Title: Development and charaterisation of 3 dimensional culture models for zebrafish (Danio rerio) skeletal muscle cells

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DEVELOPMENT AND CHARATERISATION OF

3 DIMENSIONAL CULTURE MODELS FOR ZEBRAFISH (*DANIO RERIO*) SKELETAL MUSCLE CELLS

by

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Abstract

Zebrafish (*Danio rerio*) have been extensively used over the past two decades to study muscle development, human myopathies and dystrophies, due to its higher degree of homology with human disease causing genes and genome. Despite its unique qualities, zebrafish have only been used as an *in-vivo* model for muscle development research, due to the limitations surrounding lack of a consistent isolation and culture protocol for zebrafish muscle progenitor cells *in-vitro*.

Using different mammalian myoblast isolation protocols, a novel and robust protocol has been developed to successfully isolate and culture zebrafish skeletal muscle cells repeatedly and obtain differentiated long multi nucleated zebrafish myotubes. Commitment to myogenic lineage was confirmed by immuno-staining against muscle specific protein desmin, and expression pattern of different genetic markers regulating myogenesis. In order to recapitulate the *in-vivo* bio-physiological environment for zebrafish skeletal muscle cells *in-vitro*, these cells were successfully cultured in tissue engineered three dimensional (3D) constructs based on fibrin and collagen models. Maturation of tissue engineered collagen and fibrin based constructs was confirmed using the basic parameters described in the literature i.e. collagen three times greater contraction from the original width (Mudera, Smith et al. 2010) and fibrin constructs tightly coiled up to 4mm of diameter (Khodabukus, Paxton et al. 2007).

In-vitro characterisation of zebrafish skeletal muscle cells showed hypertrophic growth of muscle mass compared to hyperplasic growth *in-vivo* as suggested for fish

species in literature (Johnston 2006), which is different from human and other mammals. Comparative analysis of zebrafish muscle cells cultured in monolayer against cultured in 3D tissue engineered constructs showed significant increase in fusion index, nuclei per myotube (two-fold) and myotubes per microscopic frame (two-fold). Cells cultured in tissue engineered construct closely resembled *in-vivo* muscle in terms of their unidirectional orientation of myotubes.

These tissue engineered 3D zebrafish skeletal muscle models could be used for various purposes such as drug screening, effect of different temperature extremes, studying underlined pathways involved in human diseases; and with further refinements it would potentially replace the need for studies on live fish in these areas.

I wish to dedicate this thesis to my Mum and Dad. It was their endless believe in

me whom helped me reach where I am today.

This is for you mummy and papa.

Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Bedfordshire.

It has not been submitted before for any degree or examination in any other university.

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Contents

X

XI

List of Figures

XV

List of Tables

List of abbreviations

ADP   Adenosine di-phosphate

ANOVA   Analysis of variance

ATP   Adenosine tri-phosphate

bp   Base pairs

BSA   Bovine serum albumin

CD56   Cluster of differentiation 56

cDNA   Complementary DNA

CAS   CRISPR associated systems

CRISPR   Clustered regularly interspaced short palindromic repeats

DM   Differentiation media

DAPI   4', 6-diamidino-2-phenylindole

DABCO   1, 4-diazabicyclo[2.2.2]octane

DMSO   Dimetyl suplhoxide

DNA   Deoxyribonucleic acid

dNTP   Deoxynucleotide triphosphates

ECM   Extracellular matrix

FBS   Foetal bovine serum

FGF   Fibroblast growth factor

FITC   Fluorescein isothiocyanate

fMyHc   Fast myosin heavy chain

GM   Growth media

ICC	Immunocytochemistry
IGF-1	Insulin like growth factor 1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
MDC	Muscle derived cell
MyoD (myoD)	Myoblast determination factor
MRF4	Myogenic regulatory factor-4
MEM	Minimum essential medium
MCadh	Myotubule-cadherin
MHC	Myosin heavy chain
MPC	Muscle precursor cell
MRF 4	Myogenic regulatory factor 4
mRNA	messenger RNA
Pax 3	Paired box transcription factor 3
Pax 7	Paired box transcription factor 7
PBS	Phosphate buffered saline
PLLA	Poly lactic acid
PLGA	Poly(lactic-co-glycolic acid)
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RPM	Revolutions per minute

RT-qPCR	Real time quantitative PCR
SR	Sarcoplasmic reticulum
sMyHc	slow Myosin heavy chain
3D	Three dimensional
2D	Two dimensional
Talen	Transcription activator-like effector proteins
TBS	Tris buffered saline
TRITC	Tetramethylrhodamine-5-(and-6)-Isothicyanate
TRIS	Trisaminomethane
UV	Ultra violet
ZMC's	Zebrafish muscle cells
ZSMC's	Zebrafish skeletal muscle cells

1. General Introduction

The experiments designed in this study were an attempt to develop, optimise and characterise a tissue engineered three dimensional culture model for zebrafish (*Danio rerio*) skeletal muscle cells which has the potential to be used in future studies related to testing different aspects of muscular dystrophies and myopathies (Bassett, Currie 2003, Guyon, Goswami et al. 2009), toxicological (Hill, Teraoka et al. 2005) and drug testing (Gibert, Trengove et al. 2013), different extremes of temperature (Scott, Johnston 2012) and in exercise physiology (Palstra, Beltran et al. 2013). Therefore the introduction shall focus on zebrafish as a model, skeletal muscle *in vivo*, embryonic development, organisation and operation of skeletal muscle. Current techniques along with their limitations for culturing or analysing skeletal muscle and adaptation both *in vivo* and *in vitro* will also be briefly highlighted, in order to replicate a more biomimetic culture model for present and future work.

1.1 Zebrafish (*Danio Rerio*) – Animal model

Zebrafish are a small (4-5 cm long) teleost fish from the *cyprinidae* family, native to the streams of the Himalayan region and found in parts of the Asian sub-continents (Mayden, Tang et al. 2007). Breeding of zebrafish is an external fertilisation process which can take place all year round and females have the capacity to produce several hundreds of eggs at a time and they can spawn every 2-3 days. Large size, translucency of the embryos (0.7 mm in diameter) and larvae allows us to understand the insights via *in vivo* visualization of the cell biological events in zebrafish (Lieschke, Currie 2007). Due to the unique properties of zebrafish such as high stocking density, rapid development, high fecundity, and external fertilization,

1

zebrafish have been extensively used for developmental biology studies such as determination of embryonic axis, cell lineage analysis, formation of the central and peripheral nervous system, signalling networks that regulate muscle development (Ochi, Westerfield 2007) and differential regulation of gene expression (Lele, Krone 1996). Recently zebrafish has been extensively used in toxicological and drug screening research due to its high degree of similarity with mammals, external fertilisation, high yield of reproduction, rapid, permeable and transparent embryonic development. In zebrafish genes or protein expression can be silenced or knock down using morpholino antisense oligo-deoxynucleotides technique (Bill, Petzold et al. 2009) and also using recently developed techniques. Genes or protein expression can also be knocked out at early embryo stage of zebrafish using techniques such as zinc finger, CRISPR (clustered regularly interspaced short palindromic repeats) (Hwang, Fu et al. 2013) or TALEN (Transcription activator-like effector nuclease) (Gaj, Gersbach et al. 2013). These techniques are very useful and efficient in research now days in order to obtain genetically modified organisms (Gaj, Gersbach et al. 2013). Specific genes can be silenced or knocked out from the genome, in order to study its effect on different pathways. TALEN has been used in literature by different researchers from last two decades due to it specificity for target sequence. Using TALENs, genes can be accurately modified via homologous recombination and this has been widely used to study gene function and mutation of interest in mice (Zu, Tong et al. 2013). Zu, Tong et al. 2013 have successfully reported homologous recombination gene modification at one cell stage and successfully transmitted through the germ line of mice. Recently CRISPR has been developed and even used

in zebrafish, to knock out specific sequence (Hwang, Fu et al. 2013). Research has suggested that it has fewer random mutations compare to other techniques by using different CAS (CRISPR associated enzymes) enzymes for cleavage and G-RNA (guide RNA) for guiding cas enzyme to the site for cleavage (Gaj, Gersbach et al. 2013). Genome of genetically modified zebrafish has been sequenced and can be easily accessed from UCSC website (Kari, Rodeck et al. 2007), which again proves it a useful model organism. Use of fish species in research has increased dramatically in the past two decades (data presented in **Table 7.1**) stating the need to increase in the number of procedures for fish species. Data in table 7.1 shows that the number of fish sacrificed is relatively higher compared to all other species and the number has been increasing every year, in part due to the reduction in use of mammals, demonstrating the need for a reduction or refinement in fish species for research.

1.1.1 *Danio rerio* in research

Over the past two decades zebrafish (*Danio rerio*) have been extensively used in scientific research (Collodi, Kamei et al. 1992) due to many of its unique qualities such as small size, low cost maintenance, easy availability and large scale genetic studies have been done previously. Most genes discovered so far in zebrafish are evolutionarily conserved and have homologues in mammals (Cerda, Conrad et al. 1998, Howe, Clark et al. 2013). Zebrafish have been widely used as a model organism to understand vertebrate development by blocking down specific genes at early embryonic stages (Langheinrich, Hennen et al. 2002). In recent years, it has been used as a model to study human myopathies and dystrophies such as *sapie* and *sapie- like* (dystrophin) (Bassett, Currie 2003, Guyon, Goswami et al. 2009), *runzel*

*(*titin*)* (Steffen, Guyon et al. 2007), *softy* (laminin b2) (Jacoby, Busch-Nentwich et al. 2009) and *candyfloss* (laminin a2) (Hall, Bryson-Richardson et al. 2007), due to its homology with human disease causing genes (Barbazuk, Korf et al. 2000, Dodd, Curtis et al. 2000).

Zebrafish research not only increases our understanding of the detailed roles of genes specific to human diseases (Howe, Clark et al. 2013) but also, recently zebrafish experiments have been designed and included for independent verification of gene activity in studies for human genetic diseases (Golzio, Willer et al. 2012, Roscioli, Kamsteeg et al. 2012, Tobin, Roca et al. 2012). In all the vertebrates sequenced so far, zebrafish have the highest number of protein coding genes, 26,206 (Collins, White et al. 2012) and they also have the highest number of species-specific genes in their genome compared to human, mouse or chicken (Meyer, Schartl 1999). In a direct comparison between zebrafish and human protein coding genes, it was found that 71.4% human genes have at least one zebrafish orthologue, as per Ensembl Compara (Vilella, Severin et al. 2009). On the contrary 69% zebrafish genes have at least one human orthologue (Howe, Clark et al. 2013). In order to find the potential disease related genes in human and zebrafish a comparison was performed with the genes bearing morbidity descriptions listed on Online Mendelian Inheritance in Man (OMIM) and it was found that 82% of these genes are related to at least one zebrafish orthologue (Howe, Clark et al. 2013). This suggests that zebrafish are an important model for studying human diseases.

1.2 Skeletal muscle structure

Skeletal muscle enables the precise and deliberate movement of the body with the help of attachments to tendons directly or indirectly, to the skeleton. Skeletal muscle fibres are longitudinally arranged in relation to the direction of movement, towards the proximal site of attachment (Smith, Shah et al. 2010). The highly organised structure of skeletal muscle has been explained as individual elongated fibres, embedded in a protein- carbohydrate rich extra cellular matrix (ECM) (Lewis, Machell et al. 2001). The hierarchy of skeletal muscle organisation (Sciote, Morris 2000) is explained in **figure 1.1**.
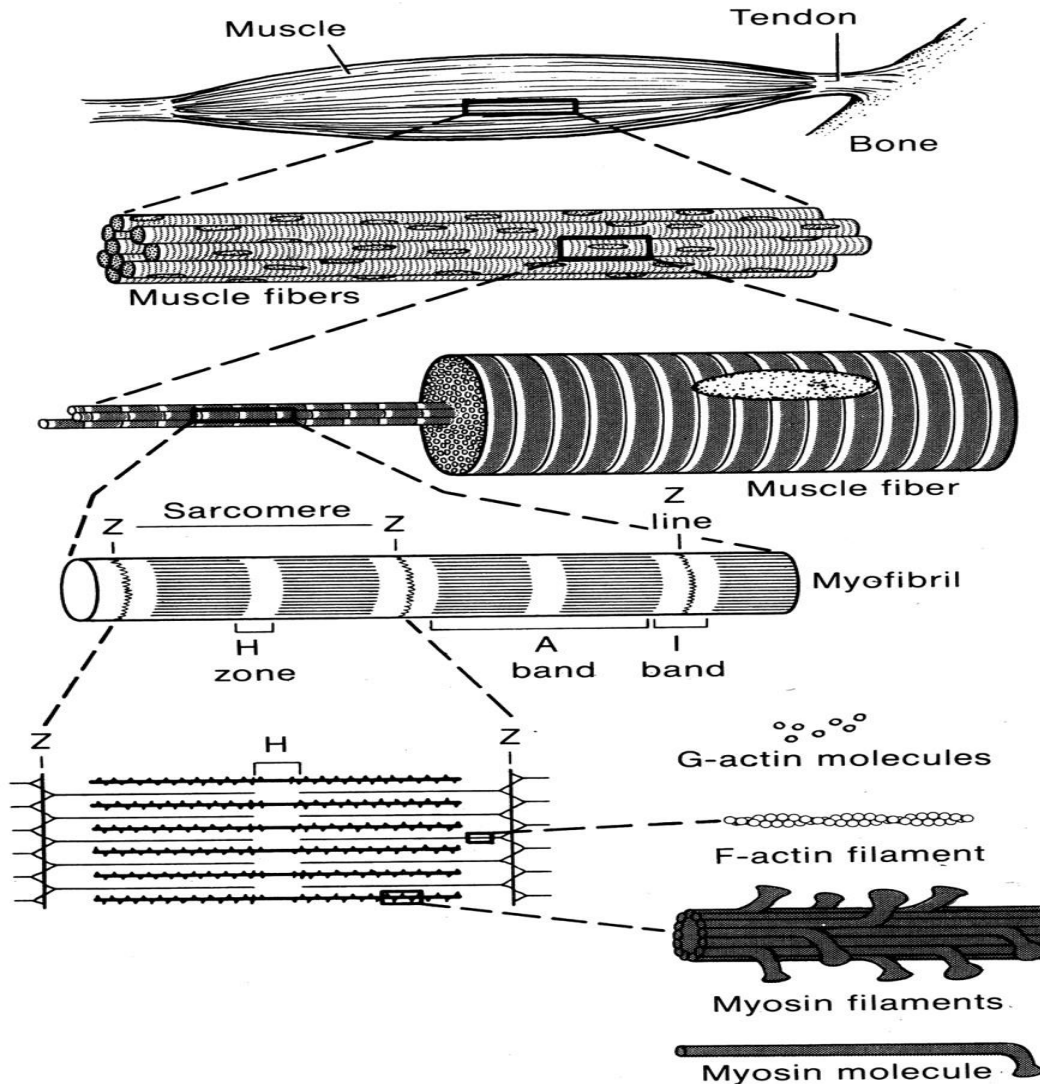
Figure 1. 1: Systematic hierarchy of skeletal muscle organisation.
Bundles of actin and myosin molecules, the smallest sub unit forms myofibrils and a bundle of myofibrils forms a muscle fibre. Myofibrils are made of sarcomere regions; area starting from one z- line to another, separated by H-zone in between. Bundles of muscle fibres form a whole skeletal muscle attached to bones via tendons. Image taken from Sciote et al. (2000).

1.2.1 Organizational and structural levels of skeletal muscle

Skeletal muscle is composed of an orderly arrangement of connective tissue, contractile cells, blood vessels and nerves. Thousands of multinucleate muscle fibres (mature muscle cells) running parallel to each other with relatively consistent diameters form a whole muscle (Lieber 2002), but fibres may differ in length

6

according to their stretching ability. The connective tissues which keep the whole muscle intact and systematically arranged are known as endomysial (around the muscle cell), perimysial (around groups of muscle cells) and epimysial (around the whole muscle) (Gillies, Lieber 2011) as shown in Figure 1.2.
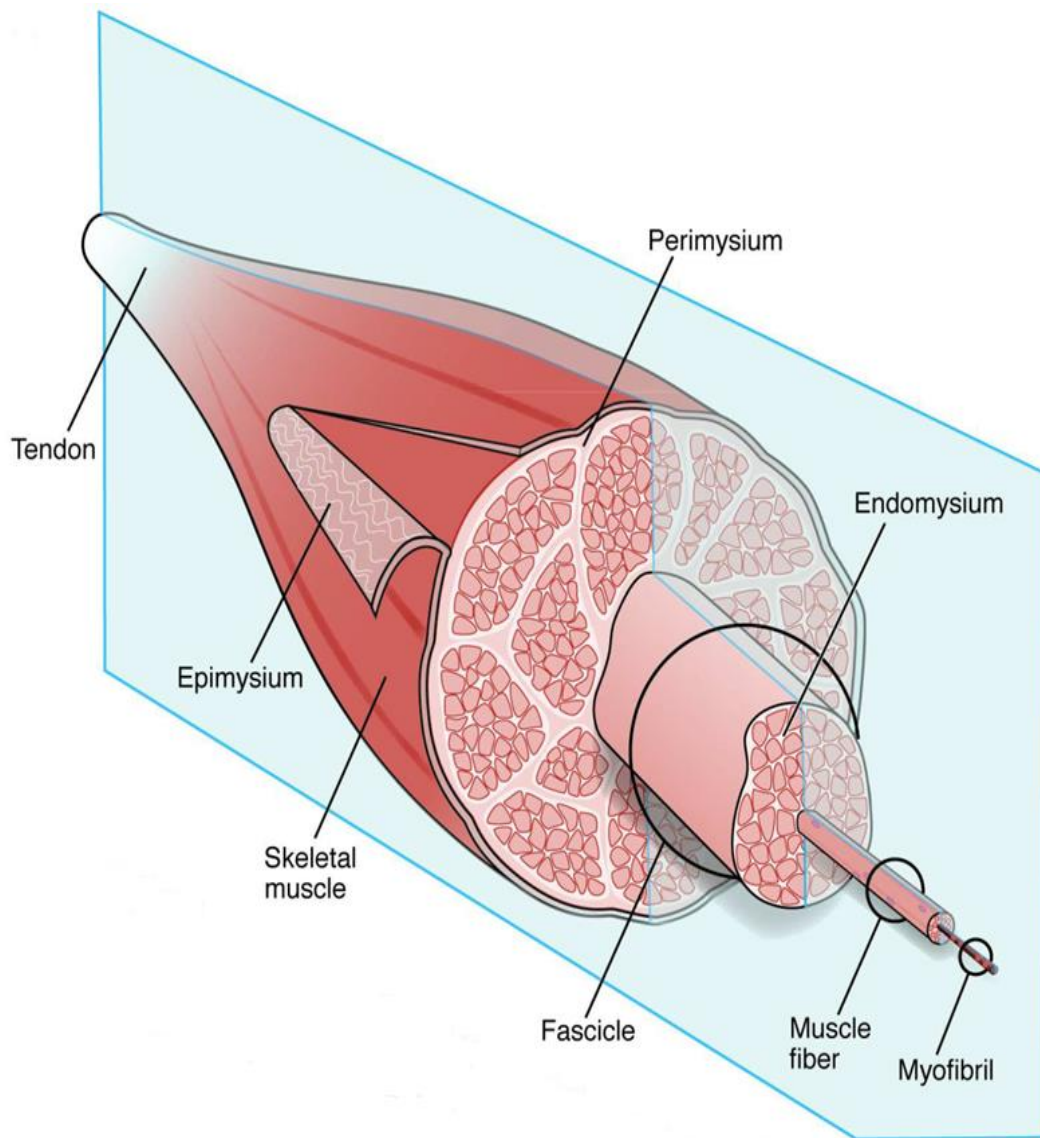
Figure 1. 2: Highly organized structural levels of skeletal muscle separated by connective tissue sheaths. In this figure whole muscle is surrounded by epimysium, bundle of muscle fibres or muscle fascicles are surrounded by perimysium and each individual single muscle fibre is surrounded by endomysium. Different levels of muscle cells are categorized by the help of different connective tissues. Image taken from Gillies and Lieber (2011).

Skeletal muscles are made of bundles of single individual muscle cells known as fascicles surrounded by connective tissue layer known as perimysium. Each individual muscle cell with-in fascicles are separated by another connective tissue layer known as endomysium and the whole muscle is surrounded with a third connective tissue layer known as epimysium (Hoppeler, Howald et al. 1985). The long skeletal muscle fibres are in fact the huge single cells formed after the fusion of many separate cells. The nuclei from the contributing cells are retained in the huge muscle cells and nuclei lie beneath the plasma membrane (Hoppeler, Howald et al. 1985).

Cytoplasm in the fused huge muscle cells is made up of myofibrils which are cylindrical bundles of long repeating contractile protein filaments normally 1-2 μm in diameter and as long as the whole muscle cell. Myofibrils are formed of long repeated chain of contractile units known as sarcomeres; each about 2.2 μm long (Cleworth, Edman 1972). Structure of sarcomere was first published in 1953 (Huxley 1953, Hanson, Huxley 1953). Sarcomere is formed of two types of specifically ordered and partly overlapping myofilaments namely actin (thin filament) and myosin (thick filament protein). Two adjacent sarcomeres; the contractile unit of muscle cell, are separated by Z lines. Along the myofibril alternating light (I bands) and dark bands (A bands) are formed according to the arrangement of thick and thin filaments (Mauro 1961). The thin filaments composed of actin protein are attached to z- disc at each end of a sarcomere and extends in towards the middle of the sarcomere where it overlaps with thick myosin filaments (See **Figure 1.1**). A- Bands are the

dark bands that contain thick filaments with an overlap of thin filaments and I- bands or the light bands contains only thin filaments (Billeter, Heizmann et al. 1981).

1.2.2 Actin and Myosin proteins in skeletal muscle cell

Muscle myosin was first discovered as concentrated solution of "albumins" in 1863 was given the name myosin in 1864 by Kuhne (Kuhne 1863, Warrick, Spudich 1987). In 1942 Albert Szent-Gyorrgyi and his colleagues validated that myosin and another protein which they termed "actin" (because it is activated by myosin) contracts in the presence of ATP (Warrick, Spudich 1987, Lowey, Risby 1971). Muscle myosin-II was the first motor protein identified which generates force during muscle contraction (about 500 kd) (Rayment, Holden et al. 1993). It's an elongated protein formed of two heavy chains approximately 230 kd each and two pairs of light chains approximately 20 kd each (Warrick, Spudich 1987). Each heavy chain is formed of a globular head domain region followed by long amino acid sequence that forms α-helical tail (See **figure 1.3 A**) (Cooper 2000). The tail-tail interaction in myosin proteins forms large bipolar "thick filaments" which have hundreds of myosin heads, leaning in opposite directions at the two ends of the thick filament (Warrick, Spudich 1987).

Actin proteins are globular, monomeric, multi-functional proteins approximately 42 kd which polymerase to form a double helical structure with long grooves throughout its length (Lieber 2002). The regulatory protein tropomyosin binds along the grooves of the actin helix and keeps the active sites of myosin covered during the resting phase (Lieber 2002). Three polymers of troponin protein molecules are also bound with tropomyosin thread named as troponin T, troponin I and troponin C (named for

their tropomyosin binding, inhibitory and Ca^{2+}-binding activities, respectively) (Lieber 2002). The regulatory function of tropomyosin depends on its interaction with troponin and its contraction occurs in response to concentration of calcium ions (Cooper 2000).

A

B
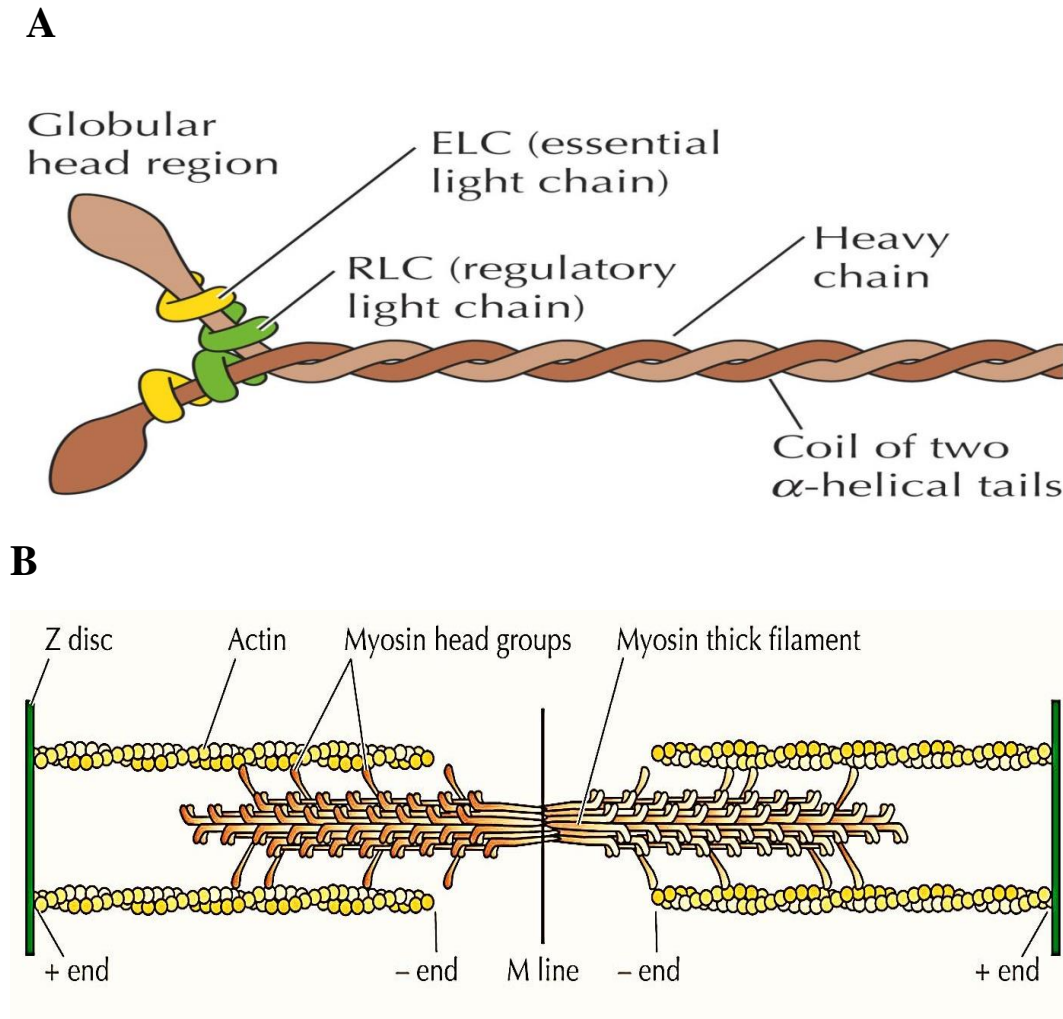
Figure 1. 3: Structure of myosin heavy chain protein. A) Represents the structure of myosin II protein. The myosin II protein formed of two pair of light chains and two heavy chains, where the heavy chains have globular head region and α-helical tail region coiled around each other forming dimers. B) Represents organization of myosin filaments. Several hundreds of myosin II proteins arranged in specific orientation forms thick filaments, where the globular head of myosin bind actin and form cross bridges between myosin and actin filaments. Myosin and actin filament orientation gets reversed at the M line, in order to keep the relative polarity same on both sides of sarcomere. Figure taken from Cooper (2000).

1.2.3 Sliding filament theory

Force is generated in skeletal muscle using actin and myosin filaments, only when the signal is passed from its motor nerve (Holmes, Geeves 2000). According to this hypothesis, when a muscle contracts the actin and myosin filaments slide passed each other and undergo cycles of attachment, pulling, detachment and reattachment (Huxley, Hanson 1943, Huxley, Niedergerke 1954). An action potential is triggered through the transverse tubules (T tubules) that extend from the muscle plasma membrane to myofibrils in the cell which activates the Ca^{2+} channels (Spudich, Huxley et al. 1972). Immediately after the signal arrives all myosin heads act in rapid succession on the same filament without interfering with one another using ATP (Spudich, Huxley et al. 1972). Influx of Ca^{2+} into cell cytosol initiates contraction of each myofibril which is performed in two steps using vast numbers of ATP; filament sliding and Ca^{2+} pumping (Spudich, Huxley et al. 1972).

In resting state of a muscle cells the Troponin I and troponin T complex which binds to the actin filament keeps tropomyosin away from its binding site on actin filament that interferes with the binding of myosin heads (Gomes, Potter et al. 2002). Once the Ca^{2+} concentration is raised reconfirmation of actin-tropomyosin structure by troponin complex takes place, helping myosin head slide along actin filaments (See **figure 1.4**) (Alberts, Johnson et al. 2007).
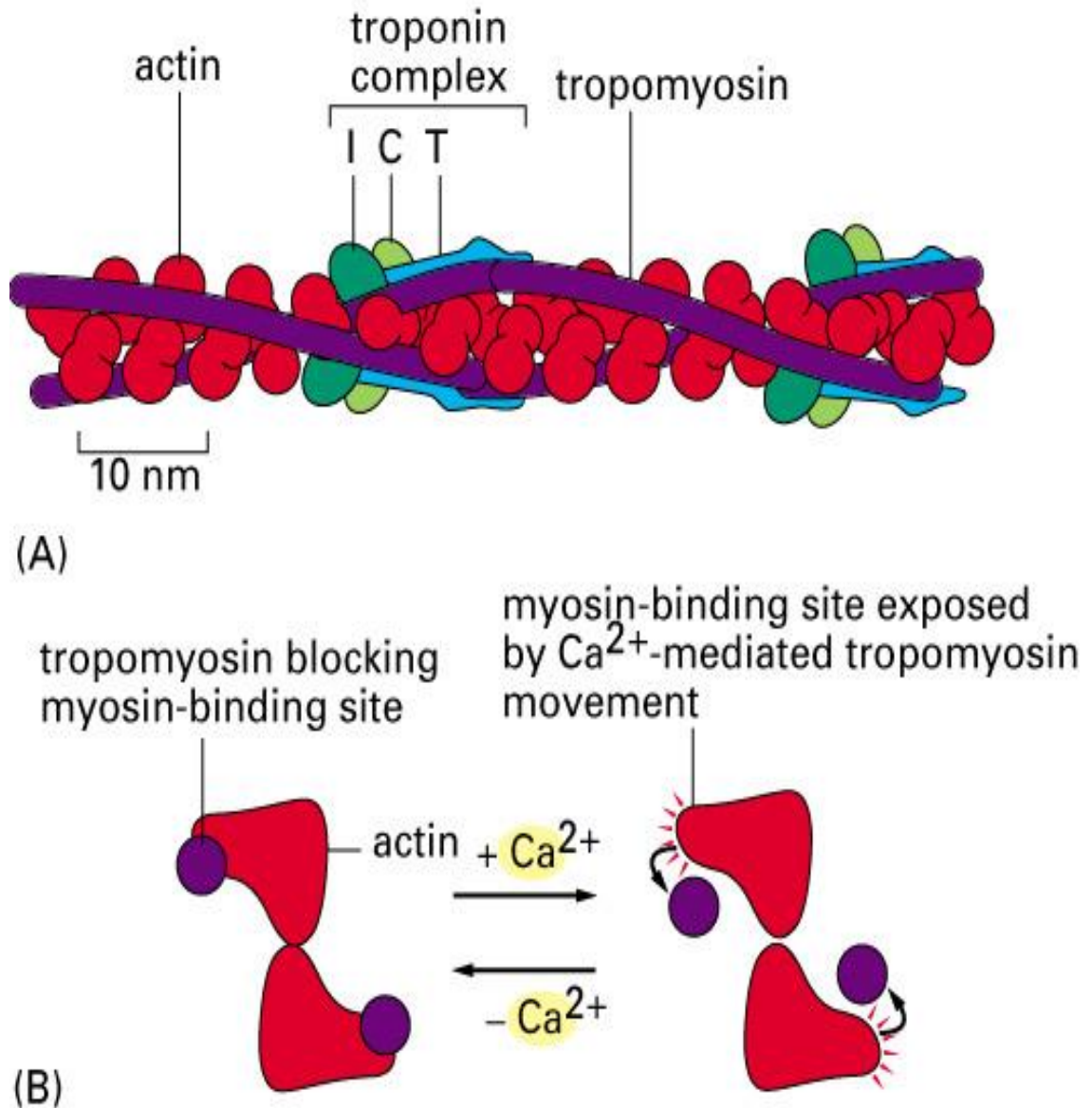
Figure 1. 4: Control of skeletal muscle contraction by troponin. *A) Diagrammatic representation of positions of troponin and tropomyosin along the actin filament. Every tropomyosin molecule normally is surrounded by seven uniformly spaced regions of homologous sequence, all of them are supposed to bind to actin monomers. B) Schematic representation of how tropomyosin blocking the myosin-binding site is released by the influx of Ca2+. Pictures taken from Alberts et al. 2007.*

1.2.4 Muscle fibre phenotype

Skeletal muscles are composed of large numbers of different types of muscle fibre, which contribute to various functional capabilities (Pette, Staron 2000). These fibre types differ from each other on the basis of their molecular, metabolic, structural and contractile properties (Pette, Staron 2001). Myosin has been termed as the most important part of contractile machinery in muscle; different isoforms contribute to the functional diversity of muscle fibres (Pette, Staron 2000). Major functional differences of myosin isoforms are due to the myosin heavy chain portion. For instance ATPase activity is performed by the globular head region of the myosin heavy chain molecule (Weiss, Schiaffino et al. 1999). Different isoforms of myosin present in myofibrils represent the contractile property of the whole muscle fibre (Sciote, Morris 2000) and the methods to determine that are myofibrillar adenosine triphosphatase (mATPase) histochemistry (Brooke, Kaiser 1970, Guth, Samaha 1969), immuno-histochemistry (Staron, Pette 1986), quantitative real time pcr (Pfaffl 2001) and electrophoretic analysis of myosin heavy chain (MHC) isoforms (Pette, Staron 2000). Myosin is a large molecule made of six amino acid chains, two myosin heavy chains (Gazith, Himmelfarb et al. 1970) and four myosin light chains (molecular weight 17-23 kd) (Perrie, Perry 1970, Lowey, Risby 1971). Functionally MHCs are structural protein as well as an enzyme which hydrolyses ATP and determines the nature of excitation-contraction in muscle movement (Barany 1967, Sciote, Morris 2000).

1.2.5 Skeletal muscle extracellular matrix

Extracellular matrix (ECM) is the most significant part of skeletal muscle in terms of development, maintenance and regeneration (Buck, Horwitz 1987, Purslow 2002). ECM plays an essential role in the macrostructure of skeletal muscle arrangement of fibres into bundles, bundles into fascicles and participating in whole muscle structure (Meyer, Lieber 2011). It is also involved in force transmission from the fibres to tendons and *vice versa* (Street 1983, Purslow, Trotter 1994, Huijing 1999, Fomovsky, Thomopoulos et al. 2010). Strength and elasticity of the ECM play a crucial role in its function; it should not break with the load of contraction and be elastic enough to bear the externally applied strains (Purslow 2002). Mainly ECM is formed of two main components carbohydrate and proteins and further these molecules can be sub-divided into four classes: collagenous glycoproteins, non-collagenous glycoproteins, proteoglycans and elastins (Smith, Shah et al. 2010, Lewis, Machell et al. 2001). According to specific skeletal muscle function in a tissue, the ECM content can vary significantly from other muscle types (Kjaer 2004).

