isoforms on the muscle cells from the muscle of patients with ALS. The concentrations for both MGF and long r^3 IGF-I were 10 ng/ml; (***) : p : <0.0005. Data were pooled for all runs and were presented as mean \pm s.d.

Treatments (growth factors)	Desmin + ve Mean (%)	Increase (%) (compare to control)
Control	4.8	-
MGF	7.2	49.8
Long r^3 IGF-I	4.9	1
MGF + long r^3 IGF-I	5.2	8

Table 3.4: Data for proliferation experiment on limb muscle cells with ALS.

3.2.3.1.2.3 Proliferation Experiment on the Muscle Cells with FSHD

Almost the same effects have been observed on myoblast proliferation derived from patients with FSHD. Figure 3.9 shows that MGF increases on myoblast proliferation in higher ratio (74%). The long r^3 IGF-I increased the proliferation in lower ratios with and without MGF (both 24%). Low ratios of myoblast were observed again in myoblasts from patients with FSHD, like in CMD and ALS. In FSHD, the ratio was low again (just 11 percent). Data can be seen from Table 3.5.

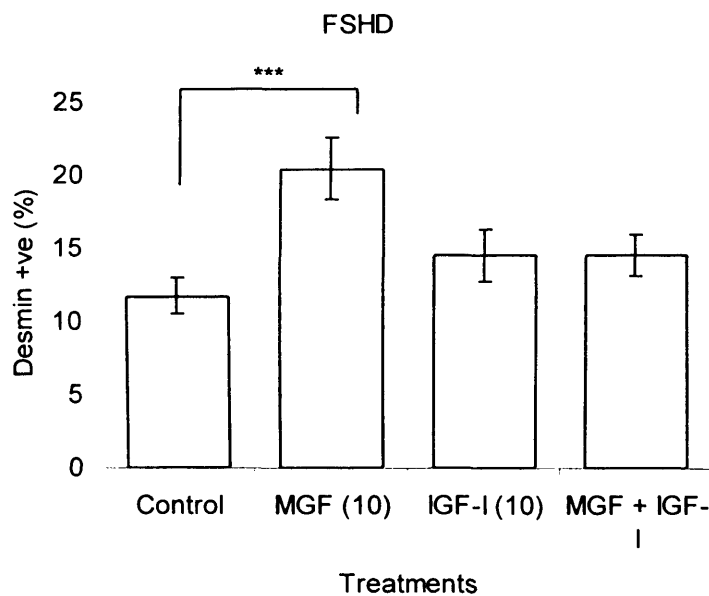


Figure 3.9: The effects of IGF-I isoforms on the muscle cells from the muscle of patients with FSHD. The concentrations for MGF and long r^3 IGF-I were 10 ng/ml; (***) : P Value: <0.05 (8.1E-07). Data were pooled for all runs and were presented as mean \pm s.d.

Treatments (growth factors)	Desmin + ve (%)	Increase (%) (compare to control)
Control	11.7	-
MGF	20.4	74.4
Long r^3 IGF-I	14.5	23.8
MGF + long r^3 IGF-I	14.5	23.8

Table 3.5: Data for proliferation experiment on limb muscle cells with FSHD.

3.2.3.1.3 Proliferation Experiments with IGF1R blocking antibody

Healthy and degenerative cells were also treated with type 1 IGF receptor (IGF1R) blocking antibody (Ab-I) in order to investigate whether or not MGF affects proliferation through IGF1R activity. For this aim, the cells from the craniofacial masseter muscle of healthy individual, and the cells from the vastus lateralis muscle of patients with CMD and ALS. When the cells had been treated with anti IGF1R blocking antibody (Ab-I), significant changes were not observed. For instance, as can be seen Figure 3.10, in healthy craniofacial muscle cells, when the myoblast percentages of untreated control cells compared to the same cells treated with Ab-I, the ratio decreased only 2 percent. Similar observation was seen in MGF treated cells. Comparing to increasing ratio of MGF treated cells, with the same cells treated with Ab-I, the increasing ratio was decreased only 3 percent.

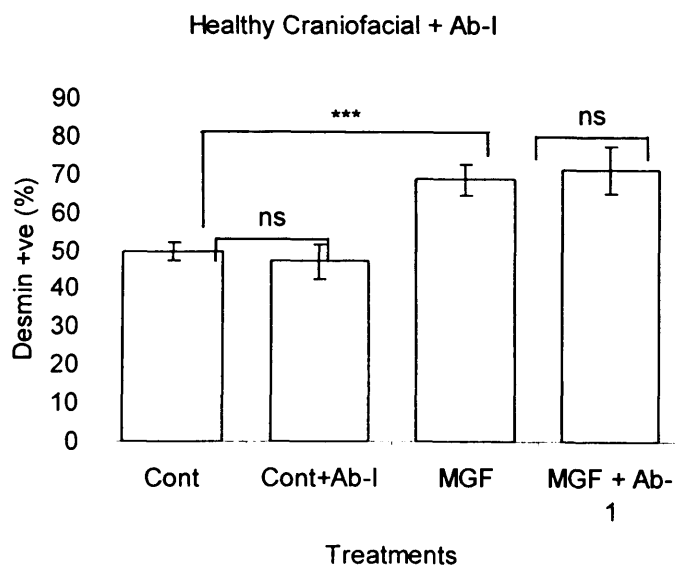


Figure 3.10: The effects of MGF and Ab-I on healthy human craniofacial muscle cells. The concentrations for MGF were 10 ng/ml; for Ab-I is 500 ng/ml (***) : $p < 0.0005$, ns: non significant (> 0.05). Data were pooled for all runs and presented as mean \pm s.e.m.

Similar effects were also observed on the cells from the muscles of patients with CMD and ALS. It was observed again that Ab-I did not change the effects of MGF. (Figures 3.11 and 3.12). Figures 3.10, 3.11 and 3.12 show that MGF does not act through IGFIR. Therefore, these experiments confirmed that MGF does not appear to affect proliferation through activity of IGFIR (Figures 3.10, 3.11 and 3.12).

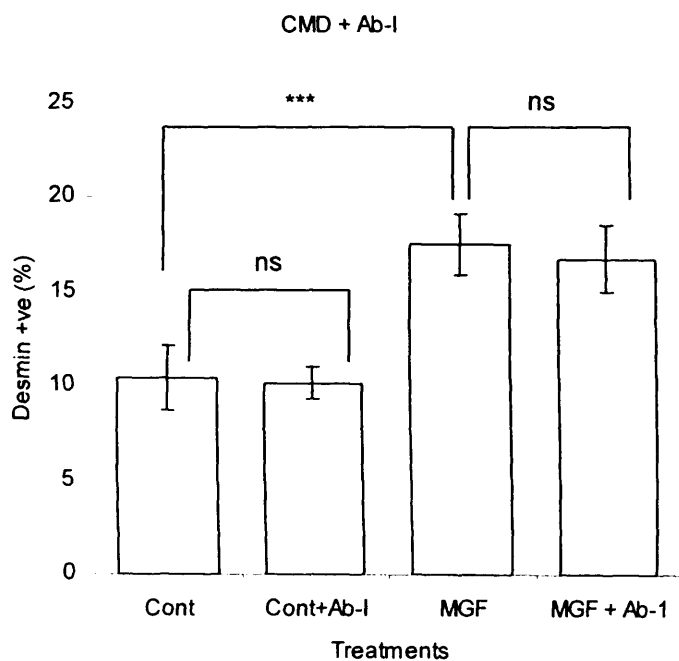


Figure 3.11: The effects of MGF with and without anti IGFIR blocking antibody (Ab-I) on the muscle cells from the muscle of patients with CMD. The concentration for MGF was 10 ng/ml and for Ab-I was 500 ng/ml. (***) : $p < 0.0005$, ns: non significant (> 0.05). Data were pooled for all runs and were presented as mean \pm s.d.

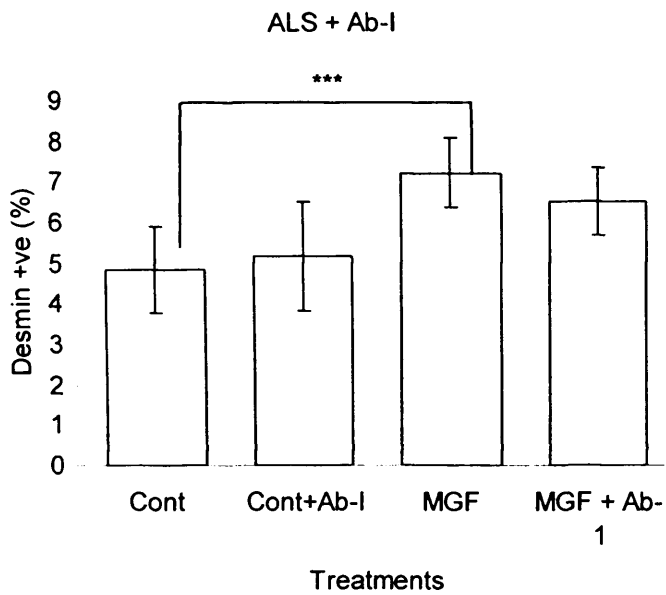


Figure 3.12: The effects of MGF with and without anti IGF-IR antibody (Ab-I) on the muscle cells with from the muscle of patients ALS. The concentrations were that MGF was 10 ng/ml and Ab-I was 500 ng/ml. (***) : $p < 0.0005$. Data were pooled and presented as mean \pm s.d.

Figure 3.13 shows all three experiments together in the same figure.

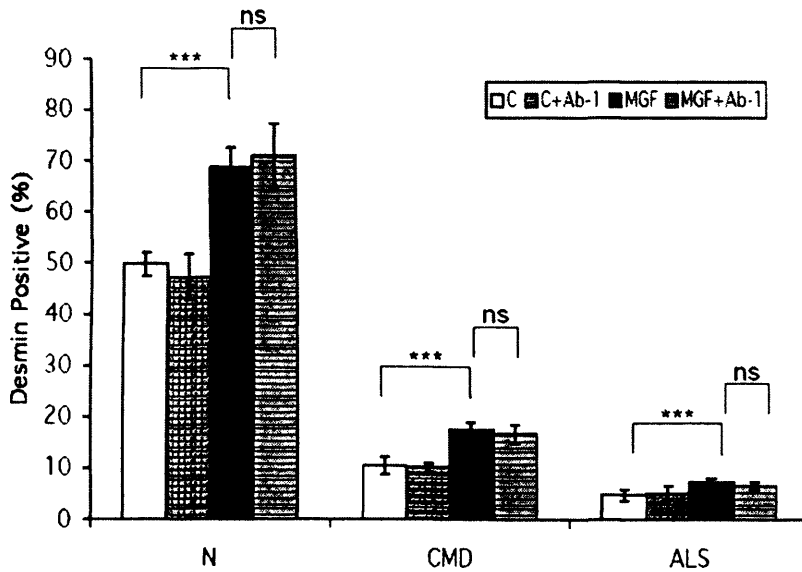


Figure 3.13: The effects of MGF and IGF-IR blocking antibody (Ab-I) on the muscle cells from muscle of healthy individuals and the cells from the muscle of patients with CMD and ALS. The figure also shows myoblast ratios in healthy and degenerative (CMD and ALS) muscle. N (normal) shows the cells from healthy individuals. The concentrations were that MGF was 10 ng/ml and Ab-I was 500 ng/ml. (***) : $p < 0.0005$. Data were pooled for all runs and were presented as mean \pm s.d.

3.2.3.2 Results for differentiation assays

3.2.3.2.1 Differentiation experiments for healthy human muscle cells

Differentiation experiments using CPK/BCA protein assays were carried out on the cells from the craniofacial masseter muscle of a healthy individual. Creatine kinase (CK) or creatine phosphokinase (CPK) is a marker of differentiated muscle fibres. Therefore, CPK/BCA protein assay were carried out to investigate differentiation state of myoblasts.

Figure 3.14 shows that MGF has no overall effect on myoblast differentiation (myotubes formation). The long r³ IGF-1 stimulates myotube formation effectively at 10 ng/ml. The MGF does not affect at doses between 10 and 100 ng/ml, but it antagonises the effect of IGF-I in a dose-dependent manner. This can be seen from Figure 3.14.

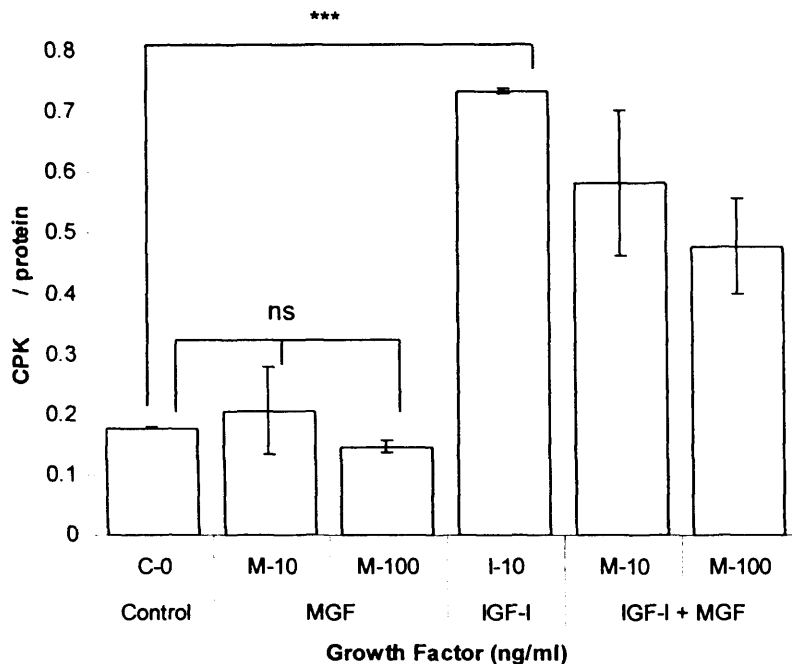


Figure 3.14: The effect of MGF with and without long r^3 IGF-I on differentiation for normal (healthy) human craniofacial muscle cells. MGF doses were 0 (for control), 10 and 100 ng/ml. long r^3 IGF-I dose was 10 ng/ml. (***) : $p < 0.0005$, ns: non significant (> 0.05). Data were presented as mean \pm s.d.

3.2.3.2.2 Differentiation experiments for human degenerative muscle cells

After completing differentiation experiments for healthy human muscle cells by CPK/BCA protein assays, Sigma company had stopped the production of CPK assays kit, therefore CPK assay kit could not be provided any longer. Thus, the remaining differentiation experiments for degenerative muscle cells were not able to be carried out by the same assay. Instead of such assay, immunocytochemistry technique was designed. As can be seen from Figure 3.15, it is possible to determine myotube formation by this technique. Differentiation would be observed by the calculation of the percentages of desmin positive multi nucleated myotubes to the percentages of single nucleated myoblasts. The study had sufficient data to suggest that

immunocytochemistry assay was a good technique for determining cellular differentiation in muscle tissue. As can be seen from Figure 3.15, some multinucleated myotubes were visualised in normal (healthy) human primary muscle cells after being immunostained with primary and secondary antibodies.

Figure 3.15 shows both mononucleated myoblasts (marked as My) and multinucleated myotubes (marked as Mt) in the same field. Both myoblasts and myotubes were seen as DAPI positive (blue stained cells -A₁ and B₁) and desmin positive (green stained - B₁ and B₂). The cells were also seen in phase contrast microscope (A₃ and B₃). Multinucleated myotubes (marked as Mt) can be seen in Figure 3.15.

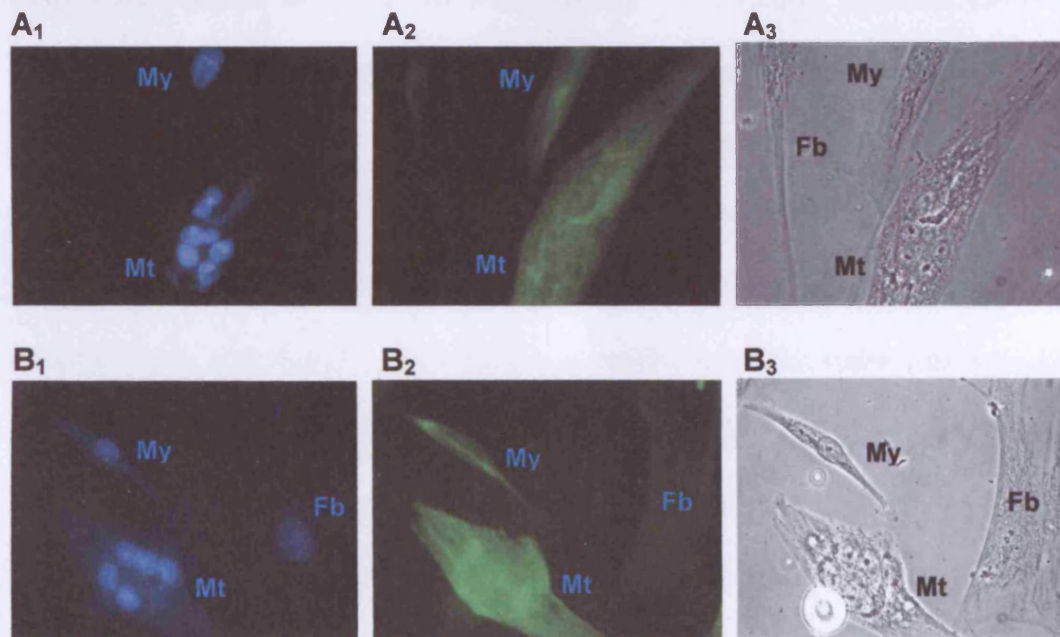


Figure 3.15: The multinuclear myotubes under epi-fluorescent and phase contrast microscopy. A₁ and B₁ show DAPI positive myoblasts, fibroblasts, (nuclei are blue); A₂ and B₂ show desmin positive myoblasts, myotubes and desmin negative fibroblast in green fluorescent; and A₃ and B₃ show phase contrast microscopy in the same fields. My: mononucleated myoblasts, Mt: multinucleated myotubes, Fb: Fibroblasts. A and B show different fields.

Although myotube formation were clearly observed and multinucleated myotubes were growth in healthy human muscle cells, they could not be seen in degenerative muscle cells. Despite all efforts, those differentiation experiments with the degenerative muscle cells by immunocytochemistry assays were unsuccessful to identify any multinucleate myotube. After adding Muscle Cell Differentiation Medium, the cells were daily checked under light microscopy until being seen the development of myotube formation in order to add different growth factors in different doses for different conditions. Even though the experiments were repeated several times with different types of degenerative muscle cells (those from CMD, FSHD and ALS patients), it was observed that myoblasts were not fused to myotubes. Cells were cultured for up to 10 weeks with no evidence of myotube formation. Although the differentiation medium was changed every 3-4 days, before adding growth factors, myotube formation was not observed. The composition of differentiation medium was also changed (from 0.5 % BSA, with low insulin [5 ng/ml] with 1 % P/S in DMEM to 2 % FBS with 1 % P/S in DMEM); the result was the same again: myotube formation did not occur. Nevertheless, some cells were fixed and immunostained without seeing myotube formation. All DAPI positive and Desmin positive cells were single nucleated myoblasts. No desmin positive multinucleated myotubes was observed. Therefore, differentiation states for degenerative muscle cells could not be determined.