ECM not only holds the structure of skeletal muscle but also serves in many other aspects of cellular physiology such as regeneration, neuromuscular transmission and myogenesis. During the complex process of muscle development it is evident from the literature available that cells of ECM are required for myoblast migration, proliferation and differentiation (Melo, Carey et al. 1996, Buchanan, Marsh 2001, Aumailley, Gayraud 1998). Laminin has been found to promote skeletal muscle cells adhesion, proliferation and myotube formation (Foster, Thompson et al. 1987, Kroll, Peters et al. 1994, Kuhl, Timpl et al. 1982), whereas blockade of these components

along with collagen type I and fibronectin inhibits muscle differentiation (Lawson, Purslow 2000, Lawson, Purslow 2001). The expression of major proteins which get upregulated during development, adaptation and regeneration of skeletal muscle are laminin-2 and -4, fibronectin, tenascin, collagens I, III and IV and dermatan (Lewis, Machell et al. 2001). Attachment of muscle fibres to ECM is performed by α7β1 integrin and the Dystrophin-Dystroglycan protein complex which navigates the sarcolemma (Smith, Shah et al. 2010, Lewis, Machell et al. 2001). These structures facilitate collaboration between muscle cells and the ECM, which are necessary in helping them to respond against any changes from peripheral mechanical cues (Burridge, Chrzanowska-Wodnicka 1996). Skeletal muscle grown *in vitro* shows the up regulation of these proteins as well and the right amount of ECM molecules have a notable effect on efficient myogenesis, which is also supported by the fact that myotubes *in vitro* form best on matrigel, ECM "cocktail" (Lewis, Machell et al. 2001, Maley, Davies et al. 1995).

1.2.5.1 Collagen

Collagens fibrous proteins are important components of ECM and are found in all multicellular animals (Shoulders, Raines 2009). Collagen is mainly produced by fibroblasts by the membrane bound ribosomes either placed in ECM or from the rough endoplasmic reticulum (Kjaer 2004). Collagen protein is a major component of skin and bone and accounts for 25% of the total protein mass of the body (Alberts, Johnson et al. 2007) and it accounts 1-10% of muscle dry weight (Bendall 1967, Dransfield 1977). Structurally, the collagen molecule is a long, stiff and triple stranded helix where three collagen polypeptide chains or α-chains are bound

together in a rope like super helix structure (Prockop 1995). In the formation of collagen's triple stranded super helix structure two amino acids: proline and glycine play very important role (Prockop 1995). Since proline has a ring structure it stabilizes the helical conformation in each α-chain whereas glycine is placed as every third amino acid in central region of α-chain (See **Figure 1.5**) and because it is the smallest amino acid it keeps the three helical α-chains packed together tightly in final collagen super-helix structure (Shoulders, Raines 2009). Proline and 4-hydroxyproline help in α-chain stabilisation and interaction with other molecules around them (See **Figure 1.5**) (Alberts, Johnson et al. 2007, Prockop 1995, Shoulders, Raines 2009).

In the human genome there are 42 different genes coding for different collagen α-chains which are expressed in different tissues of the body in different combinations (Hostikka, Eddy et al. 1990). Different combinations of α-chains form different collagen fibrils and they differ from each other on the basis of properties of resulting structures formed (Prockop 1995). Mathematically, 42 α-chains could produce thousands of triple stranded collagen molecules, but so far less than 40 collagen isoforms have been reported. Collagen molecules after being secreted in the extra cellular space, they bind together into high order polymers called collagen fibrils. These are roughly 10-300 nm in diameter and many hundreds of micrometres long in mature tissues held together in cable like bundles (Alberts, Johnson et al. 2007). Collagen polypeptide chains have to undergo an extensive number of co- and posttranslational modifications which results in the quality and stability of collagen molecules (Kjaer 2004). In the cells, translation of preprocollagen mRNA happens in

ribosomes and the procollagen assembles in endoplasmic reticulum (Marchuk, Sciore et al. 1998, McAnulty, Laurent 1987), which is further transferred to extracellular space through golgi apparatus because of its larger structure (Harwood, Grant et al. 1976, Kjaer 2004).

Collagen type I, III, VI, V, VI, XI, XIV, XV, and XVIII are mainly expressed during skeletal muscle development (Gillies, Lieber 2011, Listrat, Lethias et al. 2000) whereas fibrillar collagen type I and III are extensively found in adult endo-, peri-, and epimysium (Kjaer 2004, Bailey, Restall et al. 1979, Light, Champion 1984). Studies have shown that type I collagen forms perimysial collagen and type III collagen are found to be evenly distributed in endomysium and epimysium (Light, Champion 1984). Muscle basement membranes are mainly formed of type IV (network forming collagen) collagen network along with the presence of collagen type VI, XV, and XVIII (Halfter, Dong et al. 1998, Marvulli, Volpin et al. 1996, Myers, Dion et al. 1996, Nishimura, Ojima et al. 1997, Sanes 1982, Gillies, Lieber 2011). Type VII molecules are associated with the formation of dimers which assemble into formation of particular structures called anchoring fibrils which help in the attachment of the basal lamina to the connective tissue (Kjaer 2004). The basement membrane is assumed to be separate from the endomysium but they are intimately connected and are involved in transmission of force from myofibres to tendons (Grounds, Sorokin et al. 2005, Purslow, Trotter 1994).
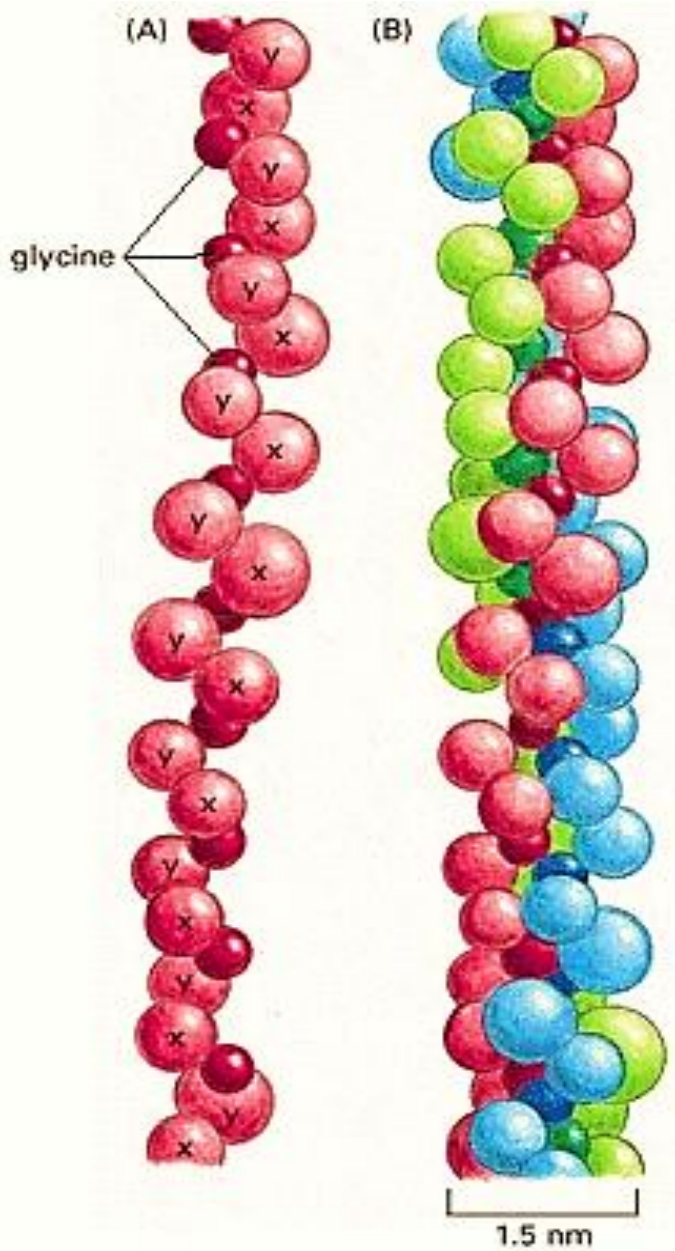
Figure 1. 5: The ultra-structure of a typical collagen molecule. *A) Model of single left handed helix α-chain of collagen molecule where amino acids are represented by spheres. There are three amino acids attached to each other following every turn where glysine is the third amino acid in each triplet and as explained in text above other two are proline and hydroxyproline. B) A small model part of whole collagen molecule where three α-chains (shown in three different colours) are coiled with each other and glycine amino acid occupying the crowded interior of triple helix. Picture taken from Molecular biology of The Cell (fifth edition) by Bruce Alberts (Alberts, Johnson et al. 2007).*

1.2.5.2 Fibrin

Fibrinogen is a glycoprotein from the extracellular matrix with high molecular weight of approximately 340 kd, it forms a fibrin polymer following thrombin cleavage, which further forms clots over wound sites in the body (Grinnell 1984, Midwood, Mao et al. 2006). Fibronectin has various functions in vertebrate organisms ensuring their normal functioning such as it is involved in cell adhesion, growth, migration and differentiation (Pankov, Yamada 2002). Fibronectin is accumulated in extracellular matrix in the form of insoluble network which separates and supports organs and tissues. Fibrin and plasma fibronectin are deposited at injury site to forms blood clot, which stops bleeding and protects underlying tissue (Grinnell 1984). Fibrin is a fibrous, non-globular protein mainly involved in blood coagulation and platelet activation; it binds to integrins - receptor proteins on the membrane and other extracellular matrix components (Pankov, Yamada 2002). Fibrin has a limited role in morphogenetic development such as adhesion factors or growth factor *in vivo* but it has a most active role when immobilized in a 3D matrix *in vitro* (Jiang, Liou et al. 1994).

Fibrin is not connected directly with mature tissue structures such as collagen or fibronectin, but as a temporary repair stage of ECM component. Commercially fibrinogen and thrombin are available, which are mixed for production of fibrin glue or matrix (Currie, Sharpe et al. 2001). Presence of significant amounts of fibronectin enhances cell attachment and migration within the fibrin matrix (Currie, Sharpe et al. 2001). Clinically fibrin as a scaffold matrix has been used for delivery of keratinocytes (Grant, Warwick et al. 2002), mesenchymal stem cells (Gelse, von der

Mark et al. 2003), spinal cord repair (Shimada, Hongo et al. 2006, Iannotti, Zhang et al. 2006) and repair of peripheral nerves (Lee, Hsiao et al. 2002).

1.3 Skeletal muscle embryonic development of zebrafish (*Danio rerio*)

In teleost fish, myogenesis is initiated at very early stages of embryo development compared to amniotes such as birds and mammals. Embryonic muscle development takes place in cellular compartments known as somites, found in pairs along the body axis separated by notochord and neural tube (Johnston, Bower et al. 2011). Zebrafish axial skeletal muscles are formed of four different fibre types; muscle pioneer cells, medial fast fibre cells, slow muscle cells and fast muscle cells (Bone 1979, Devoto, Melançon et al. 1996). All of them differ from each other in morphology, developmental properties and are also found in different regions of axial muscle as shown in **figure 1.6** (Wolff, Roy et al. 2003, Devoto, Melançon et al. 1996). Slow muscle cells are formed of red muscle whereas fast muscle cells formed of white muscle and respectively slow muscle cells are sited just under the skin whilst fast fibres are sited deeper (Ochi, Westerfield 2007). Even before the start of the gastrulation stage of zebrafish embryo development, cells which give rise to skeletal muscle arrange themselves on the marginal zone of embryo, with fast and slow muscle precursor cells occupying different locations (Ochi, Westerfield 2007, Hirsinger, Stellabotte et al. 2004). Although fast and slow muscle precursor cells differ from each other in various manner at shield stage of embryo development, their fate can be changed easily if transplanted into each other's domain which shows that at this stage they are yet not committed for a particular skeletal muscle subtype (Hirsinger, Stellabotte et al. 2004).

Muscle precursor cells based at the centre of the embryo then undergo involution and conjunction extension movements for the next stage gastrulation, where myogenesis begins shown by transcription of myogenic genes myoD and myf5 (See **Figure 1.6**) (Ochi, Westerfield 2007, Weinberg, Allende et al. 1996). As the gastrulation stage ends, muscle precursor cells arrange themselves along the sides of the notochord as shown in **figure 1.6** and continue expressing myogenic genes parallel to the formation of pseudo-epithelial cells or precursors of slow muscle cells, known as adaxial cells (See **Figure 1.6**) (Devoto, Melançon et al. 1996). Migration of these adaxial cells initiates differentiation and morphogenesis of the main fast muscle cells (Henry, Amacher 2004), situated in the posterior lateral somites. These cells trigger the expression of MRF gene expression and differentiate, elongate and fuse to each other to form long muscle fibres up to the length of the myotome (Johnston, Bower et al. 2011).
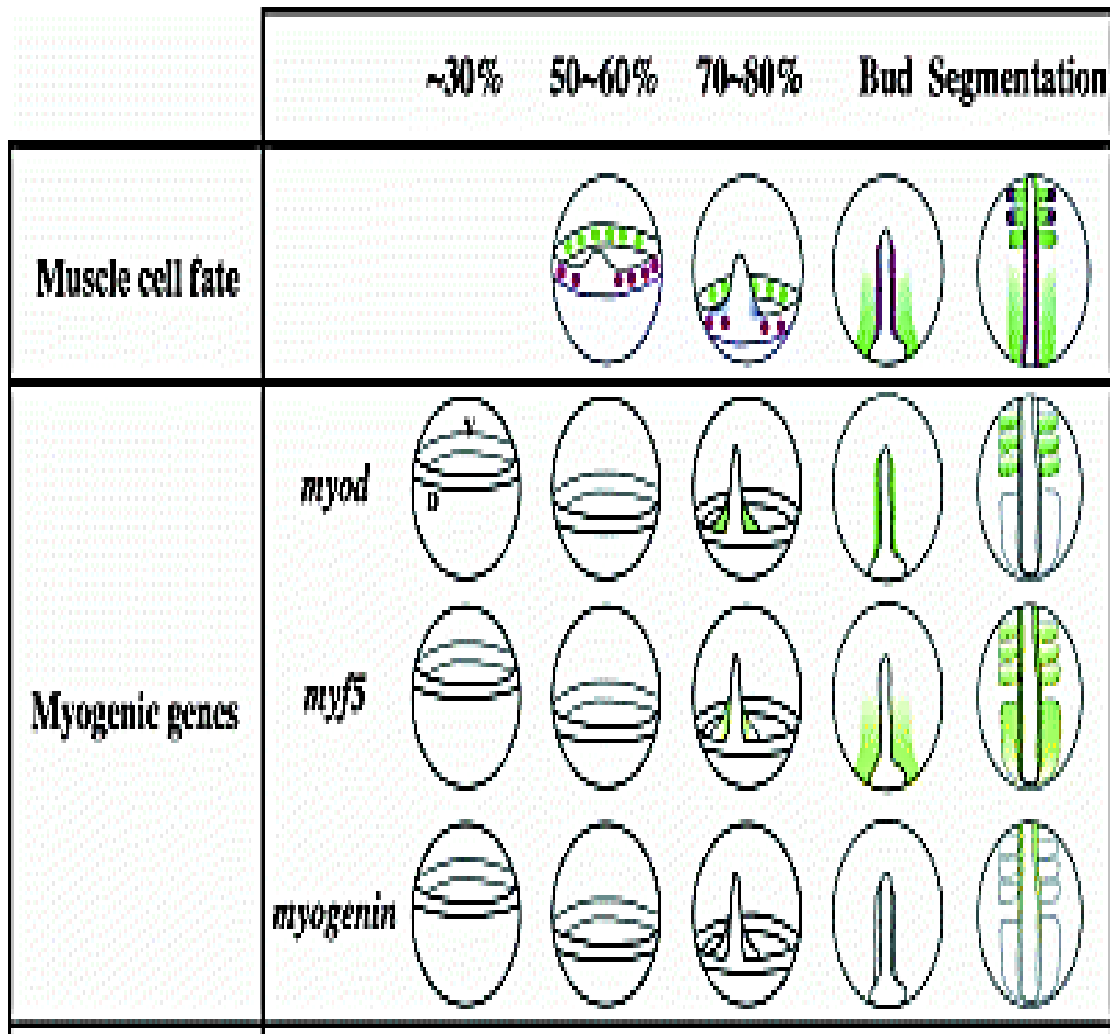
Figure 1. 6: Zebrafish muscle development at early stage embryo. *In the muscle fate section slow and fast muscle precursor cells are presented by different colour dots, arranged at different places and also showing how they arrange themselves around the notochord at the bud stage. Expression pattern of myogenic genes during muscle development; where myoD and myf5 known as early myogenic markers get expressed by 75% epiboly stage. Image taken from Ochi and Westerfield, 2007.*

These slow and fast muscle precursor cells get committed to their specific fates when they are integrated in somite stage (Devoto, Melançon et al. 1996). After integration of adaxial cells into somite stage, they move towards the lateral surface of somite where they differentiate into slow muscle fibres (Hirsinger, Stellabotte et al. 2004). Another set of adaxial cells which stays along the notochord, differentiate into muscle pioneer cells. Non-adaxial muscle precursor cells at the segmental plate differentiate into fast muscle cells followed by the somite formation (Ochi, Westerfield 2007, Hirsinger, Stellabotte et al. 2004). At the end of segmentation stage, lateral cells based between dermis and slow muscle express paired-type homeobox proteins, pax3 and pax7 (Devoto, Melançon et al. 1996). Myogenic regulatory factors (MRF's), myf5 and myogenin are also expressed; myosins which elude to the fact that external cell layer have myogenic precursors which then differentiate somewhere else into muscle (Devoto, Melançon et al. 1996).

1.3.1 Zebrafish muscle development genetic regulation

Gene expression analysis of zebrafish using *in situ* hybridization technique has provided insights into the timing and lineage specificity of MRF's. Genes such as myoD, myf5, mrf4 and myogenin are the myogenic regulatory factors of the basic helix loop helix family. Expression of myoD and myf5 has been studied in mouse proliferative myoblasts, which indicates the involvement of these MRF's in establishment and maintenance of muscle progenitor cells (Ochi, Westerfield 2007, Pownall, Gustafsson et al. 2002). Expression of myogenin and mrf4 is activated when myoblasts start differentiating and forming mutli-nucleated myotubes (Pownall, Gustafsson et al. 2002). MyoD expression in zebrafish is first noticed as small

triangular patch structure around embryonic shield (see **figure 1.6**) at 75% epiboly stage (Weinberg, Allende et al. 1996). Myf5 transcripts are expressed as circular stripe shape around the notochord (see **figure 1.6**) at about 80% epiboly stage (Coutelle, Blagden et al. 2001), whereas expression of myogenin appears at later during the segmentation stage as shown in **figure 1.6** (Ochi, Westerfield 2007).

Fibroblast growth factor (Fgf) is a signalling molecule and it also plays an important role in different aspects of muscle development through Fgf receptors and MAP kinase signalling pathway (Gotoh, Nishida 1995, Ochi, Westerfield 2007). Increase in myoblast proliferation and repression in myogenic differentiation has been studied due to the presence of Fgf (Winter, Braun et al. 1993, Yoshida, Fujisawa-Sehara et al. 1996, Edom-Vovard, Bonnin et al. 2001). On the contrary studies in chick have shown that Fgf signalling enhances muscle progenitor differentiation, instead of enhancing their proliferative capacity (Marics, Padilla et al. 2002, Ochi, Westerfield 2007).

Quiescent satellite muscle cells are found on the periphery of the whole skeletal muscle fibre as shown in **figure 1.7** in red colour. During enzymatic digestion or de-differentiation of the whole skeletal muscle fibre, the satellite cells based on the periphery become activated, resulting in their proliferation and fusion with similar kind of cells as shown in green colour in **figure 1.7**. Fusion of single satellite cells results in formation of long multi nucleated muscle fibre as shown in **figure 1.7** in red colour. From the whole lot of quiescent satellite cells activated in response to mitogens or injury, some cells keep self-renewing themselves as shown by yellow colour in **figure 1.7**. Similar kind of process will be replicated in the culture system

later where zebrafish skeletal muscle cells will be digested followed by culturing quiescent muscle cells. Satellite cells will be provided with favourable culture conditions *in-vitro* for proliferation and end to end fusion (differentiation) in order to form long multinucleated muscle fibres as shown in **figure 1.7**.
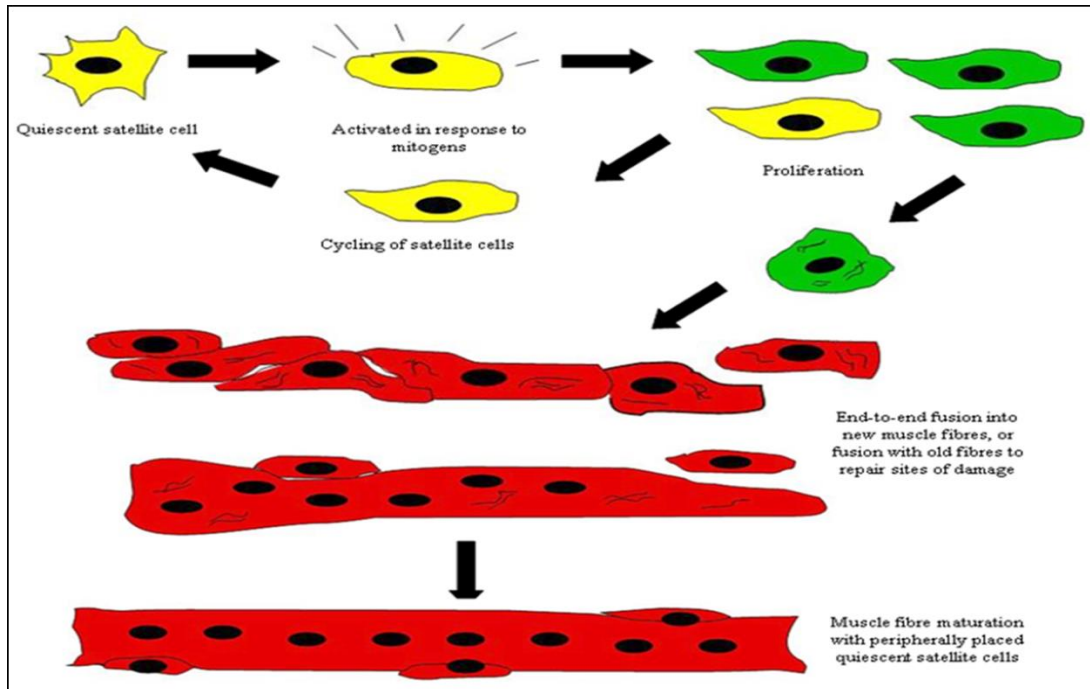
Figure 1. 7: Life cycle of satellite cells at different stages presented in different colours, once they get activated in response to mitogens. Yellow colour cells are quiescent satellite cells or myoblasts or de differentiated muscle cells, green colour cells represents proliferated satellite cells and red colour cells represents fusion or differentiated multinucleated whole muscle fibre with quiescent satellite cells on their periphery (Image taken from Lewis 2000).

In zebrafish, expression of myoD, myf-5 and Pax7a is seen in the multipotent or de-differentiated cells following enzymatic digestion, which become myoblasts (**Figure 1.8**) and commit to myogenic fate to form myogenic progenitor cells (Johnston 2006). Myogenin and mrf4 initiate the process of differentiation (**Figure 1.8**) (Rescan 2001, Chen, Goldhamer 2003, Pownall, Gustafsson et al. 2002), where myoblasts

attach to each other and form long multi nucleated muscle fibres. In a previous study, it was shown that structural muscle genes such as slow and fast myosin heavy chains (sMyHC & fMyHC) start being expressed during differentiation of satellite cells (Zammit, Partridge et al. 2006, Gabillard, Sabin et al. 2010). In other research, myosin heavy chains have been immuno-localised in embryonic zebrafish muscle cells while cultures are in progress (Bower, Johnston 2009, Greenlee, Dodson et al. 1995).
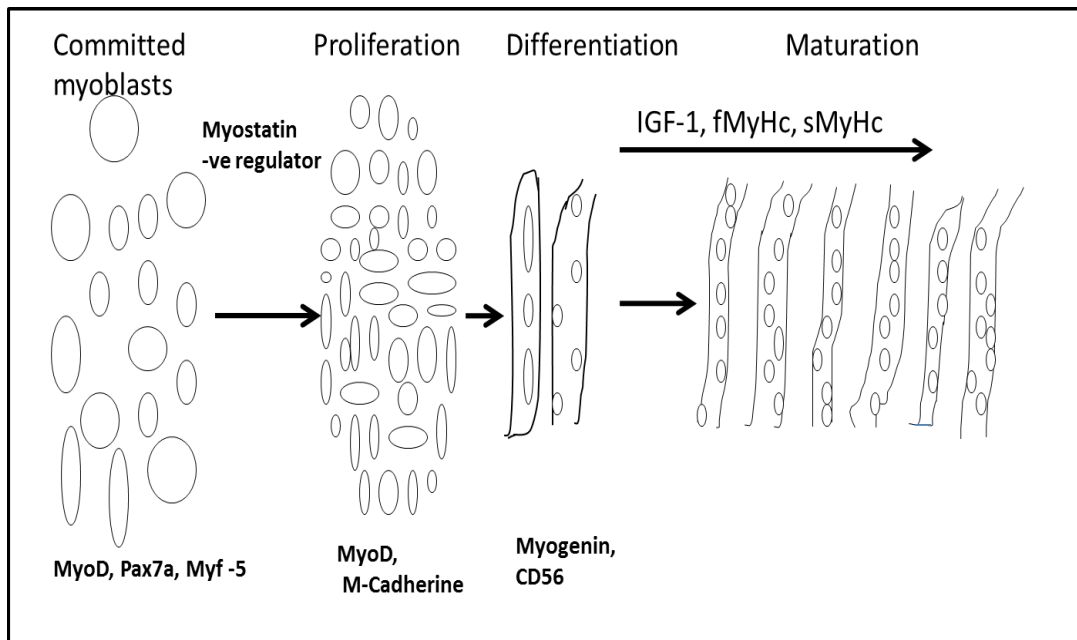
Figure1.8: Developmental stages of skeletal muscle cells with respect to genes transcribed during those phases. Figure 1.8 explains the developmental stages of skeletal muscle cells in zebrafish with respect to the genes transcribed during those stages. Such as stage one when the cells are de-differentiated or committed myoblasts or single cells, expression of MyoD, Pax7a and myf-5 is higher. Similarly during proliferation stage, when cells are dividing expression of MyoD and M-cadherine is higher, whereas myostatin during this stage acts as negative regulator. Following proliferation, differentiation takes place where cells fuse with each and form long multinucleated structures called myotubes, during which phase myogenin and CD56 expression is higher. When the myotubes are formed they further grow and mature during maturation phase and expression of IGF-1, fMyHc, sMyHc are higher during that time point. All processes explained in this figure are relative to previous figure 1.7.

1.4 Skeletal muscle post-embryonic growth

1.4.1 Satellite cells

Post-natal skeletal muscle also known as post mitotic tissue which does not have intrinsic regeneration capacity, therefore acquisition of extra nuclei, required in regeneration following damage, must come from satellite cells. Satellite cells were first described by Mauro (1961) based on their location between the sarcolemma and basal lamina of muscle fibres (Mauro 1961) and comprises approximately 2-7% of the total nuclear content of skeletal muscle (Kadi, Charifi et al. 2005). Satellite cell numbers differ according to species, age, sex and muscle fibre. In mice it is established, approximately 30% muscle nuclei in neonates which decreases 4% with age in their adult age and further reduced to 2% in senile mice (Snow 1977). Satellite cells are mononuclear progenitor, precursor to skeletal muscle cells with almost no cytoplasm found in them and have the capability to proliferate and differentiate into skeletal muscle cells (Kadi, Schjerling et al. 2004, Siegel, Kuhlmann et al. 2011). They are capable of generating new muscle fibres by fusing with each other, by joining an already existing muscle fibre suffering from trauma, or return to their quiescent state (Kadi, Schjerling et al. 2004). Formation of skeletal muscle fibres is initiated by the movement of myoblasts from the somites into muscles where they fuse and form myotubes, which mature into skeletal muscle fibres by addition of more myonuclei (Campion 1984). Growth of muscle fibres in size during the juvenile stage depends on the fusion of satellite cells with the existing myofibres (Abmayr, Pavlath 2012). When myofibres are mature, the satellite cells stabilize and return to

their quiescent state in respect to the cell cycle and their activity; (Hawke, Garry 2001, Siegel, Gurevich et al. 2013).

1.4.2 Identification of satellite cells

Satellite cells in resting state of a skeletal muscle are believed to be in quiescent or non-proliferative state. They can be identified using an electron microscope due to their unique qualities; 1) located between basal lamina and plasma membrane, 2) high nuclear to cytoplasmic volume ratio with virtually no organelles, 3) small nuclear size, 4) high quantity of nuclear heterochromatin as compare to myonuclei. On the contrary qualities of activated satellite cells are: 1) increased caveolae, 2) more organelles in cytoplasm, 3) reduction in heterochromatin (Kadi, Charifi et al. 2005, Campion 1984).

Genetically satellite cells are identified by expression of number of different proteins, but currently the literature suggests that all satellite cells express paired box transcription factors pax7 and pax3 proteins (Buckingham, Bajard et al. 2003, Seale, Sabourin et al. 2000, Bosnakovski, Xu et al. 2008), along with basic helix-loop-helix myogenic transcription factor 5 or myf5 in some studies (Cornelison, Wold 1997). Human quiescent or activated satellite cells can be identified by membrane bound neural adhesion molecule (N-CAM), a cell surface glycoprotein (Schubert, Zimmermann et al. 1989, Sinanan, Hunt et al. 2004). Activated satellite cells are harder to identify, due to the fact that their markers keep on changing according to their degree of activation, for example activated satellite cells progressively loose expression of pax7 as they enter proliferative stage, whereas later during differentiation it is highly expressed (Kjaer, Qvortup et al. 2004). After activation,

once they pass proliferation phase, high expression levels of myogenic regulatory factors MyoD, myogenin and mrf4 can be observed which are responsible for induction of myocyte- specific gene expression. Desmin, a muscle specific filamentous protein is also expressed in activated skeletal muscle cells when they proliferate and differentiate, which has also been used as a marker to identify skeletal muscle cells (Kadi, Schjerling et al. 2004).

Origin of satellite cells in zebrafish is complicated due to the difference in their embryonic development (Siegel, Gurevich et al. 2013). Zebrafish do not have a dermomyotome, therefore a different mechanism is followed for initial formation of muscle tissue or progenitor cells (Hollway, Bryson-Richardson et al. 2007). At the end of primary myogenesis, zebrafish somites also have sclerotome, myotome and dermomyotome-like external cell layer, where formation of myogenic progenitors and mature muscle take place responsible for growth and muscle regeneration as in amniotes (Stickney, Barresi et al. 2000, Morin-Kensicki, Eisen 1997). Another feature of zebrafish myogenesis is that the myogenic commitment takes place relatively early compared to amniotes (Siegel, Gurevich et al. 2013). In zebrafish myf5 and myoD perform together to drive slow myogenesis and initial fast myogenesis in medial somites, myoD is also required in pectoral fin and cranial muscle development (Hinits, Osborn et al. 2009). In zebrafish myotome is separated by lateral slow muscle and medial fast muscle population, which is again very different arrangement to amniotes where different muscle fibre types are arranged in same muscle. These differences in myogenesis between zebrafish and amniote may

be due to formation of new muscle fibres in different areas of myotome (Siegel, Gurevich et al. 2013).

resting myofiber
quiescent satellite cell

myotrauma *

myonuclei

satellite cell activation and proliferation

self-renewal

regenerated myofiber with central nuclei

chemotaxis to injured fiber

fusion to damaged myofiber (hypertrophy)

fusion to produce new myofibers (hyperplasia)
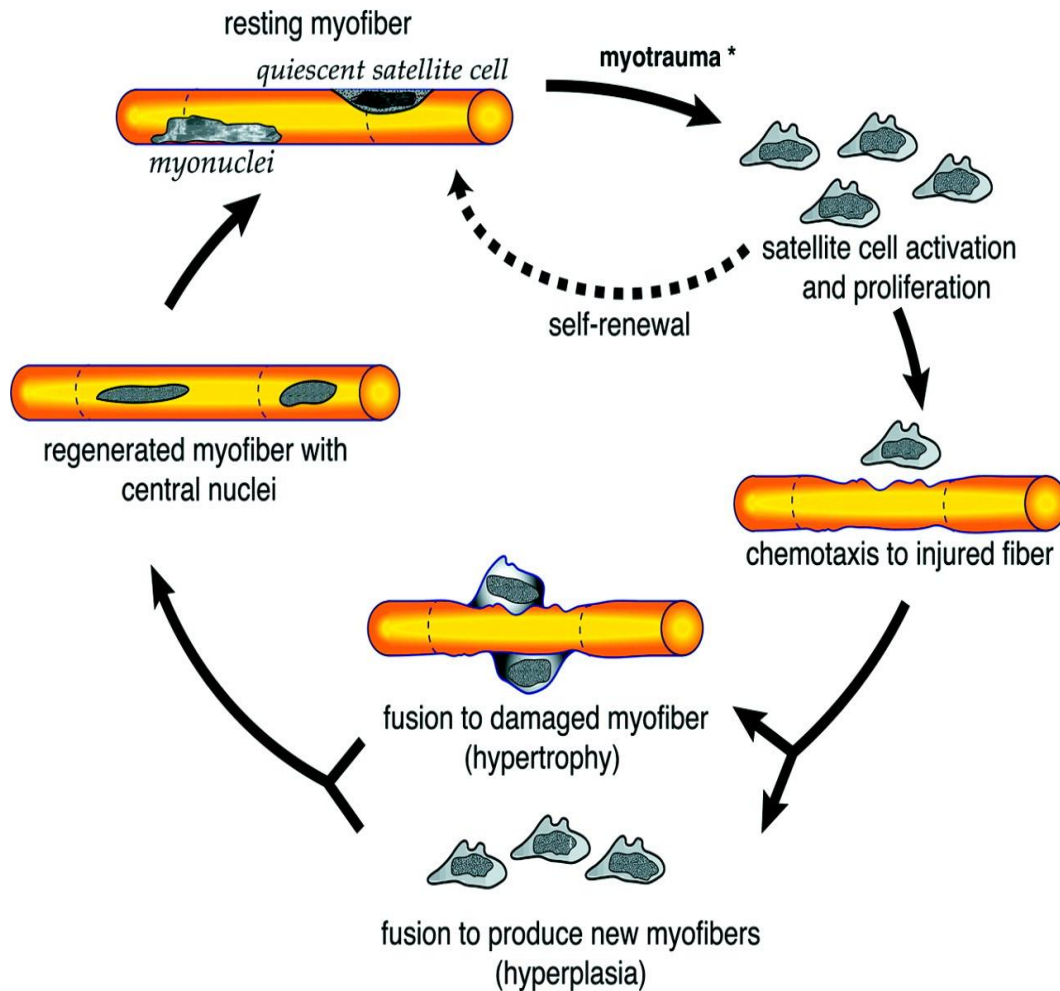
Figure 1.9: Role of satellite cells in response to myotrauma in muscle regeneration. *Satellite cells become activated and proliferate in response to any injury or exercise. A sub set of satellite cells go back in quiescent state and another set of satellite cells then migrate to damage area and fuse with existing myofibres or fuse with each other to form new myofibres. Taken from Hawke and Garry 2001 (Hawke, Garry 2001).*

1.4.3 Satellite cells in growth and repair

Physiological changes in either human or animal models such as load induced hypertrophy and resistance training promotes hypertrophic response (Williams, Neufer 1996, Rosenblatt, Yong et al. 1994). Resistance training or trauma triggers muscle hypertrophy, which is initiated with satellite cell activation, proliferation and then fusion into existing myofibres which helps in muscle growth and muscle repair (see **figure 1.9**)(Schultz, McCormick 1994). Satellite cell migration at the site of rupture of basal lamina in response to myotrauma takes place utilizing tissue bridges between adjacent myofibres (Watt, Morgan et al. 1987). When there is no rupture on basal lamina, satellite cells migrate from the proximal intact part of myofibre at the site of injury for the repair process (see **figure 1.9**) (Schultz, McCormick 1994, Hawke, Garry 2001)

In adult skeletal muscle fibres, production of mRNA and protein synthesis is controlled in specific ratio by myonuclei for a limited amount of cytoplasm (Kadi, Charifi et al. 2005). Growth of skeletal muscle can take place only up to a certain limit in presence of same number of satellite cells or skeletal muscle. After a particular limit, more satellite cells are needed for further growth and this process is known as myonuclear domain or ceiling size (Kadi, Thornell 2000, Petrella, Kim et al. 2008). Each myonucleus have its ratio to control amount of sarcoplasm and when it has reached its ceiling size, new myonuclei are required from satellite cells for further growth (Kadi, Thornell 2000, Petrella, Kim et al. 2008). This concept has been proven by the experimental fact that mRNA encoded proteins are found concentrated near myonucleous region (Pavlath, Rich et al. 1989), therefore the

expression of specific differentiation genes increase in the differentiated skeletal muscle cells.