3.2.4 DISCUSSION

This part of the study has showed that MGF has an effect on muscle cell proliferation. The MGF dramatically increased muscle cell proliferation in vitro in human primary cultured myoblasts. In humans, the MGF dramatically increased myoblast proliferation in every kind of cells, which are studied, in the cells with different embryological origins (from a head and neck muscle, and from a limb muscle), and also, the cells healthy and with degeneration from both head and neck muscle, and from limb muscle). The study showed that the E peptide of MGF induces progenitor cell proliferation significantly in primary cultured muscle cells from patients with CMD, FSHD and ALS as well as healthy individuals. In this study, E peptide of MGF increased myoblast proliferation 38.2 % in healthy craniofacial muscle cells (Figure 3.6 and Table 3.1), in healthy limb muscle cells 50.6%, (Figure 3.7 and Table 3.2), in the cells from the muscle of patients with CMD 68.1% (Figure 3.8 and Table 3.3), in the cells from the muscle of patients with ALS 49.8% (Figure 3.9 and Table 3.4) and in the cells from patients with FSHD 74.4% (Figure 3.10 and Table 3.5). All data were very significant.

On the other hand, long r³ IGF-I (systemic IGF-I) peptide increased myoblast proliferation in lower levels. The long r³ IGF-I peptide increased the proliferation in healthy craniofacial muscle cells 17.3 % (Figure 3.6 and Table 3.1), in healthy limb muscle cells 22.6%, (Figure 3.7 and Table 3.2), in the cells from patients with CMD 22.6% (Figure 3.8 and Table 3.3), in the cells from patients with ALS only 1% (Figure 3.9 and Table 3.4) and in the cells from patients with FSHD 23.8% (Figure 3.10 and Table 3.5). The increase effects of the MGF were always higher (more than

one fold) than those of systemic IGF-I one. When the cells treated with MGF and long r³ IGF-I peptide together, the effects were similar to those of long r³ IGF-I peptide alone. According to these data, it can undoubtedly say that, MGF increases in all kind of human muscle cells, head and neck or limb, healthy or degenerative. On the other hand, it was observed that the increase effects of MGF was clearly higher than those of systemic IGF-I.

In contrary, MGF had not any effect on muscle differentiation (myotube formation). As can be seen from Figure 3.14, two IGF-I isoforms, IGF-I Ea (systemic IGF-I) and MGF, have antagonist effect in differentiation. The data in this study clearly showed that MGF had not any effect on myoblast differentiation although it remarkably stimulated myoblast proliferation. Whereas, the systemic form of IGF-I had a huge effect on myotube formation while having limited stimulation on myoblast proliferation. The study also demonstrated that MGF had reduced the effect of IGF-I Ea on myoblast differentiation. When E peptide of MGF was added to long r³ IGF-I, which were used as an IGF-I Ea peptide in this study, the effect of long r³ IGF-I on myotube formation decreased (Figure 3.15). The preventative effect of E domain of MGF on myoblast differentiation was dose dependent, and the effect of IGF-I Ea decreased more when MGF was added to IGF-I Ea in higher doses. The present work therefore demonstrated that the MGF E domain had a distinct biological activity compared to long r³ IGF-I. It caused the mononucleated progenitor (satellite) cells to replicate but it has no effect on myotube formation. In contrast, long r³ IGF-I had a marked effect on such formation. Gillian Butler-Brown and Vincent Mouly suggest that systemic IGF-I starts signalling by Akt pathway after fusion competent myoblasts

fuse to myotubes, not before (Unpublished data by Butler-Brown and Mouly) (this information was obtained by Prof Goldspink from Drs Gillian Butler - Brown and Vincent Mouly during a private communication in Myores meeting, held in Rome, in November, 16th -19th, 2005). Thus, the data in this study confirmed that, MGF and long r3 IGF-I had different cellular actions: MGF increased myoblast proliferation whilst systemic IGF-I Ea induced myotube differentiation. This finding confirms Yang and Goldspink's, and Mouly and Butler-Browne's data (Yang and Goldspink, 2002; Mouly and Butler-Browne, unpublished data).

Previous studies have used BrdU or Alamar Blue, particularly those that claim IGF-I causes satellite cell replication. However, these studies have often not used markers to distinguish cells at different stages of the lineage pathway. This study found that a satellite cell activation marker, desmin, to be suitable to distinguish the muscle progenitor cell for other myoblasts. This part of the study also found that immunocytochemistry using desmin and DAPI markers is a good technique for determination of cellular proliferation and differentiation in skeletal muscle tissue. As can be seen from Figure 3.4 and 3.16, mononucleated myoblasts and fused multinucleated myotubes can be clearly seen in the culture conditions, and their proliferation and differentiation stages can be easily determined by the technique under fluorescent microscopy.

It has been known that the ratios of satellite cells drop in elderly people. This study also demonstrated that the ratios of myoblasts (progenitor or activated satellite cells) of degenerative muscles cells were also low. As can be seen from the result section of

this chapter, the ratios of myoblasts from the patients with FSHD, CMD and ALS were very low (5, 10 and 12 percent) compared to the ratios of myoblasts from healthy individuals (between 38 and 50 percent). According to data in this study, satellite cell pools are quite low in degenerative muscles compare to the cells in healthy individuals. The lack of satellite cells in degenerative muscles might be cause the decline of regeneration capacity in the patients with degenerative muscles.

The E peptide of MGF increased myoblast proliferation in the cells from a healthy limb muscle more than the cells from a healthy craniofacial masseter muscles. The ratios of increase were respectively 50 and 38 percents. Pavlath et al suggest that head and neck muscles, for instance the craniofacial masseter muscle, which used in this study, regenerate poorly compared to limb muscles (Pavlath et al. 1998). This finding might explain the differences between increase levels of two different healthy muscles.

In this study, despite extensive efforts, to obtain any muscle differentiation (myotube formation) in culture of human degenerative muscle cells was unsuccessful. Degenerative myoblasts did not fuse and myotube formation was not observed despite all efforts. Thus, differentiation state was only determined in healthy human muscles. In this study, it was observed that the ratios of degenerative myoblasts were low, compare to those of healthy cells were high. As it will discuss in details later in the general discussions, inhibition of calcineurin may play an important role in degenerative muscle pathophysiology (Stupka et al, 2004). It has been known that calcineurin is a mediator of IGF-I signalling pathway (Musaro et al, 1999). Thus,

Shavlakadze et al propose that muscle degeneration can be due to the lack of IGF-I signalling (Shavlakadze et al, 2004). As a result, myotube formation might not be occurred because of decreasing activated satellite cells ratios / myoblasts number; that of lack of IGF-I, particularly MGF, signalling; that of insufficiency of mechanotransduction, etc.

This study also demonstrated that IGF1R blocking antibody (Ab-I) did not prevent the action of MGF in myoblast proliferation. The effects of MGF alone were similar to those of MGF with Ab-I in healthy craniofacial muscle cells and also the cells from the muscles of patients with CMD and ALS. In all cells, when the data of control cells compared to those of MGF treated cells, the data always were very significant (Figures, 3.11., 3.12., 3.13 and 3.14). Whereas, the data of the cells compared to those of the cells also treated with Ab-I (control to control + Ab-I, and MGF alone to MGF + Ab-I), the data were non-significant (Figures, 3.11., 3.12., 3.13 and 3.14). According to these data, different actions of different IGF-I isoforms are probably mediated via different receptors. The data concerning the blocking of the type 1 IGF receptor in this study provided some evidence that MGF increases myoblast proliferation via a different signalling pathway, and it does not bind to IGF-I receptor. Therefore, MGF might bind to different cell surface receptor or receptors other than IGF1R; and it seems that MGF peptide is an independent growth factor to systemic form of IGF-I, although both are splice variants of the same IGF-I gene.

**An In Vivo Gene Therapy Application
for mdx Mouse Model of DMD Using cDNAs
of IGF-I Splice Variants, and the Role of
MGF in Tissue Repair Mechanism /
Satellite Cell Activation**

4.1 Roles of IGF-I Splice Variants in Muscle Growth in mdx Mouse Model of DMD

4.1.1 INTRODUCTION

Currently, there are millions of patients with neuromuscular disorders, and some other conditions associated with muscle weakness and wasting worldwide. Among them, DMD is the most common muscular dystrophy. Therefore, treatment for such diseases and conditions is crucial. Thus, the aim of this study was to attempt a novel gene therapy application for treating muscle degeneration in such disorders. Previous IGF-I delivery research have shown some anatomical and biochemical improvements in skeletal muscles (Lynch et al, 2001; Barton et al, 2002). Therefore, cDNAs of IGF-I splice variants were delivered into mdx mice skeletal muscles to drive functional improvements and restoration of muscle strength.

4.1.2 METHOD

The previous chapter of the study focused on investigating in vitro effects of IGF-I isoforms on skeletal muscle cells (myoblasts) proliferation and differentiation by cell culture model. It has been known that in vivo experimentation provides more knowledge holistically. Thus, the roles of cDNAs of IGF-I splice variants in skeletal

muscle tissue were also investigated in an animal subject with muscle degeneration, which was mdx mouse model. On the other hand, it was previously shown that mdx skeletal muscle did not produce MGF even during exercise (Goldspink and Yang, 2001; Goldspink and Yang, 2004; Goldspink, 2005; Goldspink, 2006). In addition, Prof Goldspink suggests that the production of MGF is deficient in certain diseases such as in the muscular dystrophies in which the mechanotransduction mechanism is defective. According to Prof Goldspink, deficiency of MGF production in dystrophin deficient degenerative muscles, because of insufficiency of mechanotransduction mechanism in such conditions (Goldspink, 2006). This was another reason why mdx mouse model was chosen as in vivo experimentation subject. For the same reasons, it was logical to study the transfer of MGF cDNA as an alternative gene treatment to replacing or repairing the dystrophin. Goldspink, 2005).

After three weeks (21 days) introducing cDNAs of IGF-I splice variants into one of TA muscles of young and old mdx mice, the maximum muscle forces and the weights of both TA muscles (untreated control, and treated ones) were measured in order to investigate the effects of such variants in muscle growth by strength and size. The detailed methods of in vivo experimentation section.

4.1.2.1 Calibration of the force transducer

Before maximum muscle force measurement for each TA muscle of each animal, a linear calibration of the force transducer was figured out using different weight in grams as force. Weights were as 10, 15, 25, 37.5, 50, 62.5, 75 and 100 gr.

Measurements were done with above weight by a force transducer device (Harvard

Instruments Co. Ltd) and the voltage output in V for each weight in grams were recorded into a computer using Spike 2 - Version 4 (from CED- Cambridge Electronic Design - UK) software program. After founding reading values (voltage output) for each weight, a linear standard curve were figured out (Figure 4.1). Then a formula to convert voltage output of the force in grams into maximum muscle force value in milli Newtons ($y = 0.0784 X - 0.0758$) was calculated by the computer from the data of the linear calibration plot. The force measurements were in grams, then these measurements were converted into milli Newtons (mN) by using above formula.

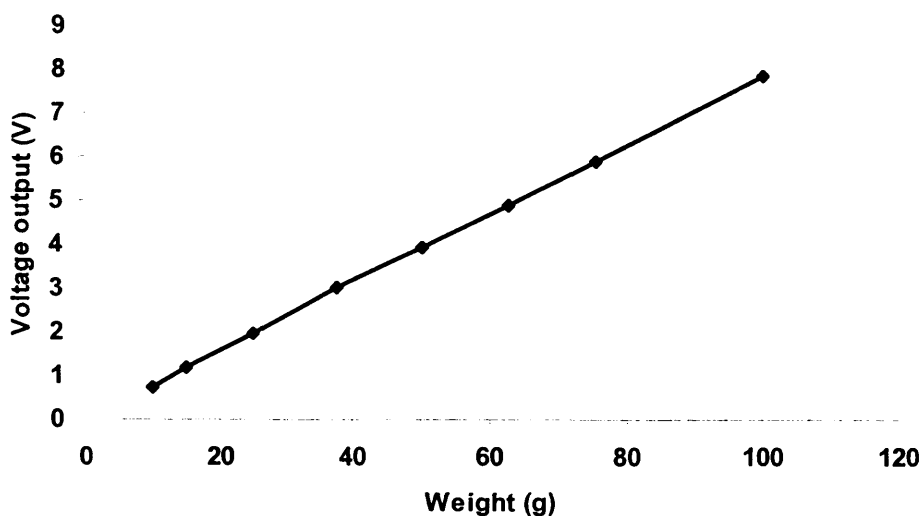


Figure 4.1. A linear calibration of the force transducer. $y=0.0784 X - 0.0758$; $R^2: 0.9994$.

As the measurements were isometric this is just multiplying contractile tension (force) by a constant. In 1938, A. V. Hill hypothesized specific relationships between the force generated by a muscle and the speed at which a stimulated muscle contracts under a given load. A stimulated muscle may contract to 1/3 its size at a particular speed. (Hill, 1938). Then Hill expressed this hypothesis in following equation:

$$(P + a)(V+b) = c.$$

P describes the force generated by a muscle, V is the speed at which a muscle contracts, and a, b, c are constants. The constant (a) describes the force expended to make the muscle contract, and (b) describes the smallest contraction rate of the muscle. Then, the equation for maximum muscle contraction can be changed as follow: (Beals et al, 1999)

$$P = \frac{c}{V + b} - a$$

4.1.2.2 The calculation of maximum muscle force:

After completing all measurements, and removing and then storing the TA muscles, the calculations for maximum muscle force values were carried out using the computer by Spike 2 software – Version 4 (CED- Cambridge Electronic Design - UK). The raw data in mV were then calculated by using above formula to determine the maximum muscle force in mN.

As an example, as can be seen Figure 4.2-A, two force measurements were made for each TA muscle, and therefore, there were two peaks (A1 and A2). The reading value of the peak of the first force (A1) for the mouse (a young mouse which MGF was injected into its right TA muscle) was 4.79887. The starting level of A1 force was below of 0 level (- 0.106026). Thus, this value was added to the peak value to calculate maximum muscle force. In this sample, the reading value of the maximum force measurement of A1 was $4.79887 + 0.106026 = 4.904896$. (Figure 4.2-A)

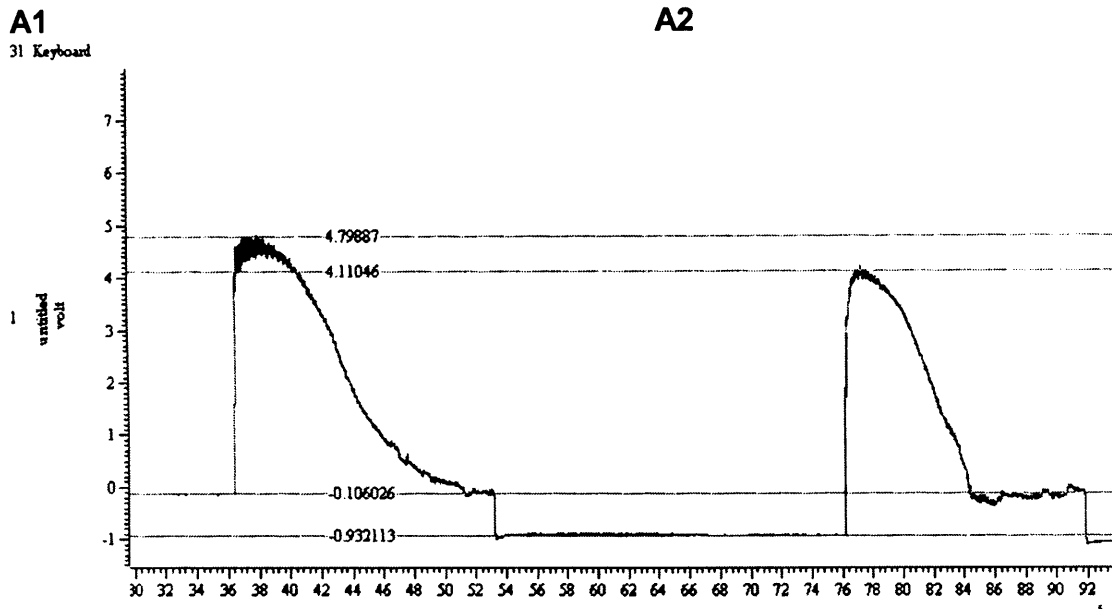


Figure 4.2: Individual maximum force of an MGF injected TA muscle from a young mdx mouse. Axes are (V) and time (sec).

In the second measurement (4.2- A2), the reading value of the peak was 4.11046, and the stimulation started from -0.932113. Then, after adding the starting reading value (-0.932113) to the reading value of the peak (4.11046), the reading value of the maximum muscle force of the second measurement was calculated as $4.11046 + 0.932113 = 5.042573$. The reading value of the A1 was 4.904896, and that of the A2 was 5.042573. Because the reading value of the second measurement (A2) was bigger than first one (A1), the reading value of A2 was selected as the reading value of the maximum force of that MGF treated animal. For this example, 5.042573 was selected instead of 4.904896.

The same calculations were also carried out for the untreated left control TA muscle. As shown from Figure 4.3, the reading value of the maximum force of untreated TA muscle of the same animal was just 4.1. In contrast, the reading value of the

maximum force of MGF injected TA muscle (right TA muscle) was 5.042573 (nearly 20% increase) (Figure 4.2 – A2). The reading values of the maximum muscle force were repeated by the same calculating method for all animals both old and young, and for both injected (control) and uninjected (experimented) TA muscles.

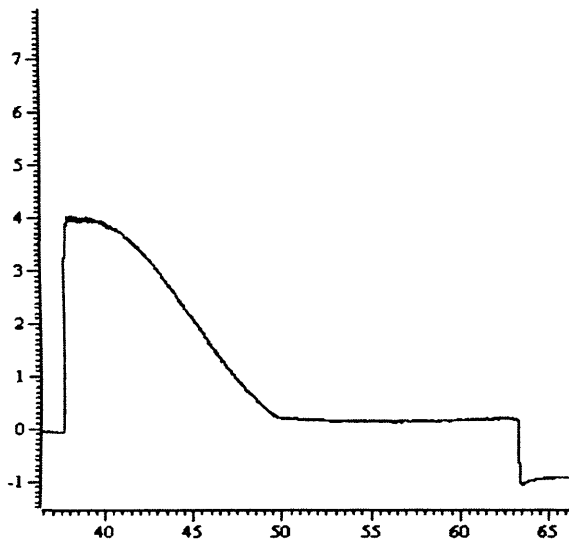


Figure 4.3: Individual maximum force of untreated TA muscle from the same MGF treated young mdx mouse. Axes are (V) and time (sec).

Figure 4.4 shows the reading value of the maximum force of an old mdx mouse. Figure 4.4-A shows MGF injected TA muscle, and 4.4-B shows uninjected TA muscle of the same animal. Differences between two maximum force measurements showed the effect of the MGF. As can be seen from the Figures 4.4-A and B, the maximum force of an MGF uninjected old mdx mouse was about 6.5 (Figure 4.4 B), the uninjected control was just about 8.5 (Figure 4.4 A).

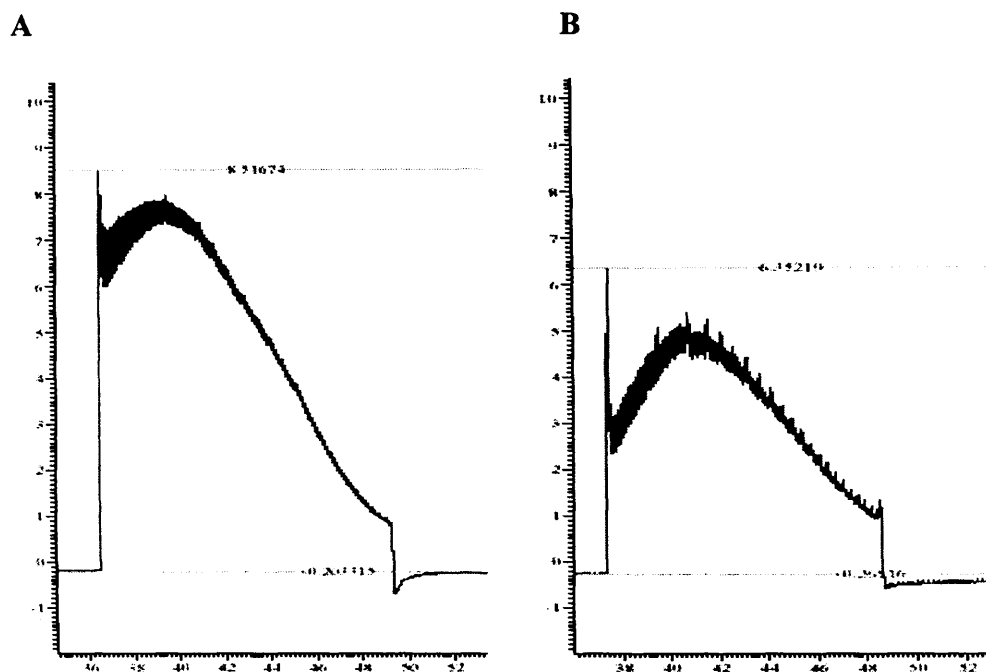


Figure 4.4: Individual maximum forces of an MGF injected and uninjected - control TA muscle of the same old mdx mouse. A: Maximum force of MGF injected TA muscle and B: Maximum force of uninjected control TA muscle. Axes are (V) and time (sec).

However, the reading values do not show the exact maximum force values. In order to find the exact maximum muscle force, a formula (the formula was calculated by a linear standard calibration) was applied to all reading values. The formula was as follows: Y (reading value) = $0.0784 X - 0.0758$;

$$\text{If, } Y \text{ (reading value)} = 0.0784 X - 0.0758;$$

$$X = Y + 0.0758 / 0.0784.$$

$$X \text{ (the exact maximum muscle force - in mN)} = y \text{ (reading value)} + 0.0758 / 0.0784.$$

For instance, the reading value (y) of above example was 5.042573 mV.

Then, the exact maximum muscle force was:

$$X = 5.042573 + 0.0758 / 0.0784 = 65.28537 \text{ mN.}$$

All calculations for both legs of TA muscles of all animals were completed using the same formula ($Y = 0.0784 X - 0.0758$ or $X = Y + 0.0758 / 0.0784$).

When all measurements were calculated, and the means and graphs were extracted from these calculations, it was observed that the means of maximum muscle forces for each group were quite different from each other, because the bodyweights and the ages of old animals varied. Therefore, in order to demonstrate the changes more clearly, the muscle force values of both treated and untreated TA muscles were recalculated again and the data were normalised to 100 for untreated control muscles. Following this, all calculations and graphs for all animals (old and young) were drawn again according to these new data.

4.1.3 RESULTS

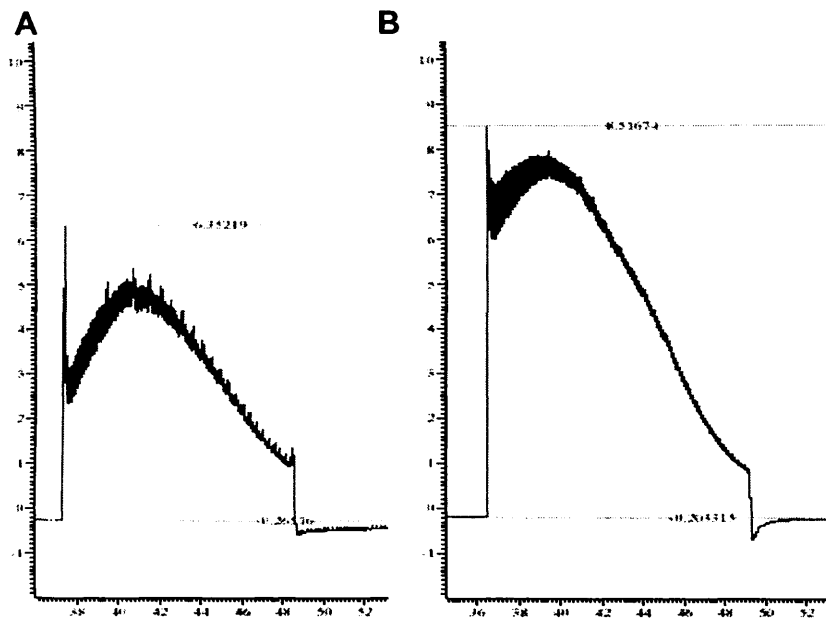
This study showed that IGF-I splice variants, particularly MGF, increased muscle forces in mdx mice in both old and young ages. MGF increased muscle strength more than IGF-I Ea in both age groups. Comparing the old and young animals, the effect of MGF was considerably high in young animals in just 3 weeks time and by only one injection. MGF also increased muscle strength in older ages, though not with same rate as with the younger animals, and the rate was in a lower rate (data was also significant statistically).

4.1.3.1 Effects of IGF-I splice variants in old mdx mice

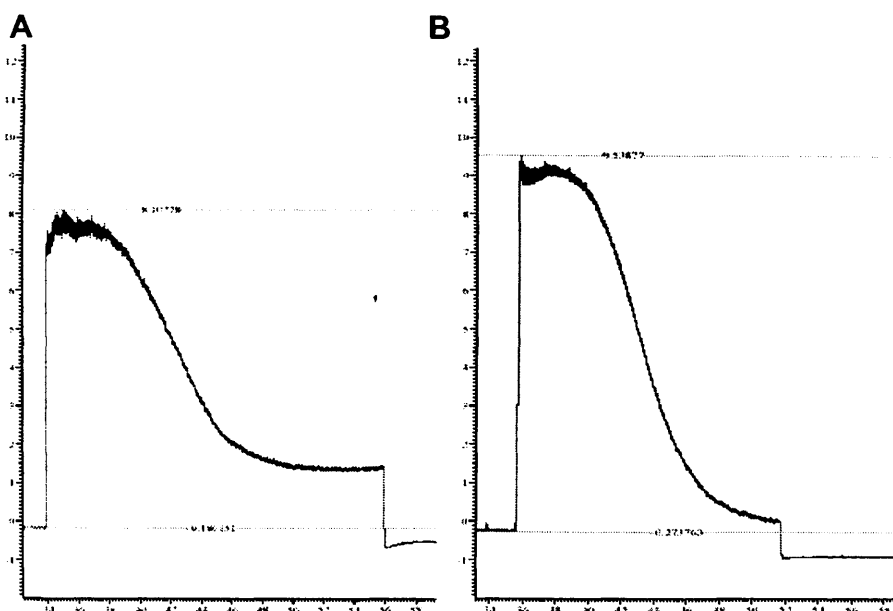
After calculations of maximum muscle force measurements and weights of all TA muscles of old mdx mice, and comparison between the uninjected controls and the injected ones, it was observed that the MGF increased muscle strength more than the IGF-I Ea although the effects of both splice variants (MGF and IGF-I Ea) were

similar to each other in old mdx mice (Figures 4.6 and 4.7). Figure 4.5 shows some individual maximum forces of single injected and uninjected control TA muscles of old mdx mice in order to show some examples.

Old MGF Group



Old IGF-I Ea Group



Old Vector-only Group

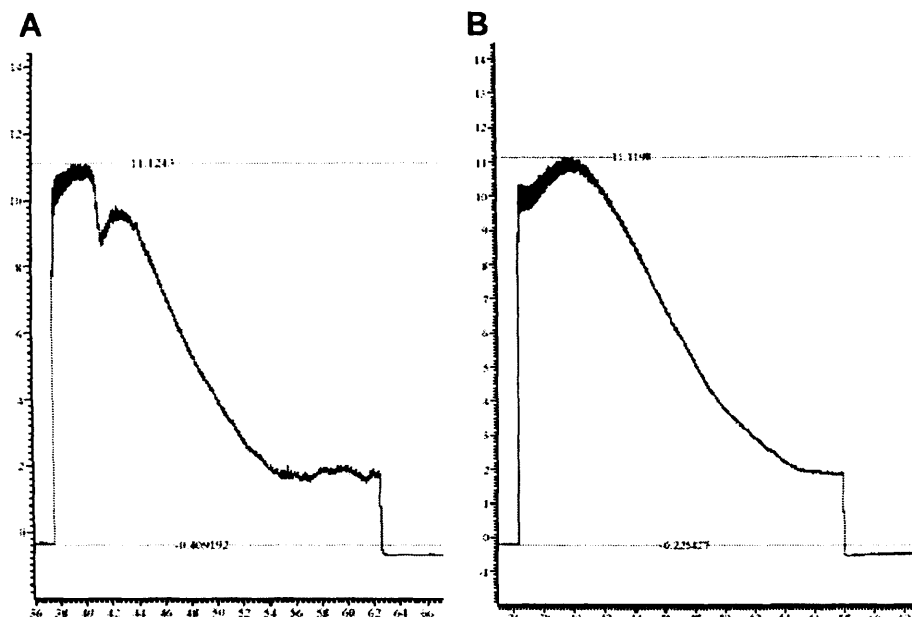


Figure 4.5: Individual maximum forces of injected and uninjected control TA muscles from MGF, IGF-I Ea and Vector-only groups of old mdx mice. A: uninjected control, B: injected TA muscle of the same animals. Axes are (V) and time (sec).

As it is evident from Figures 4.6, and 4.7, the increase caused by MGF was around 10.6 percent while the figure for IGF-I Ea was 9.5 %. On the other hand, the same figures demonstrate that a slight increase also recorded in the muscles of control group (vector-only group). The increase by vector-only control group was just 2.4 percent. Although increase rates caused by both MGF and IGF-I Ea were very similar each other (10.6 and 9.5 % respectively), the data for increase level caused by MGF was significant statistically (p value was 0.015), though the data was not significant for IGF-I Ea group (p: 0.211).

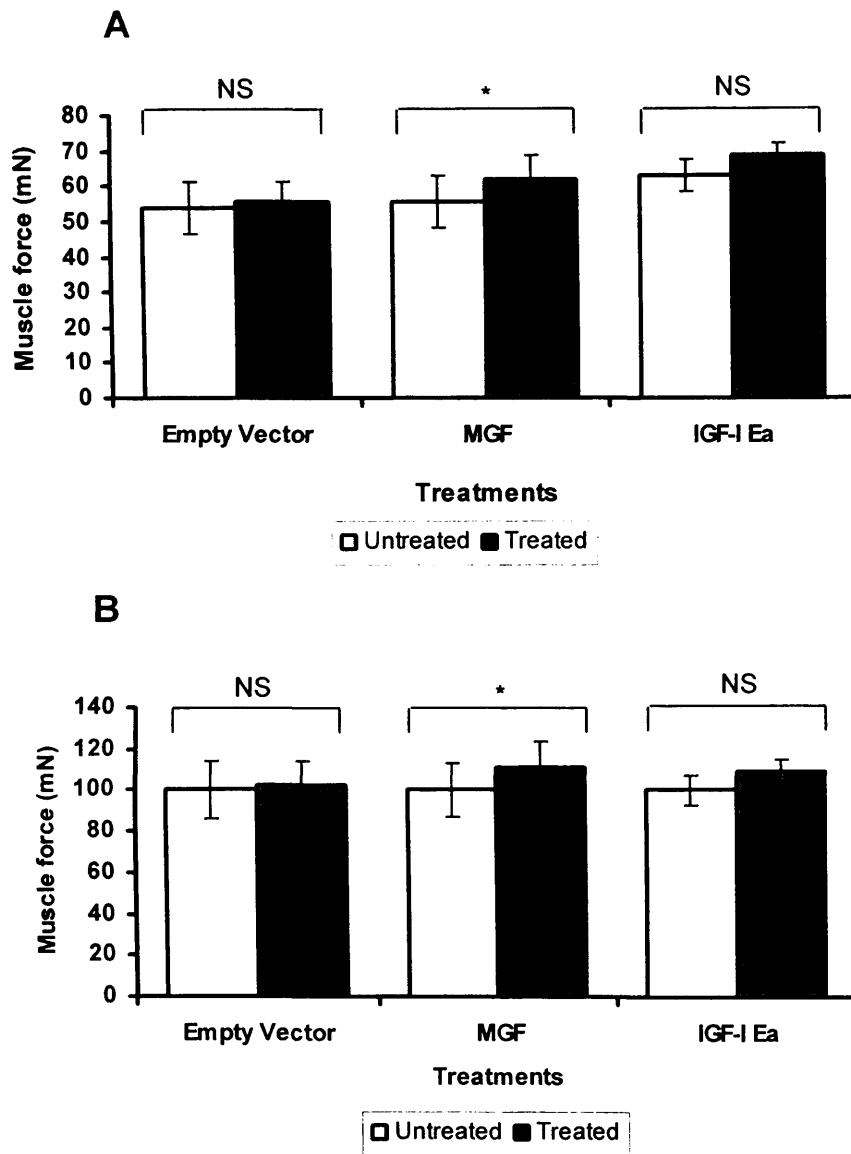


Figure 4.6: IGF-I splice variants based gene therapy application for old mdx mice. A: According to absolute data, B: According to normalised data to 100 for controls. N: Vector-only (N:6), MGF (N:7) and IGF-I Ea (N:8). P value for MGF was <0.05 (0.015), and for empty vector and IGF-I Ea were >0.05 (0.862 and 0.211 respectively). Data were presented as mean \pm s.e.m.

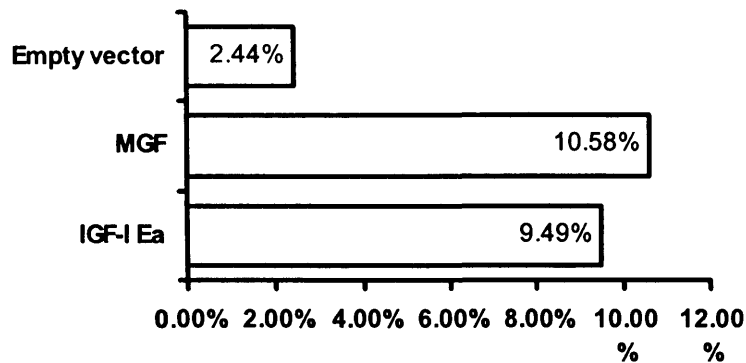


Figure 4.7: The percentage of the changes in muscle force in old mdx mice. N: Vector-only (N:6), MGF (N:7) and IGF-I Ea (N:8).

On the other hand, MGF also increased muscle mass. The changes in muscle masses were determined by weighing the treated (injected with MGF, IGF-I Ea or empty vector) and untreated (uninjected, resting) TA muscles for all groups in old and young mdx mice. Figure 4.8 shows the percentages of the changes in treated / injected (gene transferred in vector) TA muscle weights in old mdx mice comparing to those of untreated / uninjected TA muscles. As can be seen in figure 4.8, MGF increased muscle masses by 7 percent and the data was significant statistically (p value was 0.009), while IGF-I Ea increased it by only 2 percent and the data was not significant statistically (p value was 0.087). In contrast, 5 percent of decrease were observed in vector-only control group and this data was also not significant statistically (p value was 0.309).

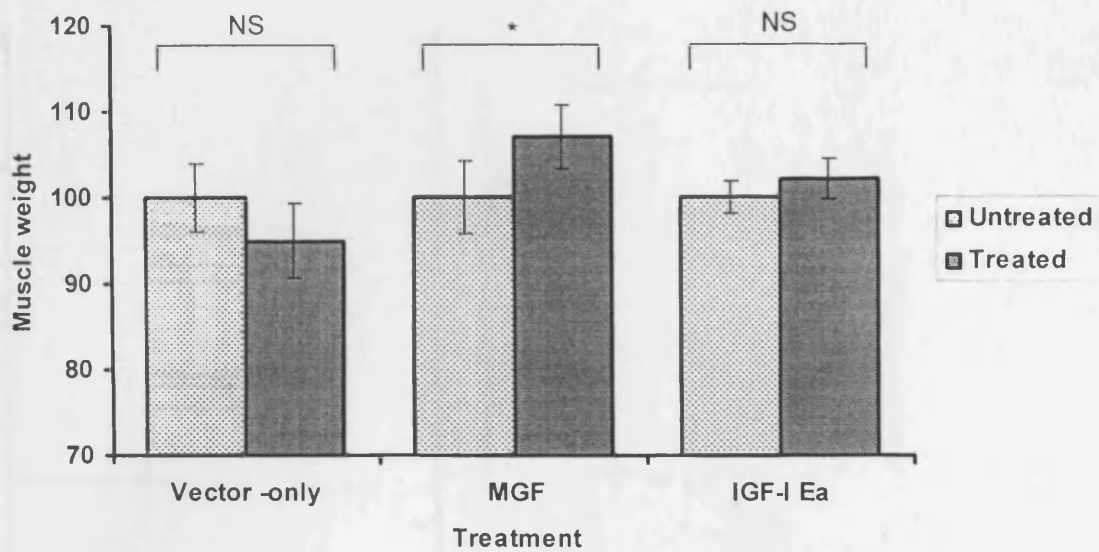


Figure 4.8: The changes in muscle weight in old mdx mice after gene transfer. Data were optimised to 100 for uninjected controls. N: Empty vector (N:6), MGF (N:7) and IGF-I Ea (N:8). P value for MGF was <0.05 (0.009), and for empty vector and IGF-I Ea were >0.05 (0.309 and 0.087 respectively). The data presented as mean \pm s.e.m.