1.5   Conventional cell culture techniques

1.5.1 Skeletal muscle cell culturing *in vitro*

In vitro culturing of primary skeletal muscle cells for both human and animals has been in practice for 30-40 years (Bischoff 1974, Yasin, Van Beers et al. 1977). Satellite cells from skeletal muscle tissue can be isolated using two techniques; either explant culturing or enzymatic digestion. In explant cultures, muscle tissue is minced in aseptic conditions using a pair of scissors or a pair of scalpels and placed in a culture flask or dish containing growth media for a week. Over time the progenitor cells migrate out of minced tissue onto the surface of the dish substrate and form mononuclear cell colonies. This explant culturing is also known as culturing a small piece of tissue itself, surrounded by its extracellular matrix, in order to mimic the *in vivo* environment (Renault, Piron-Hamelin et al. 2000, Brady, Lewis et al. 2008).

In enzymatic digestion, mononucleated cells are dissociated from whole muscle. The whole process involves releasing satellite cells from its niche by enzymatically breaking down the basal lamina (Sinanan, Hunt et al. 2004). Commonly used enzymes for isolation of satellite cells are collagenase, protease and trypsin. These enzymes need to be used at optimal concentrations for optimal times and optimal temperatures to liberate maximum numbers of satellite cells otherwise they can over digest the whole tissue along with satellite cells (Shefer, Van de Mark, Daniel P et al. 2006, Danoviz, Yablonka-Reuveni 2012). The cell population derived will be the satellite cells, responsible for growth and repair of extant multinuclear muscle fibres

in vivo (as discussed in **section 1.4.2**) (Yasin, Van Beers et al. 1977). The cells obtained will not only be pure population of myogenic cells; it will be the mixture of myogenic and different non myogenic cells types from the niche such as fibroblasts (Machida, Spangenburg et al. 2004) and pericytes (Asakura, Rudnicki et al. 2001, Valero, Huntsman et al. 2012). *In vitro* cultured myoblasts should be able to retain their hypertrophic ability and when correct stimuli is provided; they should be able to fuse and form myotubes and retain their contractile ability (Yasin, Van Beers et al. 1977).

For the *in vitro* study of skeletal muscle, formation of these multinuclear bodies is vital. Investigation of skeletal muscle metabolic and functional activity can then be easily performed using these cultures without any interference from other tissues as takes place *in vivo*. External stimuli and interaction with other cell types have a heavy influence on skeletal muscle cells but using these *in vitro* cultures such external interferences can be removed and easy investigation of specific effects of chemicals, metabolism of skeletal muscle and physical and pathological challenges on the physiology can be performed (Cosgrove, Sacco et al. 2009, Forcales, Puri 2005, Smith, Shah et al. 2010). Therefore *in vitro* culturing experiments are designed to be used for mechanistic responses. For example drugs can be added to culture medium in a controlled manner (Breen, Sanli et al. 2008), nutritional supplements can be tested (Deldicque, Theisen et al. 2007), genes can be knocked out or particular pathways can be inhibited (Rommel, Bodine et al. 2001) and other cell types can be added to investigate their interaction (Nurse, O'Lague 1975).

As mentioned earlier, cells obtained after enzymatic digestion are not only myogenic satellite cells, instead they are a mixture of myogenic and non-myogenic/fibroblast type cells (Machida, Spangenburg et al. 2004). Therefore in order to obtain pure myogenic fraction of cells from these isolations, various different techniques have been reported in the literature such as flow cytometry (Baroffio, Aubry et al. 1993), cell sorting, magnetic cell sorting (Brady, Lewis et al. 2008), differential cell plating (Rouger, Fornasari et al. 2007) and fractionation using percol/ficol density gradient (Morgan 1988, Kastner, Elias et al. 2000, Danoviz, Yablonka-Reuveni 2012). These techniques are of high importance to obtain pure myogenic cell population and also due to the fact that during *in vitro* culture myogenic cells are overtaken by non-myogenic/ fibroblast type cells over time (Machida, Spangenburg et al. 2004). Fibroblasts have their own importance for the maintenance of skeletal muscle ECM and for modulating tissue response to external cues *in vivo* (See **section 1.2.5**). Therefore there is always a danger that purification of myogenic cells will obstruct cells ability to responds to external cues. In conventional monolayer cell culturing, cells are already removed from their physical *in vivo* environment, therefore it becomes of increasing significance to move towards more biomimetic models for tissue culturing (Brady, Lewis et al. 2008).

Isolation and culture of myogenic progenitor cells are also affected by the age and sex of organism. Maria Elena Danoviz and Zipora Yablonka-Reuveni, Day and Shefer and Shefer, Van de Mark and Daniel P have shown in their study that younger mice (3-6 months old) muscle consisted of more satellite cells compare to older mice, due to the fact that numbers of satellite cells are age associated (Shefer, Van de Mark,

Daniel P et al. 2006, Day, Shefer et al. 2010, Danoviz, Yablonka-Reuveni 2012). Also with old mice they have shown the increment in non-myogenic cells which leads to modification of conditions of isolation such as duration of enzymatic digestion, extent of tissue trituration, cell straining conditions to remove debris and centrifugation speed in order to minimize the number of undesired cell types (Danoviz, Yablonka-Reuveni 2012).

1.5.2 Limitation of conventional cell culturing

Skeletal muscle cells cultured *in vitro* help us in understanding muscle development, growth and plasticity, but still *in vitro* cultured skeletal muscle cells are far from *in vivo* skeletal muscle (Engler, Griffin et al. 2004, Isobe, Shimada 1983). As explained earlier skeletal muscle cells are contractile in nature, therefore when cultured on tissue culture plastic/ glass coverslips detachment of seeded cells have been observed and myotubes mature poorly (Engler, Griffin et al. 2004). In the literature it has been reported that myotubes differentiate and mature to their optimum level when cultured in/on substrates with tissue like stiffness (Boontheekul, Hill et al. 2007, Engler, Griffin et al. 2004). Most importantly cells are removed from their biological niche and placed in an environment completely different from *in vivo* which isolates them from their extrinsic stimuli that help them in differentiation and maturation (Vandenburgh, Karlisch et al. 1988).

Whole skeletal muscles are made of unidirectional bundles of muscle fibres oriented in parallel alignment (Khodabukus, Paxton et al. 2007). Due to lack of directional tension signals, which aligns muscle fibres between the points of tension *in vivo* such as in-between tendons, they fail to recapitulate unidirectional muscle growth in

conventional two dimensional *in vitro* cultures (Eastwood, Mudera et al. 1998). Therefore, the myotubes that develop in 2D cultures are randomly arranged and have branched fibres, which is significantly different from *in vivo* tissue (Fear 1977) .

Skeletal muscles *in vivo* are incorporated with connective tissues and pericytes which help in skeletal muscle structure and function, also improved proliferation and maturation. On the contrary, when cultured *in vitro* on plastic or glass coverslips they lack the support of connective tissue which impacts on their proliferation and maturation as well (Hauschka, Konigsberg 1966, Ocalan, Goodman et al. 1988). Vandenburgh et al. (1988) observed that myotubes from 2D cultures or even from collagen coated plastic plates detach after some time due to the contractile nature of muscle (Vandenburgh, Karlisch et al. 1988). Therefore, in order to provide skeletal muscle cells more biological relevant *in vivo* environment when cultured *in vitro*, it seems logical to look forward towards more biomimetic 3D culture models. This will also help us understanding the inside mechanism of these skeletal muscle cells and obtain similar results to *in vivo* from *in vitro* experiments.

3D engineered skeletal muscle tissue models, which can produce force due to contractile property of whole muscle tissue, have been presented by different groups in recent years. Due to the lack of proper vascularisation of engineered constructs, these still cannot be compared with the native muscle tissue (Levenberg, Rouwkema et al. 2005). ECM plays an important role in formation of 3D tissue engineered constructs for attachment, alignment and differentiation of muscle progenitor cells (Levenberg, Rouwkema et al. 2005). Different studies were performed using different kinds of matrigels such as PLGA fibres, electro spun PLLA fibres, PLGA

microspheres and different sources of primary skeletal muscle cells or cells lines such as C_2C_{12}, rat muscle derived cells and primary human muscle derived cells (details explained in **Table 5.1**) (Aviss, Gough et al. 2010, Huang, Patel et al. 2006, McKeon-Fischer, Freeman 2011, Thorrez, Shansky et al. 2008). Commonly used 3D models are based on either collagen matrix or fibrin matrix. Use of collagen as a matrix to culture skeletal muscle cells in order to provide more bio- physiological environment was discovered by Vandenburgh and his colleagues (Vandenburgh, Tatto et al. 1996, Vandenburgh, Shansky et al. 2008, Vandenburgh, Karlisch et al. 1988, Vandenburgh 1987) and so far rat, mouse and human muscle cells have been cultured in collagen constructs. Tissue engineered construct based on fibrin as matrix has been discovered and manipulated by several groups as described in detail in chapter 5, later (Dennis, Kosnik II 2000, Dennis, Kosnik et al. 2001, Strohman, Bayne et al. 1990). Khodabukus and his colleagues described this model to be a more accurate and biologically relevant model for *in vitro* study of skeletal muscle (Khodabukus, Paxton et al. 2007, Khodabukus, Baar 2009, Khodabukus, Baar 2011) from rat, mouse and human skeletal muscle cells. In the present study, we have used both fibrin (chapter 5) and collagen (chapter 6) matrix to culture zebrafish skeletal muscle cells.

When current investigation was initiated there was no published literature available showing evidence of zebrafish skeletal muscle cells cultured *in vitro* even in monolayer. Therefore, a protocol needed to be optimised for culturing zebrafish skeletal muscle cells, initially in monolayer, and then moving towards culturing them in collagen and fibrin based tissue engineered constructs. During the current study,

when the protocol was developed, another group published similar protocol for isolation of zebrafish skeletal muscle cells (Alexander, Kawahara et al. 2011). Nevertheless work presented in present study surpasses their work in publication in terms of improved protocol, reproducible results, quantitative analysis of immune stained images, cost effective and time effective, which are evidenced in later chapter 3, 4 and 7.

Principles of 3R's (Replacement, Reduction and Refinement) were introduced almost five decades ago in order to framework the humane animal research. The basic idea or definition behind was to develop methods which avoid or replace the use of animals in research under Replacement. Secondly to develop methods which can reduce the number of animals used per experiment in research under Reduction and to develop methods in order to minimise suffering and increase animal welfare under Refinement. Also according to the principals of 3R's it was allowed to use young fish larvae until they can feed independently, therefore the use of fish has been increased research community in relation to other animal models. These 3D constructs will help us understanding the effects of external environment specifically on skeletal muscle cells in detail at the molecular levels. These will also help us to reducing the use of human tissues in research, as it has already been described that zebrafish has very close homology with human genome and similarity with human disease associated genes (as explained in **Section 1.1.1**). Passaging of zebrafish skeletal muscle cells *in vitro* will also make it easier to reduce the number of organisms in research, as the number of test beds prepared from the same set of organisms can be increased and used for different purposes in research. All the important aspects discussed above for

culturing zebrafish skeletal muscle cells initially in monolayer followed by culturing them in 3D models will nicely lead us to fulfil the basic principles of 3R's i.e. replacement, reduction and refinement of human and other organisms in research. As of here we are developing model or alternative approach which will avoid the use of human tissue in research and also number of animals used per experiment will also be reduced by preparing several collagen and fibrin test beds from one set of organisms which will avoid further animal use.

1.6 Aims of Thesis

The aim of this research was to develop zebrafish 3D tissue engineered skeletal muscle models.

In order to achieved by completing the following objectives:

1) Development and optimisation of a protocol for isolating zebrafish skeletal muscle cell.

2) Optimisation of culture conditions, identification and characterisation of zebrafish skeletal muscle cells in 2D culture, in terms of structure and molecular maturation.

3) Culture of zebrafish muscle cells in 3D collagen and fibrin based tissue engineered constructs and characterisation of these cells using the same characterisation parameters as in 2D.

4) Characterisation and comparison of zebrafish muscle cells cultured in 3D tissue engineered constructs and 2D, and comparison of the cells cultured in 3D with *in vivo* zebrafish skeletal muscle tissue.

2 Materials and Methods

2.1 Maintenance of zebrafish (*Danio rerio*)

Adult zebrafish of 12-14 weeks old were purchased from Aquascape Ltd. (Birmingham, UK) and were kept in 40 litre glass tanks (30 x 30 x 60 cm) at 28 ± 1° C and 12 hour light/ dark cycle. A rod shaped small heater was kept in every tank to maintain temperature. Male and female fish were housed in tanks which were fed three times a day with TetraMin® (Tetra, Germany) flake food (ingredients: processed fish and fish derivatives, cereals, yeast, vegetable protein extracts, molluscs and crustaceans, oils and fats, derivatives of vegetable origin, algae, various sugars contains permitted colorants) and once daily with freshly hatched brine shrimp (Artemia saline) (ZM systems, UK). Live brine shrimp were produced by 24 hour culture of brine shrimp cysts in salty aerated water made up with 52.5 gm sea salt (ZM systems, UK) in 1.5 L distilled water, maintained at 28 ± 1° C. Normal tap water was kept in a bucket at room temperature for two days to allow the chlorine to settle down at the bottom before using it to change fish tank water. Fish tank water was replaced every two weeks.

An automatic water circulation system (see **Figure 2.1**) was also used to maintain zebrafish. Distilled water added in the base tank which was filtered through carbon filters and UV sterilised, and then allowed to enter in the fish tanks. The water in tanks keeps circulating with the help of a small water pump in the base tank. Each 10 litre tank (35 x 17 x 26 cm) in the system was capable of holding approximately 30 fish.
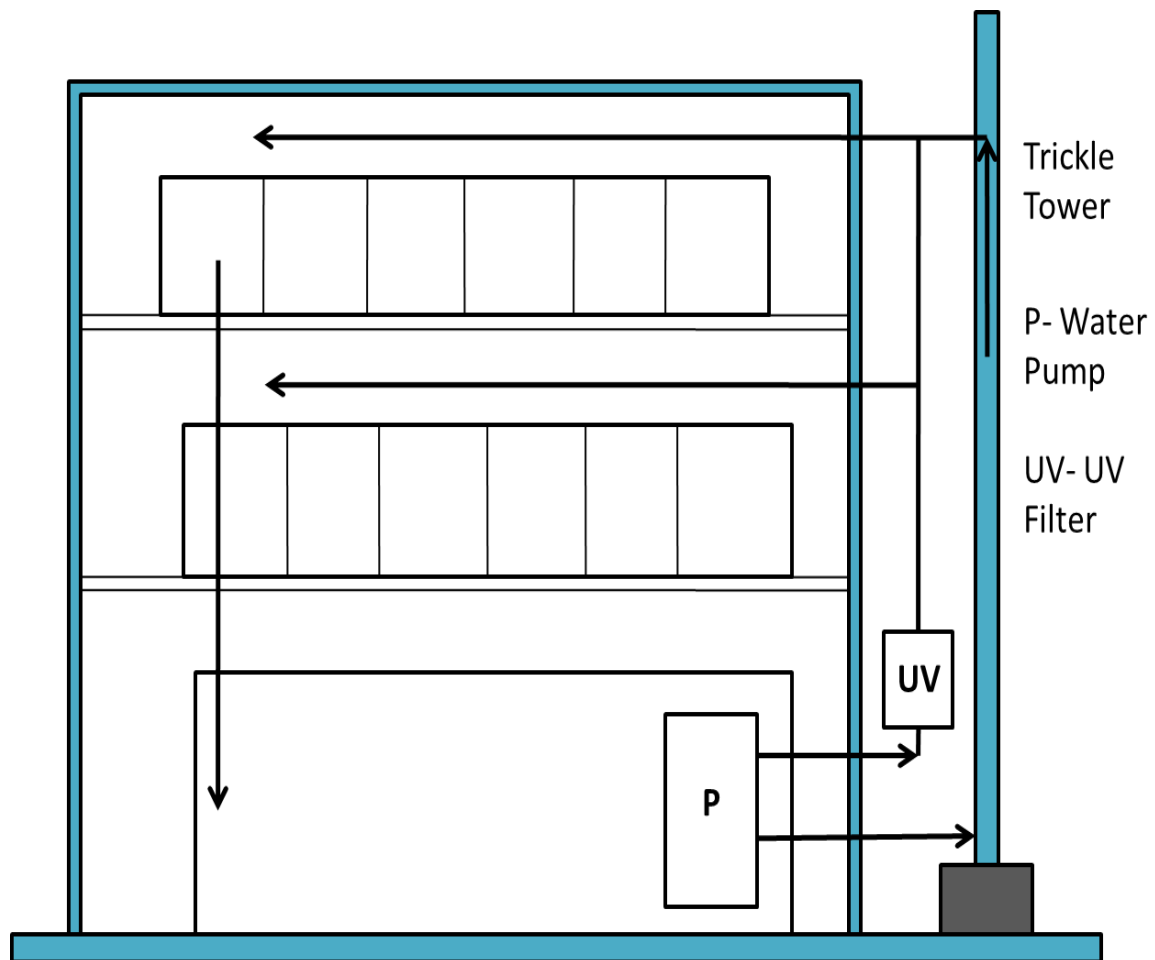
Figure 2. 1: Automatic water circulating system at iBEST- University of Bedfordshire.

2.2 Conventional Monolayer Cell Culture

All cell culture work was carried in a Class II Heraeus Biological Safety cabinet in aseptic conditions unless mentioned. Cells were incubated at 28°C without any CO_2 in a Heraeus incubator (Thermo Fisher Scientific, Rosklide, Denmark) incubator for all experimentation.

2.2.1 Culturing zebrafish muscle cells

Zebrafish muscle cells (ZMC's) were obtained using an enzymatic digestion protocol (optimised in **Section 3.3.3** of the thesis). Cells were isolated using isolation media (IM) ; Liebovitz's L15 (L15) medium supplemented with 0.8 mM $CaCl_2$, 2mM glutamine, 3% Fetal Bovine serum (FBS), 100 ug/ml penicillin/ streptomycin (Sigma-Aldrich, UK) and cultured in growth media (GM); L15 supplemented with 0.8 mM $CaCl_2$, 2mM glutamine, 20% FBS, 100 ug/ml penicillin/ streptomycin. When cells reached to 80-90% confluency GM was switched to differentiation media (DM); L15 supplemented with 0.8 mM $CaCl_2$, 2mM glutamine, 2 % Horse serum (HS), 100 ug/ml penicillin/ streptomycin.

2.2.1 Counting cells

Zebrafish muscle cells were counted using a haemocytometer using trypan blue exclusion method. Cells after isolation where re-suspended in GM, 20 µl of cell suspension was added to 20 µl of trypan blue (0.4% concentration) (Sigma, UK) solution and mixed with pipetting. 10 µl of this solution was added on the either side of haemocytometer chamber covered with coverslip and allowed the solution to fill the chamber by capillary action. Under the light microscope (CETI inverted light microscope, Thermo Fisher) the live cells are seen as white dots whereas the dead

ones stain blue with trypan blue as shown in **figure 2.2**. Live cells (shown by white arrow in **Figure 2.2**) were counted at 10X magnification from each corner quadrant of the chamber. The total value was divided by 4 to obtain an average for each corner segment. This value was further multiplied by 2 to account for the cells dilution in trypan blue, and then further by 1×10^4 to account the number of cells in 1ml. Finally, the value was multiplied by the amount of GM used to re-suspend the cells (usually 5ml) to give an estimation of the total number of cells present in the original solution.
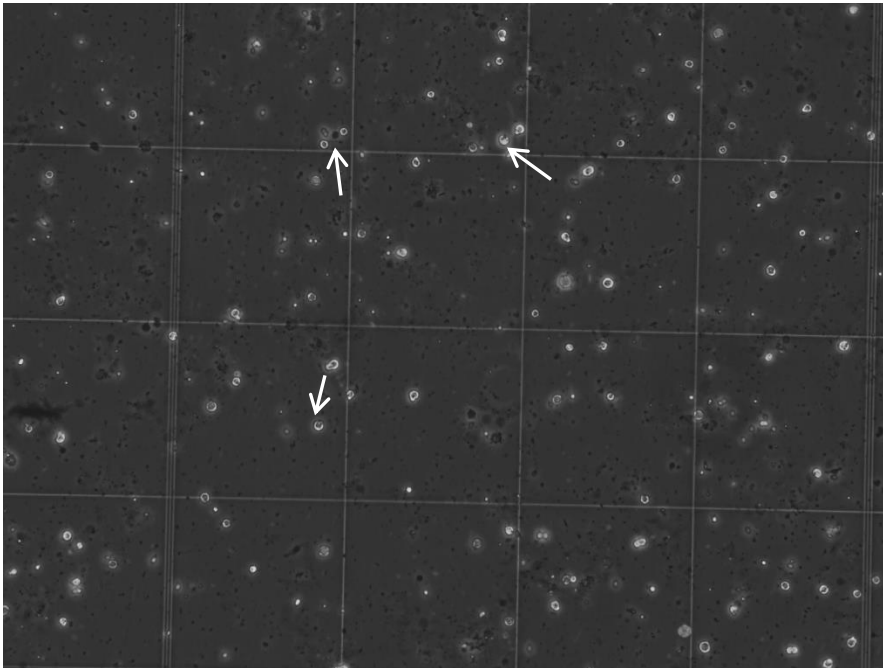
Figure 2. 2: Single quadrant of the haemocytometer used for cell counting. Each quadrant is composed of 16 squares, and the cells within each quadrant (examples shown by white arrows) are counted and used to determine the total cell number.

2.2.2 Cryofreezing of ZMC's

To freeze zebrafish muscle cells, the cell pellet was obtained via centrifugation, the supernatant was removed and the cell pellet re-suspended in FBS (90%) and Dimethyl sulfoxide (DMSO, 10%; Fisher Scientific). After thorough mixing by pipetting, the cell suspension was transferred to a 1.8ml cryovial (Fisher Scientific) at million cells per cryovial, labelled, and placed in a 'Mr Frosty' (Fisher Scientific). The 'Mr Frosty' was then placed in a -80°C freezer overnight. The 'Mr Frosty' containers allow for the slow (-1°C/minute) freezing of the cell suspension. The vials were then transferred to liquid nitrogen for storage.

When ZMC's were resuscitated from cryopreservation, vials were quickly thawed and plated at an appropriate density on gelatin (bovine) (Sigma, UK) coated six well plates (Sigma, UK) with coverslips in each well.

2.3 Tissue Engineering Skeletal Muscle Constructs

2.3.1 Tissue Engineered Fibrin Constructs

2.3.1.1 Preparation of Reagents

Powdered fibrinogen (batch number 029K7635, Sigma-Aldrich) was made up to a concentration of 20mg/ml by dissolving in F12K medium (Sigma-Aldrich) for approximately 4 hours at 37°C in water bath, with occasional swirling. The fibrinogen was then separated into 5ml aliquots and frozen at -20°C until needed for use. The fibrinogen was strictly thawed at room temperature for minimum of 3-4 hours and sterile filtered through 0.2 μm syringe filters (Sigma-Aldrich, UK) just prior to being used for gel formation. Each aliquot was only used once and never freeze-thawed.

Powdered thrombin (batch number 105K7700, Sigma-Aldrich) was made up to a concentration of 200U/ml in DMEM (Sigma-Aldrich, UK), sterile filtered using 0.2 μm syringe filters and then frozen down into 500μl aliquots for future use.

Aprotinin (Sigma-Aldrich) was dissolved in deionised water (Fisher Scientific) at a concentration of 10mg/ml and sterile filtered before being stored at -20°C in 50μl aliquots.

2.3.1.2 Preparation of Fibrin Plates

35mm plastic Petri dishes (VWR, UK) were coated with approximately 2ml of Sylgard (Sylgard 184 elastomer Kit, Dow Corning) and left to cure at room temperature for at least one week before being used. Silk suture thread was cut into 6mm strips, and two strips per plate were pinned into place using minutien pins, with a 12mm gap between sutures as shown in **figure 2.3**.

Before use, plates were UV sterilised in a tissue culture hood for 90 minutes. Following UV exposure, plates and lids were filled with 70% absolute ethanol and left in a tissue culture hood for 30 minutes. The ethanol was then removed from the plates by aspiration and the plates were left in UV light to dry for at least 1 hour before being used for experimentation.
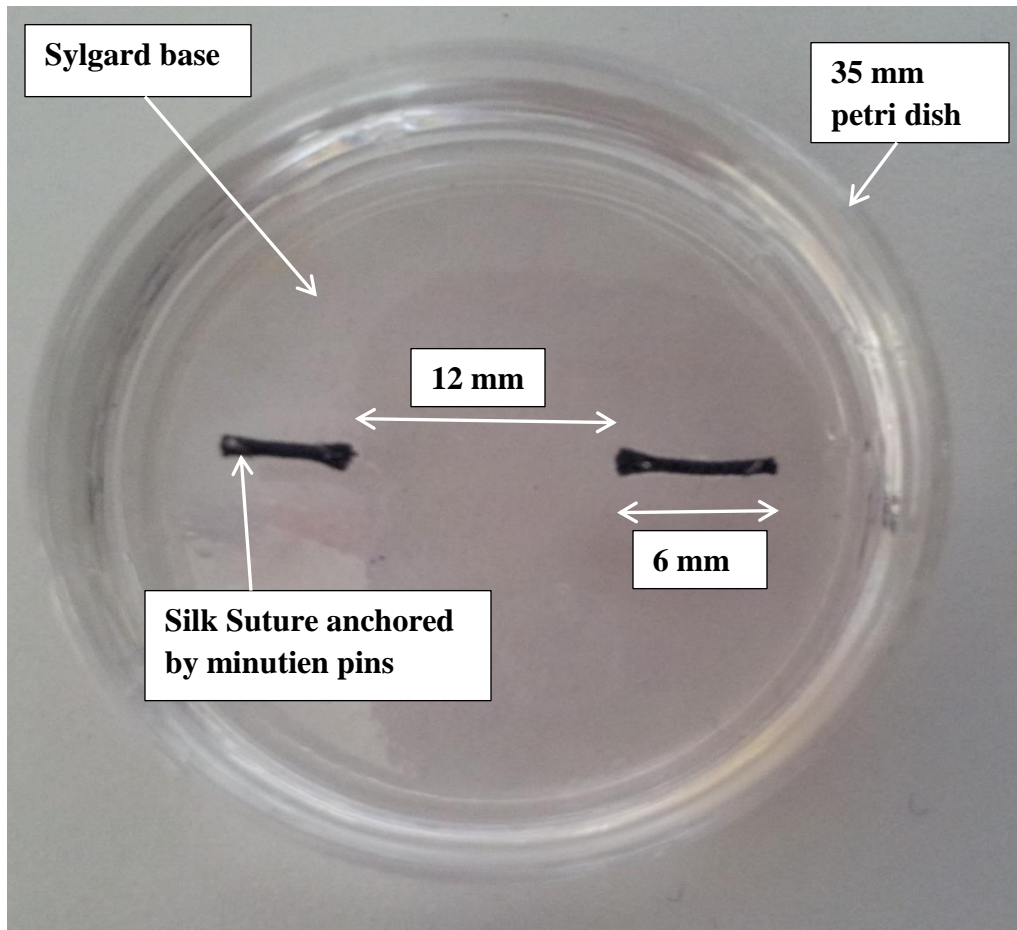
Figure 2. 3: Fibrin based tissue engineered construct set up. *35 mm Falcon dish plate is coated with 2 ml of Sylgard approx. before anchoring sutures using minutien pins.*

2.3.1.3 Formation of Fibrin Constructs and Subsequent Culturing

500µl of GM containing 10U/ml thrombin and 20µg/ml aprotinin was filled in each petri dish drop by drop ensuring by tapping the plate on surface of hood that it is

spread evenly over the whole surface and the sutures were fully covered. 200µl of the stock fibrinogen solution was then added drop by drop to the plate, and was agitated gently to ensure even distribution and then left to incubate for 10 minutes at room temperature before being transferred to the incubator (28°C) for one hour. ZMCs were then seeded at appropriate densities in 2ml of GM. GM was changed every 48 hours until cells were confluent across the gel surface, at which point the media was switched to differentiation medium (DM) consisting of L-15 media supplemented with 2% horse serum. DM was changed every 48 hours until the cessation of the experiment, the determination of which was dependent upon the intended outcome of the experiment itself.

2.3.2 Tissue Engineered Collagen Constructs

2.3.2.1 Preparation of reagents

The collagen constructs used in the present study were based upon previously published protocols (Mudera, Smith et al. 2010, Brady, Lewis et al. 2008, Smith, Passey et al. 2012, Cheema, Yang et al. 2003). The 3D collagen gels were formed by seeding the cells in collagen solution neutralised by Sodium hydroxide (NaOH) and allowing it to polymerise within a mould between two fixed points. These fixed points were custom made structures termed floatation bars and A-frames (see **Figure 2.4**). The flotation bars were constructed using small pieces of polyethylene plastic mesh (Darice Inc, Strongsville, Ohio, US) and stainless steel wire of 0.3 mm diameter (Scientific Wire Company, Great Dunmow, UK, **Figure 2.4**). Slygard mould was used in order to divide glass chamber and make the collagen gels smaller in size (see **Figure 2.4**). Two 3 x 2 mm sections of mesh were bound by two lengths of 0.3 mm

wire. The A-frames were made to fit for the glass chamber system, which were constructed using 0.7 mm wire (Scientific Wire Company). All floatation bars and A-frames, along with the glass chambers and Sylgard moulds were sterilised before experimentation using 70% ethanol (ETOH) and UV sterilised on the biological safety cabinet.

2.3.2.2 Collagen Gel Preparation

0.2 ml of 10 times MEM (Gibco) was added to 1.2 ml of type 1 rat-tail collagen (First Link, Birmingham, U.K, prepared in 0.1 M acetic acid, protein concentration 2.035 mg/ ml) in a 50 ml Falcon tube and mixed by swirling. Titration was done where sodium hydroxide (NaOH, Fisher Scientific) was used in 5 M and 1 M concentrations to neutralise the solution in a drop-wise fashion and swirling with every drop, until colour of solution change (yellow to citrus pink) was observed. Following neutralisation, a cell suspension of the correct number of cells for experimentation, were added and mixed thoroughly in a volume of 0.1 ml GM.

The collagen-cells suspension was set in either a Chamber Slide (Lab-Tek, Fisher Scientific, UK) or a Derilin mould (G.W. Cowler Ltd, Mead Lane, Hertford, UK), divided in half using the Sylgard mould, between two custom made polyethylene mesh flotation bars ('A-frames'). The construct was placed in a 28°C incubator and allowed to gel for 15 min. Once set, the construct was physically detached from the side and base of the mould using a syringe needle and floated in standard growth medium.

The 'A-frames' provided two attachment points which lead to the development of lines of longitudinal isometric strain. The result was a 3D tissue possessing uni-

axially aligned myoblasts capable of differentiating and performing directed

contraction, analogous to *in vivo* skeletal muscle (see **Figure 2.4**).
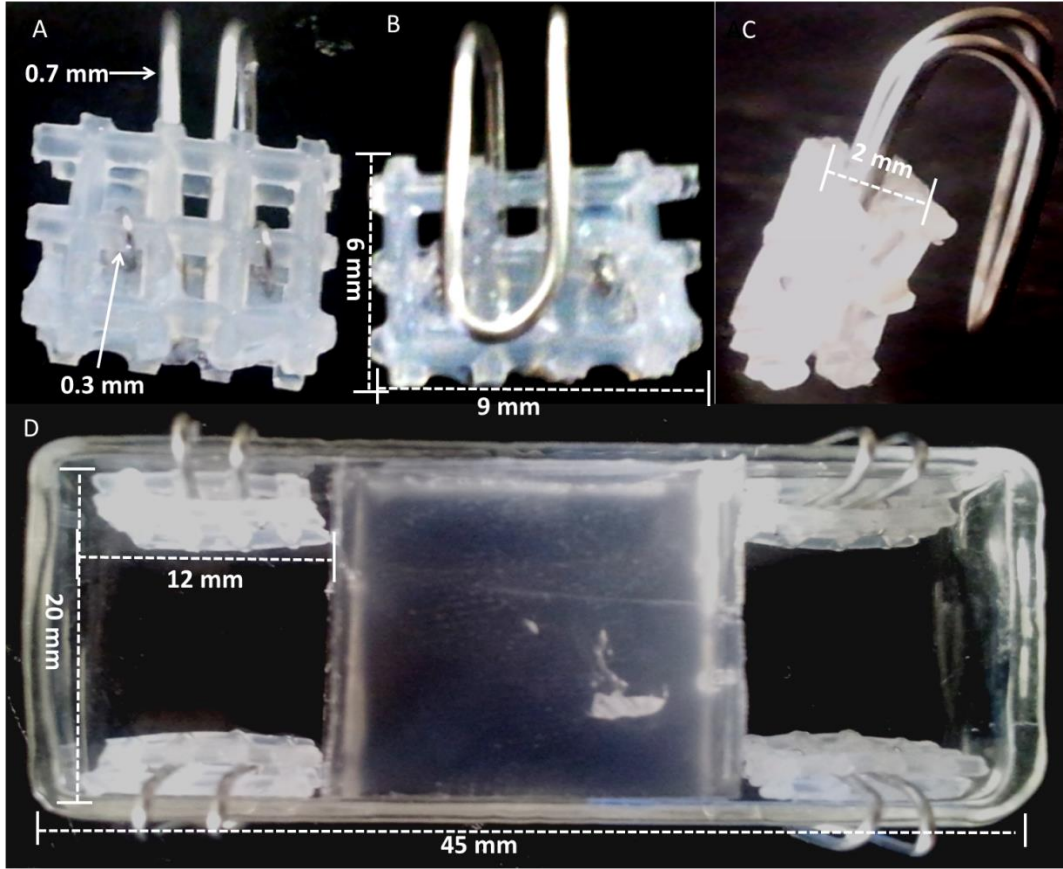
Figure 2. 4: Floatation bars and A-Frame set up for 0.75 ml collagen gels. *A, B and C represents the front, back and side view of custom made floatation bar with A-Frame. D) Whole set up for two 0.75ml collagen gels in a chamber separated by Sylgard spacer which is removed once the gels are polymerised. Dotted lines represent the dimensions of A-Frames, whole chamber and the gels. Solid lines with arrows annotate the grade of wires used for making A-Frames.*

2.4 Immunohistochemistry and Microscopy

2.4.1 Principles of Immunohistochemistry

Immunohistochemistry (IHC) is a process which refers to detection of antigens (proteins) present in cells of whole tissue, by antibody binding specifically to the antigen in the cells. IHC is widely used in research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of tissue. This process could be either one step or two steps depending on the type of antibody being used, if the antibody is tagged along with the fluorophore it is known as direct immunofluorescence. In indirect immunofluorescence which is used throughout this project antigens present in cells bind to unlabelled primary antibody and then a labelled secondary antibody binds with primary antibody. The only thing to remember is that the primary antibody should be specific against the antigen in the cells, whereas the secondary antibody must have been raised against the IgG of the animal species in which primary antibody is raised. This method is more sensitive than direct because the signal gets amplified due to the fact that many secondary antibodies can bind to each primary antibody.

2.4.2 Sample Preparation

Firstly the tissue or cells need to be fixed in order to maintain the structural morphology of a cell; therefore it's necessary to choose the fixative carefully. Organic solvents such as alcohols and acetone remove water and lipids from the cells whilst maintaining cell architecture. Another kind of fixatives are cross-linking agents such as paraformaldehyde which form intermolecular bridges creating a network of linked antigens, and preserves cell structure better than organic solvents. Next step

after fixation is permeablisation of cells in order to allow the antibodies to penetrate in the cells. This step is not required if organic solvents are used for fixation because they permeablise the cells as well. A detergent such as triton is used as a permeabliser in blocking solution which helps in evenly distribution of antibodies. Cells if fixed with paraformaldehyde need to be premeablised. After permeablisation blocking is done using goat serum (Sigma-Aldrich) which blocks the non-specific binding sites in the cells which reduces the background staining/ fluorescence and also allows antibody to bind only at the sites where they are needed.

Two types of antibodies can be used for IHC; Monoclonal and Polyclonal antibodies.

(i). Polyclonal antibodies. These are produced from the serum of an animal following the injection of the antigen of interest and they are not specific to one epitope. For example, if a foreign desmin antigen was injected in an animal, the immuno system will produce antibodies specific for desmin. These antibodies can be subsequently purified and used in IHC.

(ii). Monoclonal antibodies. Monoclonal antibodies take longer to be produced because they are relatively more sensitive and attach to one epitope only therefore monoclonal antibodies are more expensive as well. For example again to produce monoclonal antibodies, an antigen is injected into an animal. The B-lymphocytes are then isolated from the host animal spleen and fused *in vitro* with cancer cells to form hybridoma. Single hybridomas are cloned. This population of cells will then be used to produce identical antibodies for specific for one epitope only.
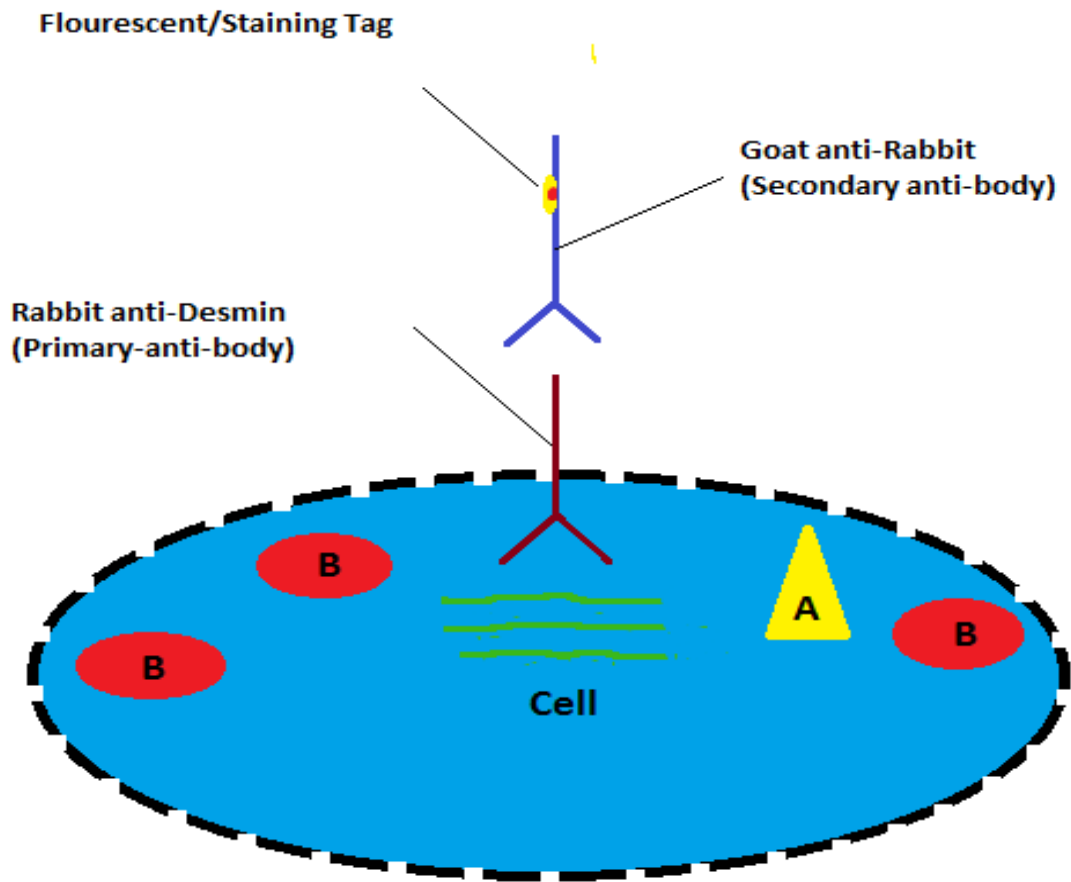
***Figure 2. 5: Principal of IHC showing indirect immuno staining of specific desmin protein in a cell**. Diagram of a whole cell represented in blue colour with dotted line representing fixed membrane of cell, green filaments representing desmin protein, structure A and B representing different organelles in the cell.*

2.4.3 IHC on Glass Coverslips

The media was removed from each well of 24-well plate containing 13mm coverslips and the cover slips were washed twice in PBS. After washing, the cells were fixed for 10 minutes in 50% PBS and ice cold 1:1 methanol-acetone (Fisher Scientific) solution. Thereafter, the cells were exposed to 100% 1:1 ice-cold methanol-acetone solution for 5 minutes.

Once fixed, each coverslip was then treated with 100μl of blocking solution; consisting of 1 x Tris Buffered Saline (TBS, pH 8.5), 5% goat serum (Sigma-Aldrich) and 0.2% Triton-X 100 (Fisher Scientific). After 45 minutes the blocking solution was removed from the coverslip and coverslips were washed twice with TBS. Each coverslip was then treated with 100μl of primary antibody solution; consisting of TBS, monoclonal goat anti-mouse desmin (Sigma, UK) in chapter 3 whereas rabbit anti-zebrafish desmin (Abcam, UK) for rest of chapters at a concentration of 1 in 200, 2% goat serum and 0.2% Triton –X 100. The coverslips were kept in primary antibody solution for overnight, next day each coverslip was washed three times with TBS before the addition of the secondary antibody, consisting of TBS, rabbit anti-goat IgG (Sigma, UK) for chapter 2 and TBS and goat anti-rabbit IgG, TRITC (Abcam, UK) at a concentration of 1 in 200, 2% goat serum and 0.2% Triton-X 100. After 1 hour incubation, the secondary antibody was removed from the coverslips and washed three times with PBS. A chemical compound 4',6-diamidino-2-phenylindole (DAPI) (Sigma, UK) was used to stain nuclei, diluted at 1 in 3000 in deionised water and left to incubate for 10 minutes. Finally the coverslips were mounted onto glass microscope slides (Fisher Scientific) using a drop of MOWIOL (MOWIOL 4-88,

Sigma-Aldrich) containing the anti-fade agent DABCO (Sigma-Aldrich) and the coverslips were viewed and imaged using an inverted confocal microscope (Leica, UK).

2.4.4 IHC of Tissue Engineered Constructs

Both fibrin and collagen tissue engineered constructs were fixed similarly as coverslips (see **section 2.4.3**), instead the incubation time was increased to 20 minutes initially (50-50 solution of methanol: acetone (1:1) and PBS) and a further 20 minutes incubation with methanol: acetone solution alone. Following fixation, gels were detached from their fixed points; sutures in fibrin or A-frames in collagen. Gels were mounted on poly-l-lysine coated microscope slides (Fisher Scientific) and the constructs were ringed with PAP pen (Fisher Scientific) to minimise the amount of antibody used. Gels were then blocked for 3 hours using the same solution made-up as in **section 2.4.3**, before being washed three times using TBS. The primary antibody solution sufficient to cover the gels was added to the gel and incubated overnight in a humidified staining chamber to avoid evaporation of antibody and drying out of the constructs. The primary antibody was removed and the construct washed three times in TBS before the addition of the secondary antibody solution (see **section 2.4.3**) for 3 hours incubation, the secondary antibody was removed and the construct washed three times with PBS before the addition of 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualization for 30 minutes followed by three washes with PBS. The gels were mounted with glass coverslips (fisher Scientific) using MOWIOL containing DABCO and allowed to dry overnight before visualisation. Immuno-stained engineered constructs were imaged upside down using a Leica confocal microscope.

2.5 Gene Expression Analysis

Gene expression measurement is a multi-step process which includes RNA extraction, reverse transcription, primer design and optimisation, standard generation, polymerase chain reaction (PCR), and analysis of PCR product using agarose gel, quantitative real time polymerase chain reaction (qRT-PCR). All the instruments and surfaces used for any RNA or cDNA work were thoroughly cleaned using RNase Zap (Sigma-Aldrich, UK) and rinsed in deionized water (Sigma-Aldrich, UK) prior to use.

2.5.1 RNA extraction

RNA was extracted using the TRIzol (Invitrogen/Life technologies, UK) method following the manufacturer's protocol. Media was removed from the wells containing cells for PCR analysis and washed twice with PBS and further cells were scraped using a P1000 tip in presence of 500 µl of TRIzol from each well. The TRIzol along with homogenised cells solution was further transferred to 1.5 ml sterile RNase free eppendorf tubes (Fisher Scientific, UK).

Tissue engineered collagen and fibrin constructs were also washed twice with PBS initially and removed from the fixed points (A-frames for collagen and sutures for fibrin) to be transferred to RNase free 1.5 ml eppendorf tubes. Further 500 µl of TRIzol reagent was added to each tube before homogenising the constructs using hand a held stick homogeniser (IKA T10 Fisher Scientific, UK). After this point both cells from monolayer and tissue engineered constructs were treated in a similar way.

100 µl of chloroform (Sigma-Aldrich, UK) was added to the tubes containing TRIzol reagent with scraped/homogenised cells and were shaken vigorously for 4-5 minutes until it turned cloudy pink colour at room temperature. The samples were then

centrifuged at 12000g for 15 minutes at 4°C, after which eppendorf will look similar to **figure 2.6** where the upper aqueous layer was transferred to fresh RNase free 1.5 ml eppendorf tube.

Aqueous Layer (RNA, chloroform and a component of TRIzol). This layer is **CLEAR.**

Milky Layer (DNA and Protein**).** This layer is **MILKY/WHITE**.

TRIzol Layer (TRIzol and Cell Debris). This layer is **RED**.
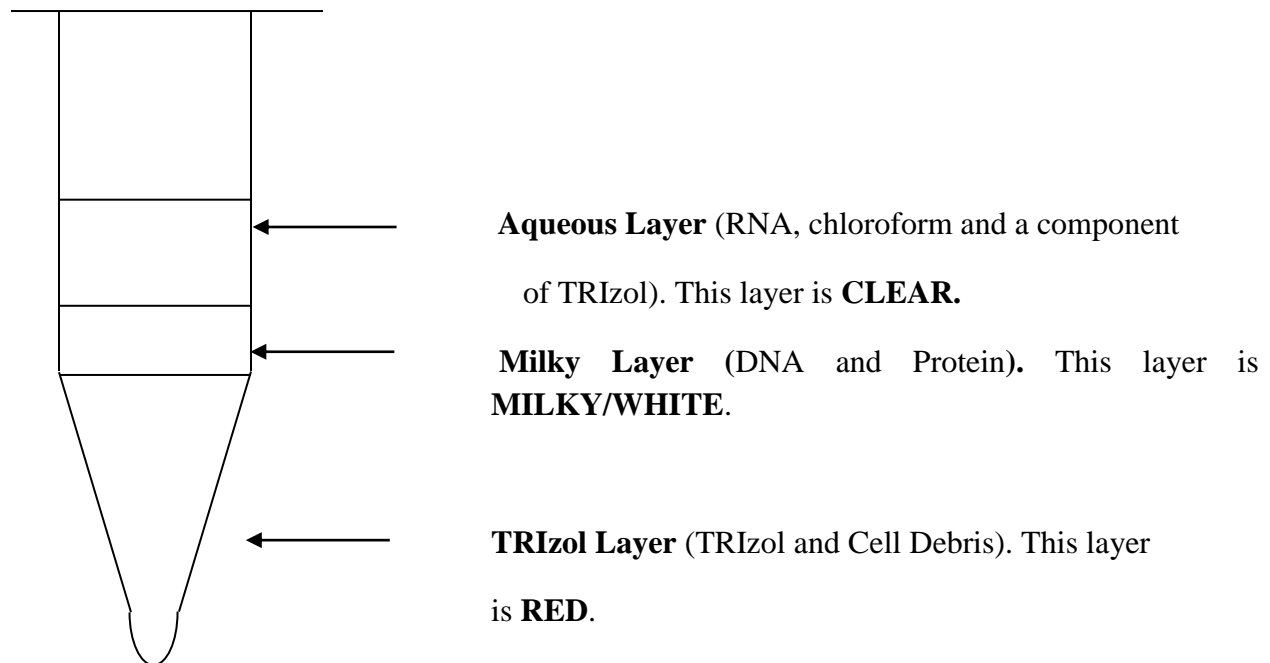
Figure 2. 6: Schematic presentation of three different layers of aqueous, milky and TRIzol obtained after spin.

The samples were treated with isopropyl alcohol (Fisher Scientific, UK) in 1:1 ratio to the amount of aqueous solution obtained after centrifugation and incubated for 10 minutes at room temperature. The rest of the original sample not transferred to a fresh tube was discarded at this point. Samples were centrifuged at 12000g at 4°C for 10 minutes followed by discarding the supernatant and pellet being treated by 75% ethanol (diluted in RNase free water). Samples were centrifuged for 8 minutes at the speed of 7,500g at 4°C followed by removal of supernatant and pellets were left to

dry for 15-20 minutes. The purified RNA was finally resuspended in 50 µl of nuclease free water and frozen at -80 ˚C until further analysis.

2.5.1.1 Total RNA Quantification

RNA yield was quantified using a Nanodrop ND 3000 spectrophotometer (Fisher scientific, UK). Nuclease free water was used as a blank and 1 µl of RNA was quantified. The absorbance was read at 260 nm and 280 nm, ratio range from 1.6 to 2.1 was considered as pure RNA and concentration of the original RNA sample was calculated using equation:

(Absorbance at 260nm x RNA coefficient) = µg/ml of RNA

Where RNA coefficient was 40 ng/µl.

The purity of RNA was also calculated by Nanodrop, which was the absorbance ratio at 260 nm and 280 nm. The pure RNA samples produced absorbance ratios between 1.6 to 2.1.

2.5.2 cDNA synthesis (Reverse transcription)

Total mRNA was converted into cDNA using Precision nanoScript reverse transcription Kit (Primerdesign, UK) according to the manufacturer's instructions. The whole protocol was a two-step protocol. First, 5 µl RNA sample (~1 µg) was mixed with oligodT primers (1 µl) and final mixture volume was made up to 10 µl using RNAse/DNAse free water. The RNA mixture was incubated in a Thermocycler PCR machine (Techne, UK) at 65°C for 5 min and chilled for 2 min on ice. In the second step RNA sample from step one, plus nanoscript buffer (2 µl), dNTP mix (10 mM each -1 µl), DTT (100 mM – 2 µl), nanoscript reverse transcriptase (1 µl), the

final volume was made up to 20 µl using DNAse/RNAse free water. The reaction mixture was incubated in PCR machine at 55 °C for 20 min, followed by termination of the reaction by inactivating enzyme at 75° C for 15 min. No RNA template controls and no reverse transcriptase reactions were also performed alongside to check reaction purity. For conventional PCR, undiluted DNA was used subsequently. For real time PCR experiment, cDNA was diluted 1:3 times in molecular biology grade water (SIGMA, UK) and stored at -80°C.

2.5.3 Conventional polymerase chain reaction (PCR)

PCR was performed for each gene along with internal control housekeeping genes. For each gene specialized primer pairs were designed using primer design software on PubMed website, after taking in consideration of primer length, small product size, GC content, annealing temperature and no primer dimer forming. Specificity of primers was evaluated by product size on agarose gels as well as with the help of melt curve obtained after real time PCR run. Forward and reverse primers designed with their respective annealing temperatures and product sizes are given in **Table 2.1**. In table 2.1 below the large pcr product size for IGF-1 gene was due to the fact that all primers were designed considering that they are spanning only on exon-exon region which resulted in higher base pair size for IGF1 gene.

Table 2.1: PCR primers designed with annealing temperature and PCR product size used in the present study to examine zebrafish (*Danio rerio*) muscle cells and maturation.

Genes		Forward/Reverse Primers	Annealing Temp. ˚C	PCR product size
fMyHC	**F:**	GGGAGAAGGCCAAGAAGGCCA		
	R:	CAGACGGTGCTGCAGGTCCT	58.5	137 bp
sMyHC	**F:**	AGAGGCTGAGGAACAGGCCA		
	R:	CCTTTCTTGGGTCCTGAATCACGG	57	147 bp
Igf-1	**F:**	GGGATGTCTAGCGGTCATTT		
	R:	CTACATGCGATAGTTTCTGC	58.5	489 bp
M-Cadherin	**F:**	CCCCAGTGACATGCTTCCAGCC		
	R:	AGCAGCGTCCAAGCCAACATTGA	60	181 bp
MRF4	**F:**	GCAGGACCTCTTGCATTCGCTGG		
	R:	AGACTCCAACACGGCTCCTTCTC	59	193 bp
Myostatin	**F:**	TGGAGAAGAAGGACTGCAACCCT		
	R:	ATTGGCCTTGTAGCGCTTGGGG	58	190 bp
MyoD	**F:**	GGCTGCCCAAAGTGGAGATTCTGA		
	R:	TGGGCCCATAAAATCCATCATGCCA	59	137 bp
Myogenin	**F:**	GCTCCACATACTGGGGTGTCGT		
	R:	AGATCCTCGTGGGCGGAGCT	59	125 bp
β actin	**F:**	CGAGCTGTCTTCCCATCCA		
	R:	TCACCAACGTAGCTGTCTTTCTG	59	86 bp
EF1 - α	**F:**	CTGGAGGCCAGCTCAAACAT		
	R:	ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87 bp

The master mix for conventional PCR reaction was made up in 50 µl reaction tubes which had NH4 PCR buffer (Bioline, UK), 200 µM dNTPs (Bioline, UK), 1.5 mM MgCl2 (Bioline), 2 U BIOTAQ™ DNA polymerase (Bioline), 0.5 µM each primer (forward and reverse) (see **Table 2.1**), 1 µg c-DNA template and PCR water (Sigma, UK). PCR conditions for all genes were identical except the difference in annealing temperature as well as the number of amplification cycles. Number of cycles for each gene was different due to low/high expression levels. PCR conditions which were standard for all genes were: initial denaturation at 94 °C for 5 min (1 cycle), amplification step contains different number of cycles at 94° C for 30 seconds, at annealing temperature (see **Table 2.1**) for 30 seconds, 72° C for 30 seconds followed by 1 cycle of additional extension step at 72 °C for 10 min. PCR reaction was performed for all the genes at the same time along with control housekeeping genes EF1-α and β-actin.

2.5.4 Agarose Gel Electrophoresis

To analyse the PCR product, they were run on agarose gel electrophoresis using Ethidium Bromide (EtBr) staining. Agarose gel was prepared by dissolving 2 % agarose powder in sterile TAE buffer (Sigma-Aldrich, UK) and warming the solution until it was clear. 0.5 µg/mL EtBr (Sigma-Aldrich, UK) was added to agarose solution, mixed by swirling and poured onto gel cast and left to set. Before pouring the gel, a comb was put in the cast to form wells where the samples were loaded. Gel loading buffer (Sigma-Aldrich, UK) was mixed with PCR samples before being loaded into well along with HyperLadder™ V (Bioline, UK). Gel was run at constant

voltage at 100V for 2 hours. Bands on the gel were viewed using Genosmart UV Gel

Documentation System (VWR, UK).

2.5.5 Generation of standards for qRT-PCR

The standards for quantitative real time PCR for all the genes along with

housekeeping genes EF1-α and β actin were generated using conventional PCR.

Using the primer sequences given in the **Table 2.1** PCR reaction was performed

following the protocol mentioned in **section 2.5.3**. PCR products were run on 2%

agarose gel as explained in **section 2.5.4** and gel bands of the correct product size

were excised. DNA was isolated from excised bands using EZNA Gel Extraction Kit

(Omega Bio-Tek) according to manufacturer's instructions. The flow diagram of the

protocol followed is given in **figure 2.7**. DNA isolated from the bands was quantified

using Bio-Photometer (Eppendorf, UK) at 260 nm. The concentration of DNA

obtained for each gene was diluted to 2 ng/µl followed by 10- fold serial dilutions to

generate standards for qRT-PCR.

500 µl Binding Buffer was added to 1.5 ml eppendorf tube containing excised agarose gel fragment and incubated at 55-60° C for until it dissolves.

↓

Solution was put through spin column containing 2 mL collection tube and centrifuged at 13000 g for 1 minute.

↓

Column was washed with 300 µl of Binding buffer and washed twice with 700 µl of Wash Buffer followed by centrifugation for 2 min at 13000 x g

↓

DNA was eluted with 30µl and followed by 20µl of elution buffer

Figure 2. 7: Protocol for DNA isolation from excised agarose gel PCR product bands.

2.5.6 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

All real time PCR work was performed on RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor. Each reaction was formed of 7.5 µl of sensimix 2X Reaction buffer (contained heat activated DNA polymerase, Ultrapure dNTPs, $MgCl_2$, SYBR® Green I), 333 nano molar of each reverse and forward primers (Table 2.1) and 2 µl of cDNA sample and the final volume made up to 15µl with PCR water (sigma, UK). The standard reaction conditions for all the genes were 1 cycle at 95° C for 10 min, followed by denaturation 50 cycles at 95° C for 10 sec, at respective annealing temperature (**Table 2.1**) for 15 sec and extension at 72° C for 15 sec. Data were acquired on FAM/SYBR channel after the end of each extension cycle. To

check any mispriming or primer dimers melt curve was also analysed and amplification efficiency was calculated from a standard curve (R^2 should be close to 1). All the different time points for different genes were analysed using real time PCR. Relative gene expression data for each gene normalised against house-keeping genes Ef1 α and β actin was obtained using Rotor Gene software (Version 1.7, Corbett research) and calculations were performed in Microsoft Excel. Calculation of relative expression is explained in appendix I with example (**See appendix I**).

The chemistry behind SYBR Green I dye is that it only fluoresces when it's bound to double strand DNA. The standards prepared at 10 fold dilution of DNA should show equally spaced curves on florescence graph as shown in **Figure 2.8a**. As mentioned earlier at the end of each extension cycle step, fluorescence data was measured (see **Figure 2.8b**). PCR products should give only one peak on melt curve graph as shown in **Figure 2.8c**, several peaks on a melt curve represents mispriming or primer dimers. The cycle number at which threshold level is reached can be used to create a standard curve from which sample data can be quantified (see **Figure 2.8d**).

A)

B)

C)

D)
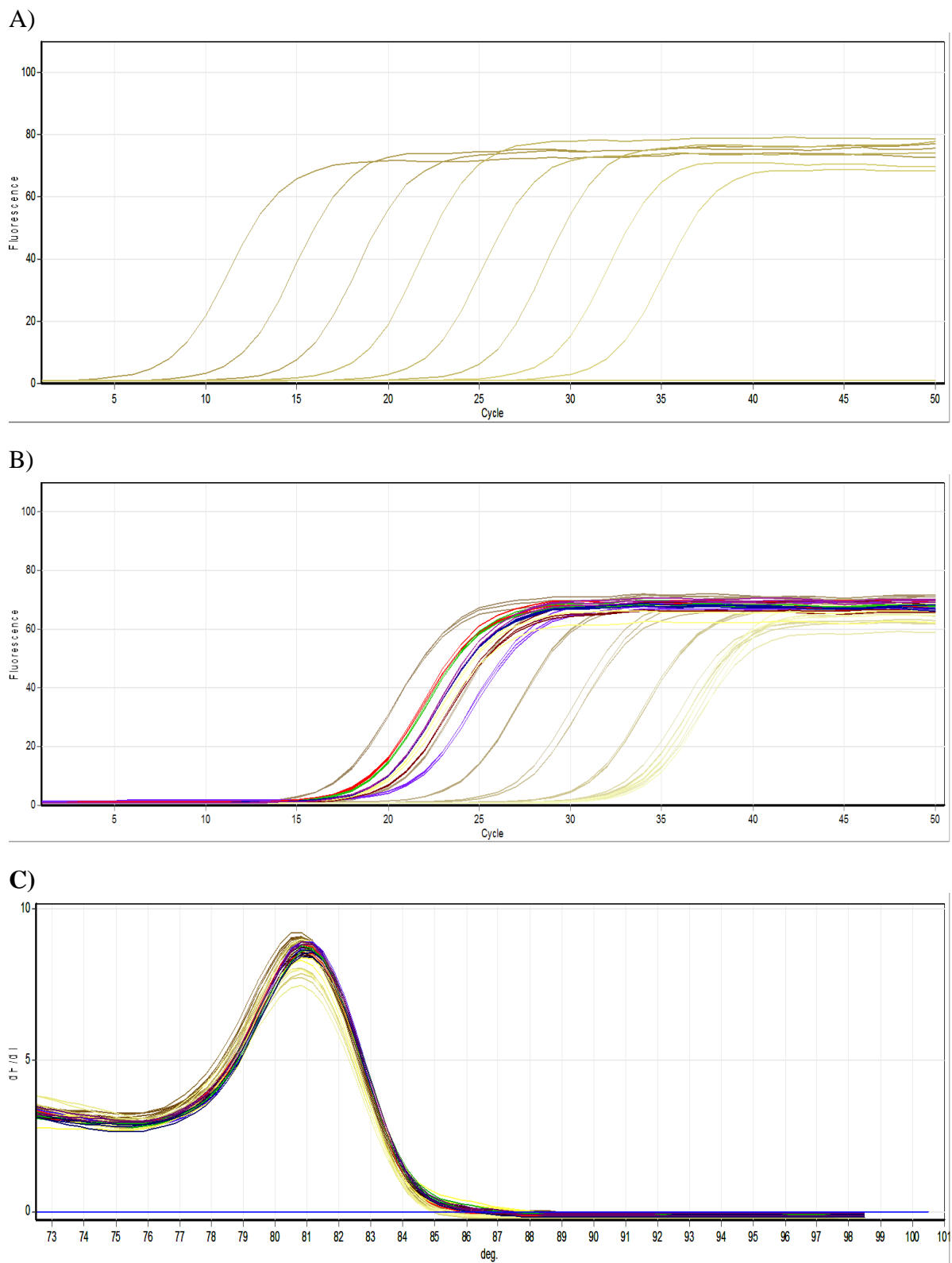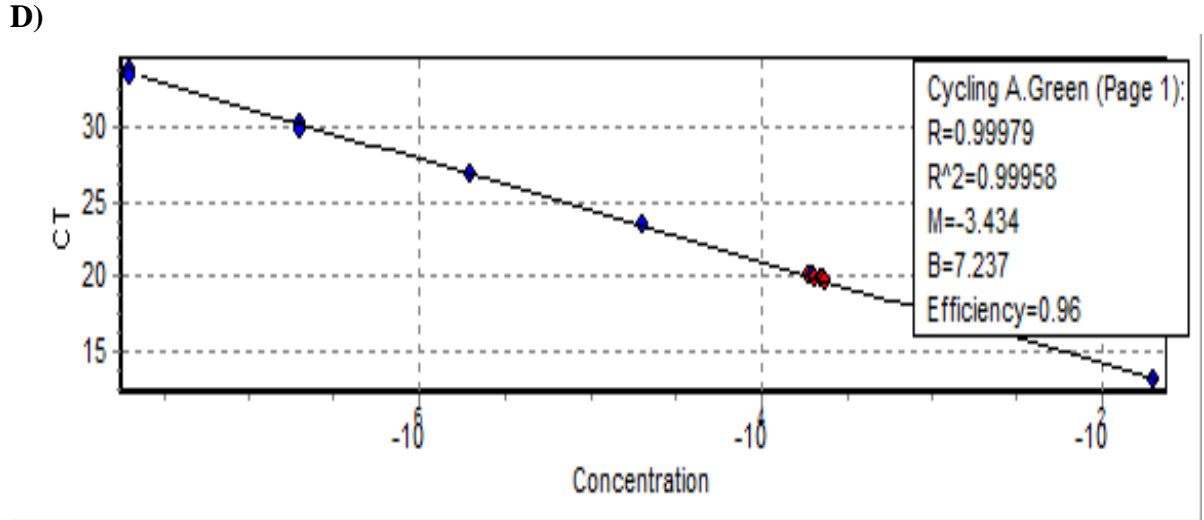
Figure 2. 8: Representative graphs obtained during real time PCR run. *A) Graph representing standards spaced equally, B) Fluorescence graph from where fluorescence is measured after each extension step, C) Melt curve with one peak, D) Standard curve graph produced from 10 fold diluted standards and sample, showing $R^2=0.99958$.*

2.5.7 Relative Quantification

Relative gene expression levels for each gene were calculated using the two standard curve quantification method with kinetic PCR efficiency correction (see equation below) in the Rotorgene software (Pfaffl 2003b). All gene expression values were normalised against values at time zero and the expression levels were also normalised with internal housekeeping genes Ef1 α and β actin. The complete process for calculations is explained in details with example in Appendix-I.

$$\text{Relative quantification} = \frac{(E_{\text{target}})^{\Delta CP\,(\text{target})\,(control-sample)}}{(E_{\text{ref}})^{\Delta CP\,(ref)\,(control-sample)}}$$

Where E = real time PCR efficiency

ΔCP = Difference in threshold cycle (CT) values between unknown sample and reference sample (house-keeping genes) (Pfaffl 2001).

3. Protocol development and optimisation for isolation of zebrafish skeletal (*Danio rerio*) muscle cells

3.1 Introduction

Molecular biology, stem cell biology and tissue engineering research primarily involve culturing of cells on flat plastic dishes. In 1885, Wilhelm Roux was the first person who developed this technique by culturing medullary plate of embryonic chicken in warm saline for several days in on flat glass plate (Sander 1997). This technique is now known as two- dimensional cell culture which involves utilisation of culturing laboratory flasks, conical and disposable bags used in bioreactors.

In order to obtain a genetically identical cell population, raised in a uniform sterile culture environment *in vitro* cell culture methods are deployed. These cell populations are ideal for chemical manipulation and cell cultures are convenient for quantification of cellular phenotypes, tissue specific gene expression studies and observation of subcellular structures (Myhre, Pilgrim 2010). To understand the mechanism behind tissue specific cellular differentiation to investigate early developmental processes, culture models are particularly useful (Myhre, Pilgrim 2010). In the literature these studies are mainly done on immortalised cell lines, which can be passaged multiple times to increase uniformity and amplify cell number. But on the contrary these immortalised cell lines are likely to be altered from their native form and function, therefore cannot be used in studying the progression of gene expression during normal *in vivo* development (Pan, Kumar et al. 2009, Obinata 1997). Few zebrafish immortalised cell lines have been reported and used in

different studies such as fibroblast like cell line, Z4F, PCA2 and SJD (He, Salas-Vidal et al. 2006, Driever, Rangini 1993, Chen, Burgess et al. 2002, Paw, Zon 1998), but until now there has been no specific skeletal muscle cell line reported from zebrafish, which can be used to study the mechanistic myogenesis of zebrafish skeletal muscle cells.

As discussed in chapter one, the use of zebrafish has increased dramatically in recent years as a model organism to study molecular pathways in normal and diseased states (Lieschke, Currie 2007). Zebrafish has also been termed as an excellent model to study developmental myogenesis *in vitro,* which allows us to understand the insights of molecular determination of the timing events during somatogenesis till formation of the whole mature myofibres (Devoto, Melançon et al. 1996). Myogenetic evolutionary similarities between mammals and zebrafish are very high as discussed in chapter one (See **Section 1.1.1**), which makes zebrafish a good model for rapid and economic experiments such as loss of function (morpholinos) or gain of function (transgenic fish) (Guyon, Steffen et al. 2007). In literature, there are many approaches discussed for treating muscular dystrophies and myopathies such as drug therapy where drugs can be used either to mitigate the symptoms of disease or to provide with the missing chemical or protein needed. Secondly, protein therapy used to supply the missing gene or protein using DNA vectors or by transplanting whole cells (cell therapy) (Guyon, Steffen et al. 2007). All these potential therapies have their own disadvantages, for example, it would not be easier to identify the correct drug and also drugs might have positive effect on one specific cell type but the same drug can have adverse effects on non-target cells (Guyon, Steffen et al. 2007). Among

them all, cell based therapy has been seen as most promising alternative (Kunkel, Bachrach et al. 2006) to overcome both immune reactivity and integration problems associated with gene therapy (Ali, Lemoine et al. 1994). During cell based therapy, therapeutic cells or stem cells cultured *in vitro* are transferred in the host recipient to treat the symptoms or cause of disease. Recent research from Myhre and Pilgrim (2010) have used blastomeres isolated from single zebrafish embryo to produce genotypically and phenotypically similar cell population to study the specific manipulations of gene expression on subsequent myogenic differentiation in cultures (Myhre, Pilgrim 2010).

In a review by Jeffrey R. Guyon and colleagues (2007), the potential of zebrafish in modelling human disease has been described. During muscular dystrophy, degeneration of muscle occurs faster than the rate it can be repaired. It is caused because of mutation in dystrophin gene known by the name of Duchenne muscular dystrophy (Hoffman, Fischbeck et al. 1988). In humans, the dystrophin gene transcribes a large protein that localises into the intracellular part of the muscle sarcolemmal membrane and which is also shown in zebrafish by immunohistochemistry (Guyon, Mosley et al. 2003, Chambers, Dodd et al. 2001). Inhibition of dystrophin gene in zebrafish embryo resulted in disorganised sarcomeres and body defects (Dodd, Chambers et al. 2004). Therefore, zebrafish have been extensively used as a model to study human dystrophies and their treatments. In the study of Guyon and steffen et al (2007), dystrophic affected cells from mice were co-cultured with normal muscle cells and they found that, the

affected cells started recovering and performing normally, illustrating the positive affect on dystrophic mice muscle cells.