4.1.3.2 Effects of IGF-I splice variants in young mdx mice

After all calculations, it was observed that the MGF dramatically increased muscle fibre mass by strength and size in young mdx mice. Figure 4.9, 4.10 and 4.11 show some individual tetanic forces of single injected and uninjected control TA muscles of young mdx mice in order to show some examples.

Figure 4.10: Individual tetanic forces of injected and uninjected control TA muscles from mdx mice. Graph of young mdx mice. A: uninjected control. B: injected TA muscle of the same animal. C and D: the TA muscle of different animals. Axes are (Y) and time (sec).

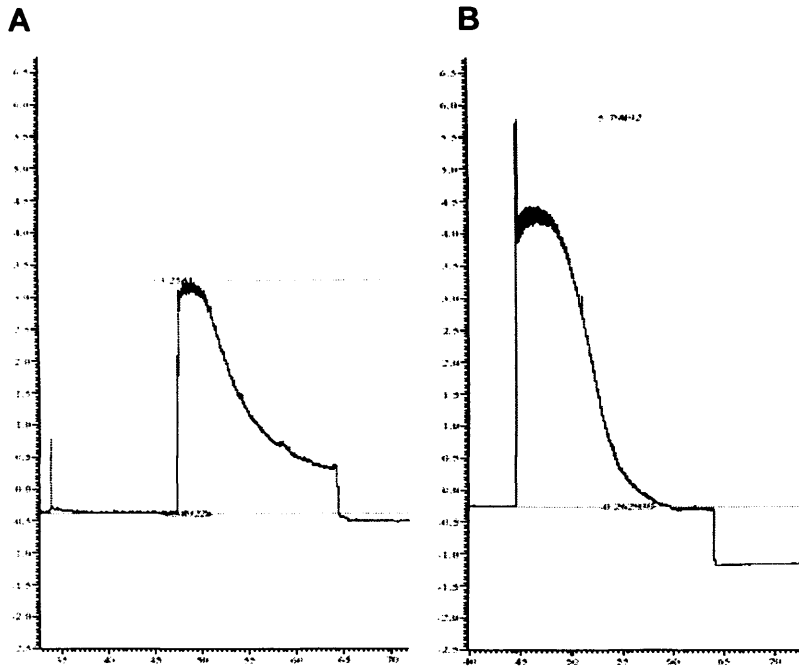


Figure 4.9: Individual tetanic forces of injected and uninjected control TA muscles from MGF group of young mdx mice. A: uninjected control, B: injected TA muscle of the same animals. 1 and 2 are the TA muscles of different animals. Axes are (V) and time (sec).

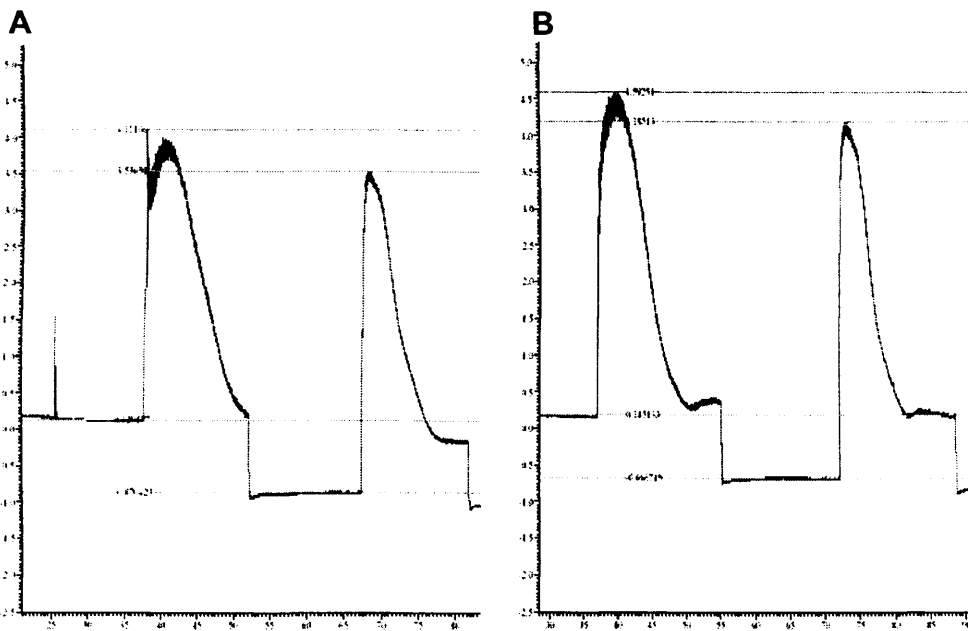


Figure 4.10: Individual tetanic forces of injected and uninjected control TA muscles from IGF-I Ea group of young mdx mice. A: uninjected control, B: injected TA muscle of the same animals. 1 and 2 are the TA muscles of different animals. Axes are (V) and time (sec).

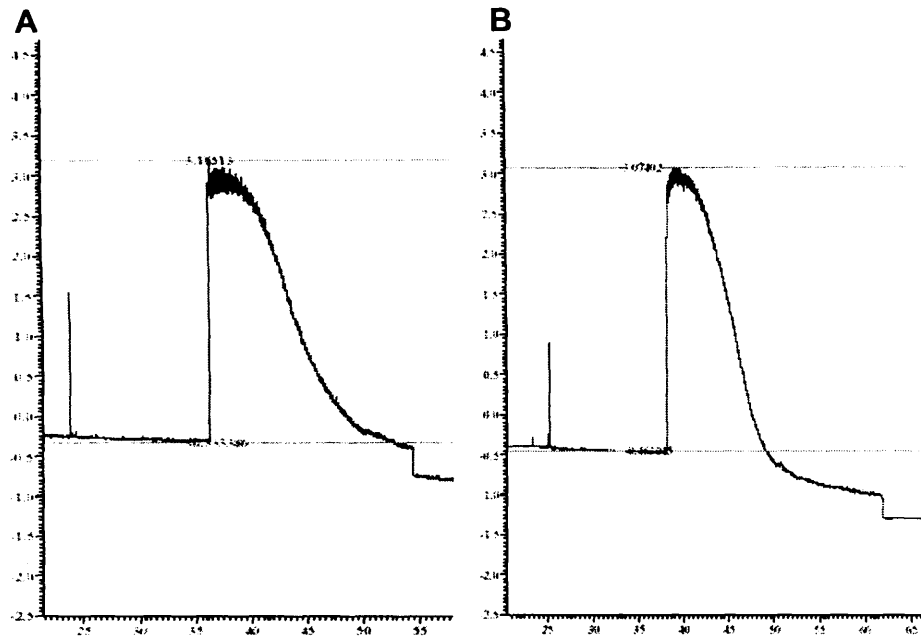


Figure 4.11: Individual tetanic forces of injected and uninjected control TA muscles from vector-only control group of young mdx mice. A: uninjected control, B: injected TA muscle of the same animals. 1 and 2 are the TA muscles of different animals. Axes are (V) and time (sec).

Figures 4.12, and 4.13 show that MGF has a marked effect on young mdx mice. The MGF increased muscle force of young animals by more than 37 percent in 3 weeks and the data was significant statistically (p value was 0.014). In contrast, IGF-I Ea increased the muscle force only by 14 percent (but not significant). The effect of MGF in muscle force was 2.5 fold higher than that of IGF-I Ea. Again, there was not a significant change in vector-only control group. In the young muscles of the vector-only control group, the maximum muscle force decreased 3.6 percent after 3 weeks from the injection. Although the maximum muscle force changes between injected and uninjected TA muscles of vector-only control and IGF-I Ea groups were not significant statistically (p: 0.783 and 0.292 respectively), the changes of MGF group were clearly significant statistically with a p value less than 0.05 (0.014).

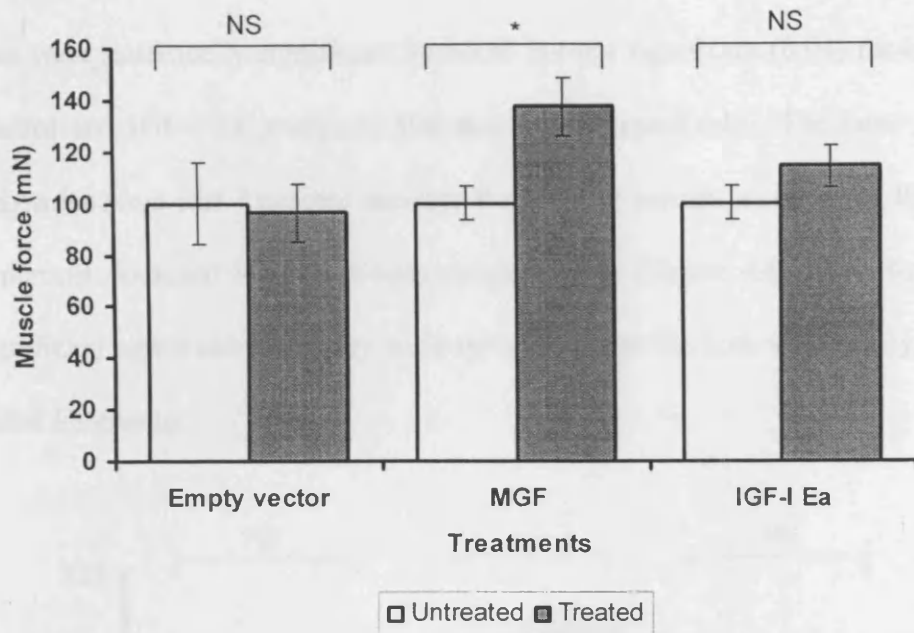


Figure 4.12: Gene therapy application by IGF-I splice variants for young mdx mice. Values are normalised to 100 for uninjected control TA muscles N: Vector-only (N:7), MGF (N:8) and IGF-I Ea (N:8). *: P value. P values for Vector-Only and IGF-I Ea groups were $P > 0.05$ and non significant (0.783 and 0.292 respectively); and for MGF: $P < 0.05$ and significant (0.014). Data were pooled and presented as mean \pm s.e.m.

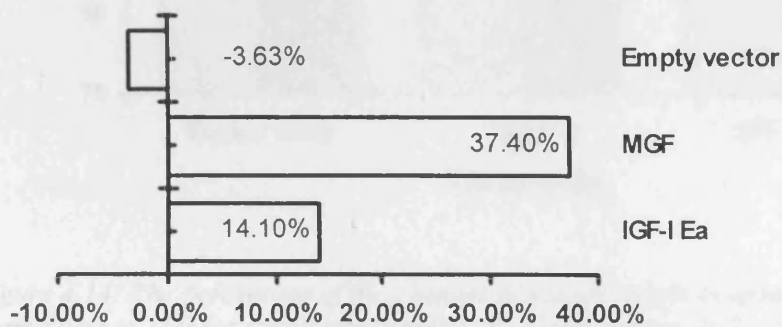


Figure 4.13: The percentage of the changes in muscle force by gene therapy in young mdx mice. N: Vector-only (N:7), MGF (N:8) and IGF-I Ea (N:8).

4.1.4 DISCUSSION

The MGF also increased muscle mass in higher rate in TA muscles of young mdx mice. Figure 4.14 shows the percentages of the changes in treated TA muscle weights in young mdx mice comparing to those of untreated TA muscles. As can be seen the figure 4.14, MGF increased muscle masses by 13 percent while IGF-I Ea increased it

by only 6 percent. The increase in empty vector control group was just 2 percent. The data were statistically significant for MGF but not significant (0.04) for empty vector control and IGF-I Ea groups (0.598 and 0.079 respectively). The same data for old mdx mice were just 7 percent increase for MGF, 2 percent increase for IGF-I Ea, and 5 percent decrease for vector-only control group (Figure 4.8). Data for MGF was significant again although they were not significant for both vector-only control and IGF-I Ea groups.

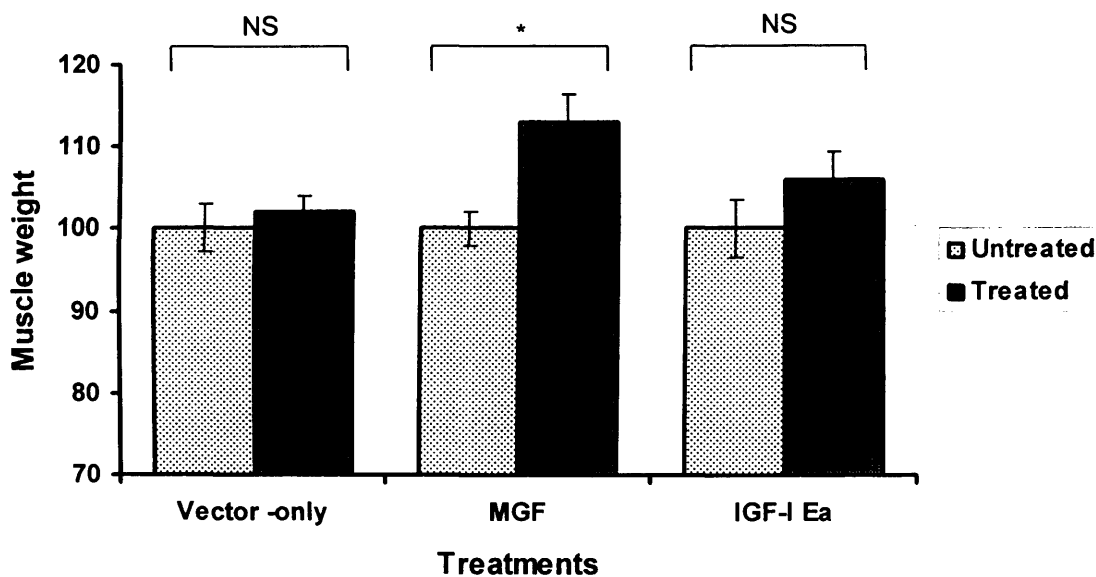


Figure 4.14: The percentage of the changes in muscle weight in young mdx mice. Values were normalised to 100 for uninjected controls. N: Empty vector (N:7), IGF-I Ea (N:8) and MGF (N:8). P value for MGF was <0.05 and significant (0.04), and for empty vector and IGF-I Ea were >0.05 and non significant (0.598 and 0.079 respectively). The data presented as mean ± s.e.m.

4.1.4 DISCUSSION

This study found that cDNAs of MGF, which were transferred into TA muscles of mdx mice in a plasmid vector, increased muscle growth of dystrophic mdx mice in strength, and that this was much more effective in younger animals. MGF

dramatically increased muscle strength in young mdx mice by average of 37.4%, only 3 weeks after injection. Whereas the increase effect of IGF-I Ea in maximum tetanic muscle force in young animals was lower than that of MGF. cDNA of IGF-I Ea slightly increased maximum muscle force (14.1 %). On the other hand, while the increase effect of MGF was significant, the effect of IGF-I Ea was not significant. Therefore, the study clearly showed that MGF splice variant of IGF-I was considerable more effective than systemic variant of the gene (IGF-I Ea) in restoring muscle strength.

MGF also increased muscle force in old mdx mice although the increase level was lower than that in young animals. The ratios of the increase of MGF and IGF-I Ea were very close in old mdx mice; they were for MGF 10.6 %, and for IGF-I, increase ratio was 9.5 % (they were 37.4% and 14.1% in young animals). Similar to the data of young animal experimentation, the data of old animals also were statistically significant for MGF group. The data of IGF-I ea and vector only groups were not significant statistically again.

Similar effects were also observed in TA muscle weights in both young and old animals. The MGF also increased muscle mass in higher rate (13%) in TA muscles of young mdx mice when comparing treated muscles to untreated ones. The increase ratio of IGF-I Ea was only 6% in young animals (Figure 4.14). Whereas MGF increased muscle mass in old animals only 7 percent, and IGF-I Ea increased the mass 2% (Figure 4.8). MGF data for both young and old animals were statistically significant (0.04 and 0.009 respectively). Whereas, as can be seen from Figures 4.14

and 4.8, vector-only control and IGF-I Ea groups for both young and old animals, data were not significant statistically.

Comparing this observation in old mdx mice to the effect of MGF in young animals, it can be said that the effects of cDNAs of MGF in muscle growth of old mice is not high. This is in accord with the findings of Wells and Goldspink (1992) who reported that the age significantly influenced the level of expression of the injected plasmid DNA (Wells and Goldspink, 1992). They found that the highest levels of plasmid DNA expression were obtained from the 4-6 weeks age young mice, and they were significantly higher than those of from the mice older than 10 weeks (Wells and Goldspink, 1992). In this study, young mdx group was age of 4-8 weeks, whereas, the age of the old mdx mice were between 15-26 months old.

It is known that, in degenerative skeletal muscle states, including DMD, motor nerve innervation reduces in advanced age; satellite cell numbers and proliferative potential significantly decrease; skeletal muscle's regenerative capacity and contractility diminishes; particularly in DMD, regenerative capacity is exhausted (Jejurikar and Kuzon, 2003; Shi and Garry, 2006). In addition to above observations, plasmid distribution drops in advanced ages. The animals in old mdx groups of in vivo part of this study had two disadvantages: They were old and also had degenerative muscles (mouse model of DMD). The lower effect of cDNAs of MGF in muscle growth in old mdx mice can be explain under all above conditions (degenerative muscles and age concern).

4.2 Roles of IGF-I Splice Variants in Satellite Cell Activation

4.2.1 INTRODUCTION

A number of studies suggest that satellite cells are involved in repair and regeneration following local injury and/or damage of muscle fibers. When satellite cells are activated by a variety of external stimuli (e.g. loading, injury, disease, age), daughter cells (muscle precursor cells) are generated that go on to repair muscle fibers by supplying extra nuclei.

In healthy adult undamaged muscle, the satellite cells are quiescent. The quiescent satellite cells are activated and begin proliferating in response to stimuli. Hill and Goldspink suggest that MGF activates quiescent satellite cells in vivo in rats (Hill and Goldspink, 2003, Hill et al, 2003). Therefore, it is crucial to investigate the role of IGF-I splice variants, particularly MGF, in satellite cell activation mechanism in degenerative muscles.

4.2.2 METHODS

mRNA transcripts levels of the activated satellite cell markers, which are MyoD and Myogenin (comparing with that of internal control β -actin), were investigated by quantitative real-time RT-PCR after three weeks introducing cDNAs of IGF-I splice variants. The detail of the quantitative real time RT-PCR method can be found from chapter 2.2 of this study.

4.2.2.1 Analysing of data

Although real-time RT-PCR is the most sensitive technique for detection mRNA targets, and its application is broadly used to quantitate biological changes in mRNA levels, there remain some problems associated with its use. RT-PCR specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. These errors become particularly important when the samples have been obtained from different individuals (or animals), and they can result in the misinterpretation of the expression levels of the target gene/genes (Bustin, 2000; Bustin and Nolan, 2004; Huggett et al, 2005).