One of the advantages of this single-embryo zebrafish embryonic blastomere (seZEB) culture system is that, zebrafish as a developmental model is available in thousands of different strains carrying different mutations in genes of molecular and developmental interest (Postlethwait, Talbot 1997, Pichler, Laurenson et al. 2003). It will be of great interest to study the myogenic differentiation in specific mutated or transgenic fish primary cell cultures developed from a genetically uniform cell population (Myhre, Pilgrim 2010).

In the literature, there are protocols for isolation, differentiation and culturing of muscle precursor cells from different large fish such as the Atlantic salmon (*Salmo salar*) (Bower, Johnston 2009), rainbow trout (*Oncorhynchus mykiss*) (Gabillard, Sabin et al. 2010), crab (*Cyprinus carpio*) (Koumans, Akster et al. 1990), shark (Kryvi 1975) and eel (Willemse, van den Berg 1978). However, regardless of the fact that the increased use of zebrafish to study muscle development and diseases, when the present study was initiated there was no published protocol for isolation and characterisation of myogenic progenitor cells from adult zebrafish skeletal muscle cells (Dodson, Zimmerman et al. 2000, Alexander, Kawahara et al. 2011). In order to develop a robust protocol for isolation and culturing of zebrafish muscle precursor cells, several published protocols using different fish were studied thoroughly and experiments were performed for optimisation of the major steps as explained in later chapters to generate a short, simple and precise protocol for isolation, culture and differentiation of zebrafish skeletal muscle cells.

3.1.2 Aims of this chapter are:

1. Develop a robust protocol for isolation of zebrafish progenitor cells from adult zebrafish.

2. Optimise the culture conditions and culture media ingredients for monolayer culturing of zebrafish skeletal muscle cells *in vitro.*

3. Phenotypic and genotypic identification of zebrafish skeletal muscle cells cultured in monolayer using specific techniques.

3.2 Materials and Methods

3.2.1 Cell culture

Cells isolated from adult zebrafish were cultured at 28˚C on sterile-autoclaved glass cover slips placed in either six well plates or 24 well plates, both pre-coated with 0.2% gelatin in heraeus incubator. Cells were cultured for different time points according to the demand of experiments for different stages of cell cycle such as commitment, proliferation, differentiation and maturation (See **Figure 1.7**)**.**

Cells were counted using a haemocytometer (see **Section 2.2.1**) and plated at different plating densities as per the requirement of experiment.

3.2.2 Immunocytochemistry

At the end of every experiment, cells were fixed and blocked according to protocol explained in **section 2.4.3**. Cells were then stained for desmin protein, which is a cytoskeletal intermediate filament protein specifically expressed by skeletal muscle cells through all the developmental stages. For negative control, cells were stained for rhodamine phalloidin (Invitrogen, UK) for 20 minutes which is a stain against actin filament and counter stained for DAPI to visualise nuclei. Cells were imaged using Leica SP5 confocal microscope and checked for different stages of cell cycle i.e. sub-confluent cells, confluent cells, early myotubes and mature myotubes. A myotube was defined as a long multi- nucleated desmin positive cell incorporated with three or more than three nuclei.

3.2.3 PCR analysis

For PCR analysis wells were designated parallel to immunocytochemistry wells. Cultures were homogenised in TRIzol reagent and the RNA was purified using the protocol described previously (see **Section 2.5.1**) and quantified as explained in **section 2.5.1.1**.

3.2.3.1 Conventional PCR analysis

RNA was converted into cDNA as explained in **Section 2.5.2** and cDNA samples were amplified by PCR and run on 2% agarose gels according to protocol described earlier (see **Section 2.5.4**). PCR was used as a technique to identify the cells cultured for the expression of muscle specific genes such as MyoD and myogenin expressed at different stages of cell cycle. Housekeeping genes EF1 α and β-Actin were always run parallel to gene of interest as a control and gels were view as described earlier in **Section 2.5.4.** The list of primer sequences used in these experiments is provided in **Table 2.1**.

3.2.3.2 Quantitative PCR analysis

Once the c-DNA sample preparation was complete (see **Section 2.5.6**), the samples were loaded into a Rotogene 6000Q (Qiagen) using a 72 well round plate. All reactions were performed in triplicates and included a no reverse transcriptase control and no template control. Fluorescence at the end of each extension step was measured using excitation at 470 nm and emission at 510 nm as explained in **Section 2.5.6**.

3.2.4 Statistical Analysis

All experiments were repeated at least three times using isolation done on different days from different lot of fish every time. Statistical analysis was carried out using SPSS V.19 (IBM, USA) and Microsoft Excel (Microsoft Corp. USA). To find whether the data obtained for all genes was normally distributed or not either before or after logarithmic transformation, one-sample Kolmogorov-Smirnov test was performed. Gene expression data which was normally distributed was analysed using one-way ANOVA along with Tukey's and bonferroni post hoc tests. Where data was non-parametric, a non-parametric test Kruskal- Wallis along with the Mann-Whitney U analysis was used to find where the difference occurred. All data were expressed as mean ± SEM and P values of less than 0.05 were considered to be significant.

3.3 Results

3.3.1 Protocol Optimisation

After thoroughly reading all the literature available for isolation of muscle cells from different fish species, either by enzymatic digestion or explant; different experiments were designed and performed to optimise a robust protocol for isolation of progenitor muscle cells from adult zebrafish. In the absence of any published protocol for isolation of skeletal muscle cells from zebrafish, experiments were started by adapting a published protocol as shown by the flow diagram in **figure 3.1** for isolation of muscle cells from Atlantic salmon (Bower & Johnston 2009).
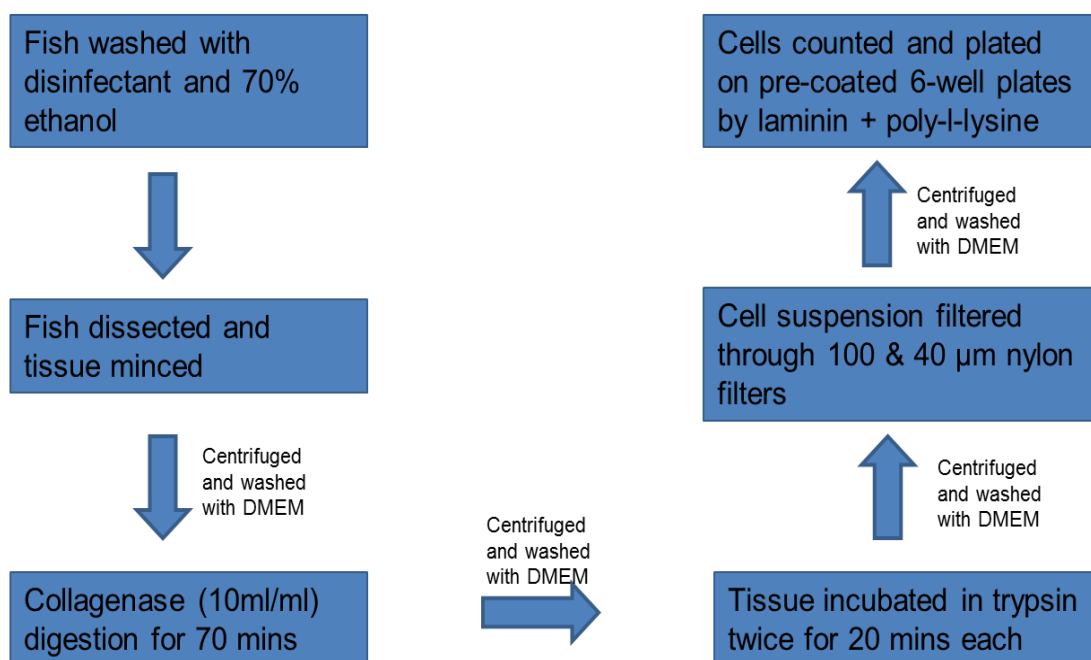
Figure 3.1: Protocol for isolation of skeletal muscle cells. A flow diagrammatic presentation of the protocol designed to isolate zebrafish muscle cells adapted from Bower & Johnston 2009 study for isolation of muscle cells from Atlantic salmon.

Initially zebrafish skeletal muscle cell isolations were performed using the protocol from Bower and Johnston study on Atlantic salmon (**Figure 3.1**) resulted in very few cells with lot of debris. Further optimisation was required firstly because of the inconsistency of cells and secondly because the number of cells obtained per isolation was not enough to design further experiments. Therefore the protocol was optimised at different steps as described below; Main parameters and their sub-parameters optimised were:

- Preparation of samples

- Enzymatic digestion

  ➢ Collagenase digestion

  ➢ Trypsin digestion

- Culture conditions

  ➢ Culture medium

  ➢ Coating dishes

  ➢ Avoidance of contamination

3.3.1.1 Preparation of sample

Work bench was cleaned using ethanol wipes prior starting dissection of zebrafish. 15-20 adult zebrafish per isolation were collected from fish tank where they were maintained (see **Section 2.1**) and washed twice with distilled water. Fish were sacrificed with a lethal dose of tricane (Ethyl 3-aminobenzoate methanesulfonate) (Sigma- Aldrich, UK), followed by washing them with 0.5% sodium hypo chloride ((NaOCl - commonly used in bleach) Sigma-Aldrich, UK) diluted in ddH_2O for 45

seconds to remove any kind of contaminates/oils from the surface of fish. To remove

bleach residue, anesthetised fish were washed in PBS. Fish were decapitated,

skinned, gutted and fins and internal organs were also removed on a sterile petri dish

using the sterile scalpel as shown in **Figure 3.2**. Again fish tissues were sterilised in

0.5% sodium hypo chloride for 45 seconds followed by washing in PBS. Dissected

soft dorsal part of fish with bone (see **Figure 3.2**) was moved to isolation media

(mentioned in **Section 3.3.1.3.1**) supplemented with antibiotics in a 50 ml falcon

tube, kept on ice for further processing.
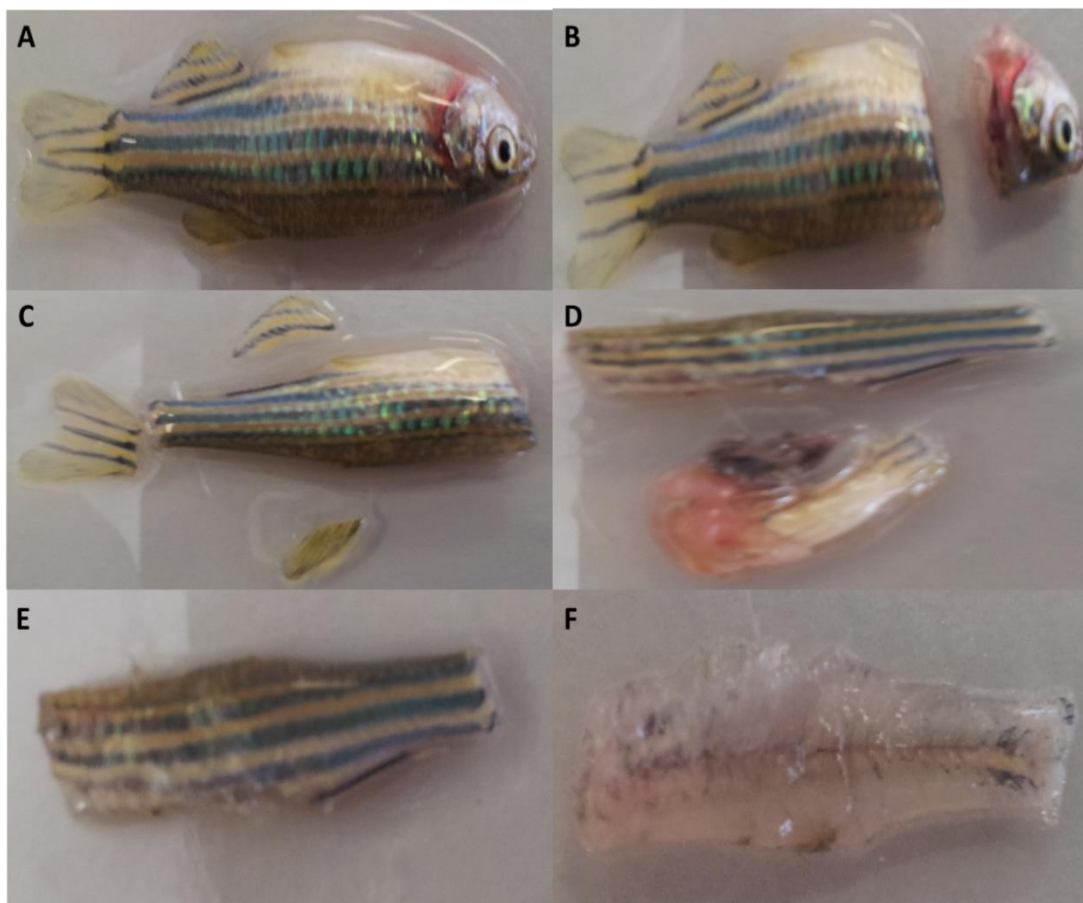
Figure 3.2: Zebrafish images during sample preparation for isolation protocol. *A)
Image of an anesthetised whole adult zebrafish (length ranges from 4-6 cm). B)
Image of a decapitated zebrafish. C) Image of zebrafish with removed fins and tail.
D) Removal of guts and other organs in stomach of a zebrafish. E) Zebrafish tissue
after removal of fins, stomach and de-capitated. F) Zebrafish muscle tissue after
removal of skin.*

3.3.1.2 Enzymatic Digestion

Digestion of tissue is an integral part of any isolation protocol following enzymatic digestion method. Digestion of zebrafish tissue kept in isolation media from the previous step was done in order to release the progenitor skeletal muscle cells from the basal layer. Therefore double digestion was performed according to this protocol firstly using collagenase type 1A (Sigma- Aldrich, UK) and secondly with the help of trypsin (Sigma-Aldrich, UK). This step of digestion needed optimisation in terms of concentration of enzyme and time required, for to develop a robust protocol.

3.3.1.2.1 Collagenase Digestion

Collagenases are the enzymes obtained from a bacteria *clostridium* that break peptide bonds of collagen in the cells body itself to stop formation of large structures. Commercially it is used to digest connective tissues of muscle cells or other tissues (Williams et al. 1995) to free the satellite cells. But if the concentration is higher or lower than required, satellite cells will be either digested due to enzymatic activity or the intra cellular bonds of collagen will not be digested to liberate cells respectively. Therefore an experiment was designed to determine the optimum incubation time and concentration of collagenase type1A enzyme needed to break down cellular adhesion of cells and liberate satellite cells. A range of different concentrations of collagenase from 2mg/ml to 10mg/ml were tested on same number of fish for each concentration, parallel to different time points ranging from 20 minutes to 60 minutes (**see table 3.1**). Collagenase was prepared in distilled water and filtered through 0.2 μm filters prior to use.

Table 3.1: Impact of collagenase in milligram/millilitre on cultured cells in monolayer for different time points. Key for + signs used here is + : 0-1 million cells obtained, + + : A range of 1-5 million cells were obtained, + + + : 5-10 million cells were obtained (n=20).

Conc. Time	2 mg/ml	4 mg/ml	5 mg/ml	7 mg/ml	10 mg/ml
20 minutes	+	+	+ +	+ +	+
45 minutes	+	+	+ + +	+	+
60 minutes	+	+ +	+ +	+	+

Results from the experiment showed that 5 mg/ml concentration of collagenase type 1a enzymatic digestion for 45 minutes liberates maximum number of cells per digestion compare to all other concentrations of collagenase for different time periods. Therefore it was concluded to use 5 mg/ml concentration of collagenase type1a for 45 minutes for further experiments. Additional primary data for all the experiments has been provided in the log sheet for isolations in appendix-II.

3.3.1.2.2 Trypsin Digestion

The isolation protocol used for isolating muscle cells in this study was originally designed for Atlantic salmon fish (Bower & Johnston 2009). In the literature trypsin digestion has been used to dissociate rigid bonds of cells to other cells and bones. Trypsin is mainly found in the digestive system of vertebrates where it breaks down proteins, termed as serine protease. It breaks the peptide chains from their carboxyl end at lysine or arginine amino acids except when any of them are followed by proline (Finehout, Cantor et al. 2005). In the protocol for isolation of satellite cells from Atlantic salmon where the size of fish was normally 70- 80 cms, trypsin digestion method was effective which might not be necessary in case for zebrafish which are normally 3-5 cms in size. Therefore an experiment was performed to investigate the effect of trypsin digestion of zebrafish muscle tissue on the yield of cells. During the whole isolation process a small amount of tissue or cell suspension was taken out at different stages which were; 1) after mincing tissue, 2) after collagenase digestion, 3) before trypsin digestion, 4) after trypsin digestion and stained with DAPI for nuclei.

The result of this experiment showed nuclear degeneration and thereby a decrease by 60-70% in number of nuclei between before and after trypsin digestion. It is observed from the set of pictures in **figure 3.3**, that there was a noticeable difference in number of nuclei which were stained with DAPI, in the cells suspension taken out before and after trypsin, so it was interpreted that trypsin was digesting zebrafish muscle cells. Therefore trypsin treatment was removed from the zebrafish isolation protocol.
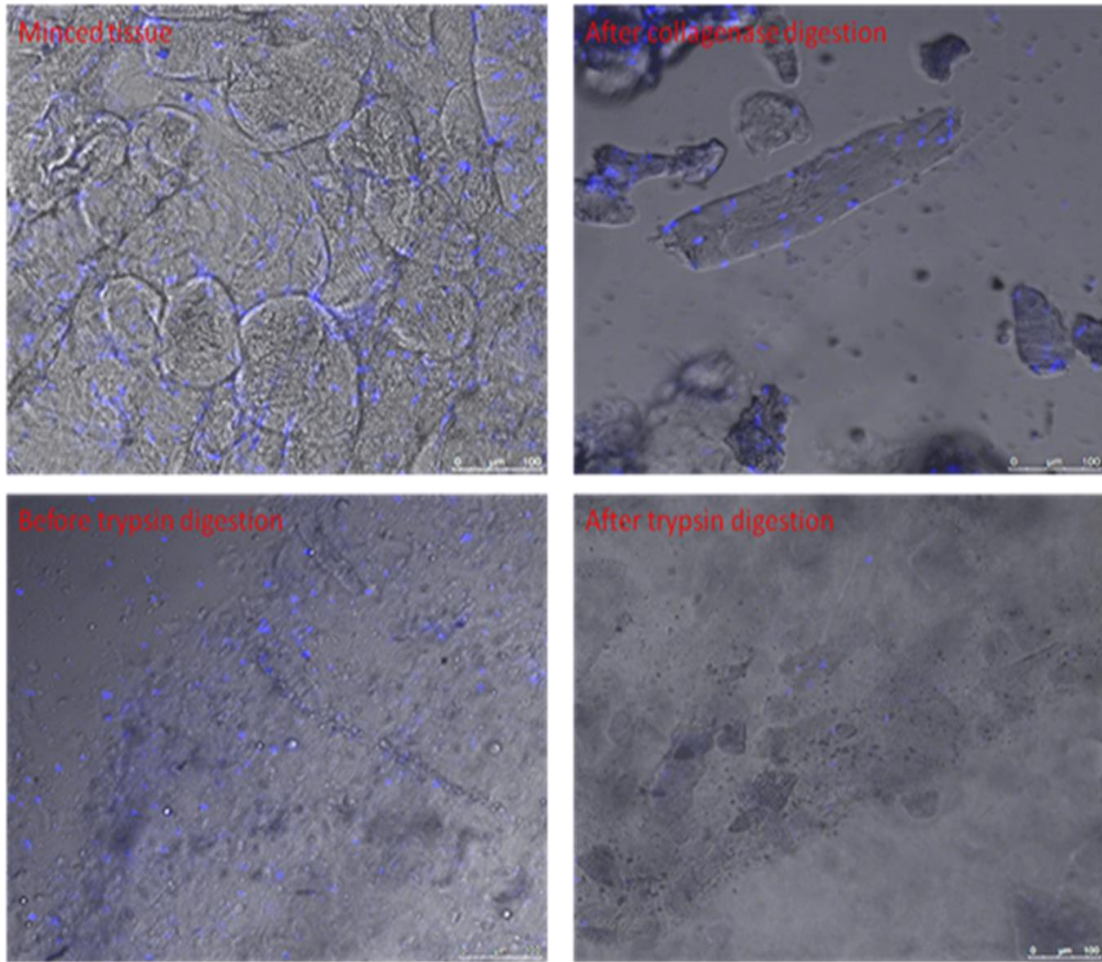
Figure 3.3: Zebrafish tissue or cells suspensions during the isolation protocol stained with DAPI for nuclei (shown in blue colour) at 100X magnification. 1) Image of zebrafish tissue after mincing, 2) Cell suspension taken out of the isolation protocol after collagenase digestion step, 3) Cell suspension before trypsin digestion, 4) Cell suspension after trypsin digestion.

3.3.1.3 Culture Conditions

Cells grown *in vitro* need appropriate temperature, growth medium, plating density and adhesion matrix to attach and proliferate as they do *in vivo*. These culture conditions differ for each cell type with respect to their phenotypes.

3.3.1.3.1 Growth Medium

Culture medium provides all vital components for the growth and nourishment of cells, so it was quite important to find the right culture medium with appropriate supplements and the glucose level. All the work previously reported in the literature used different species and they also used different growth media to isolate and culture skeletal muscle cells in-vitro. Every species has different requirements for glucose and other supplements to grow, therefore different growth media were tested to isolate and culture zebrafish muscle cells such as Dulbecco's Modified Eagles's Medium (DMEM) F12 (D6421, Sigma-Aldrich, UK), DMEM (D6429, Sigma-Aldrich, UK) high glucose medium, DMEM (D7777, Sigma-Aldrich, UK) high glucose and Liebovitz's (L15) (L4386, Sigma-Aldrich, UK) medium. In the literature DMEM (D6429), DMEM (D7777) and DMEM-F12 (D6421) were termed as mammalian medium whereas; Leibovitz's medium (L-15) was fish media. All medium were supplemented with l-glutamine, fetal bovine serum (FBS) and antibiotic prior use (media preparation described in **Section 2.2.1**). FBS batch screening was performed previously by other group members mentioned in Darren player thesis UoB 2013, therefore afterwards same batch of FBS was brought for the whole lab and used in the present study as well.

Table 3.2: Impact of different culture media on survival and confluency of zebrafish skeletal muscle cells in monolayer cultures. *The number of + sign denotes the quality of cultures obtained with respect to the media used, key for signs shown in* ***Figure 3.4***.

Isolation number	Length of culture (in days)	Media used	Sign indicating quality of culture
#003	2 days	DMEM, high glucose (D6429)	+
#004	11 days	DMEM-F12, low glucose (D6421)	++
#005	11 days		
#006	9 days		
#007	11 days	DMEM, high glucose (D7777)	++
#008	10 days		
#009	11 days		
#036	14 days	L-15 media, high glucose	+++
#040	12 days		
#043	15 days		
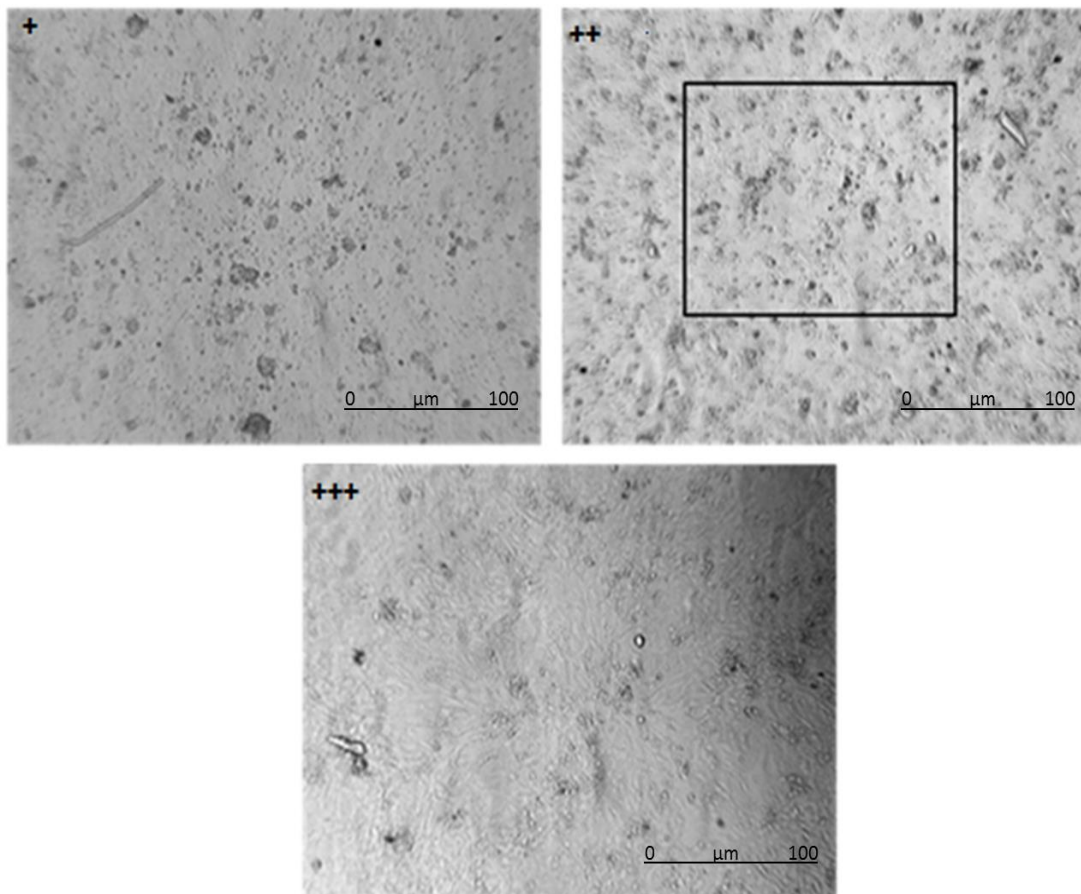
Figure 3.4: Images of different cultures represents different respective sign keys used in Table 3.2. Keys for sign are; +) 1-5% cells attached, ++) 5-30% confluent cells with debris, +++) 40-70% confluent cells attached with debris. Images were captured at 100X magnification.

The results showed that zebrafish muscle cells in culture survived for longer time period, also the number of cells obtained during isolation with L-15 high glucose media was higher than all other media tested. After experimenting with different culture media explained above, fish media L15 supplemented with l-glutamine, fetal bovine serum and antibiotics was found optimum to culture zebrafish muscle cells as explained by the results from **table 3.2** and **figure 3.4**.

3.3.1.3.2 Cell Adhesion Matrix

When zebrafish skeletal muscle cells were isolated and cultured, they needed a matrix to adhere to, so that they can get support / attachment to proliferate normally. According to the protocol from Ian Johnston and Neil Bower (Bower & Johnston 2009) which was followed initially, a two layer coating of poly-l-lysine and laminin was used on six well plates or cover slips placed in them where the cells attached and proliferate. To coat six well plates with poly-l-lysine and laminin at least 24 hours incubation was required. Therefore gelatin was also investigated to find the adherence of zebrafish skeletal muscle cells.

Results showed that zebrafish muscle cells attach similarly on 0.2% gelatin (see **figure 3.8**) (Sigma Aldrich, UK) coating as they do on poly-lysine and laminin (see **figure 3.4** +++) coating. Gelatin only needs half an hour incubation to prepare the matrix for cells to adhere and it is cost effective as well compare to laminin and poly-lysine; therefore laminin and poly-l-lysine coating was replaced with gelatin in the protocol for future experiments.

3.3.1.3.3 Avoidance of contamination

Sterilisation plays the most important part in cell culturing. While culturing if the cells get contaminated then contaminant cells for example bacteria, can overtake desired cells and pH of media changes to acidic. Initially, various different types of contaminants were observed in the cultured zebrafish muscle cells. The morphological analysis suggested that the source of contamination were various bacteria and fungi. In the next step, therefore a combination of various antibiotic and

antifungal drugs such as penicillin, streptomycin, amphotericin B, gentamycin and

betadine ((vinyl pyrrolidone)-iodine complex) were tested.

Results showed that zebrafish muscle cells were not able to survive/ proliferate

without any antibiotics as the bacterial population took over the muscle cells

population in cultures as shown in **figure 3.5 (A)**. Whereas in presence of

amphotericin B and gentamycin along with penicillin- streptomycin solution as

shown in **figure 3.5 (B)** the contaminants were not present but the cell were not able

to proliferate as well. Cells cultured only in presence of penicillin- streptomycin

solution were proliferating without any contaminants contaminating the cultures as

well.
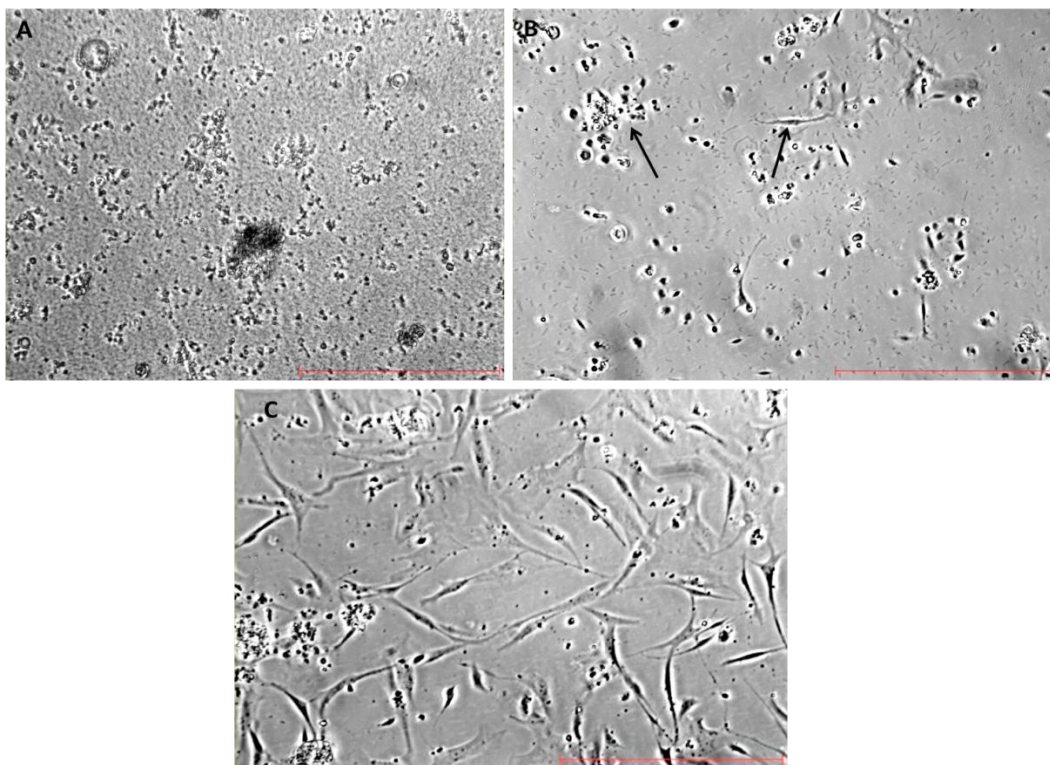
Figure 3.5: Impact of use of different antibiotic and antifungal drugs and their effect on cell survival taken at 200X magnification. A: Cells cultured without any antibiotics, B: Cells cultured with amphotericin B and gentamycin along with penicillin- streptomycin solution, C: Cells cultured with only penicillin- streptomycin solution.

Therefore it was concluded that after initial wash of the fish muscle tissue before isolation with betadine ((vinyl pyrrolidone)-iodine complex) followed by 100 µg/ml of penicillin and streptomycin solution in media would be used in subsequent experiments to avoid contamination and culture the cells for longer time.

3.3.2 Revised Protocol

Using results and interpretation from all above mentioned experiments a revised protocol was formulated to isolate and culture skeletal muscle cells from zebrafish which is described below with the help of flow diagram in figure 3.6.

Sterilisation of fish which includes washing with NaOCl, PBS, ddH$_2$O and anesthetised in tricane.

Fish decapitated, skin, guts and fins removed.

All steps from here onwards were performed in laminar flow hood.

Tissue washed twice with PBS before digested in 5 mg/ml collagenase for 45 minutes.

Cell suspension filtered through 40 and 100 µm nylon strainers.

Cell suspension spun down and pellet washed with sterile PBS.

Cells counted and plated at required density in growth media on six well plates pre-coated with gelatin.

Figure 3.6: Flow diagram of the revised protocol for isolation of zebrafish progenitors from adult zebrafish skeletal muscle.

Phase contrast images of cells obtained using the revised protocol
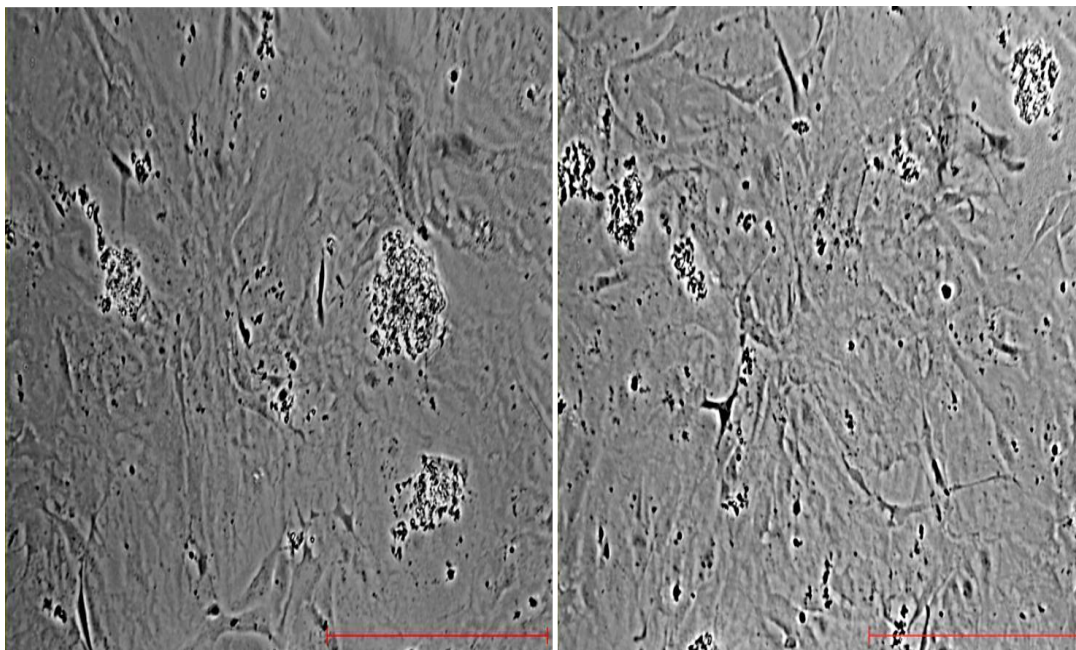
Figure 3.7: Zebrafish skeletal muscle cells cultured in monolayer using revised protocol. Zebrafish cells were isolated and cultured on glass coverslips in six well plate using revised protocol and images were taken every day using phase contrast microscope to keep record of cell growth and confluency. Images taken at 100X magnification.

Pictures shown in **figure 3.7** were obtained using the revised protocol formulated using all the results obtained from experiments explained in **section 3.3.1**, results from which were more consistent compare to the protocol used from Bower and Johnston (2010). But still the drawback with this revised protocol was presence of debris in isolations which can be noticed in **figure 3.7** and **figure 3.4** (+++). Debris present in cultures can be potential source of contamination during the analysis of cells using PCR techniques and also it can limit the growth of cells due to the proteins released by them. According to recent studies Ficoll-density gradient can be used to remove the heavy particles from mono nuclear cells in culturing protocols

(Garbade, Schubert et al. 2005, Lafrance-Corey, Howe 2011, Alexander, Kawahara et

al. 2011). Ficoll-density gradient's principal is that it works according to the density

of particles, for example when particles with different densities are layered on top of

a Ficoll layer and spun down, the heavier particles will settle at the bottom under

Ficoll layer whereas the lighter particles will make a layer on top of Ficoll layer as

shown in **figure 3.7**. Therefore in order to make cultures cleaner, another step was

investigated in the protocol which separates mono nuclear cells from the heavy

debris. After introduction of Ficoll step in protocol, cleaner cultures were obtained as

shown in **figure 3.7** and optimisation of ideal protocol was done.
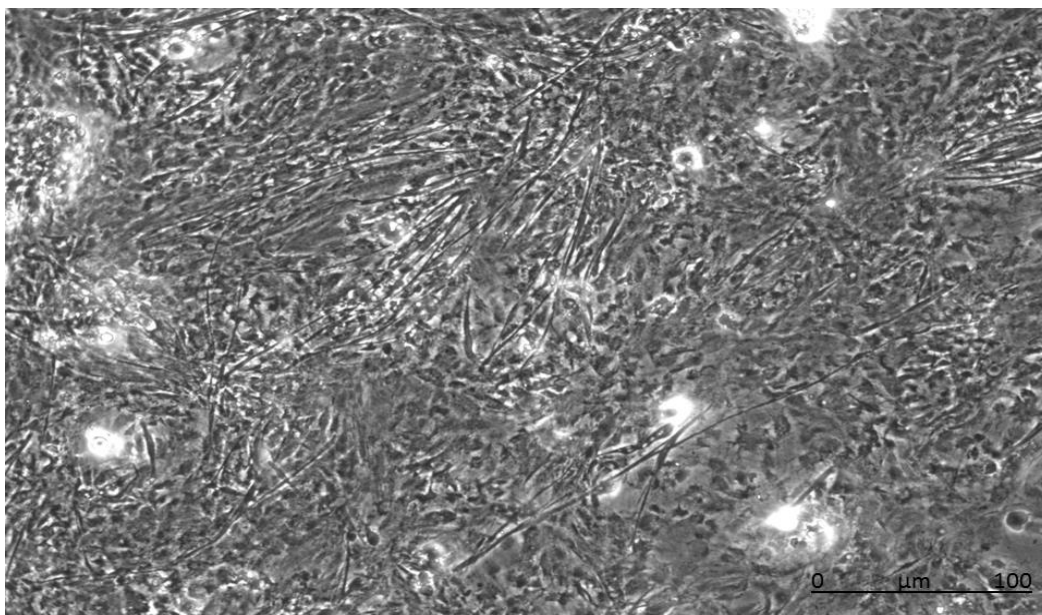
Figure 3.8: Zebrafish skeletal muscle cells cultured with Ficoll step included in the protocol. Image captured at 100X magnification.
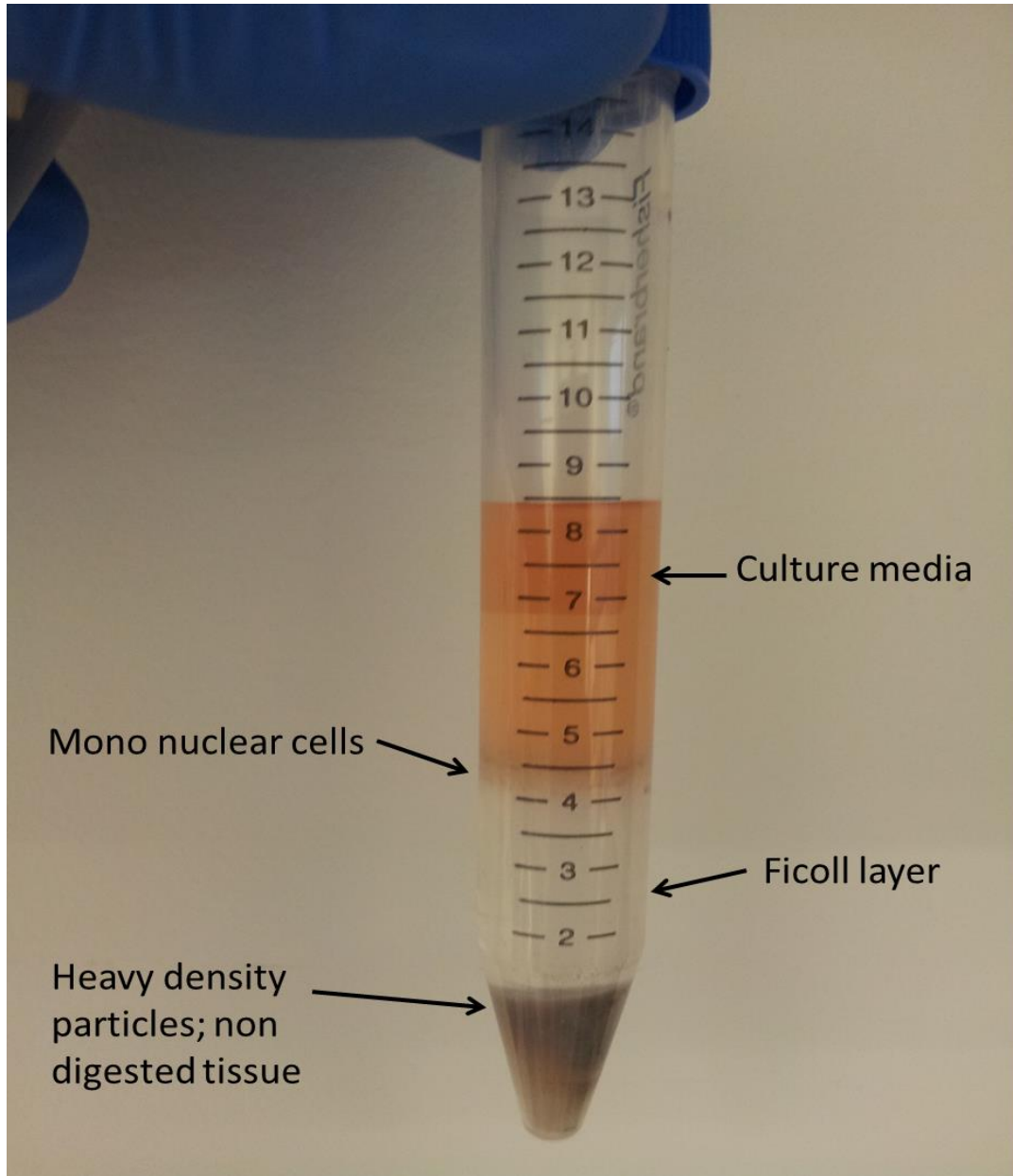
Figure 3.9: Separation of mononuclear cells from heavier density cells debris particles using Ficoll density gradient.

3.3.3 Formulation of Ideal protocol

Using the results and interpretation from all above mentioned experiments in **section 3.3.1** a robust protocol was formulated to isolate and culture cells from zebrafish skeletal muscle tissue which is described below:

Fish were collected and sterilised as explained in **section 3.3.1.1**. Fish soft dorsal muscle tissue was moved to a laminar hood and washed in PBS twice. Tissue was further minced with sterile scalpels in a petri dish following a general rule of thumb, 1 minute mincing for 1 fish. Minced tissue was incubated in a sterile 50 ml falcon tube with 10 ml of 5mg/ml collagenase type 1A solution at room temperature for 45 minutes on a shaker at 200 rpm. 20 ml of isolation media (L15 supplemented with 0.8 mM CaCl2, 2mM glutamine, 3 % FBS, 100 µg/ml penicillin/ streptomycin) was added after incubation to stop collagenase activity followed by cell suspension filtered through 100 and 40 µm nylon strainers (Fisher Scientific, UK) respectively. The filtrate was centrifuged at 1600 rpm for 10 minutes and the supernatant was discarded. The pellet was re-suspended in 4 ml PBS prior to layering over 4 ml of Ficoll solution in a 15 ml centrifuge tube and centrifuged at 3750 rpm for 45 minutes. Mono nuclear cells were collected from top of Ficoll layer and washed with PBS and then rsuspended in 10 ml growth media which is L15 supplemented with 0.8 mM CaCl2, 2mM glutamine, 20 % FBS, 100 µg/ml penicillin/ streptomycin. The process is represented diagrammatically in **figure 3.9**.

Cell density was measured at this point before plating cells, using a haemocytometer by diluting the cell suspension in trypan blue (see **Section 2.2.1**). Cells were plated at a desired plating density on sterile 6-well plate pre-coated with 0.2% gelatin and

three sterile cover slips were placed in each well. Cells were cultured at 28 °C in a

tissue culture incubator and culture medium was changed on alternate days. On

average 8-10 million cells were obtained per isolation using 20 adult zebrafish.
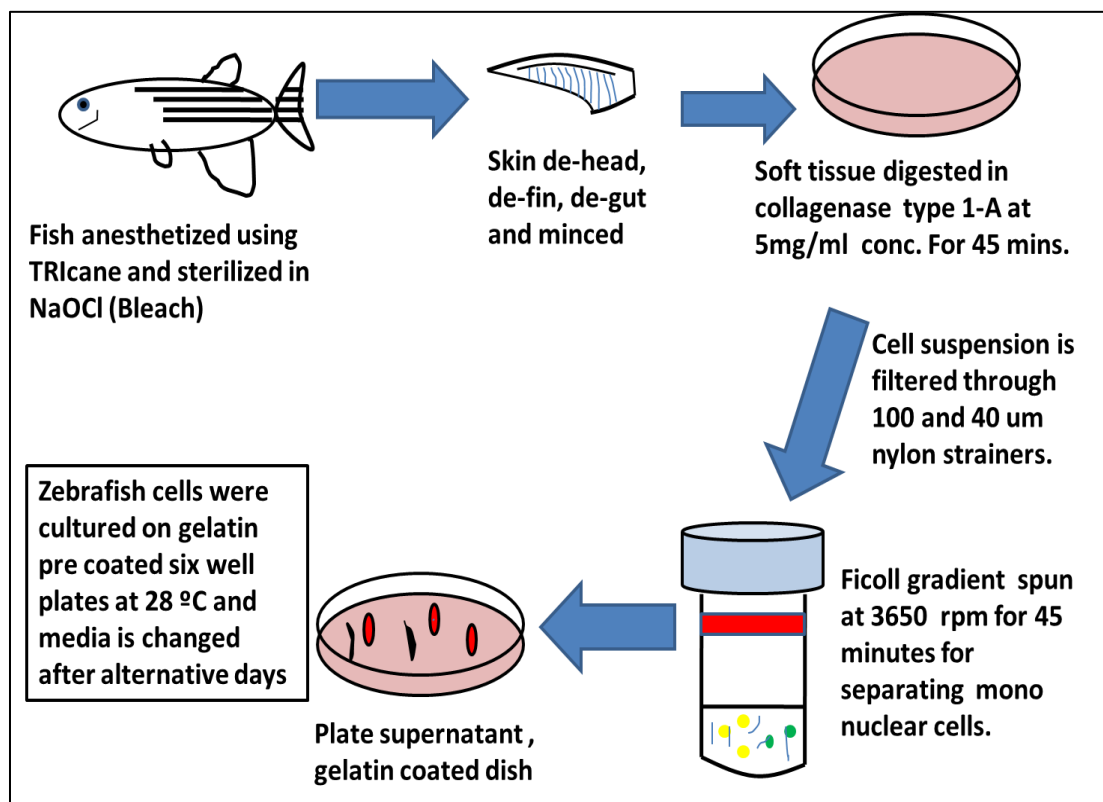
Figure 3.10: Schematic presentation of ideal protocol for isolation of zebrafish progenitor cells from adult zebrafish skeletal muscle. Diagram represents the whole protocol, starting from anesthetising zebrafish and removing head, skin, fins and guts. Soft tissue was further digested in collagenase, followed by Ficoll spin. Cells were collected from top of Ficoll and plated on gelatin pre coated six well plates.

Growth and division of cells can be seen in phase contrast images in **figure 3.9** from

zebrafish muscle cell culture results, it is shown that cells were growing in size and

increasing in number from day 1 to day 14, where after approximately day 6-7 when

cells are 90% confluent they were switched to low serum differentiation media (L15

supplemented with 0.8 mM CaCl2, 2mM glutamine, 2 % horse serum, 100 µg/ml

penicillin/ streptomycin) to fuse and form myotubes.
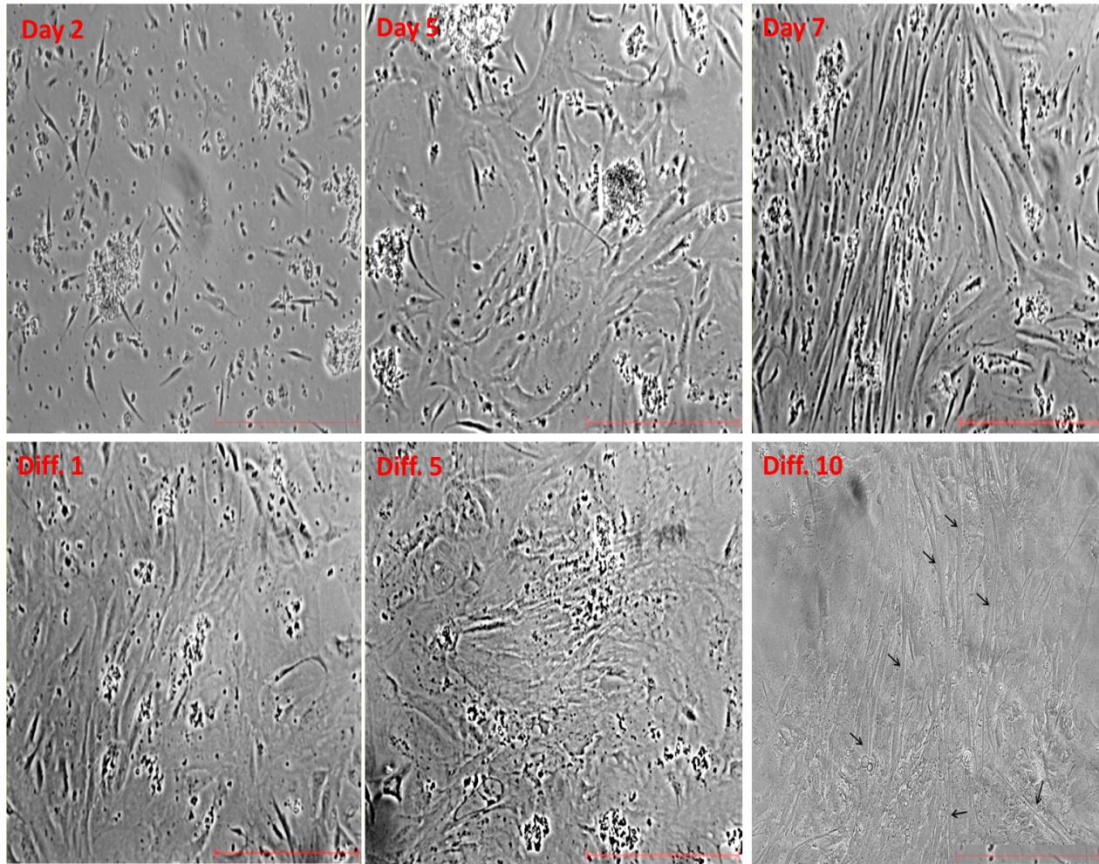
Figure 3.11: Zebrafish skeletal muscle cells cultured at different time points in monolayer starting from day1 till day 14. Cells isolated were cultured on gelatin coated glass coverslips in six well plates and monitored by taking images using phase contrast microscope. Here are images from day2, 5 and 7. At day 7 cells were switched to differentiation media when they start fusing and form myotubes, images during differentiation are diff 1 represents cells in differentiation media at day 1similarly diff 5 and diff 10 represents, in differentiation media after day 5 and day 10. Images were captured at 100X magnification and the arrows in diff. 10 image are denoting myotubes formed in the monolayer culture.

3.3.4 Identification of cells

The identity of cells cultured from zebrafish muscle tissue was investigated by

immunocytochemistry and qRT-PCR. For immunocytochemistry purpose cells

cultured were fixed and stained (see **Section 2.4.3**) for different stages of cell cycle;

sub-confluent cells, confluent cells, myotubes and mature myotubes. Desmin, is a

muscle marker for filamentous protein subunit of skeletal muscle cells (Li, Mericskay

et al. 1997), therefore cells cultured were stained with monoclonal anti-desmin

antibody specific for zebrafish (for desmin protein). Phalloidin (for actin filaments)

and DAPI (for nuclei) staining were also used as a positive control as explained in

section 2.4.3. Gene expression for myoD and myogenin gene which are genes

expressed only in cells of the skeletal muscle lineage were quantified for the same

stages of cell cycle parallel to immunocytochemistry to confirm genotype of muscle

cells using qRT-PCR. Experimental set up is shown in **figure 3.12** below.
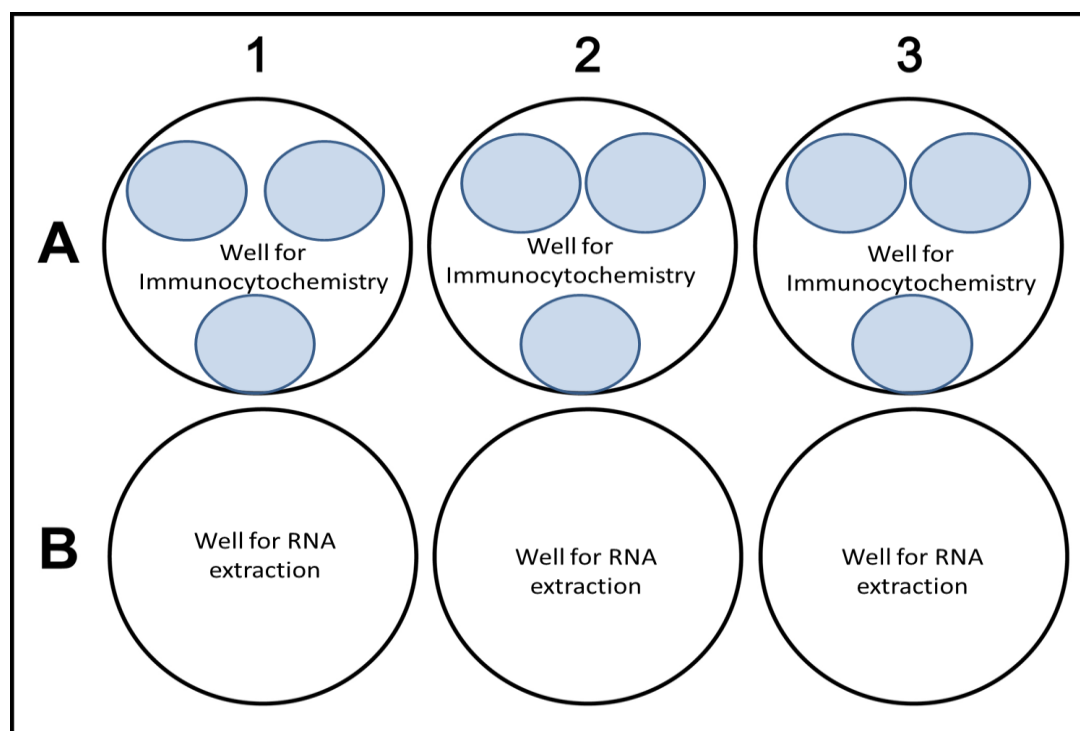
Figure 3.12: Diagrammatic presentation of the experiment conducted for identification of cells cultured from zebrafish muscle tissue using ICC and PCR. In top 3 wells coverslips (shown in round blue colour) were placed for ICC whereas in bottom 3 wells coverslips were not placed which were used for RNA isolation and later for PCR.

3.3.4.1 Immunocytochemistry

To determine the nature of cells obtained from optimised protocol, cover slips were

collected from cultures at different time points when the cells were at a sub-confluent

state, confluent state ready to be switched for differentiation and after formation of

myotubes. Cover slips were fixed and stained as described in material and methods

chapter two **section 2.4.3**.
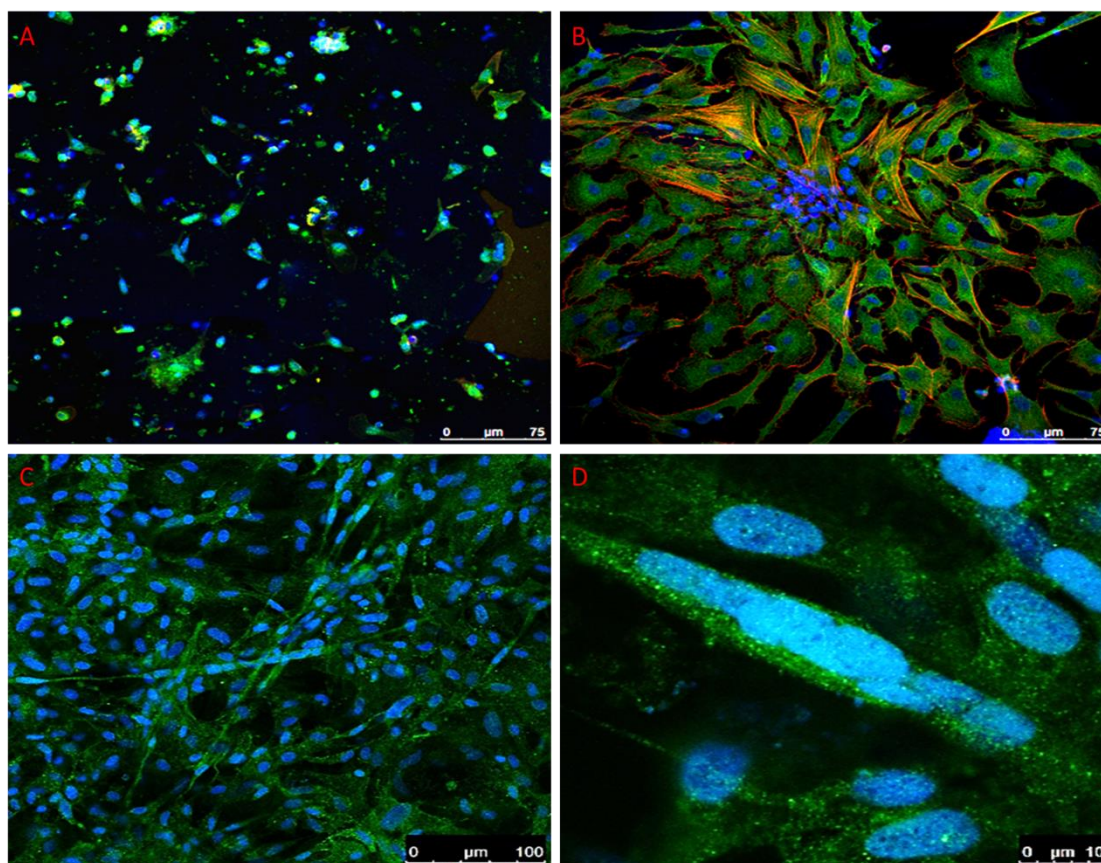
Figure 3.13: Images of zebrafish cells stained for desmin (desmin protein) in green colour, phalloidin (actin filaments) in red colour and DAPI (nuclei) in blue colour at different time points. A) Zebrafish muscle cells after one day in culture, B) Confluent zebrafish muscle cells (after 6-7 days in culture media) culture, C) Zebrafish muscle cells forming myotubes after being switched to differentiation media for 6-7 days culture, D) Zoomed-in view of zebrafish myotube (after 7 days in differentiation media. Red channel for action stain was reduced in C and D images in order to enhance green representing desmin in myotubes. Scale bars shown on individual images.

Results in above set of pictures (**Figure 3.13**) shows zebrafish cells stained at different time points with desmin showing in green colour for desmin protein, rhodamine phalloidin showing in red colour for actin filaments and DAPI showing in blue colour for nuclei. Cells cultured were confirmed to be zebrafish muscle cells, after staining them with desmin antibody specific for zebrafish skeletal muscle protein. When cells were approximately 80-90% confluent they were switched to differentiation media. Differentiation media was changed on alternate days and the cultures were monitored on phase contrast microscope for formation of more multi nucleated elongated cellular structures. These long multi nucleated elongated cellular structures were confirmed to be myotubes when stained with desmin (muscle specific protein) and DAPI (stain for nuclei), shown in figure 3.13.

3.3.4.2 qRT-PCR

At this stage only two genes were selected for the identification of zebrafish skeletal muscle cells base on their function i.e. myoD (marker for muscle cells); myoblast determination factor and myogenin (marker for differentiation). RNA was harvested from cells at different time points parallel to the immunohistochemistry time points and converted into cDNA (as explained in **section 2.5.2**). Expression levels for myoD and myogenin normalised to housekeeping gene β – actin were found using quantitative real time polymerase chain reaction at different time points i.e. control (just after isolation), after 2 days, 2 days in differentiation media and 5 days in differentiation media. RNA from cells just after isolation was run alongside different time points of cells cultured in mono layer and used as calibrator and termed as zero days on graphs (see **figure 3.14 & 3.15**). Primers sequences for myoD, myogenin and

β-actin were shown in table 2.1 in chapter 2 with their annealing temperature and

product size.
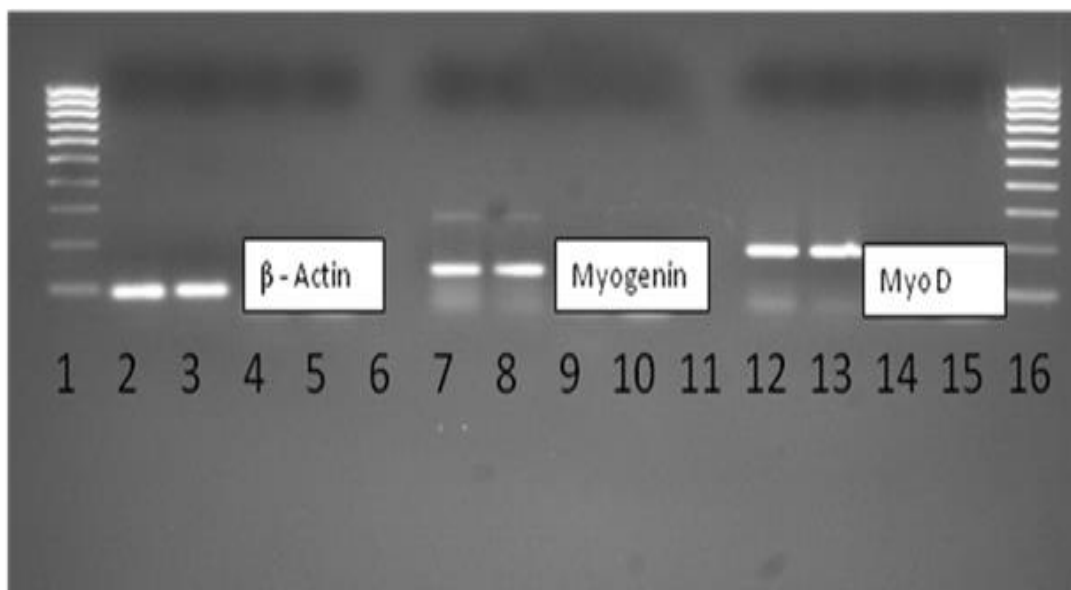
Figure 3.14: Micrograph of 2% agarose gel stained with ethidium bromide (5µg/ml) confirming different genes at their specific base pair sizes. Lanes 1 and 16 represents hyperladder IV (100 bp), lanes 7-8 and lane 12-13 represents myogenin (125bp) and myoD (176 bp) respectively along with internal control β-actin in lane 2-3 (98 bp).

Firstly expression of myoD, myogenin and β-actin genes was detected in the samples

run on agarose gel, which confirmed that the cultured cells were muscle cells as the

product size matched to those expected from sequence database as shown in **figure**

3.13. Expression pattern of myoD and myogenin at different time points was studied

using qRT-PCR. RNA was extracted and reverse transcribed into cDNA as explained

in chapter two, **section 2.5.3**.
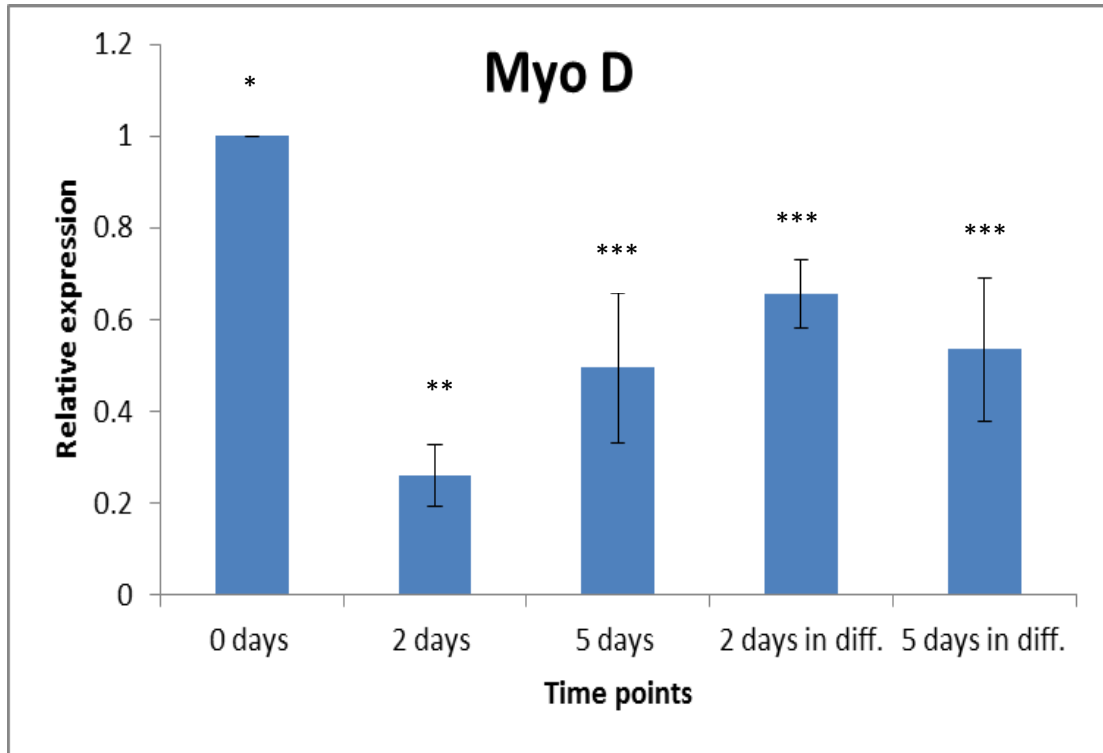
Figure 3.15: Relative gene expression pattern in zebrafish muscle cells cultured in monolayer for gene myoD. *Relative expression in plotted on Y-axis and time points in days on x-axis (n=3). Data was normalised against housekeeping gene β-actin and calibrated against zero days (cells just after isolation). Cell were cultured for first five days in growth medium until they were confluent, then taken out from cell cycle and switched to low serum differentiation media to help them in myotube formation. Statistically data was different from each other as p-value was less than 0.05 indicated by different letters.*

Gene expression pattern for myoblast determination factor- myoD in zebrafish skeletal muscle cells cultured in monolayer after one way anova statistical analysis was observed to be significantly decreased from 0 days to 2 days in whereas it increased from 2 days to 2 days in differentiation and at 5 days in differentiation ended at same level.
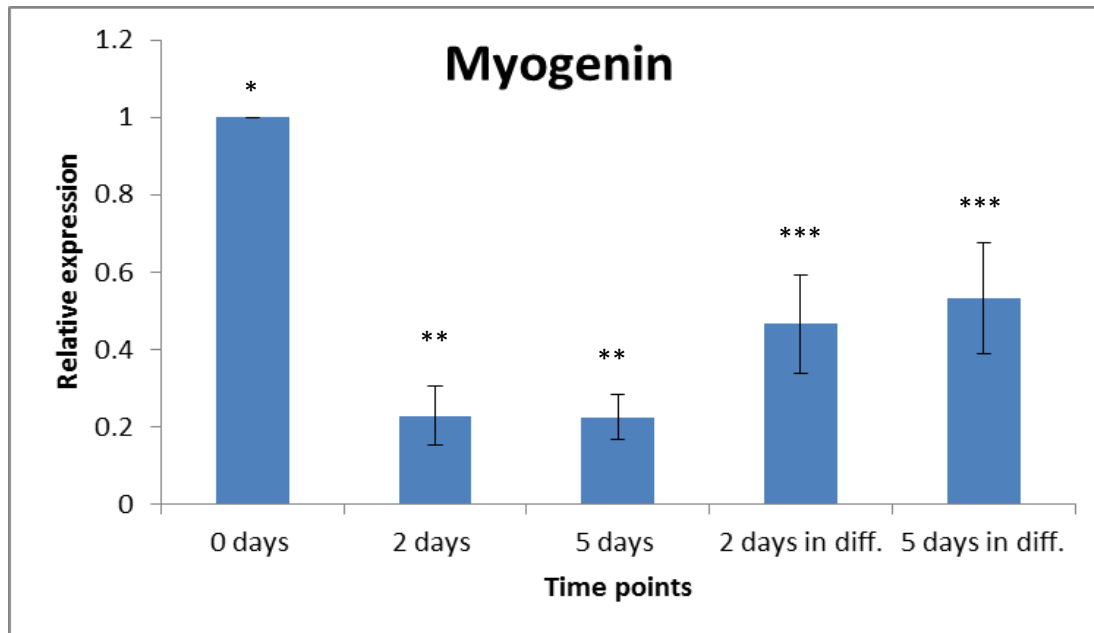
Figure 3.16: Relative gene expression pattern for gene myogenin in zebrafish muscle cells cultured in monolayer. Relative expression is plotted on y-axis and time points in days on x-axis (n=3). Data was normalised against housekeeping gene β-actin and calibrated against zero days (cells just after isolation). Cell were cultured for first five days in growth medium until they were confluent, then switched to low serum differentiation media to help them in myotube formation. Statistically significant differences were observed along different time points from each other as p-value was less than 0.05.

Cells grown in differentiation media were also confirmed differentiating by PCR results using myogenin gene marker for differentiation in muscle cells. Cells were cultured in normal growth media until day 5 and then switched to differentiation media. Results show (**Figure 3.16**) the sudden average mean increase in expression level of myogenin gene when cells were switched to differentiation media after 5 days in growth media, which concluded that zebrafish muscle cells were differentiating. In the literature it has been established that expression of myogenin peaks when skeletal muscle cells are confluent and ready to fuse therefore it was

expected that the expression of myogenin will increase during the differentiation as

shown in figure 3.15 and also confirmed by the ICC where formation of myotubes is

increased at same time point (shown in f**igure 3.12**).

3.4 Discussion

This work aimed to develop a novel isolation protocol for zebrafish skeletal muscle

cells. Cells isolated from zebrafish dorsal muscle firstly needed to be cultured in

monolayer and needed to optimise the culture conditions and essential media

ingredients. Cells cultured then needed to be identified morphologically and for

genetic traits of skeletal muscle cells using the specific primers designed for muscle

specific gene sequence of zebrafish.