Therefore, it is important to choose an accurate normalisation method to control for the possibility of misinterpretation of the data (Huggett et al, 2005). One of most commonly used normalisation methods is to use an internal control / reference / housekeeping gene by dividing absolute copy numbers of target genes against corresponding absolute copy numbers of internal reference / housekeeping gene (Huggett et al, 2005). In this study, as an internal reference or housekeeping gene, β -actin was selected, because β -actin is one of most commonly used internal control / housekeeping / reference gene (Bustin, 2000; Bustin and Nolan, 2004; Huggett et al,

2005; Bustin and Mueller, 2006; Nolan et al, 2006) and its mRNA is expressed at moderately high levels in most cell types including muscle cells and it encodes an ubiquitous cytoskeleton protein (Bustin, 2000). Therefore, the β -actin mRNA was amplified to ensure equal quantities of starting RNA before performing RT-PCR. Then after RT-PCR were performed with target mRNAs (MyoD and myogenin), mRNA expressions of MyoD and myogenin were compared with that of β -actin by calculating their absolute gene copy numbers. For this aim, copy numbers of MyoD, myogenin and β -actin for each sample, both injected and uninjected ones, were calculated separately. Each of the calculated copy numbers for either MyoD or myogenin was normalized against the corresponding β -actin copy numbers by dividing absolute gene copy numbers for either MyoD or myogenin to the absolute gene copy number of β -actin of the same sample.

As can be seen from the materials chapter (chapter 2.2), the primers, which were used this study, were MyoD myogenin and β -actin. Their primer sequences, the sequences of their amplified fragment and molecular weights are as follow:

MyoD (NCBI NM 010866) 123 bp Mw: 38.139 g/mol KD ssDNA

Primer:

Upperstream (forward) – TTTCTTCACCACACCTCT -

Downstream (reverse) – TTAAC TTTCTGCCACTCC -

Amplified fragment (123 bp):

5'TTTCTTCACCACACCTCTGACAGGACAGGACAGGGAGGAGGGGTAGAG
GACAGCCGGTGTGCATTCCAACCCACAGAACCTTTGTCATTGTACTGTTGG
GGTTCCGGAGTGGCAGAAAGTTAA - 3'

Myogenin (NCBI NM 031189) 102 bp Mw: 31.547 g/mol KD ssDNA

Primer:

Upperstream (forward) – AGACGAAACCATGCCCAA -

Downstream (reverse) – TAAAAGCCCCCTGCTACAGA -

Amplified fragment (102 bp):

5'AGACGAAACCATGCCCAACTGAGATTGTCTGTCAGGCTGGGTGTGCATG
TGAGCCCCCAAGTTGGTGTCAAAGCCATCACTTCTGTAGCAGGGGGCTT
TTA -3'

β-actin (GenBank AB117093) 186 bp mw 41.695 g/mol KD ssDNA

Primer:

Upperstream (forward) – GACGATGATATTGCCGCACT -

Downstream (reverse) – GATACCACGCTTGCTCTGAG -

Amplified fragment (186 bp):

5'GACGATGATATTGCCGCACTCGTTGTTGACAATGGATCCGGTATGTGCA
AGGCCGGCTTCGCGGATGATGCTCCACGTGCTGTCTTCCCATCCATCGTGG
GGCGCCCAAGACATCAGGGTGTGATGGTTGGCATGGGCCAGAAAGACAG
CTACGTTGGTGAAGCTCAGAGCAAGCGTGGTATC – 3'

4.2.2.2 Calculation of Copy Numbers

Copy number of each PCR product (MyoD, myogenin and β-actin) was calculated separately. Calculations of copy numbers were made as follow: As an example, the reading value (the value for fluorescent absorbance) of myogenin mRNA expression in young MGF treated sample was 5.94×10^{-6} ng/μg. The reading value was then multiplied by 10^{-9} to establish the number in grams. $5.94 \times 10^{-6} \times 10^{-9} = 5.94 \times 10$

$^{-15}$ g. Therefore, the final weight was divided by molecular weight of myogenin: 102 bp (size of product for myogenin) x 31.547 g/mol (weight of ssDNA in g/mol) = 3217.794 g/mol. $(5.94 \times 10^{-15} \text{ g}) / 3217.794 \text{ g/mol} = 1.84 \times 10^{-18}$ mols. This was then multiplied by the Avogadro constant which is 6.0221415×10^{23} . $(1.84 \times 10^{-18}) \times (6.022 \times 10^{23}) = 1111678.39$. Thus, 1111678.39 molecules were presented in 2 μl of 30 μl solution containing 0.75 μg of RNA [2 x (30x0.75)]. To calculate the copy number per μg of RNA, the value (1111678.39) was multiplied by 2 and then by 22.5 (30x0.75). An finally, absolute copy number for above sample was: $1111678.39 \times 2 \times 22.5 = 50025526 (5 \times 10^7)$ copies / μg RNA.

An example for copy number calculation:

Reading value of myogenin mRNA expression: $5.94 \times 10^{-6} \text{ ng}/\mu\text{g}$

$$5.94 \times 10^{-6} \times 10^{-9} = 5.94 \times 10^{-15} \text{ g}$$

$$(5.94 \times 10^{-15} \text{ g}) / 3217.794 \text{ g/mol} = 1.84 \times 10^{-18} \text{ mols}$$

$$(1.84 \times 10^{-18}) \times (6.022 \times 10^{23}) = 1111678.39$$

$$(1111678.39) \times 2 \times 22.5 = 50025526 (5 \times 10^7) \text{ copies} / \mu\text{g RNA}$$

An example for the calculation of ratios of copy numbers of myogenin and β -actin (myogenin copy number / β -actin copy number):

Myogenin copy number for an MGF treated sample in young mdx mice group was = 3815546.304

β -actin copy number for the same sample was = 17104850

Myogenin copy number / β -actin copy number = $3815546.304 / 17104850$
 = 0.223068 (2.23E-01).

Copy numbers and the ratio of copy numbers of target gene mRNA and internal reference gene mRNA of all samples (uninjected and injected of both, young and old mdx mice groups) were separately calculated by this way, and the results were interpreted.

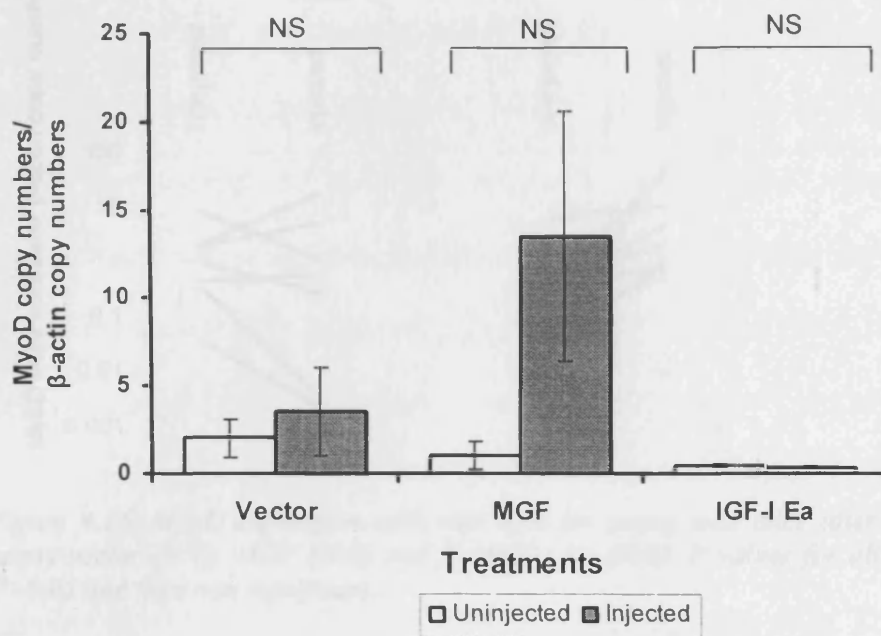
4.2.3 RESULTS

4.2.3.1 Satellite cell activations in the young mdx mice.

4.2.3.1.1 MyoD expression

As can be seen from Figures 4.15A, 4.15B and 4.16, the MyoD expressions in the samples of the treated TA muscles in the young MGF group were higher than untreated ones. Although the data were statistically not significant, Figure 4.15A, 4.15B and 4.16 show that MyoD expression was clearly increased in the young MGF group comparing with untreated controls. As can be seen from such Figures and from the raw data in appendix 5, the normalised absolute MyoD gene copy numbers in MGF injected samples was increased by more than 13 folds (increased from 1.02 to 13.07) than uninjected samples in the same animals. Whereas, normalised MyoD copy numbers of IGF-I Ea and empty vector injected samples were almost similar when comparing them to uninjected samples of the same animals. A slight increase occurred in empty vector injected samples, and a small decrease occurred in IGF-I Ea injected samples.

A



B

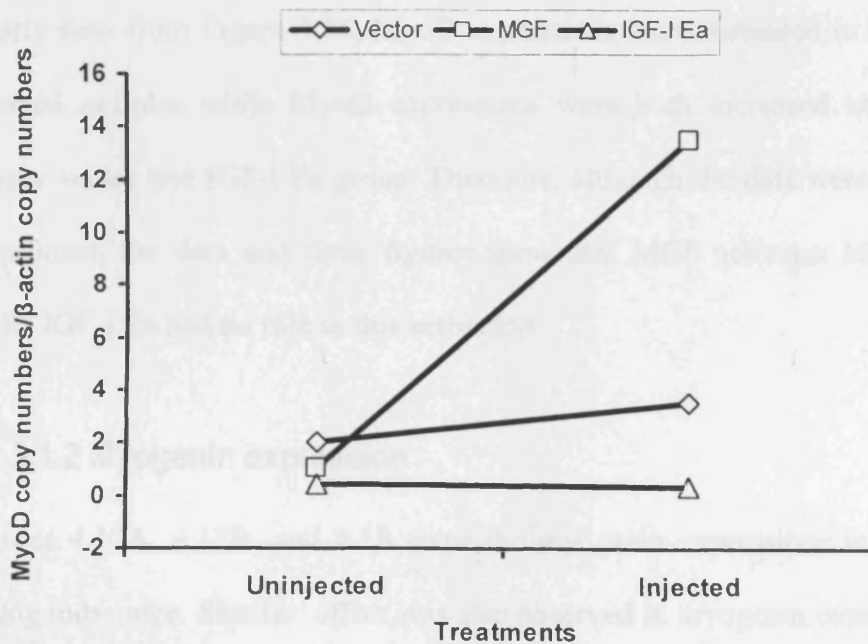


Figure 4.15. MyoD expression for young mdx mice after gene transfer: A shows the data in column chart, and B shows the same data in line chart. N: for empty vector (N:7), MGF (N:8) and for IGF-I Ea (N:8). Data were statistically analysed by paired t-test and presented as mean \pm s.e.m.. *: P values. P values for all three group were $P > 0.05$ and then non significant.

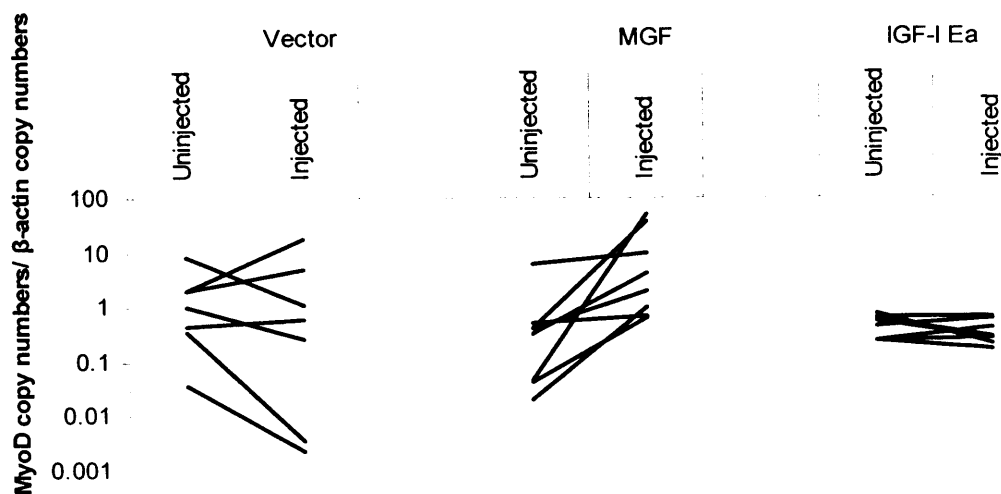


Figure 4.16. *MyoD* expression with raw data for young *mdx* mice after gene transfer: *N*: empty vector (*N*:7), MGF (*N*:8) and for IGF-I Ea (*N*:8). *P* values for all three group were $P > 0.05$ and then non significant.

Although the data were not statistically significant for MGF group, As it can be clearly seen from Figure 4.16, *MyoD* expressions were increased in almost all MGF injected samples while *MyoD* expressions were both increased and decreased in Empty vector and IGF-I Ea group. Therefore, although the data were not statistically significant, the data and three figures show that MGF activates *MyoD* expression while IGF-I Ea had no role in this activation.

4.2.3.1.2 Myogenin expression

Figures 4.17A, 4.17B, and 4.18 show the myogenin expressions in the samples of young *mdx* mice. Similar effect was also observed in myogenin expression in young MGF group. Figures 4.17A, 4.17B, and 4.18 show that MGF clearly increased myogenin expression in young *mdx* mice. Normalised Myogenin copy numbers from MGF treated samples were quite higher than untreated ones. As can be seen from such Figures and from the raw data in appendix 5, normalised absolute Myogenin

copy numbers from MGF treated samples were almost 25 folds higher (increased from 0.39 to 9.83) than those of uninjected samples of the same animals. The data were also significant statistically (p ; 0.009). Again, similar to MyoD, normalised MyoD copy numbers of IGF-I Ea and empty vector injected samples were almost similar when comparing them to uninjected samples of the same animals. In addition, while the data for MGF group was significant statistically (0.009), The data for both empty vector and IGF-I Ea groups were also not significant. Thus, above three figures clearly show that MGF also activates Myogenin expression in young mdx mice whereas IGF-I Ea had no role in this activation. Figure 4.18 also show that myogenin expression were increased in all samples of MGF group, while MyoD expressions were both increased and decreased in Empty vector and IGF-I Ea group

A

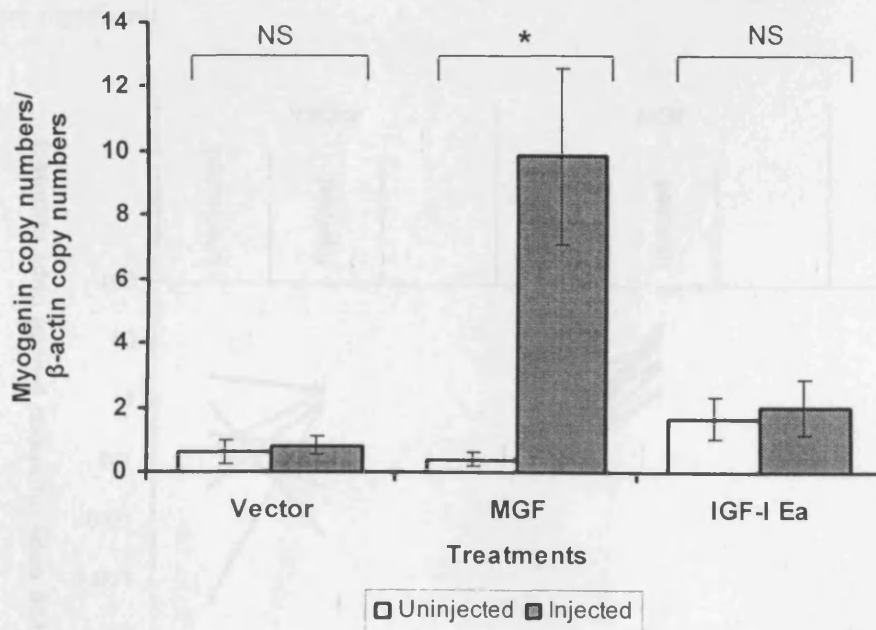


Figure 4.18 Myogenin expression in mdx mice after gene transfer. The empty vector (E), MGF (M) and the IGF-I Ea (I) groups. P values for MGF group was $P=0.009$ ($P=0.009$ and $P=0.009$), and for both empty vector and IGF-I Ea groups were $P=0.0$ and they were not significant.

B

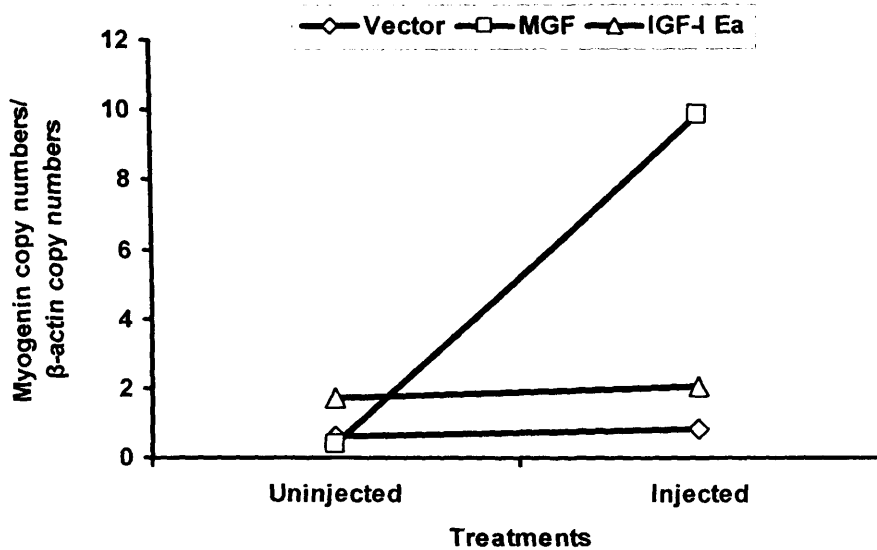


Figure 4.17. Myogenin expression for young mdx mice after gene transfer: A shows the data in column chart, and B shows the same data in line chart. N: for empty vector (N:7), MGF (N:8) and for IGF-I Ea (N:8). Data were statistically analysed by paired t-test and presented as mean \pm s.e.m. *: P values. P values for MGF group was $P < 0.05$ (0.009) and significant, and for both empty vector and IGF-I Ea groups were $P > 0.0$ and then non significant.