A protocol for isolating and culturing zebrafish muscle cells was developed,

optimised and tested during this chapter. All the basic steps for isolating and

culturing skeletal muscle cells from different species were optimised for zebrafish as

described through-out this chapter. Most important was the tissue digestion step

where the muscle tissue is digested to break the basal lamina and release the satellite

cells which give rise to muscle progenitor cells. During the enzymatic digestion of

tissue timing and concentration of enzymes are very important factors, due to the fact

that the muscle tissue can either get digested for too long which can kill satellite cells

or tissue might not get digested enough to release the satellite cells (Williams,

Mckenney et al. 1995). Different culture media were tested to find the optimum

components for culturing zebrafish skeletal muscle cells as explained in **section**

3.3.1.3.1. As media supplemented with different serum and antibiotics provide

cultured cells nutrients and kills pathogens respectively, therefore different

combination from the literature were tested and later it was found that fish media L15

supplemented with l-glutamine, fetal bovine serum (FBS) and antibiotics was

optimum to culture zebrafish muscle cells also supported by results from **table 3.2**

and **figure 3.4**. All the primary data was documented in a log sheet in accordance to

particular isolation regarding the design of isolation and results observed every day,

shown in appendix-II.

To summarize, the present study was started with a published protocol for isolation of

skeletal muscle cells from salmon, which was further optimised for the different

parameters such as preparation of tissue, digestion of tissue, culture medium, coating

of dishes and use of antibiotics. Finally in order to obtain cultures without debris or

non-digested tissue in culture Ficoll density gradient step was included and reliable

protocol isolation and culturing for zebrafish muscle cells was developed. Using the

optimised protocol zebrafish muscle cells were cultured in gelatin coated six-well

plates containing glass coverslips. Cultured cells were recognized to be muscle cells

by two approaches; firstly using immunohistochemistry by staining with desmin,

phalloidin and DAPI for intermediate muscle specific desmin protein, actin filaments

and nuclei respectively as shown in **figure 3.13**, secondly by agarose gel

electrophoresis (**figure 3.14**) followed by quantitative polymerase chain reaction

using molecular markers for muscle cells such as myoD (**figure 3.15**) and myogenin

(**figure 3.16**). Whilst the optimisation was being undertaken during this study, for

isolation and culturing zebrafish muscle cells, a similar work was published by

Alexander and his colleagues (Alexander, Kawahara et al. 2011). That work was

published in March 2011, however the protocol optimised during the present study is

a significant improvement from that of Alexander and Kawahara et al 2011 published

protocol. In Alexander's study, during the enzymatic digestion step, collagenase type

IV along with protease for digestion of dorsal muscle tissue of zebrafish, whereas, in

the current study, we have optimised the use of collagenase type-I. Removal of

protease from the digestion step makes the current protocol cost effective without any

effect on the number or quality of zebrafish skeletal muscle cells isolated using the

protocol. The protocol optimised during the current study is time saving because in

the published protocol zebrafish muscle cells after isolation were initially pre-plated

for 12 hours, whereas, it was experimented and removed from the protocol optimised

during the current study, without compromising the features and number of muscle

cells. In the protocol devised by Alexander and his colleagues, zebrafish skeletal

muscle cells in culture were not found to be fusing and forming myotubes effectively

as shown from the immuno stained images in the article. In the present study,

however, cells were found to be fusing and forming myotubes as confirmed by

immuno-stained images presented in later chapters. A much improved protocol for

isolating and culturing zebrafish skeletal muscle cells have been developed during the

present study which, is a significant improvement from the protocol published by

Alexander et al (2011). Further, results obtained from the protocol optimised in the

current study have been compared with those of Alexander et al 2011, in chapters 4

and chapter 7.

Cells were stained with desmin antibody for filamentous desmin protein to confirm

their myogenicity (Bockhold, Rosenblatt et al. 1998) as shown in **figure 3.13**. Myod

and myf-5 have been considered to be first genes activated in single myogenic

satellite cells (Cornelison, Wold 1997, Smith, Janney et al. 1994). Therefore results were also confirmed by the help of qRT-PCR data (**figure 3.14**) which showed the expression of myoD gene (myoblast determination factor) in cultured zebrafish cells, which was found to be consistent throughout all the time points apart from after 2 days in differentiation media when it peaked. Statistically all the data was normally distributed which was tested using one-sample Kolmogorov-Smirnov test where the p-value was found to be less than 0.05, therefore one-way ANOVA along with Tukey's and bonferroni post hoc tests to find the significant differences among the samples. Therefore all data was found to be statistically different from each other in both genes i.e. myoD and myogenin. Similarly fused multi nucleated elongated cellular structures were also stained with desmin antibody for desmin protein to prove them elongated muscle fibres known as myotubes as shown in **figure 3.13**. Again myotubes were confirmed by qRT-PCR data for myogenin, marker for differentiated cells. Previous studies suggested that myogenin is a marker which increases its expression in differentiated cells (Edmondson, Olson 1989, Wright, Sassoon et al. 1989). Similar results were shown with differentiated zebrafish muscle cells in **figure 3.13**, where the expression level of myogenin was raised by 2 folds from day 2 to 5 days in differentiation when cells were switched to differentiation media shown in **figure 3.15**.

To conclude this chapter it can be said that a novel protocol for isolation of zebrafish skeletal muscle cells has been developed. Isolated zebrafish skeletal muscle cells were successfully cultured *in vitro* and monitored for different time points and were capable of forming long multi nucleated myotubes. These cells expressing muscle

specific protein and genes, confirmed using immunohistochemistry and qRT-PCR.

The next step was to characterize the cells and their differentiation ability further

(chapter 4), before putting a step forward towards the big aim of culturing and

characterizing them in a more physiological relevant 3 dimensional tissue engineered

constructs (chapter 5 and 6).

4. Characterisation of Zebrafish Muscle Cells in 2D monolayer Culture

4.1 Introduction

Development of a novel protocol for isolation of zebrafish skeletal muscle cells in the previous chapter (chapter 3) leads to the requirement for extensive characterisation of the muscle cells obtained from zebrafish using their 2D monolayer culture. To understand zebrafish skeletal muscle cell behaviour cultured monolayer it was necessary to characterize them on basic parameters such as the plating density, fusion efficiency, time required reaching confluence and percentage of desmin positive cells obtained per isolation.

Previous literature has suggested the importance of cell plating density on the mechanism of myogenesis using different skeletal muscle culture cell lines such as mouse myogenic cells, C_2C_{12} and human muscle cells (Blau, Webster 1981, Kishioka, Thomas et al. 2008, Bhasker, Friedmann 2008). The community effect phenomenon has been suggested to be involved in myogenesis where muscle precursor cells should contact sufficient number of like neighbours to achieve coordinated differentiation (Tanaka, Sato et al. 2011). Cell-cell contact which is mediated by cell surface molecule, cadherin-mediated adhesion promotes myogenic differentiation, whereas neural cell adhesion molecule (NCAM) have small role in myogenesis (Tanaka, Sato et al. 2011). Most biochemical studies on cultured muscle cells *in vitro* have been obtained either through explant cultures or dissociated monolayer of primary cells (Blau, Webster 1981). With both the techniques muscle cells were found

contaminated with diverse cell types including nerves, adipocytes and fibroblasts

(Blau, Webster 1981) and the ratio of muscle to non-muscle cells influences the

behaviour of muscle cells.

Skeletal muscle development process is defined using terms such as proliferation, cell

cycle withdrawal and differentiation followed by fusion into multinucleated

myofibres (Charge, Rudnicki 2004). Post-natal muscle growth which means addition

of myogenic cells to myofibres is a process called hypertrophy. Satellite cells stay in

a quiescent form until they receive a signal through neurons about injury, when the

quiescent satellite cells get activated to respond and attach at the injured myofibre or

attach with each other to form new myofibres, a process called hyperplasia

(Gabillard, Sabin et al. 2010). The expression of myoD is triggered as soon as the

cells get activated and start proliferating, followed by expression of myogenin at the

onset of differentiation (Yablonka-Reuveni, Rivera 1994). Initiation of differentiation

also triggers the expression of structural genes including both isoforms of myosin

heavy chain (Zammit, Partridge et al. 2006) and IGF, a gene responsible for

hypertrophy (human and mammalian cell lines) (Bower, Johnston 2009, Bower,

Johnston 2010). Myostatin, which belongs to the transforming growth factor beta

family, is a negative regulator of muscle mass (Johnston 2006). In a study with

transgenic zebrafish, where myostatin was over expressed, a small significant

increase in muscle fibre number was found relative to wild type zebrafish; with no

change in fibre size (Xu, Wu et al. 2003).

In fish species such as trout and carb (*cyprinus carpio*), where post larval muscle

growth is rapid and continuous, there is muscle hypertrophy and hyperplasia

involving the strong activity of satellite cells (Johnston 2006). The growth of fish muscle is completely different from mammals and birds, where hyperplasia plays in important role in muscle growth even after the juvenile stage (Koumans, Akster et al. 1990). However in mammals and birds the addition or formation of new muscle fibres stops shortly after birth and further muscle growth is mainly due to hypertrophy (Koumans, Akster et al. 1990).

Therefore the aim of this chapter is to characterize zebrafish skeletal muscle cells isolated using the protocol developed in the previous chapter and cultured in monolayer. Extensive characterisation of zebrafish skeletal muscle cells will be achieved using immunohistochemistry and quantitative real time pcr.

4.2 Material and methods

4.2.1 Cell Culture

Zebrafish skeletal muscle cells isolated were cultured and maintained as explained in chapter three. Zebrafish skeletal muscle cells were grown to eighty to ninety percent confluency and switched to low serum differentiation media, which helps them to withdraw from cell cycle and also to fuse and form long multi nucleated myotubes. Cells were fixed or RNA was extracted from different stages of cell differentiation cycle for immunohistochemistry and for qRT-PCR experiments to access the presence of different markers for muscle cells at different stages (see **Figure 1.7**). Zebrafish muscle cells grown to ninety percent confluence were tried to sub-culture through passaging them.

Each isolation performed yielded a different number of cells due to the fact that a different pool of zebrafish were sacrificed each time, therefore cells were counted using a haemocytometer (**Section 2.2.1**) and plated on gelatin coated coverslips in six well plates at 100,000 cells/cm^2. Zebrafish skeletal muscle cells were fixed using ice-cold methanol-acetone as described in **section 2.4.2** at different stages of cell differentiation such as sub-confluent, confluent cells, early myotubes and mature myotubes. Fixed coverslips were subsequently used for immunocytochemistry.

4.2.2 Immunohistochemistry

Cultured zebrafish skeletal muscle cells were immuno-stained (according to **Section 2.4.3**) for desmin protein: a filamentous protein specifically expressed by cells committed to the myogenic lineage and counter stained with DAPI for nuclei. Zebrafish specific monoclonal antibody was purchased from Abcam, UK and used at

1 in 200 dilutions for overnight primary staining (As explained in **Section 2.4.3**),

followed by staining with TRITC as secondary antibody. Stained cells were imaged

using Leica confocal microscope and analysed for the number of muscle precursor

cells by calculating the total number of nuclei co-expressing desmin as a proportion

of the total nuclei. Myogenicity of differentiated muscle cells was analysed by

calculating the number of nuclei incorporated into myotubes as a proportion of the

number of total nuclei. A myotube was defined as a desmin positive cell with three or

more nuclei. Myotubes formed were also analysed by measuring their length and

width and total number of myotubes counted per frame.

4.2.3 PCR

RNA was extracted and collected from different stages of cell developmental stages

using TRIzol method as explained in section 2.5.1. RNA was converted into cDNA as

explained in **Section 2.5.2** and cDNA samples were amplified by PCR and run on 2%

agarose gels according to protocol described earlier (see **Section 2.5.4**). PCR was

used to identify the expression of different markers of skeletal muscle cells cultured

at different stages of cell cycle. Housekeeping genes EF1 α and β-Actin were always

run parallel to gene of interest as a control and agar gels were viewed as described

earlier in **Section 2.5.4.** The list of primer sequences used in these experiments is

provided in **Table 2.1**.

4.2.3.1 Quantitative PCR analysis

Once the c-DNA sample preparation was complete (see **Section 2.5.6**), the samples

were loaded into a Rotogene 6000Q (Qiagen) using a 72 well round plate. All

reactions were performed in triplicates and included a no reverse transcriptase control

111

and no template control. Fluorescence at the end of each extension step was measured using excitation at 470 nm and emission at 510 nm and the fluorescence values were converted to relative expression values as explained in **Section 2.5.6**.

4.2.4 Statistical Analysis

All experiments were repeated at least three times using isolations carried out on different days from different lot of fish every time. For all zebrafish muscle cell cultures, Shapiro-Wilk tests were conducted in order to determine if desmin positivity and myogenicity data was normally distributed. Where data was not normally distributed, Kruskal-Wallis test was used to analyse differences between different cultures and Mann-Whitney post-hoc tests were conducted to determine inter-data differences. Where data were normally distributed and variation was homogeneous one-way ANOVA was used with a bonferonni post-hoc test. Significance was taken at an alpha value of .05 for parametric tests, and was adjusted according to the number of pairwise comparisons for non-parametric tests (.05/number of comparisons). The number of nuclei per myotube were analysed by univariate multiple factorial, where as well significance was measured at an alpha value of 0.05. All statistical analyses were performed using SPSS version 19.

4.3 Results

In this chapter zebrafish skeletal muscle cells were isolated from zebrafish muscle tissue using enzymatic digestion method (as explained in chapter 3) and cultured in monolayer. The cellular, molecular and physiological characteristics were examined and analysed extensively by immunohistochemistry and quantitative real time polymerase chain reaction.

4.3.1 Passaging zebrafish skeletal muscle cells

Zebrafish muscle cells were grown up to 80-90% confluence detached off the substrate; cells were again plated at the original plating density in order to sub-culture them. Zebrafish skeletal muscle cells were passaged three times from three different isolations without any success. After passage survival rate per isolation was between 80% to 90% but only 2-5% of cells were found to be attached from the total number of cells plated and they also survived for couple of days only (as shown in **Figure 4.1**).
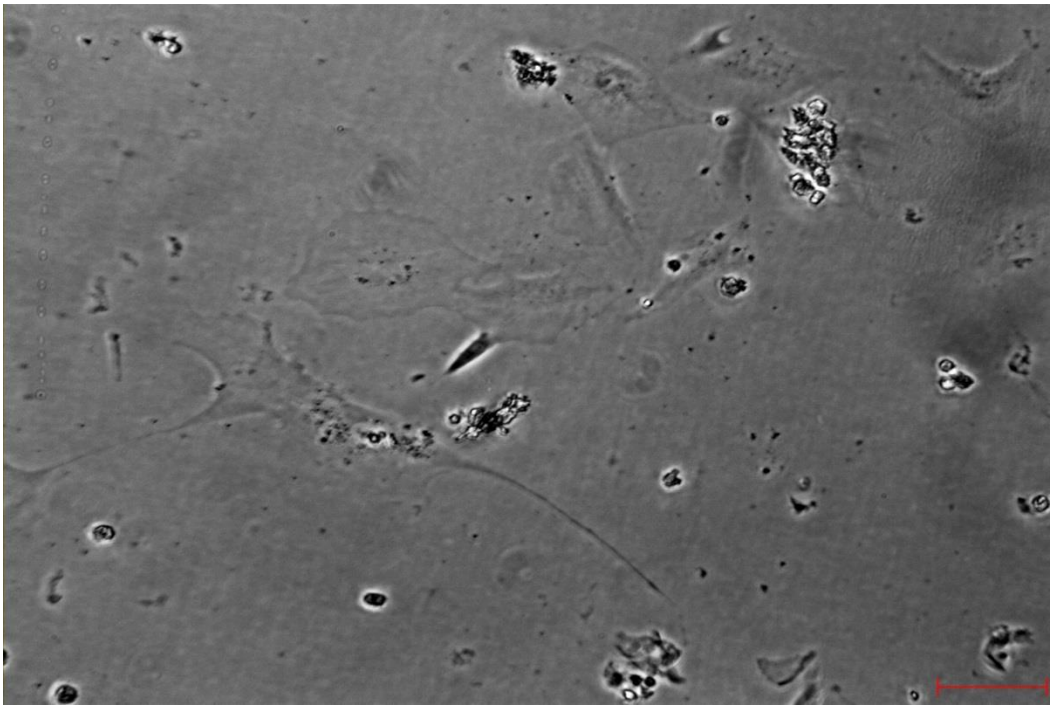
Figure 4.1: Effect of passaging on zebrafish skeletal muscle cells in culture for 3 days in monolayer. *From the image (taken at 100X magnification) it can be observed that cells are widely spread in search for other cells because of the fact that most of cells plated after passage didn't adhere to the matrix.*

4.3.2 Cryo-freezing of zebrafish skeletal muscle cells

Zebrafish skeletal muscle cells were grown up to 80-90 % confluence and detached

off from the substrate using 0.25 % trypsin, centrifuged and pelleted. Cell pellet was

re-suspended in 90% FBS and 10% DMSO (as explained in **Section 2.2.2**) in a cryo-

vial and placed in Mr. Frosty (equipment used for slow cooling) for slow cooling in

freezer. Cryopreserved zebrafish skeletal muscle cells were resuscitated via quick

thawing and plated on gelatin coated glass coverslips placed in six well plates.

Cryopreservation of zebrafish muscle cells was repeated at least three times using

three different batches of zebrafish each time. After resuscitated and counted using

haemocytometer, when plated, it was observed that only 2-5% of zebrafish muscle

cells were able to attach to the substrate. After 3-5 days in culture these 2-5% of

attached zebrafish muscle cells also reach the cell senescence (as shown in Figure

4.2).
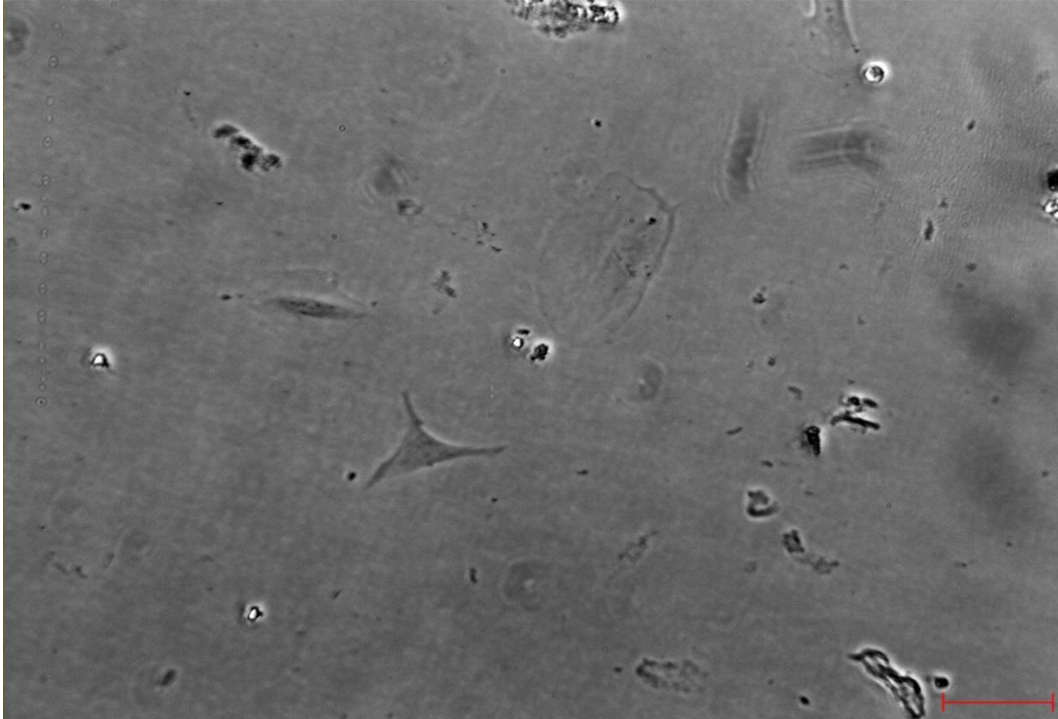
***Figure 4.2: Cryo-freezed zebrafish skeletal muscle cells resuscitated and
cultured for 3 days.*** *Approximately 90-95% of Zebrafish skeletal muscle cells
were not able attach to the substrate therefore died and the rest which attached
reached cell senescence after 3-5 days in culture. Image captured at 100X
magnification.*

4.3.3 Immunocytochemistry of ZMC's

Zebrafish skeletal muscle cells were fixed and blocked using acetone: methanol method as previously explained in **Section 2.4.3**, for different time point of a cell differentiation which were; sub- confluent cells, confluent cells, early myotubes and mature myotubes. Fixed cells were stained for monoclonal desmin antibody, for muscle specific desmin protein and counterstained for DAPI for nuclei. In order to determine the number of microscope images required for morphological analyses to obtain valid data, a cumulative frequency analysis was performed. In these analyses, the outcome variable from each image e.g. desmin positivity, was averaged until the error bar became negligible. A total of 25 images per cover slip were captured using Leica confocal microscope at 20x objective magnification (Leica, UK) and analysed using Image J (Java) software (National Institutes of Health, USA).
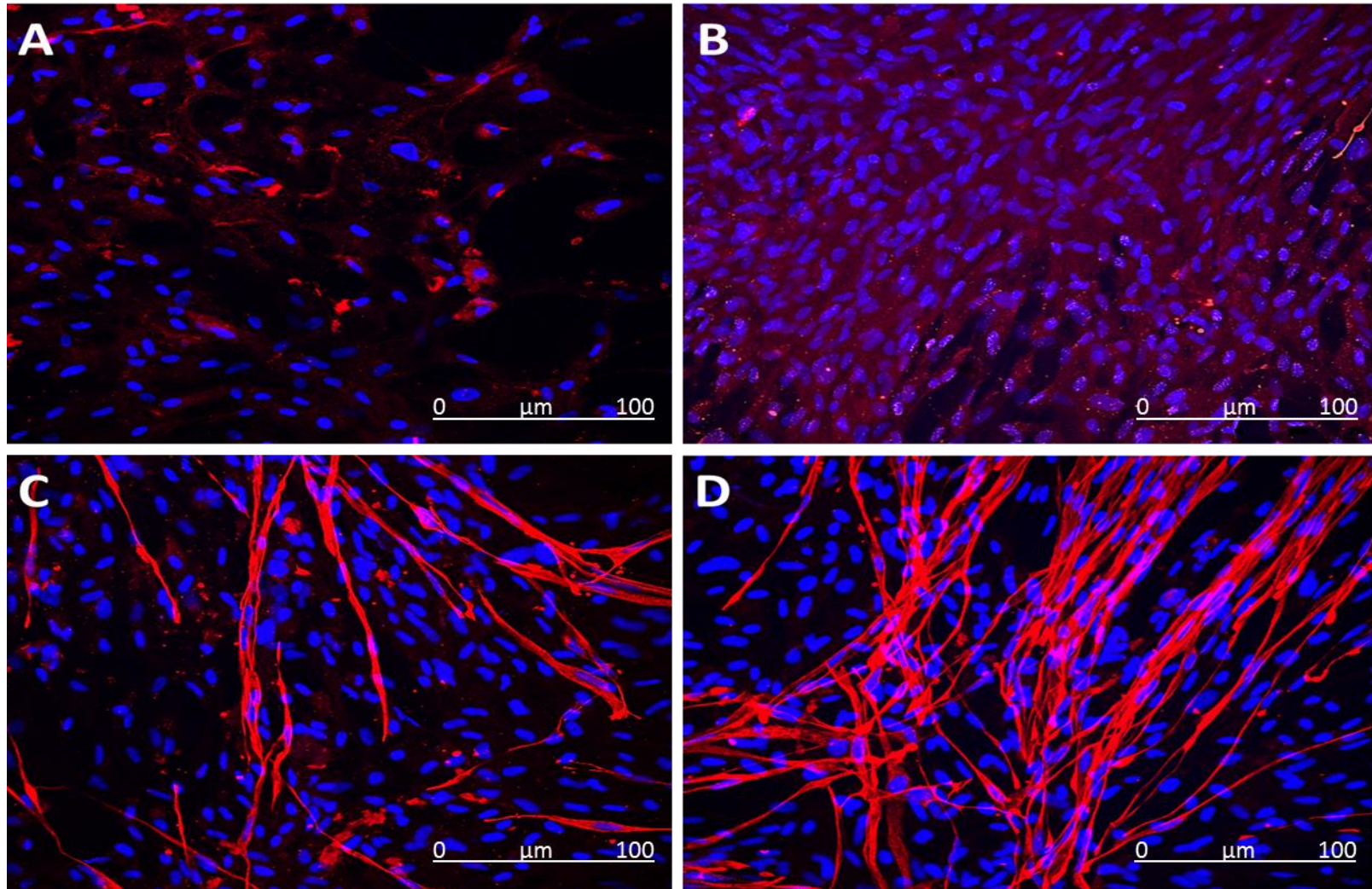
Figure 4.3: Immuno-stained images of zebrafish skeletal muscle cells in monolayer at four different stages. *Red
represents desmin stained by desmin antibody and blue represents nuclei stained by DAPI. A) Zebrafish skeletal muscle
cells at sub-confluent stage, B) Cells at confluent stage, C) Cells at early myotube stage, D) Cells at late myotube
stage. Images captured at 100X magnification.*

117

4.3.3.1 Desmin positivity

Desmin positivity of the isolation was measured as a ratio between desmin positive nuclei and total number of nuclei. Repeatability of cultures was determined by observing the consistency of desmin positivity per isolation which was accounted by average of desmin positivity from 25 images per isolation. The table 4.1 below shows desmin positivity of 15 different cultures measured and analysed using Image J software. Desmin positive cells obtained per isolation were 23.67 ±2 on average, which was fairly consistent throughout.

Table 4.1: Average desmin positivity per isolation for different cultures. *Desmin positivity was measured from all isolations to confirm the effect of other parameters on muscle cell in culture.*

Isolation number	Total number of cells obtained	Average desmin positivity
#001	10 million	26.6%
#002	9 million	23.6%
#003	10 million	23.4%
#004	14 million	23.5%
#005	10 million	23.8%
#006	15 million	23.3%
#007	15million	23.6%
#008	16million	23.8%
#009	18 million	23.3%
#010	16 million	24.1%
#011	13 million	23.6%
#012	15million	23.6%
#013	15 million	23.7%
#014	16 million	24.2%
#015	15 million	23.9%

4.3.3.2 Fusion index of cultures

Desmin positivity of a culture does not necessarily define the fusion capability of muscle cells in culture therefore fusion index of isolation was measured. Fusion index is measured as a ratio between number of nuclei present in myotubes and total number of desmin positive nuclei. Fusion index was measured and compared from the images captured from early myotube (3 days in differentiation media) and mature myotube (10-12 days in differentiation media) stages. Coverslips were taken from same isolations at different time points and stained for this experiment. Statistically there was no significant difference found between stages at early and mature myotube fusion index (p=0.780). Average of n=3 different isolations, from 25 images captured at early and late myotube time point were considered for statistical analysis (as shown in **Figure 4.4**).
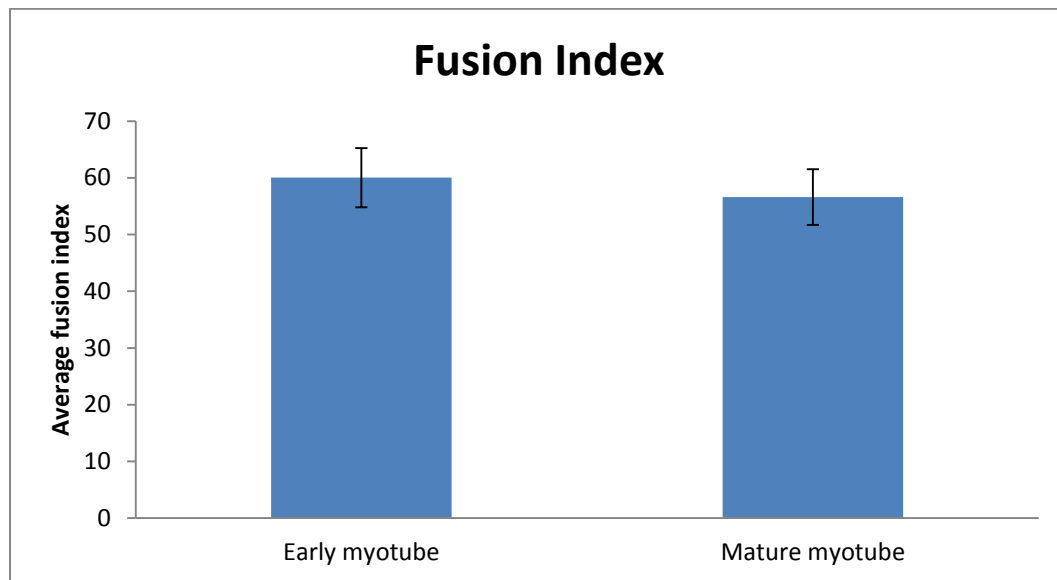
Figure 4.4: Fusion efficiency of zebrafish skeletal muscle cells in-vitro at two different time points (n=3). *Statistically no significant difference (p>0.05) was found in the fusion efficiency of cells at early and mature myotube stage.*

4.3.3.3 Myotube number and measurements

A myotube is a cellular structure formed by the fusion of single myoblasts and should have encapsulated at least 3 or more than three nuclei. Formation of myotubes in *in-vitro* cultures bring them closer to the *in vivo* muscle structure, therefore the higher the number of myotubes formed, the greater the degree of success of monolayer cultures. Total number of myotubes were counted from images captured from early and mature myotube stages from three different isolations (n=3). Independent sample t-test was performed to find any statistical difference between the two groups. A total of 25 images per time point were analysed for statistical analysis. Statistically it was found that there was no significant difference between the number of myotubes at early and mature myotube stage (p=0.912).
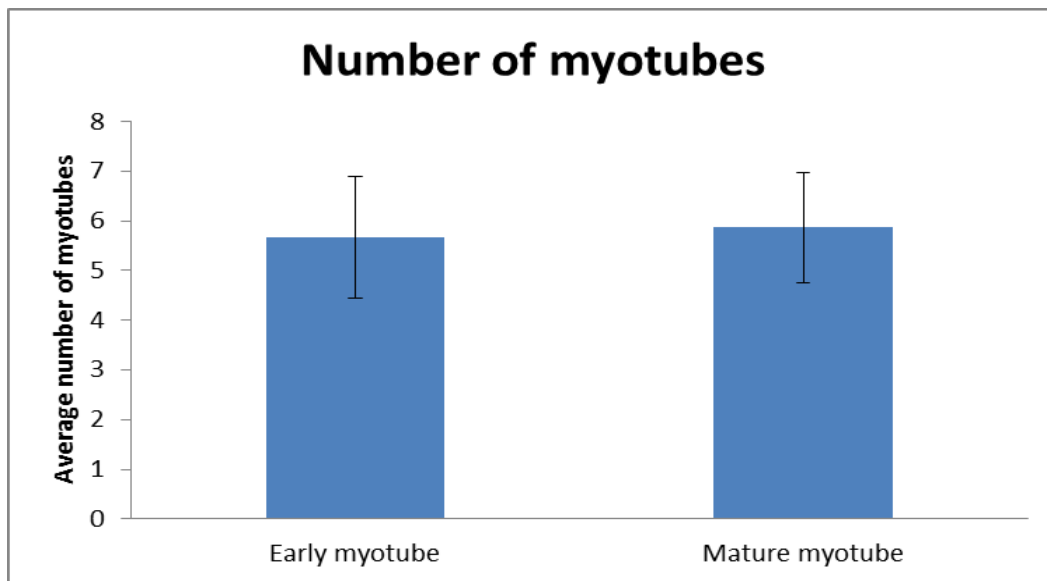
Figure 4.5: Graphs illustrates the average number of myotubes formed at early and mature myotube stages (n=3). *Statistically no significant difference was found in the data from early and mature myotubes stages (p>0.05). Error bars represents mean standard deviation.*

4.3.3.3.1 Nuclei per myotubes

Number of nuclei per myotube was counted to in order to determine whether the number of nuclei per myotube is increasing post differentiation or the myotubes are only increasing in length and width (**Section 4.3.3.3.2**). Therefore a total number of nuclei per myotube were counted from 25 images captured from early and mature myotube stage from three different isolation (n=3). Univariate factorial anova was performed in order to find any significant difference between the groups of different number of nuclei's for early and mature myotubes. Statistically there was no difference found between early and mature myotubes (p=0.721) within different groups of number of nuclei's, but there was significant difference observed in between different groups of number of nuclei's. There was significant difference observed between a combined group of early and mature myotubes of 3 nuclei/myotube from combined group of early and mature myotubes of 6 (p=0.002), 7 (p=0.00) and 8/8+ (p=0.001) nuclei/myotube. Similarly significant difference was observed between combined group of early and mature myotubes for 4 nuclei/myotube from a combined group of early and mature myotube for 6 (0.035), 7 (0.001) and 8/8+ (0.011) nuclei/myotube.
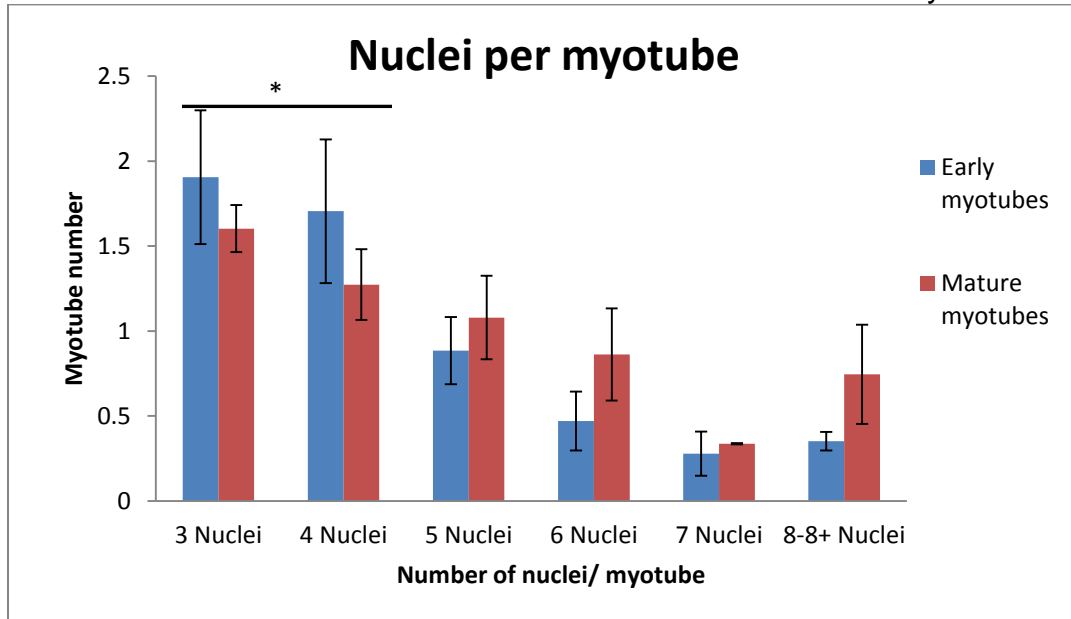
***Figure 4.6: Comparison between early and late myotube groups across with groups
of different number of nuclei.*** *Graph illustrates number of nuclei on the x-axis and
myotube number on y-axis (n=3). Statistically there was no difference found between
early and mature myotubes for different groups of number of nuclei's (p>0.05). But
statistical difference was observed between the combined groups of early and mature
myotubes for different groups of number of nuclei.*

4.3.3.3.2 Length and Width of myotube

Myotubes were measured in order to determine the average length and width of monolayer cultured zebrafish skeletal muscle myotube. Length of a myotube was measured using Image J software and width of a myotube was determined by measuring diameter of myotube from three equidistant points on each myotube such as one from right end, one from left end and one from middle of a myotube, to obtain the mean value for average width of a myotube.