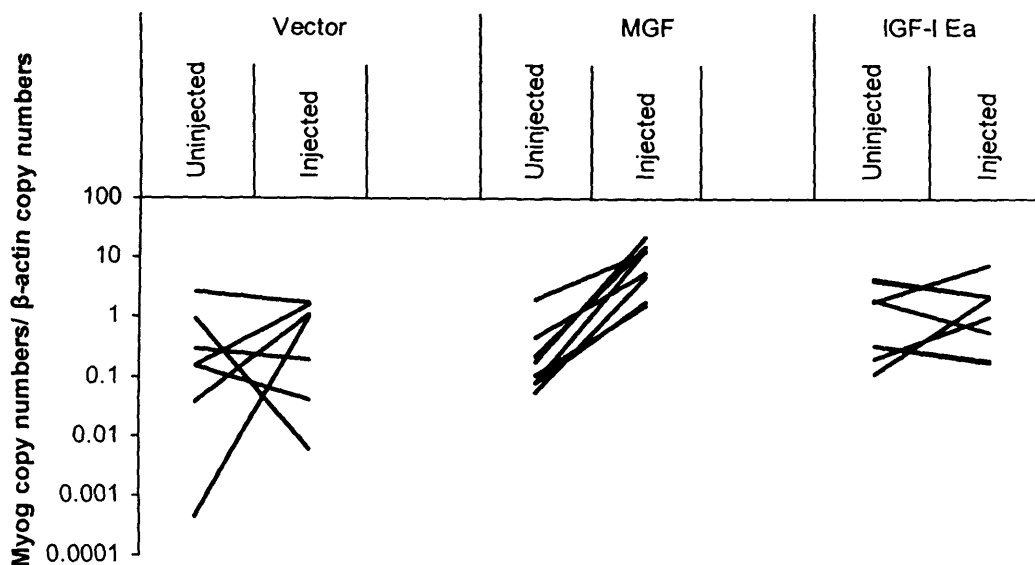


Figure 4.18. Myogenin expression with raw data for young mdx mice after gene transfer: N: empty vector (N:7), MGF (N:8) and for IGF-I Ea (N:8). P values for MGF group was $P < 0.05$ (0.009) and significant, and for both empty vector and IGF-I Ea groups were $P > 0.0$ and then non significant

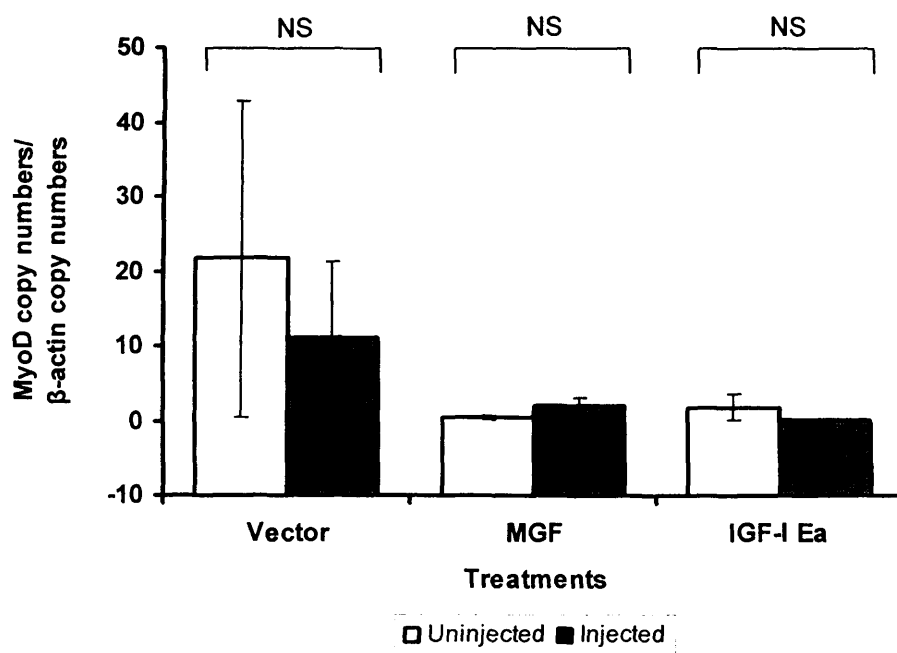
4.2.3.2 Satellite cell activations in the old mdx mice.

Similar effects were also observed in old mdx mice myofibre samples. As it can be seen from Figures 4.19 to 4.22, MGF increased both MyoD and myogenin expressions in TA muscles of old mdx mice after 3 weeks introducing cDNAs of MGF. Similar to young mdx experimentation, while the data of MGF were statistically significant for myogenin expression, they were not significant for MyoD expression again.

4.2.3.2.1 MyoD expression

Figure 4.19A and B, and 4.20 shows that MGF also increased MyoD expression by 4.5 folds in old mdx mice, despite the data were not significant statistically. On the other hand, MyoD expressions were slightly decreased in both groups, in empty vector, and IGF-I Ea injected animals. The data for them were also not significant.

A



B

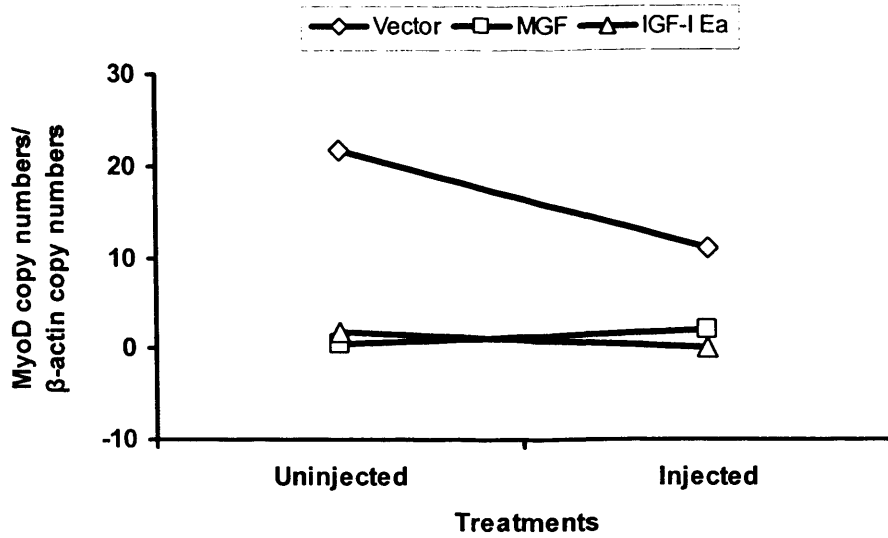


Figure 4.19. MyoD expression for old mdx mice after gene transfer: A shows the data in column chart, and B shows the same data in line chart. N: for empty vector (N:5), MGF (N:7) and for IGF-I Ea (N:7). Data were statistically analysed by paired t-test and presented as mean \pm s.e.m.. *: P values. P values for all three group were $P > 0.05$ and then non significant.

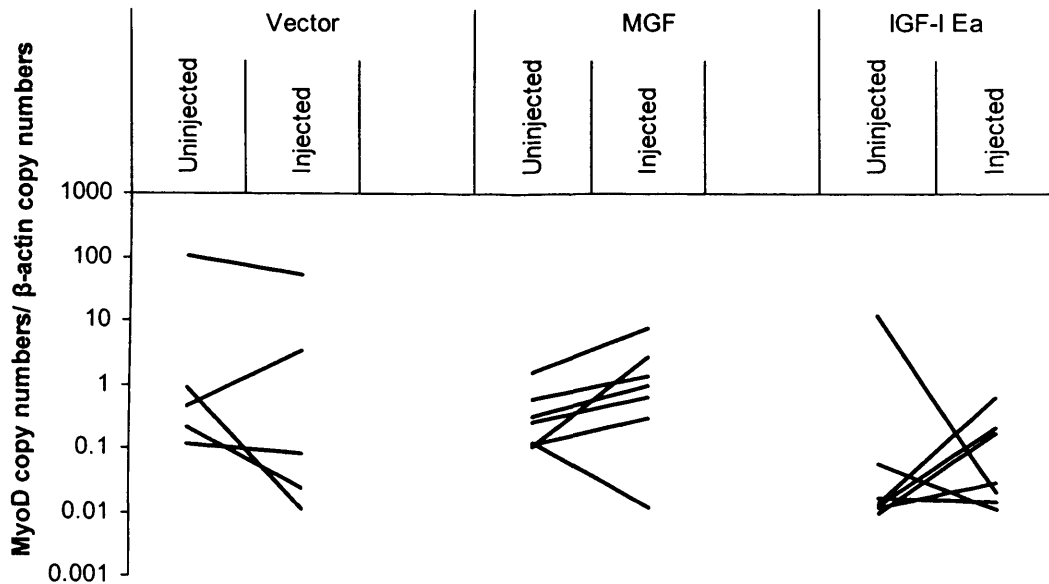
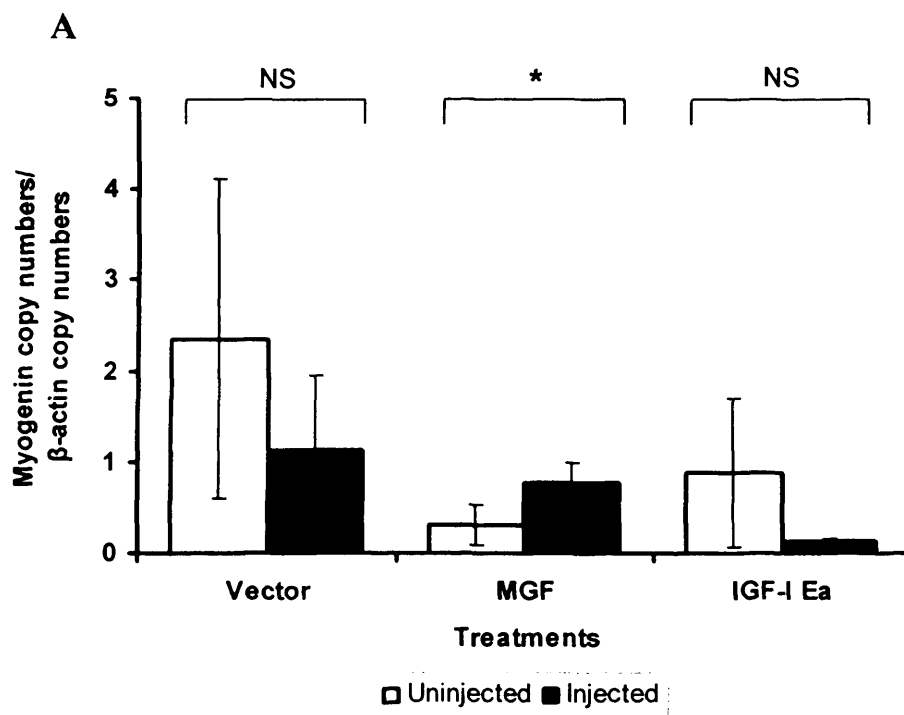


Figure 4.20. MyoD expression with raw data for old mdx mice after gene transfer: N: empty vector (N:5), MGF (N:7) and for IGF-I Ea (N:7). P values for all three group were $P > 0.05$ and then non significant.

4.2.3.2.2 Myogenin expression

Similar effects were also observed in myogenin expression in old animals.

Figures 4.21A, 4.21B, and 4.22 show that MGF clearly increased myogenin expression in old animals as well. The Figures show that MGF increased myogenin expression 2.5 folds while IGF-I Ea sharply decreased. Myogenin expression was also decreased in empty vector injected samples when compared to injected samples. The data for MGF were also significant statistically ($p: 0.04$). On contrary, the data for both empty vector and IGF-I Ea groups were also not significant. Figure 4.22 also show that myogenin expression were increased in all samples of MGF group, while Myogenin expressions were both increased and decreased in Empty vector and IGF-I Ea group



B

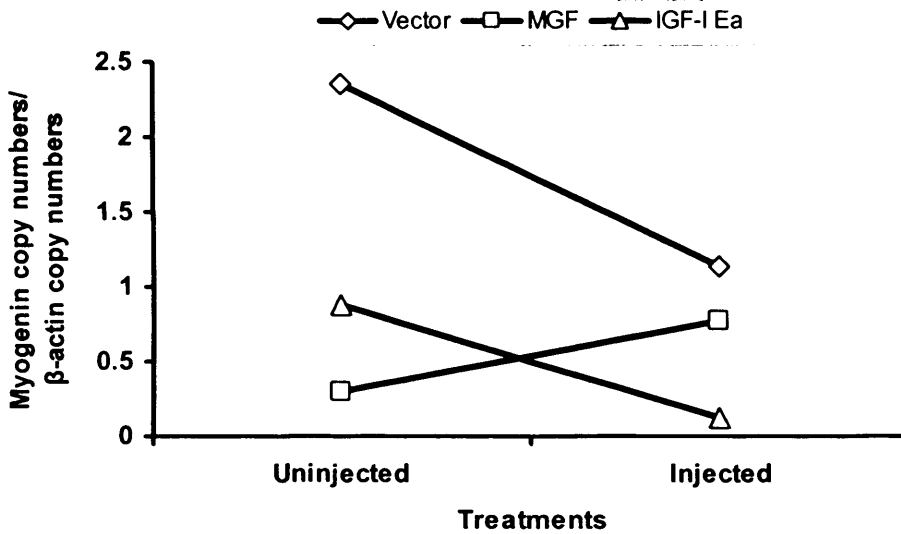


Figure 4.21. Myogenin expression for old mdx mice after gene transfer: A shows the data in column chart, and B shows the same data in line chart. N: for empty vector (N:5), MGF (N:7) and for IGF-I Ea (N:7). Data were statistically analysed by paired t-test and presented as mean \pm s.e.m.. *: P values. P values for MGF group was $P < 0.05$ (0.044) and significant, and for both empty vector and IGF-I Ea groups were $P > 0.0$ and then non significant.

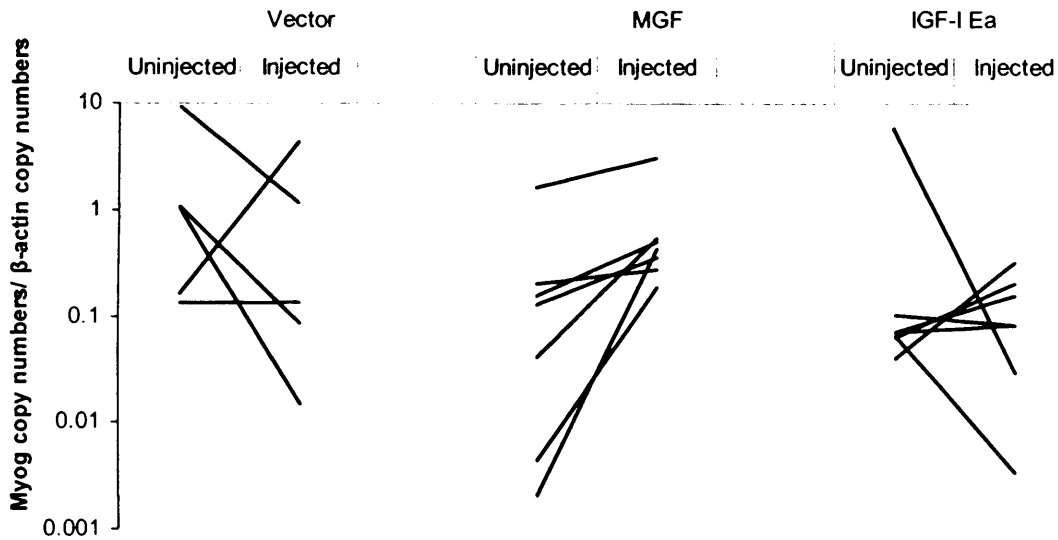


Figure 4.22. Myogenin expression with raw data for old mdx mice after gene transfer: N: empty vector (N:5), MGF (N:7) and for IGF-I Ea (N:7). P values for MGF group was $P < 0.05$ (0.044) and significant, and for both empty vector and IGF-I Ea groups were $P > 0.0$ and then non significant.

4.2.4 DISCUSSION

The data in this study showed that the effect of transfer of the MGF on satellite cell activation by investigating satellite cell markers expression in young mdx mice was higher and than those of old animals. Similar effects with muscle growth were also observed in the same experiments. In all analysis, the effects of MGF in muscle growth and satellite cell activation in young mdx mice was higher than in old ones.

When uninjected control muscle samples were compared with injected muscle samples, the study demonstrated that transfer of MGF activated satellite cells, however IGF-I Ea did not. The study showed that both MyoD and myogenin transcript levels were significantly higher in TA muscle tissues from MGF treated young mdx mice when compared to uninjected control muscles of the same animals. In this way, the study indicated that MGF activated muscle satellite cells of mdx mice, particularly in young animals, although the data were not significant statistically for MyoD expression, they were significant for myogenin expression in young animals. On the other hand, IGF-I Ea were ineffective in both activated satellite cell markers expressions, MyoD and myogenin.

As they can be seen from figures of all young and old animals (figures, 4.16, 4.18, 4.20 and 4.22), both MyoD and myogenin expressions in all samples of MGF injected muscles were always increased when compared to those of uninjected ones. Whereas, in samples of IGF-I Ea and in those of empty vector, both MyoD and myogenin expression were increased or decreased or did not change.

On the other hand, in old mdx mice, while MGF slightly increased both MyoD and myogenin expressions, IGF-I Ea and empty vector decreased the expression levels of MyoD and myogenin. MyoD is expressed in the early stage of satellite cell activation process and then indicates myoblasts proliferation. Whereas, myogenin is expressed in the late stage of the satellite cell activation process, and therefore it indicates differentiation state. In the in vivo experimentation part of this work, plasmid vectors were injected into the right TA muscles of both young and old animals. Plasmid vectors contained either cDNAs of MGF or IGF-I Ea, or empty vector (for control group). The injections might cause some damage to muscle tissues. Therefore, the damage might decrease MyoD and myogenin expressions for a while in all animals in all groups. However, expression might be increased again a while later due to effects of intrinsic and extrinsic signalling factors. Furthermore, in old mdx group, because the satellite cell pools decrease in advanced age, there might be not enough quiescent satellite cells to proliferate and then differentiate and therefore to express MyoD and myogenin. As a result, due to the damage, MyoD and myogenin expression in IGF-Ea and empty vector injected muscle samples in old animals might be decreased. On the other hand, because MGF stimulated the activated satellite cell markers (MyoD and myogenin), both expression might be increased again. The data in this study and the figures 4.19, 4.20, 4.21 and 4.22 confirm this observation. MGF data for myogenin expressions were statistically significant for both young and old animals (p: 0.009 and 0.044 respectively).

A recent publication suggests that plasmid distribution in muscle tissue drops by the 15th day (Coelho-Castelo et al, 2006). The same researchers also suggest that plasmid

/ naked DNA (pcDNA) distribution is dose dependent, and the distribution time decreases in lower doses (Coelho-Castelo et al, 2006). Plasmid DNA doses introduced into animals in this study was 1 µg/g. The average weight of old mdx mice was 26 g, therefore, less than 30 µg pf plasmid DNAs were transferred into TA muscles of each old animal. According to Coelho-Castelo et al, this amount of plasmid DNA is low. Therefore, along with reduced satellite number and activation process, plasmid DNA distribution might be ended after 21 days, and RNA extracted from them for investigating MyoD and myogenin expression by RT-PCR. Therefore, on the day, on which the tissues samples were taken, cDNAs of IGF-I splice variants might be stopped or decreased, and thus satellite cell activation might be ended. As a result, the doses and time point used in the in vivo gene transfer part of this study might cause the low effects of MGF in satellite cell activation.

On the other hand, the data for MyoD expression of both young and old mdx mice were not significant statistically. As explained before, firstly, MyoD is expressed in satellite cell activation process and then myogenin is expressed. MyoD also indicates myogenin expression. Therefore, on the day, on which the tissues samples were taken, the effect of MGF might be stopped or decreased, and thus MyoD expression by MGF might be ended.

As can be seen from Figures 4.19 to 4.22, it is observed that, the data were very variable, particularly in old animals,. Because the expression levels for all three genes (MyoD, myogenin and beta-actin) largely varied, the error bars for all groups were high. Particularly in old mdx mice, all animals were in different ages, and some of

them were too old (some of them were 26 months old) and as a result, they were too weak. This age differences can be a reason for high error bars for old animals.

In addition, in this part of the study, an internal control / reference / housekeeping gene (β -actin) was used to normalise target mRNA expressions. The β -actin mRNA was amplified to ensure equal quantities of starting RNA before performing RT-PCR. Then, each of the calculated copy numbers for either MyoD or myogenin was normalized against the corresponding β -actin copy numbers by dividing them to the absolute gene copy number of β -actin. The β -actin expressions in all samples had very large variation. Many publications suggest that transcription levels of β -actin can vary widely in response to experimental manipulation in different human and animal cells (Bustin, 2000). In addition, after large volume liquid delivery to muscle cells, β -actin variations widely increase in muscle cells (Prof G. Goldspink, May 2007, personal communication). In this study, β -actin expression varied widely in injected muscle samples when compared them to injected control ones. In order to normalise them the corresponding internal reference / housekeeping gene, absolute copy numbers of MyoD (and those of myogenin) were divided to the absolute gene copy number of β -actin. The ratios varied widely and therefore, error bars were very high. Another reason of non-significant states of MyoD data for MGF group might be this β -actin variation.

Hill and Goldspink previously showed that MGF activated quiescent satellite cells in wild type rats (Hill and Goldspink, 2003, Hill et al, 2003). This study indicated that MGF also activated satellite cells in mdx mice. MGF initiates the activation of muscle

satellite (stem) cells, as well as up regulating protein synthesis. The action of MGF is more specific than that of systemic IGF-I Ea.

Chapter 5:

General Discussion

5 General Discussion

5 General Discussion

Numerous publications have so far shown that IGF-I is involved in several cellular processes, including proliferation, differentiation, repair and maintenance. For some time, it has been also known that alternative splicing of the IGF-I gene within human skeletal muscle tissue occurs leading to isoforms that have different E domains, including pro-IGF-IEa, pro-IGF-IEb, and pro-IGF-IEc (MGF) (Goldspink and Yang, 2004). For a long time, it has been proposed that E peptide of these pro-IGF-I precursors may act as independent growth factors (Siegfried et al, 1992; Yang and Goldspink, 2002; Goldspink and Yang, 2004).

In recent years, the physiological effects of two main splice variants of the IGF-I gene in muscle cells were studied and it has been shown that one of these variants (MGF) significantly increased myoblast proliferation in some vertebrates, including rabbits and mice (Yang et al, 1997; Yang and Goldspink, 2002). However, the physiological functions of the splice variants of the gene on human muscle in in vitro culture models have not been previously elucidated. Therefore, this study has, for the first time ever, investigated the E domain in a human in vitro cell culture model.

This study has strongly showed that MGF has a remarkable effect in muscle cell proliferation. The MGF dramatically increased myoblast proliferation in vitro in both animals and humans (in mouse C2C12 myoblast cell lines and human primary myoblasts). In humans, MGF increased myoblast proliferation in all muscle-derived cell cultures studied, including muscles with different embryological origins, and the cells from muscles of patients with degenerative diseases.

This study showed that MGF has a significant effect on mouse myoblast proliferation. The study also showed that the proliferation effect of MGF on C2C12 was dose dependent, and the effect was not high in lower doses. This study clearly showed that the effect of MGF was higher than the systemic form of IGF-I, IGF-I Ea. Four years ago, Yang and Goldspink suggested that MGF increased mouse C2C12 myoblast proliferation, and its effect was higher than IGF-I Ea (Yang and Goldspink, 2002). This study confirmed their suggestion.

The study also showed that the MGF did not affect myoblast proliferation in rat myoblast cell lines (L6 E9). Rosen et al suggests that IGF-I expression is very low or nonexistent in the neonatal rat L6 cell line (Rosen et al, 1993). On the other hand, Braun et al suggests that L6 cells are restricted in their expression of myogenic factors (Braun et al, 1989), and MyoD is not expressed in L6 cells (Braun et al, 1989; Rhodes and Konieczny, 1989). MyoD is vital for myogenesis, and myoblast proliferation cannot start without MyoD. Therefore, MGF might be stimulating L6 E9 cells, but the proliferation could not occur due to lack of MyoD expression.

This study showed that two IGF-I isoforms, IGF-I Ea and MGF, have antagonist affect in muscle differentiation. The data in this study showed that MGF had not any effect on myoblast differentiation (myotube formation) although it remarkably stimulated myoblast proliferation. Whereas, the systemic form of IGF-I (IGF-I Ea) had a huge effect on myotube formation while having limited stimulation on myoblast proliferation. IGF-I Ea might be taking desmin positive cells out of stem cell pool into myogenic differentiation.

The study also showed that MGF had reduced the effect of IGF-I Ea on myoblast differentiation. When E peptide of MGF was added to long r³ IGF-I, the effect of long r³ IGF-I on myotube formation were decreased. The preventative effect of E domain of MGF on myoblast differentiation was dose dependent, and the effect of IGF-I Ea decreased more when MGF was added to IGF-I Ea in higher doses.

Previous studies have used BrdU or Alamar Blue, particularly those that claim IGF-I causes satellite cell replication. However, these studies have often not used markers to distinguish cells at different stages of the lineage pathway. This study found that a satellite cell activation marker, desmin, to be suitable to distinguish the muscle progenitor cell for other myoblasts. It demonstrated that the E peptide of MGF induces progenitor cell proliferation significantly in primary muscle culture from patients with CMD, FSHD and ALS as well as healthy individuals. Additionally, MGF did not affect myotube formation, a process that IGF-I accelerates significantly. The present work therefore demonstrated that the MGF E domain had a distinct biological activity compared to long r³ IGF-I. It caused the monocleate progenitor

(satellite) cells to replicate but it has no effect on myotube formation. In contrast, long r³ IGF-I had a marked effect on such formation. Therefore the data in this study confirmed that, MGF and long r³ IGF-I had different cellular actions: MGF increased myoblast proliferation whilst systemic IGF-I Ea induced myotube differentiation and was in accord with that of Yang and Goldspink's (2002), and Mouly and Butler-Browne's data (Yang and Goldspink, 2002; Mouly and Butler-Browne, unpublished data).

In this study, as mentioned above, MGF did not have any effect in proliferation in rat myoblast cell lines (L6 E9). Whereas it increased myoblast proliferation 2 fold (more than 100 percent) in C2C12: 38.2 percent increase for healthy primary cultured craniofacial myoblasts; 50.6 percent for healthy primary cultured limb muscle myoblasts. It was observed in this study that C2C12 cells grow quiet easily and rapidly in high levels. Therefore, a more than 100 percent of increase in MGF E Domain treated proliferating cells was not surprising. Again, it was observed in this study that human primary cultured muscle cells grew more slowly.

Gillian Butler-Brown and Vincent Mouly suggest that systemic IGF-I starts signalling by Akt pathway after fusion competent myoblasts fuse to myotubes, not before (Unpublished data by Butler-Brown and Mouly) (this significant information was obtained by Prof Goldspink from Drs Gillian Butler - Brown and Vincent Mouly during a private communication in Myores meeting, held in Rome, in November, 16th -19th, 2005). The results of this study clearly confirm these findings: MGF increase myoblast proliferation by activating of the quiescent satellite cells, whilst systemic

IGF-I induces differentiation. On the other hand, a while ago, it has been suggested that MGF is expressed before systemic IGF-I following muscle damage (Yang and Goldspink, 2002; Haddad and Adams, 2002; Goldspink and Yang, 2004). This study indicated that MGF stimulated myoblast proliferation and IGF-I Ea stimulates myoblast differentiation (myotube formations). Proliferation stage is earlier stage of myogenesis than differentiation. Therefore, the findings of this study also support above information.

In this study, differentiation experimentation in human degenerative muscle cells was unsuccessful. Degenerative myoblasts did not fuse and myotube formation was not observed despite all efforts. Thus, differentiation state was only determined in healthy human muscles. In this study, it was observed that the ratios of degenerative myoblasts were very low (between 5 and 12 percents) compare to those of healthy cells (between 38 and 50 percents). Inhibition of calcineurin may play an important role in degenerative muscle pathophysiology (Stupka et al, 2004). Calcineurin also is a mediator of the IGF-I signalling pathway (Musaro et al, 1999). Thus, Shavlakadze et al propose that muscle degeneration could be due to a lack of IGF-I signalling (Shavlakadze et al, 2004). As a result, because of decreasing activated satellite cells / myoblasts number; lack of IGF-I, particularly MGF, signalling; insufficiency of mechanotransduction, etc, myotube formation might not be occurred.

For a long time, it has been known that, IGF-I is a neurotrophic factor and possesses potential clinical applications, in neurodegenerative disorders, particularly in ALS (Wilczak and Keyser, 2005). To date, systemic delivery of human recombinant IGF-I

has been used to treat ALS (Wilczak and Keyser, 2005). Most recently, Kasper et al (2005) found that exercise, when combined with IGF-I gene therapy by AAV2 vector, have some synergistic effects in treatment of ALS (Kaspar et al, 2005). The findings of Kaspar et al support the findings of this study, because MGF is expressed only in response to mechanical stimuli, including exercise.

This study demonstrated that different actions of different IGF-I isoforms were probably mediated via different receptors. The data concerning the blocking of the IGF-I receptor in this study provided sufficient evidence that MGF increases myoblast proliferation via a different signalling pathway, and MGF does not stimulate proliferation through IGF1R. Therefore, MGF might be bound to different receptor or receptors other than IGF1R. Thus, it seems that E domain of MGF is an independent growth factor to systemic form of IGF-I, despite both are splice variants of the same IGF-I gene.

Muscle satellite cells were one of the first tissue specific stem cells to be studied (for review see Dhawan and Rando, 2005). However, many studies have been carried out on transformed cells because of the availability of muscle cell lines and little work has been carried out using primary human muscle culture. In human muscle, the lineage and signalling associated with the activation process from quiescent, mononucleated to progenitor cells has been studied. Muscle satellite (stem) cells appear to be residual myoblasts that have not fused to form myotubes/muscle fibers. These are seen in juxtaposition with the muscle fibers following embryological differentiation of the tissue. Although, residual myoblasts are believed to be the main source of muscle

satellite cells, myoblast is a generic term for any cell that gives rise to muscle, and in primary cell cultures, all mononucleated cells are not satellite cells. There is a need therefore to have a marker to denote the stage in the progenitor cell lineage such as desmin (Sinanan et al, 2004; Dhawan and Rando, 2005).

Muscle loss is one of the main causes of death in patients with certain neuromuscular diseases. Goldspink and Yang suggested that in some cases, the muscle loss can be linked to the inability to express MGF (Goldspink and Yang, 2001; Goldspink, 2005), and they observed that muscles of mdx dystrophic mice, a model of human Duchenne Muscular Dystrophy, are unable to express MGF even during mechanical stimuli (Goldspink and Yang, 2001; Goldspink and Yang, 2004; Goldspink, 2005). De Bari et al observed that, when mesenchymal stem cells were introduced into dystrophic muscles of mdx mouse, the sarcolemmal expression of dystrophin and also that MGF are restored (De Bari et al, 2003). Therefore, it seems that MGF is required for activation of stem cell lineage.

In addition, Stupka et al suggest that calcineurin has an important role in the pathophysiology of degenerative muscle (in dystrophic mdx mouse) (Stupka et al, 2004). Inhibition of calcineurin resulted in 300 – 400 % more necrotic myofibers and reduced regeneration (Stupka et al, 2004). It has been known that the calcineurin is a mediator of IGF-I signalling pathway (Musaro et al, 1999). Therefore, Shavlakadze et al propose that muscle degeneration in dystrophic mdx mouse can be due to the lack of IGF-I signalling (Shavlakadze et al, 2004). Furthermore, Prof G. Goldspink suggests that the production of MGF may involve some type of mechanotransduction

mechanism (Goldspink, 2005). According to this study, it seems that both mechanisms, insufficiency of satellite cells and inability to express MGF, have some role in the occurrence of defective muscles mechanism.

This study found that cDNAs of MGF, which were transferred in a plasmid vector, significantly increased muscle growth of dystrophic mdx mice both in strength and mass, and that this was much more effective in younger animals.

The data in this study shows that MGF increases muscle force slightly in old mdx mice. The ratio of the increase of MGF was 10.6 %, and IGF-I increases 9.5 %. Comparing this observe to the effect of MGF in young animals, it can be said that the effects of cDNAs of MGF in muscle growth of old mice is not high when comparing it to those of IGF-I Ea one. This is in accord with the findings of Wells and Goldspink who reported that the age significantly influenced the level of expression of the injected plasmid DNA (Wells and Goldspink, 1992). They found that the highest levels of plasmid DNA expression were obtained in the 4-6 weeks age young mice, significantly higher than in mice older than 10 weeks (Wells and Goldspink, 1992). In this study, young mdx group was age of 4-8 weeks, whereas, the age of the older mice were between 15-26 months.

It has been known that the ageing process causes a reduction in the regenerative capacity of skeletal muscles eventually leading to reduced muscle strength, and to take place muscle weakness and also wasting (sarcopenia). On the other hand, satellite cells are the primary source of regenerating muscle fibers, and satellite cells must first

be activated from quiescent state to produce myoblast progeny and to start regeneration (Zammit et al, 2006). The mechanisms causing the impaired regenerative response to injury or degeneration observed in skeletal muscle of aged animals are not known yet. So far, different hypothesis have been proposed. For instance, Jejurikar et al, suggests that apoptosis may be a mechanism responsible for the depletion of satellite cells in old animals and then impairing the regenerative response (Jejurikar et al, 2006). The same researchers therefore proposes that aging increases the susceptibility of skeletal muscle derived satellite cells to apoptosis (Jejurikar et al, 2006). On the other hand, it is known that satellite cell activation is controlled by the Notch signalling pathway that is initiated by the rapid increase in expression of the Notch ligand, Delta, following injury. Conboy and Rando suggest that this up-regulation of Delta is blunt and thus satellite cell activation is remarkably reduced in old skeletal muscles (Conboy and Rando, 2005). Again, Hameed et al found that MGF expression in elderly women and men dropped significantly (Hameed et al, 2002; Hameed et al, 2003; Harridge, 2003; Hameed et al, 2004).

As a result, degenerative skeletal muscle, including that of DMD, states motor nerve innervation; satellite cell numbers and proliferative potential significantly decrease in advanced age; regenerative capacity and contractility of skeletal muscle exhaust in degenerative muscles of neuromuscular diseases, particularly in DMD (Jejurikar and Kuzon, 2003; Shi and Garry, 2006). On the other hand, plasmid distribution decreases in advanced age. The lower effect of cDNAs of MGF in muscle growth in old mdx mice can be explain under all these negative conditions.

For many years, it has been broadly known that the satellite cells in skeletal muscle provide the extra nuclei for post-natal growth. (Moss and Leblond, 1971; Schultz, 1976; Zammit et al, 2006). The satellite cells are the primary stem cells in adult skeletal muscle; they are responsible for postnatal muscle growth, regeneration, repair and hypertrophy (Dhawan and Rando, 2005, Zammit et al, 2006). Therefore, the satellite cells are also called muscle stem cells (Collins and Partridge, 2005).

The mononucleated / activated satellite cells express M-Cadherin and CD 34. When the satellite cells are activated, they commence to co-express c-met, MyoD, myf5, Desmin, Pax-3, Pax-7 and later myogenin, and become intermediate progenitors and fusion competent myoblasts, subsequently they fuse and differentiate to myotubes (Dhawan and Rando, 2005). Two families of myogenic basic helix-loop-helix (bHLH) transcription factors regulate above biochemical differentiation: (a) the myogenic regulatory factors (MRFs) including MyoD, myogenin, Myf-5 and MRF4, and (b) myocyte enhancer factor 2 (MEF) proteins, and other transcription factors such as Pax-3 and Pax-7. MyoD and myogenin are among the most important factors of those regulatory factors. Therefore, the levels of mRNA transcripts of such markers demonstrated satellite cell activation in this study.

Since quantitative real-time RT-PCR technique was first developed by Higuchi et al in 1993 (Higuchi et al, 1993), the technique has being increasingly used for the quantification of specific mRNA expression. The quantitative real-time RT-PCR technique with SYBR Green I fluorescent dye is highly sensitive and it allows quantification of transcripts even in very low abundance.

This study demonstrated that both MyoD and myogenin transcript levels were higher in MGF treated young mdx mice muscle tissues compared to uninjected control muscles of the same animals. In this way, the study showed that MGF activated muscle satellite cells of mdx mice, particularly in young animals. In contrary, transfer of IGF-I did not stimulated the expression of satellite cell markers. The effectiveness of MGF was apparently in the satellite cell activation. Hill and Goldspink also showed this effect in rats. Hill and Goldspink's data showed that MGF clearly activated quiescent satellite cells (Hill and Goldspink, 2003a, Hill et al, 2003b). This study indicated that MGF initiates the activation of muscle satellite (stem) cells. The study also showed that the effect of MGF in satellite cell activation by investigating satellite cell markers expression in young mdx mice was higher and / or clearer than those of old animals.

In summary, this study found that MGF significantly increased myoblast proliferation in animal myoblast cell lines and primary cultured human myoblast, in both healthy and degenerative muscles from CMD, FSHD and ALS. The study also found MGF probably have another signalling pathway, and it used another receptor rather than type 1 IGF receptor. The study showed again that MGF increased muscle growth by gene transfer way, and also increased satellite cell activation. The hypothesis of in vitro part of this study was that the E domain of MGF, in comparison with IGF-I mature peptide, influences cell proliferation and prevents myogenic differentiation in primary cultured muscle cells from patients with CMD, FSHD and ALS, and rodent muscle cell lines. In vitro data of this study confirmed this hypothesis.

On the other hand, second hypothesis was that the coding sequence of MGF, in comparison to the coding sequence of IGF-I Ea, improves the maximum tetanic contractile force of muscles by activating satellite cells in the mdx mouse. In vivo data of this study also confirmed this hypothesis.

Consequently, one aims of this study was to find a way to treat degenerative muscles in neuromuscular disorders. This study showed that MGF has a potential as a therapeutic agent to treat muscle degeneration in neuromuscular disorders, such as DMD, CMD, FSHD and also ALS.

Chapter 6:

Conclusion

6.1 Conclusion

6.1 Conclusion

Skeletal muscle is highly regenerative tissue, and for a while, it has become main target for gene therapy applications. This study also attempted to develop a novel gene therapy application to treat muscle degenerating in neuromuscular disorders using IGF-I splice variants. For this aim, the effects of IGF-I splice variants (IGF-I Ea and MGF) were investigated in animal and human skeletal muscles in vitro and in vivo. In order to study the effects of the splice variants of the gene in vitro, along with rodent myoblast cell lines, primary cultured healthy and degenerative human muscle cells were investigated. This study found that MGF had a dramatic effect on myoblast proliferation, both in mouse and human muscles. MGF increased myoblast proliferation significantly in both healthy and degenerative muscles from CMD, FSHD and ALS patients. The study also demonstrated that MGF and IGF-I have antagonist effect. IGF-I Ea increased myoblast differentiation (myotube formation), whilst MGF increased myoblast proliferation and prevent the differentiation. Moreover, the study showed that MGF had a different signalling pathway than systemic / liver type IGF-I variant. In addition, the study found that the ratios of

myoblast of degenerative muscles were very low when compare them to those of healthy muscles.

The effects of such splice variants were also investigated in in vivo animal model of most common neuromuscular disorder, DMD, which was mdx mouse. After cDNAs of MGF and IGF-I Ea were introduced into skeletal muscles of young and old mdx mice in plasmid / naked DNA, which is the main non-viral gene delivery system, the roles of the variants, particularly that of MGF were studied in muscle growth in strength and mass and satellite cell activation. The study showed that MGF dramatically increased muscle strength, particularly in young animals. MGF also increased satellite cell activation. In conclusion, the study firmly proposes that MGF has a potential as a therapeutic agent to treat muscle degeneration in neuromuscular disorders, such as DMD, CMD, FSHD and also ALS. Thus, gene therapy applications using MGF transfer into muscle can be an effective technique.

6.2 Suggestions for Future Investigations

In this study, the effects of MGF were investigated only in muscle cells and tissues. However, recent data shows that MGF has also similar effects on different tissues and organs, including damage to cardiac muscles (G. Goldspink and S. Y. Yang, unpublished data; Dr Paul Goldspink of Department of Physiology and Biophysics, University of Illinois College of Medicine at Chicago – USA, unpublished data) the CNS (Dluzniewska, 2005), periphery nerves (S. Y. Yang, unpublished data), endometrium (Prof Goldspink group, unpublished data), tendons and ligaments (Olesan et al 2006; Heinemeier et al, 2007). Recent years, MGF has become regarded as a general tissue repair factor. However, the data about the action of MGF on different tissues and organs are still very limited. Therefore, in vitro and in vivo actions of MGF on such tissues and organs with certain conditions should be investigated.

Recent investigations found that MGF also has some neurotrophic effects, and it significantly increases motor neuron survival (Aperghis et al, 2004; Dluzniewska, 2005). The in vitro data in this study was also supported these findings. MGF dramatically increased activated mononucleated satellite cell proliferation in primary

muscle cell from ALS patients. These effects should be investigated in vivo animal models of ALS, such as SOD1 mouse. The MGF expression in ALS might be also investigated in resting and mechanically stimulated muscle and neuron cells from both human and animals with and without ALS.

This study demonstrated that MGF probably has a different receptor-mediated pathway to the systemic liver type IGF-I (IGF-I Ea). The pathways of MGF (both receptor-mediated and signalling pathways) are unknown. In order to learn effectiveness mechanism of MGF, to investigate MGF pathways therefore vital.

Up to date, E domains of IGF-I peptides, including MGF, has not been demonstrated in blood circulation. Only mature IGF-I peptide has been seen in circulation. However, it is known that the level of mature IGF-I peptide increases after heavy exercise, and major IGF-I supply tissue is skeletal muscle during exercise. However, this has binding proteins which bind the IGF-I domain which stabilize it and act as a time release mechanism. It seems that during exercise, MGF expression significantly increases in skeletal muscle during heavy exercise and then, E domain of MGF are probably cleaved at specific sites by some proteases and this may stay within the basal lamina of the fibres. The mature peptide of MGF may leave the cell and enter the blood circulation whilst the E domain of MGF probably goes to only a little further to activate the satellite cells that are within basal lamina of each fibre. These mechanisms should also be investigated.

It appears that MGF signalling is different to IGF-I Ea; there is much work needed to investigate their different cellular mechanisms. Because of their therapeutic potential for treating muscle wasting, this effort would be worthwhile.

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Appendix

Appendix

Appendix – 1: Additives for MCGM (Muscle Cell Growth Medium)

ADDITIVES	STOCK SOLUTIONS	FINAL SOLUTIONS
EGF	20 µg/ml	10 ng/ml
bFGF	2 µg/ml	2 ng/ml
Low Insulin	100 µg/ml	5 ng/ml
Dexamethasone	390 µg/ml	390 ng/ml
Feutin	50 mg/ml	50 µg/ml
Vitamin E (Trolox)	25 mg/ml	25 µg/ml
Vitamin C	100 mg/ml	50 µg/ml
Vitamin H (D-Biotin)	100 mg/ml	250 ng/ml
Transferrin	5 mg/ml	5 µg/ml
Sodium Selenite	100 µg/ml	5 ng/ml
Albumax-1	20 % sol. (200 mg/ml)	500 µg/ml
Penicillin / Streptomycin	10 000 U/ml or (50 mg/ml)	500 µg/ml
Long r ³ IGF-I	2 mg/ ml	various
MGF	1 mg/ml	various
DMEM	Serum free medium	-

Appendix – 2: In Vitro Primary Cell Culture Data

HEALTHY CRANIOFACIAL MUSCLE	Cont	Cont + Ab-1	MGF	MGF + Ab-1	IGF-I	MGF + IGF-I
	53.09735	52.58621	70.12448	76.99531	55.88235	60.43478
	50.93633	49.79079	72.58065	77.19298	52.17391	56.50558
	52.22672	50.36765	71.21951	75	58.41121	58.54701
	45.89372	47.44526	70.12448	62.67281	63.30645	56.84647
	47.34513	41.12554	66.26506	69.41581	59.82143	61.70213
	49.37238	42.22222	69.09722	65.38462	66.28352	60.96491
	50.2439		67.17557		59.07173	61.27451
	50.93633		66.51584		58.59031	
	48.0198		59.03084		54.43548	
			69.62617		55.81395	
			73.55769			
			65.91928			
			73.23232			

HEALTHY LIMB MUSCLE	Control (%)	MGF (10) (%)	IGF-I (10) (%)	MGF (10) + IGF-I (10) (%)
	35.55556	55.14019	43.63636	38.68613
	39.86486	59.00621	52.43902	38.28125
	40.41096	54	48.40183	39.49045
	41.04046	60.37736	43.18182	
	39.24051	55.26316	46.26866	
	35.06494	61.70213	48.6911	
	41.08108	55.05051		
	35.20408	62.56983		
	38.38384	57.79221		

CMD	Cont (%)	Cont+Ab-1 (%)	MGF (%)	MGF + Ab-1 (%)	IGF-I (%)	MGF + IGF-I (%)
	9.615385	10.52632	19.71831	16.39344	11.66667	12.69841
	13.33333	8.62069	17.1875	14.51613	13.11475	12.90323
	9.433962	10.16949	15.71429	18.84058	15.87302	11.47541
	9.803922	10.34483	16.39344	17.1875	13.46154	14
	11.53846	10.14493	18.42105		11.76471	14.58333
	8.695652	11.11111				

ALS	Cont (%)	Cont + Ab-1 (%)	MGF (%)	MGF + Ab-1 (%)	IGF-I (%)	MGF + IGF-I (%)
	6.5	5	7	7	3	5
	5	7	8	6	3	5
	3	3	7	6	5	3
	4	5	8	6	5	5
	5	5	6	6	6	6
	5	6	7	8	6	6
	6		8		6	6
	4		8		5	6
	5		6			5

FSHD	Control (%)	MGF (10) (%)	IGF-I (10) (%)	MGF + IGF-I (%)
	12	22	14	13
	12	22	15	14
	13	23	15	13
	10	21	16	15
	13	19	12	16
	12	17	15	16
	10	19	12	

Appendix – 3: TA Muscle Force Measurements after Gene Transfer

Young mdx muscle force measurements

	Vector – only group		IGF-I Ea group		MGF group	
	Untreated (control)	Treated (Exp)	Untreated (control)	Treated (Exp)	Untreated (control)	Treated (Exp)
1	170.6097	155.176	48.5282	41.02167	100.3291	95.99235
2	46.50255	81.57908	50.65128	36.95866	86.42602	90.3801
3	134.0026	104.4107	24.36758	38.93613	106.1964	117.676
4	129.5383	70.99235	32.66584	43.32933	69.46173	115.5077
5	96.375	125.7117	42.48676	41.51977	95.60969	112.574
6	68.95153	80.30357	40.89591	55.22658	114.2321	178.773
7	77.625	80.04847	50.46212	48.79844	71.375	135.1505
8			35.1604	65.28537	72.14031	137.3189
Mean	103.3721	99.74599	40.65226	46.38449	89.4713	122.9216
Inc (%)		- 3.63%		14.10%		37.38%
p val.		(NS) 0.783		(NS) 0.292		(S) 0.014

Old mdx muscle force measurements

	Vector – only group		IGF-I Ea group		MGF group	
	Untreated (control)	Treated (Exp)	Untreated (control)	Treated (Exp)	Untreated (control)	Treated (Exp)
1	24.11499	38.04082	63.56138	69.46647	59.78204	66.63198
2	52.22341	59.30957	57.18371	62.85267	62.61654	69.46643
3	62.85264	60.96906	47.57806	63.79747	69.23028	64.97724
4	45.84587	46.08208	65.92334	62.61645	62.38033	68.28546
5	60.72684	80.10179	90.48305	81.27668	12.77804	20.09949
6	78.28647	47.45061	58.60106	85.05608	64.01909	72.99375
7			56.71138	59.07347	59.54579	69.21746
8			62.75821	66.39575		
Mean	54.00837	55.32565	62.85002	68.81688	55.76459	61.6674
Inc (%)		2.44%		9.49%		10.58%
p val.		(NS) 0.862		(NS) 0.211		(S) 0.015

Appendix – 4: Mdx TA Muscles Weights after Gene Transfer

Young Mdx TA Muscles Weights

	VECTOR-ONLY		IGF-I Ea		MGF	
	Cont. (mg)	Exp. (mg)	Cont. (mg)	Exp. (mg)	Cont. (mg)	Exp. (mg)
1	40	40	48	48	36	43
2	30	39	48	55	46	56
3	28	29	49	55	32	33
4	34	32	53	53	36	33
5	47	43	55	61	41	48
6	23	29	48	57	29	29
7	37	37	76	80	34	46
8	44	41	65	60	43	46
Mean	35.4	36.2	55.2	58.6	37.1	41.7
Inc (%)	100	102	100	106	100	113
p val.		(NS) 0.598		(NS) 0.079		(S) 0.040

Old Mdx TA Muscles Weights

	VECTOR-ONLY		IGF-I Ea		MGF	
	Cont. (mg)	Exp. (mg)	Cont. (mg)	Exp. (mg)	Cont. (mg)	Exp. (mg)
1	59	61	91	92	88	90
2	85	70	89	95	91	95
3	75	64	88	92	92	99
4	78	79	88	87	76	86
5	87	90	80	81	65	70
6	75	74	80	80	70	80
7			92	94	89	89
8						
Mean	76.5	73	86.8	88.7	81.6	87
%	100	95	100	102	100	107
p val.		(NS) 0.309		(NS) 0.087		(S) 0.009

Appendix – 5: Expression of Satellite Cell Markers after Gene Transfer

Young Mdx MyoD Expression Data by RT-PCR (MyoD copy number / β -actin copy number ratios)

Vector-only			IGF-I Ea			MGF		
	Untreated	Treated		Untreated	Treated		Untreated	Treated
1	9.95E-01	2.57E-01	1	5.63E-01	2.63E-01	1	3.99E-01	3.86E+01
2	4.41E-01	5.88E-01	2	6.44E-01	6.62E-01	2	6.36E+00	9.38E+00
3	8.09E+00	1.11E+00	3	2.43E-01	4.27E-01	3	4.56E-02	5.12E+01
4	2.03E+00	5.10E+00	4	7.34E-01	2.08E-01	4	4.43E-02	6.27E-01
5	3.75E-02	2.30E-03	5	2.43E-01	1.70E-01	5	5.27E-01	6.91E-01
6	2.04E+00	1.77E+01	6	2.44E-01	2.72E-01	6	3.38E-01	4.28E+00
7	3.62E-01	3.68E-03	7	6.10E-01	2.90E-01	7	4.24E-01	1.98E+00
8			8	4.47E-01	6.08E-01	8	2.04E-02	1.02E+00
Mean	2.00E+00	3.54E+00		4.66E-01	3.62E-01		1.02E+00	1.35E+01
St Err	1.06E+00	2.46E+00		7.11E-02	6.52E-02		7.66E-01	7.03E+00
p val.		(NS) 0.578			(NS)0.285			(NS)0.125

Young Mdx Myogenin Expression Data by RT-PCR (Myogenin copy number / β -actin copy number ratios)

Vector-only			IGF-I Ea			MGF		
	Untreated	Treated		Untreated	Treated		Untreated	Treated
1	1.52E-01	3.94E-02	1	2.08E-01	1.03E+00	1	2.23E-01	1.60E+01
2	4.41E-04	1.00E+00	2	1.90E+00	7.67E+00	2	2.04E+00	1.29E+01
3	2.82E-01	1.90E-01	3	1.11E-01	2.14E+00	3	5.51E-02	4.68E+00
4	2.60E+00	1.71E+00	4	4.51E+00	2.31E+00	4	7.68E-02	1.79E+00
5	3.69E-02	1.13E+00	5	4.10E+00	2.31E+00	5	1.02E-01	1.57E+00
6	9.86E-01	5.72E-03	6	3.28E-01	1.79E-01	6	4.40E-01	5.43E+00
7	1.53E-01	1.60E+00	7	3.30E-01	1.81E-01	7	1.71E-01	2.31E+01
8			8	2.02E+00	5.73E-01	8	7.98E-02	1.33E+01
Mean	6.01E-01	8.11E-01		1.69E+00	2.05E+00		3.98E-01	9.84E+00
St Err	3.56E-01	2.76E-01		6.31E-01	8.66E-01		2.38E-01	2.72E+00
p val.		(NS) 0.59			(NS) 0.707			(S) 0.009

**Old Mdx MyoD Expression Data by RT-PCR
(MyoD copy number / β -actin copy number ratios)**

Vector-only			IGF-I Ea			MGF		
	Untreated	Treated		Untreated	Treated		Untreated	Treated
1	1.07E+02	5.15E+01	1	1.26E+01	2.05E-02	1	1.08E-01	2.63E+00
2	4.69E-01	3.23E+00	2	1.17E-02	3.00E-02	2	1.12E-01	2.91E-01
3	1.13E-01	8.32E-02	3	1.36E-02	6.53E-01	3	1.20E-01	1.22E-02
4	8.76E-01	1.12E-02	4	5.61E-02	1.09E-02	4	2.56E-01	6.33E-01
5	2.16E-01	2.34E-02	5	1.24E-02	2.20E-01	5	1.55E+00	7.67E+00
6			6	1.65E-02	1.47E-02	6	5.78E-01	1.41E+00
7			7	9.77E-03	1.69E-01	7	3.12E-01	9.48E-01
Mean	2.17E+01	1.10E+01		1.81E+00	1.60E-01		4.33E-01	1.94E+00
St Err	2.12E+01	1.02E+01		1.79E+00	8.81E-02		1.09E+01	3.34E-01
p val.		(NS) 0.391			(NS) 0.398			(NS) 0.12

**Old Mdx Myogenin Expression Data by RT-PCR
(Myogenin copy number / β -actin copy number ratios)**

Vector-only			IGF-I Ea			MGF		
	Untreated	Treated		Untreated	Treated		Untreated	Treated
1	9.36E+00	1.14E+00	1	5.77E+00	2.87E-02	1	2.07E-03	4.30E-01
2	1.59E-01	4.31E+00	2	7.02E-02	8.20E-02	2	1.27E-01	3.55E-01
3	1.31E-01	1.31E-01	3	3.93E-02	3.14E-01	3	4.46E-03	1.89E-01
4	1.08E+00	8.41E-02	4	1.04E-01	8.11E-02	4	2.03E-01	2.73E-01
5	1.05E+00	1.50E-02	5	6.17E-02	2.03E-01	5	1.59E+00	3.12E+00
6			6	6.35E-02	3.31E-03	6	1.55E-01	4.99E-01
7			7	6.96E-02	1.56E-01	7	4.13E-02	5.40E-01
Mean	2.35E+00	1.14E+00		8.83E-01	1.24E-01		3.04E-01	7.72E-01
St Err	1.76E+00	8.20E-01		8.15E-01	4.10E-02		2.17E-01	3.94E-01
p val.		(NS) 0.573			(NS) 0.396			(S) 0.044

Appendix – 6: List of Publications

Peer reviewed papers:

1- **Kenan Ates**, Shi Yu Yang, Richard W. Orrell, Andrea C. M. Sinanan, Paul Simons, Andrew Solomon, Steven Beech, Geoffrey Goldspink and Mark P. Lewis. IGF-I splice variant, (MGF) increases progenitor cells in ALS, dystrophic and normal muscle. *FEBS Letters* (in press).

2- **Ates, K.**, Yang, S. Y., Lewis, M., and Goldspink, G. Changes in muscle regulatory factors in mdx murine skeletal muscle following intramuscular transfer of the cDNA of the IGF-IEa and MGF splice variants of the IGF-I gene. *Journal of Physiology* (submitted, under consideration).

3- Yang, S. Y. *, **Ates, K. ***, Beech., S., and Goldspink, G. Increased contractile force in mdx murine skeletal muscle following intramuscular transfer of the cDNA of two splice variants of IGF-I. *Journal of Physiology* (submitted, under consideration).

(* both authors made an equal contribution)

Published abstracts:

1- G. Goldspink, **K. Ates**, S. Y. Yang, R. T. Jaspers, J. Weaden R. W. Orrell. (2005) MGF (a splice variant of IGF-I) increases satellite cell number and kick starts the hypertrophy process in normal and atrophic muscle. FASEB Summer Research Conference 2005, 4rd International Conference on Skeletal Muscle Satellite and Stem Cells, Omni Tucson National Golf Resort & Spa, Tucson, AZ, US (June 11-16, 2005)

2- **K. Ates**, S. Yang, A. Sinanan, G. Goldspink and M. Lewis. (2006) Effects of MGF on human muscle stem cell proliferation in dystrophic, ALS and healthy human muscle, *FEBS Journal*. 273 (s1), 128. 31. International FEBS Congress on Molecules in Health and Disease, Istanbul, Turkey (24-29 June 2006)