Independent sample t-test was performed to find any statistically significant difference in the length and width of zebrafish myotubes at early and mature myotube stages. Length of myotubes was found to be significantly increased (p=0.02) from early to mature myotubes stage and width of myotubes was also found significantly increased (p=0.00).
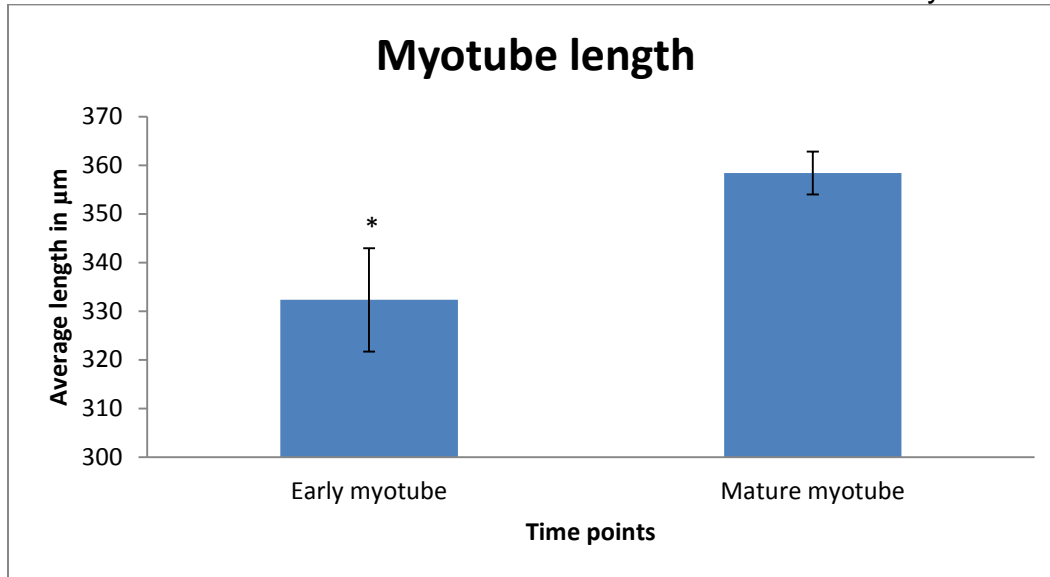
***Figure 4.7: Average length of zebrafish muscle myotubes measured in micrometres
at early and mature myotube stage (n=3).*** *Statistically there was a significant
difference observed in between two groups (p=0.02).*
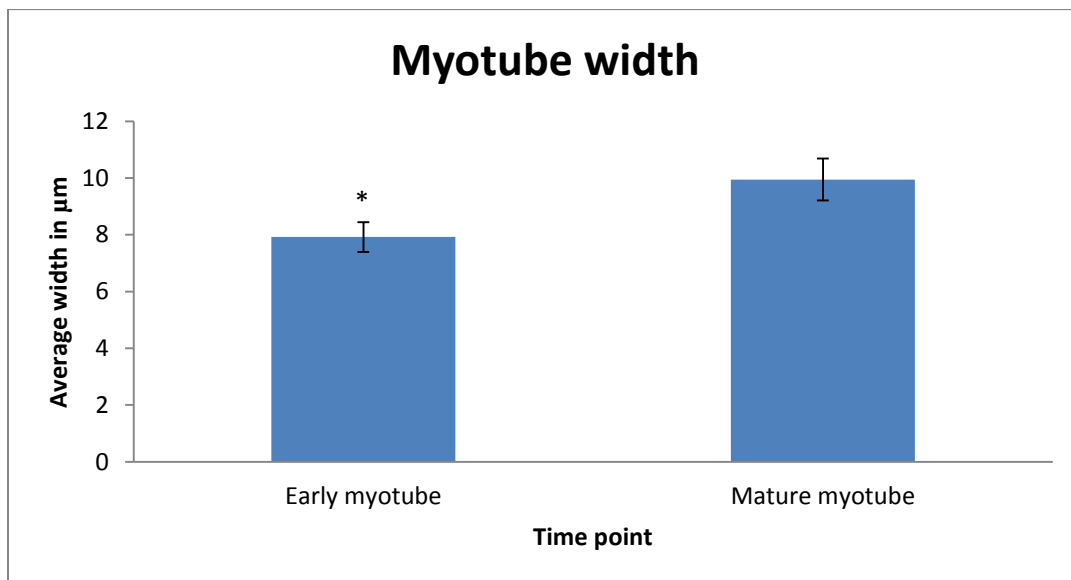
***Figure 4.8: Average width of myotubes measured from three equidistant points
from early and mature myotubes (n=3).*** *Statistically data suggests that there is a
significant increase in width of myotubes from early to mature stage (p=0.00).*

4.3.4 Importance of IGF in zebrafish muscle cell culturing

Insulin like growth factor (IGF) has been reported in the literature as an important ingredient in culturing and differentiating muscle cells to form myotubes for different species (Gabillard, Sabin et al. 2010). An experiment was designed and performed to determine the effect of insulin on zebrafish skeletal muscle cells cultured in monolayer by adding IGF (human recombinant IGF at 10ng/ml) in differentiation media. Two different combination of differentiation media tested were L15 media + horse serum + IGF + antibiotics and L15 media + horse serum + antibiotics. Results showed that addition of IGF in differentiation media kills zebrafish muscle cells as shown in **figure 4.9** below.
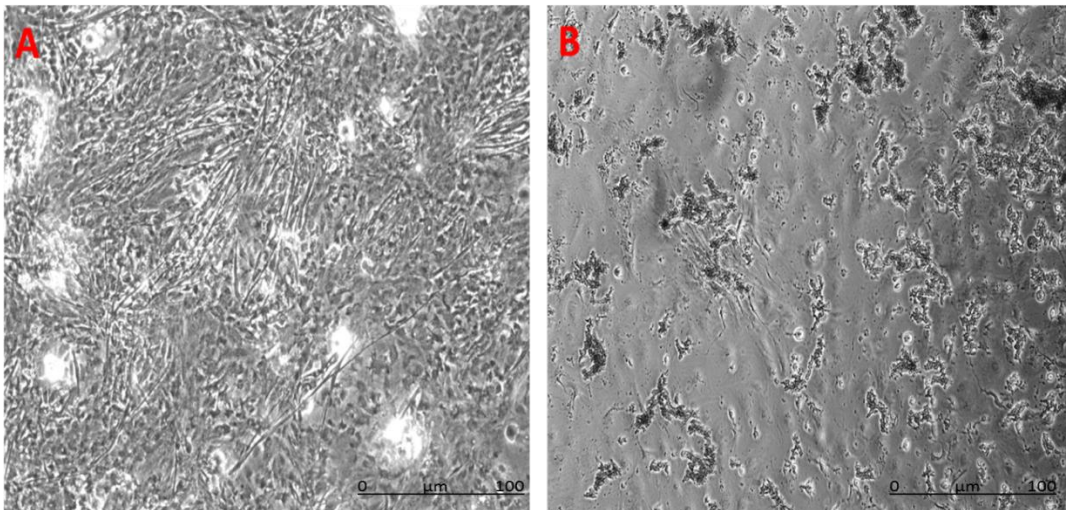
Figure 4.9: Impact of IGF on zebrafish skeletal muscle cells cultured in monolayer after 7 days in differentiation media with or without IGF. A) Representing image from cells differentiated using L15 + horse serum, B) Representing image from cells differentiated using l15 + horse serum + IGF. All images were captured at 100X magnification using phase contrast microscope.

4.3.5 qRT-PCR

Zebrafish skeletal muscle cells were sampled for RNA extraction (explained in **Section 2.5.1**) at different time points parallel to time points selected for immunohistochemistry; after isolation, sub-confluent, confluent cells, early myotubes and late myotubes. RNA was converted to cDNA (see **Section 2.5.2**) and used for analysis or measure expression of different genes involved in deciding myogenic fate, muscle development, maturation and differentiation during those phases of cell cycle.

Expression of Myod at early myotube stage was found to be significant higher from all the other time points (see **figure 4.10**). mRNA expression for myogenin at after isolation and early myotube was found significant different from all other time points apart from after isolation being not significantly different from myotubes, whereas at sub-confluent and confluent stages expression was lowest compare to all other time points and confluent stage was also not statistically different from mature myotube stage (**Figure 4.10**). Myostatin mRNA expression at after isolation was significantly higher from all other time points followed by confluent and myotube stages, where expression was found similar but myotube stage expression was different from sub confluent and early myotube stage, however the expression level at sub confluent, confluent and early myotubes stages was found significantly similar (**Figure 4.10**). mRNA expression for M-Cadh was found significantly similar at all the time points apart from early myotube stage where it was significantly higher from all the other time points (**Figure 4.10**).
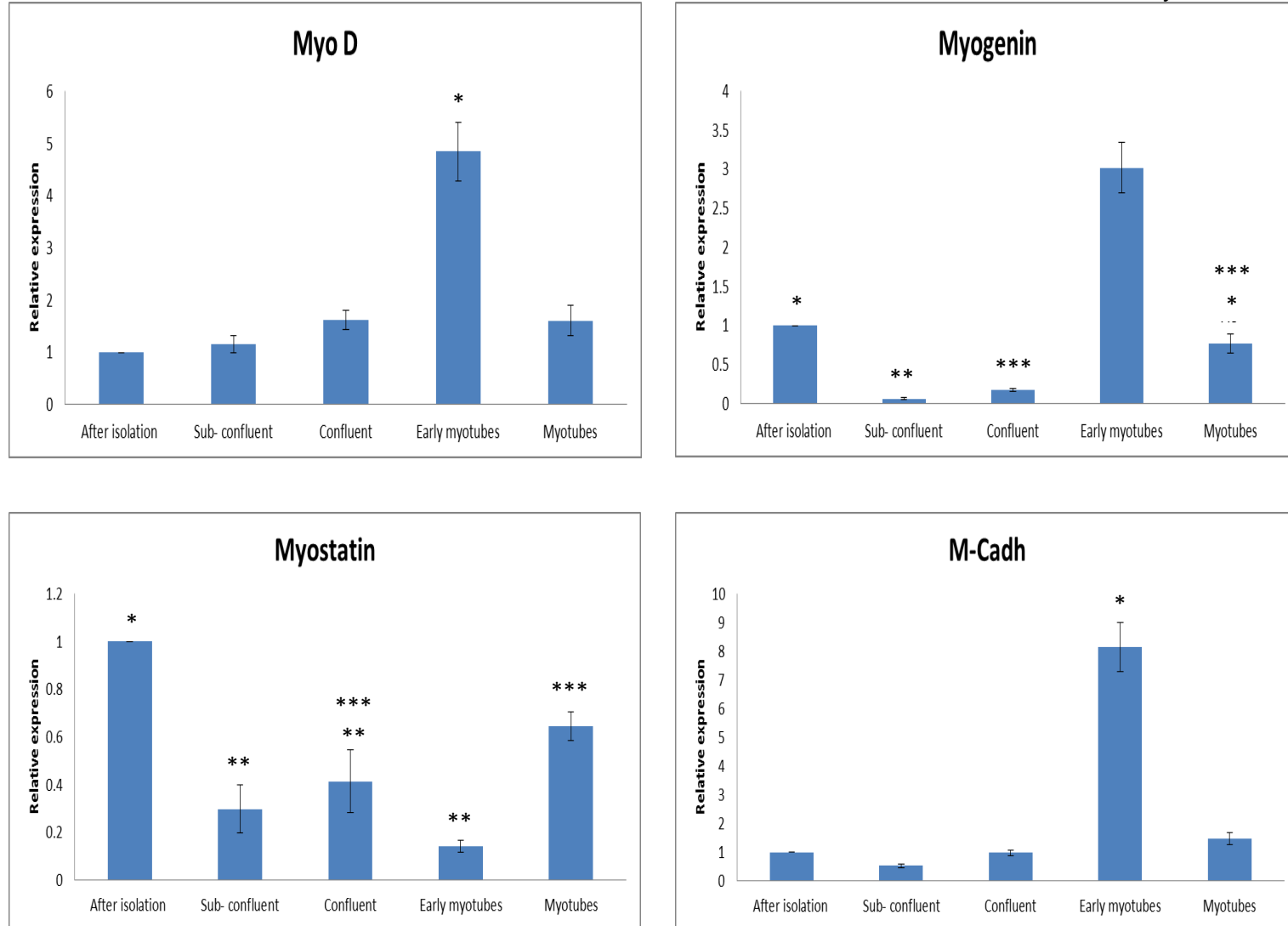
***Figure 4.10: mRNA expression analysis for MyoD, Myogenin, Myostatin and M-Cadh in monolayer at different
time points.*** *N=3 at each time point. Data presented as mean ±SD with relative expression on y axis and time points on
x axis. Significant difference was measured at p>0.05 and represented by different letters.*
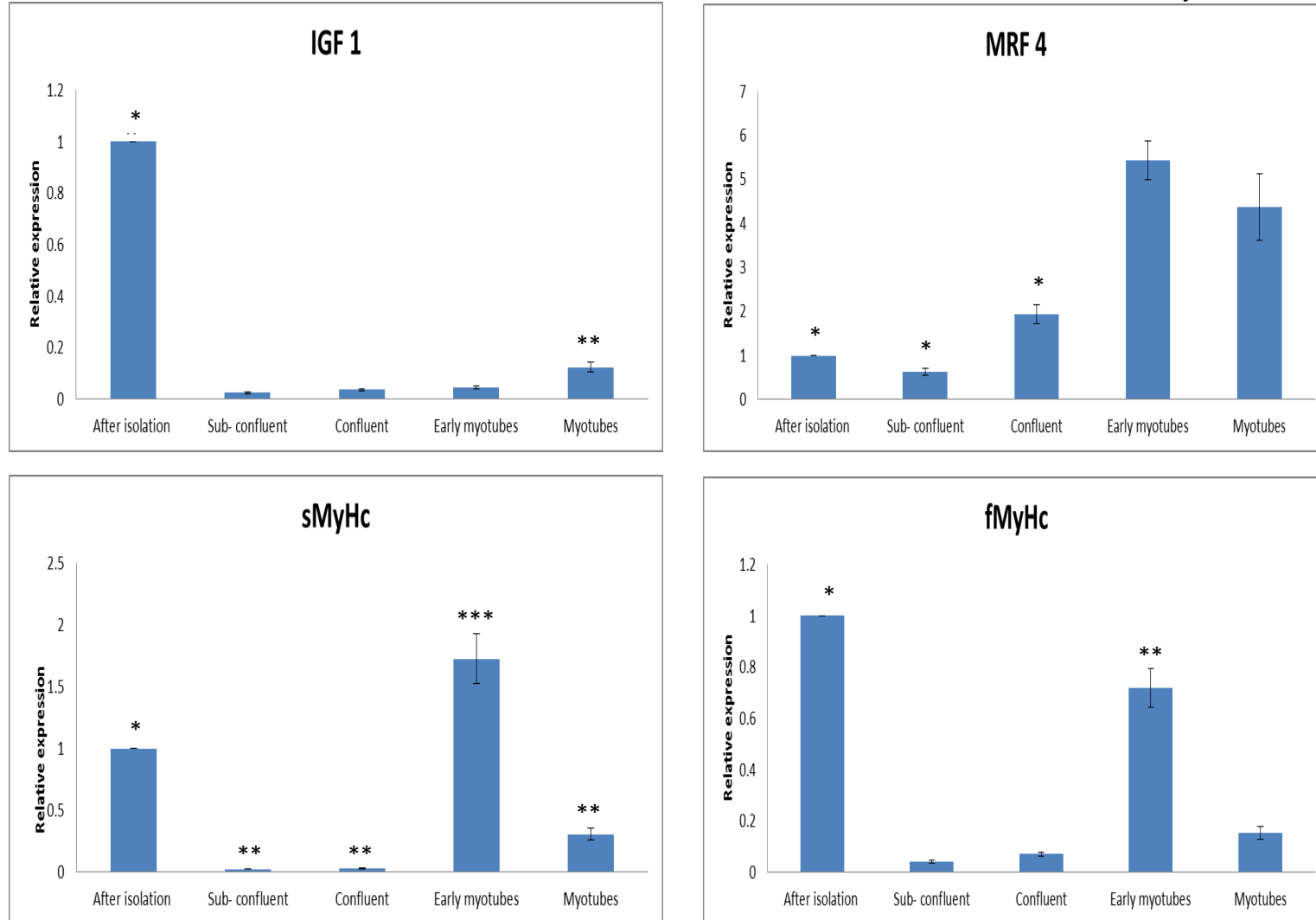
Figure 4.11: mRNA expression analysis for IGF1, MRF4, sMyHc and fMyHc in monolayer at different time points. N=3 at each time point. Data presented as mean ±SD with relative expression on y axis and time points on x axis. Significant difference was measured at p>0.05 and represented by different letters.

Expression of IGF1 at after isolation stage was found significantly higher from all the other stages, whereas sub confluent, confluent and early myotube stages showed similar expression levels of IGF1 which was significantly lower than after isolation and myotube stages expression (see **figure 4.11**). MRF4 expression was found significantly similar at after isolation, sub confluent and confluent stages but it increased at early myotube and myotubes stages which were also similar to each other but different from other time points.

mRNA expression for sMyHc and fMyHc had a similar kind of expression pattern where after isolation and early myotube stage were significantly different from each other and all other time points. However mRNA expression at sub confluent, confluent and early myotube were significantly similar to each other and lower from other time points. Expression of sMyHc was found higher at early myotube stage from after isolation stage whereas, it was reverse in case of fMyHc (see **figure 4.11**).

4.4 Discussion

Zebrafish skeletal muscle cells were successfully isolated and cultured in 2 dimensional monolayer cultures, followed by characterisation of these zebrafish skeletal muscle cells in this chapter.

4.4.1 Cryo-freezing and passaging of zebrafish skeletal muscle cells

Zebrafish skeletal muscle cells were attempted to cryo-freeze and passage with minimal success in section **4.3.2** and **4.3.1** respectively. The pattern for the failure of both these experiments looked very similar for zebrafish skeletal muscle cells as in both the experiment they failed to re- adhere to the substrate in monolayer. Zebrafish skeletal muscle cells were successfully detached off from the substrate for passaging them but after the cell count using haemocytometer, when plated again on the gelatin coated plates 95% of cells failed to attach and died eventually (as shown in **Figure 4.1**). Similar to passaging, zebrafish skeletal muscle cells were successfully cryo-freezed, resuscitated and counted using haemocytometer before plating them on gelatin coated plates, where again only 5% zebrafish skeletal muscle cells were able to adhere again as shown in **Figure 4.2**.

In a study from Dodson and his colleagues on rainbow trout and yellow perch fish, they found it challenging to cryopreserve, thaw and re-plate their satellite cells with minimal success rate of 5-27% and 14-30% respectively for both species (Dodson, Kinkel et al. 2008).

4.4.2 Morphological analysis of Zebrafish skeletal muscle cells

Zebrafish skeletal muscle cells were cultured and immuno-stained at different time points and morphologically analysed for better understanding. Most of the analysis

performed on immuno-stained zebrafish skeletal muscle cells was on later stages of culture i.e. early and late myotubes. Fusion efficiency of zebrafish skeletal muscle cells at early and late myotube stages was not found statistically different from each other (p=0.780), which suggests that the number of desmin positive nuclei fused to form myotubes does not increase over time. Fusion efficiency data was confirmed when number of nuclei per myotube was also found not significantly different at early and late myotube stages. However myotubes formed of 3 or 4 nuclei were found significantly higher in both early and late myotube stages from myotubes formed from 5, 6, 7 and 8 nuclei per myotube.

No significant difference in myotube number, fusion index, nuclei per myotube of Zebrafish skeletal muscle cells in monolayer culture at early and late myotube stages suggests that the number of nuclei once committed to formation of myotube does not increase over time, therefore hyperplasia is not responsible for muscle growth as suggested in previous literature for fish species by Koumans and his colleagues (Koumans, Akster et al. 1990) supported by other previous literature in the article. Hypertrophy is defined as growth in the size of skeletal muscle mass due to increase in its cellular components whereas hyperplasia is increase in number of skeletal muscle cells or increase in specific number of muscle cells (Johnston 2006). Data obtained for hypertrophy of zebrafish muscle cell shows significant increase in size of cells from early to late myotube stage suggesting that myotubes once formed in culture increase in length and width but there no difference in number of nuclei per myotubes, which again conflicts with literature where it has been suggested that in

fish muscle cells growth is mainly due to hyperplasia not hypertrophy as compare to

mammals and birds (Koumans, Akster et al. 1990, Johnston 2006).

4.4.3 IGF kills zebrafish muscle cells

In the literature IGF has been used extensively for different mammalian and non-

mammalian species in differentiation media for skeletal muscle cells to enhance the

formation of myotubes when myoblasts reach confluence (Gabillard, Sabin et al.

2010, Martin, Passey et al. 2013). In a study by Gabillard et al. 2010, they found five-

fold increase in cell proliferation of trout satellite cells when 50 nM IGF1 was added

to media and four fold increase in differentiation after addition of IGF1 (Gabillard,

Sabin et al. 2010). Role of IGF-1 in fish skeletal muscle cells is still unclear but

Castillo Juan and his colleagues have shown that IGF-1 enhance the glucose uptake in

primary cultures of rainbow trout myosatellite cells; and it further increases as the

cells develop into myotubes *in vitro* (Castillo, Codina et al. 2004). They have also

shown that IGF-1 enhances the L-alanine uptake compare to insulin and this decrease

as the cells differentiate and form myotubes (Castillo, Codina et al. 2004). IGF-1 has

also been noticed to stimulate the proliferation of trout muscle cells in their model

whereas insulin does not.

Jean C. Gabillard and his colleagues have shown in their study that the proliferation

and differentiation of trout muscle cells is highly responsive to growth factors

(Gabillard, Sabin et al. 2010). They observed two fold increases in stimulation of

trout muscle cell proliferation in DMEM with 50 nM IGF-1 and the same dose of

IGF-1 lead to five-fold increase in proliferation when cells were incubated in F10

media instead of DMEM. Trout satellite cell proliferation was observed to be

increased by four-fold with 50 nM IGF-1 and two-fold with FGF-2 under same

conditions (F10 + 2% FCS) (Gabillard, Sabin et al. 2010). In another study, it has

been established that IGF-1 is myotoxic for C2 immortal cell line (Saini, Al-Shanti et

al. 2008), where they found that exogenous addition of IGF1 with or without TNF-α

induces cell death regardless of its concentration (Saini, Al-Shanti et al. 2008). In the

present study, however, human recombinant IGF (Sigma-Aldrich, UK) at 10 ng/ml

was found toxic for zebrafish muscle cells cultured in monolayer; whereas, it was

observed that with differentiation media in the absence of IGF, zebrafish muscle cells

were fusing and forming myotubes efficiently. The data was also supported to some

extent by the qRT-PCR relative expression measured, where IGF1 was found

significantly higher in satellite cells isolated from fish but it decreased 10 fold after

two days in culture at sub confluent stage of cells. The expression of IGF1 in

zebrafish skeletal muscle cell cultures did significantly increase at later time points,

when the cultured cells formed myotubes, but it was five times less than the

expression measured from the cells just isolated. There is still more scope to study the

effect of IGF on *in vitro* cultured zebrafish skeletal muscle cells at lower

concentrations in order to find out, if the lower concentration of IGF in the

differentiation media is not toxic and helpful for zebrafish muscle cells. There is a

possibility that IGF at lower concentration can enhance or shift the differentiation

process of zebrafish skeletal muscle cells. Since the primary aim of the present study

was to isolate and culture zebrafish skeletal muscle cells *in vitro*, much emphasis was

not put to test different concentrations of IGF on zebrafish skeletal muscle cells *in*

vitro. Also, our protocol was in line with the protocol published by Alexander et al.

2011, where the differentiation media used for differentiation of zebrafish skeletal

muscle cells does not contain IGF (Alexander, Kawahara et al. 2011).

Previous literatures also suggest that rapidly growing fish species such as rainbow

trout and salmon have continuous muscle growth and perseverance of hyperplasia

during post larval growth, showing the high involvement of satellite cells in their life

time (Gabillard, Sabin et al. 2010, Johnston 2006). However, in the present study with

zebrafish skeletal muscle cells cultured *in vitro* and quantified for the first time shows

growth of cells to be hypertrophic instead of hyperplasic. The hypertrophic growth of

zebrafish skeletal muscle cells can be due to the fact that they are induced to follow a

different pathway where IGF is toxic for proliferation and differentiation. Therefore,

further investigation in this field of research need to be undertaken to identify where

the effect of human recombinant IGF is down to species specific IGF or concentration

of IGF.

4.4.4 qRT-PCR analysis

Relative expression of myoD gene was used as key marker in determining skeletal

muscle cells lineage and its function is also exerted in early stages of muscle

development (Bower, Johnston 2010, Jimenez-Amilburu, Salmeron et al. 2013).

Expression of myoD was found consistently increasing over time until myotubes are

completely formed and defined. It significantly increases by at least 2 fold, when

muscle cells are in early myotube formation stage, which is also in line with data by

Bower and Johnston's publications (Johnston 2006, Bower, Johnston 2010,

Macqueen, Johnston 2008). M-cadherin (M-cadh) determines the protein responsible

for adherence of skeletal muscle cells and slow cell migration, along with their early

development and proliferation processes (Cortes, Daggett et al. 2003). Expression

pattern of M-cadh also shows significant increase at early myotube stage when cells

interact with each other or for the formation of myotubes they do slow migration. The

differentiation of myoblasts is characterized by the expression of myogenin and

MRF4 proteins (Bower, Johnston 2010, Jimenez-Amilburu, Salmeron et al. 2013),

which has shown significant increase at early myotube stage. It has been studied

previously in gilthead sea bream myocytes that myogenin and mrf4 are highly

expressed during differentiation phase, when the myotubes are already present, which

suggests their late role in process of muscle development (Jimenez-Amilburu,

Salmeron et al. 2013). Myostatin is termed as negative regulator particularly in fish

muscle cell growth in the literature with different paralogues (Garikipati, Rodgers

2012). Previously expression of myostatin has been studied to be ubiquitous at all the

stages but it is expressed highly when myoblasts start differentiating, it inhibits the

expression of myoD and myf5 and enhances the expression of myogenin which helps

in differentiation (Froehlich, Galt et al. 2013, Seiliez, Sabin et al. 2012). Expression

of myostatin was found in line with the previous literature here as well, where its

expression was found universal throughout all stages, but peaked at confluent cell

stage. Maturation of zebrafish skeletal muscle cells was analysed by the expression of

two isoform of myosin heavy chain i.e. slow myosin heavy chain and fast myosin

heavy chain. Expression pattern of both slow and fast isoforms of myosin heavy chain

was found almost similar, where the expression peaked during early myotube stage.

Expression at early myotube stage for sMyHc increased by almost 15 fold from sub-

confluent stage and confluent cells, whereas it went down again almost 5 fold in later

myotube stage. However expression of fMyHc increased by only 8 fold at early myotube stage from sub-confluent and confluent stage and decreased by 4 fold at the later myotube stages. This expression pattern of myosin heavy chain suggests that sMyHc isoform is expressed more compare to fMyHc. The expression patterns for most of the genes tested from zebrafish skeletal muscle cells were found to be following similar expression pattern to mammalian cells, excluding the fact that they need IGF for their growth and differentiation.

Results obtained from the current protocol were found to be more consistent and reliable compared to the results of Alexander et al (2011). Myotubes obtained in the published article by Alexander et al (2011) looked like a cluster of muscle cells, these cells were not even stained for the nuclei. A myotube is defined as a long multinucleated (more than two nuclei) structure of cell stained for muscle specific protein desmin and nuclei, but this definition was not fulfilled in the published images. In the current study, multiple long multinucleated myotubes were obtained repeatedly from every culture and were stained for desmin and nuclei; representative images were shown in **figure 4.3**. No quantitative data was presented in the published paper by Alexander et al (2011), whereas, in the current study, more quantitative results such as number of myotubes, length and width of myotubes, desmin positivity and fusion index were obtained and presented.

To conclude this chapter, zebrafish skeletal muscle cells cultured in monolayer have been successfully characterized morphologically as well as genetically. Now after understanding them thoroughly, they can be cultured in three dimensional tissue engineered constructs or used for other mechanistic analysis. As now a baseline for

measuring 3D cultures against monolayer is also available and test suitability of two

matrices i.e. collagen and fibrin in three dimensional constructs.

5. Zebrafish skeletal muscle cells in fibrin based three dimensional tissue engineered construct

5.1 Introduction

Tissue engineered skeletal muscle have offered a novel approach and potential alternative for the replacement of tissue after severe damage due to traumatic injury, congenital defects, tumor ablation and prolonged denervation (Rossi, Pozzobon et al. 2010). Tissue engineering approaches have also been termed to be useful in muscle dysfunction which includes skeletal myopathies such as Duchenne muscular dystrophy (DMD), where progenitors or stem cells are required to be delivered (Rossi, Pozzobon et al. 2010). However, the transfer of myoblasts into the injured muscle is still a challenge. Transplantation of tissue engineered skeletal muscle constructs required for clinical purposes remains elusive due to the fact that constructs have not been engineered of the correct size and also have failed to produce active force of the correct magnitude as *in vivo* tissue (Bian, Bursac 2008). Two main components are required for successful skeletal muscle regeneration which are cells with regenerative potential and an appropriate delivery vector i.e. polymer required for cell embedment (Rossi, Pozzobon et al. 2010).

Tissue engineered skeletal muscle constructs also benefits applications besides transplantation, for example Vandenburgh (2010) identified their application in medical *in vitro* drug-screening, where these constructs were exposed to drugs and their effects monitored by various functional and cellular and molecular outputs (Vandenburgh 2009). Such applications could reduce the use of number of small organisms sacrificed for research. Establishment of tissue engineered constructs also

plays an important role in studying muscle development in physiology and pathology

of skeletal muscle (Khodabukus, Paxton et al. 2007). Furthermore, there are

comparatively minor ethical issues with skeletal muscle constructs to perform

invasive studies.

As explained previously in chapter one, *in vivo* skeletal muscle cells were formed of

bundles of highly differentiated, multi-nucleated muscle fibres (myotubes) oriented

uniaxially, surrounded in three dimensions by connective tissues in order to facilitate

contraction of the tissue in one plane (Smith, Shah et al. 2010). Therefore it is

essential to promote differentiation of muscle precursor cells (myoblasts) into

myotubes and the orientation of these muscle fibres into densely packed "fascicle"

like structure for an *in vitro* engineered skeletal muscle construct in order to replicate

the organization of skeletal muscle cells *in vivo*.

5.1.1 Synthetic scaffolds

Scaffolds serve as a synthetic extracellular matrix to align cells in a three-dimensional

architecture and to present stimuli, which direct growth and formation of the desired

tissue (Drury, Mooney 2003). Uniaxial cell alignment of skeletal muscle cells has

been the primary aim of groups using synthetic scaffolds and substrates by

manipulating their topography in order to generate aligned cultures of myotubes.

Along with their ability to produce aligned cultures they should be reproducible and

large scale manufacture which make them useful for tissue engineered skeletal

muscle cells. The summary table 5.1 below shows a section of synthetic scaffolds

reported in the literature for skeletal muscle tissue engineering applications.

Typical synthetic scaffolds include polylactic acid (PLA). PLA is a commonly used

synthetic material, which degrades easily in the human body to form lactic acid:

naturally occurring chemical and can be easily removed by the body.

Table 5.1: Summary of selected studies used synthetic scaffolds for tissue engineering skeletal muscle. Adapted from Neil Martin (UoB, 2013) thesis.

Substrate and topography	Cell Type	Key Findings	Reference
Electrospun PLGA fibres	C_2C_{12}	• Uniaxial cellular alignment compared with random alignment on glass coverslips • Improved fusion index versus glass coverslips • Sarcomeric protein organisation comparable with glass coverslips	(Aviss, Gough et al. 2010)
Electrospun PLLA fibres (randomly aligned fibres used as controls)	C_2C_{12}	• Uniaxial cellular alignment • Improved myotube length compared with randomly orientated PLLA fibres • Cross-striations displaying sarcomeric protein organisation	(Huang, Patel et al. 2006)
Electrospun PLLA fibres and gold nanoparticles	Primary rat MDCs	• Improved cellular proliferation over PLLA alone. However proliferation was at best equal to that on conventional tissue culture plastic	(McKeon-Fischer, Freeman 2011)
Polyacrylamide films micro-patterned with adhesion proteins to form specific lanes	C_2C_{12} and primary human MDCs	• Cells adhere only to the patterned areas • Lane width effects proliferation and differentiation (narrow lanes =↑ proliferation, wide lanes =↑ differentiation)	(Zatti, Zoso et al. 2012)
PLGA fibre mesh	Primary rat MDCs	3 hours post-cell seeding, constructs implanted into Fisher mice for 4-6 weeks. • Implanted cells survive and form muscular networks on the constructs • Some evidence of host vascularisation	(Saxena, Marler et al. 1999, Saxena, Willital et al. 2001)
PLLA and PLGA sponges	Primary mouse MDCs and HUVECs	• *In vitro*, myotubes were observed alongside vessel like structures • Addition of embryonic fibroblasts improved vascularisation • When implanted *in vivo* to nude mice, cells survived, differentiated further and host vessels invaded the constructs	(Levenberg, Rouwkema et al. 2005)
PLGA microspheres	Primary human MDCs	• *In vitro*, myotubes developed which expressed tropomyosin • When implanted *in vivo* to nude mice, cell survival was poor.	(Thorrez, Shansky et al. 2008)

PLGA= Poly-lactide co-glycolide, PLLA= Poly(L-lactide), HUVECs= Human umbilical vein endothelial cells, MDCs= Muscle derived cells

Similar to PLA are polyglycolic acid (PGD) and polycaprolactone (PCL) which can also

be used as scaffolds with their degradation rate faster and slower respectively to PLA.

Engineering new skeletal muscle tissue *in vitro* and then implantation depends on the host

for vascularization, which has achieved success with many other tissues but not in case of

thick and highly vascularized muscle tissue (Levenberg, Rouwkema et al. 2005). Skeletal

muscle consists of long, cylindrical multinucleated individual muscle fibres arranged in

parallel to each other surrounded by connective tissue (Levenberg, Rouwkema et al.

2005). Levenberg and colleagues, who studied whether embryonic endothelial cells can

induce endothelial vessel networks in engineered skeletal muscle tissue if an appropriate

environment is provided (Levenberg, Rouwkema et al. 2005). This model improved the

cell survival, along with complexity of the model by incorporating multiple cell types

which brought engineered tissue *in vitro* another step closer to biomimetic culture system

(Levenberg, Rouwkema et al. 2005) as previously engineering new skeletal muscle tissue

in vitro and then implantation depends on the host for vascularization.

Michelle Peckham (2008) reported the importance of myoblast alignment to be able to

fuse and form myotubes efficiently (Peckham 2008). There are a number of techniques

discussed in the literature which helped the myoblasts in *in vitro* culture alignment such as

uniaxial stretching (Huang, Patel et al. 2006), micro-patterning (Huang, Patel et al. 2006),

electrospinning of aligned fibres (Smit, Bűttner et al. 2005) or use of mandrel as a

collector plate (Theron, Zussman et al. 2001). Electrospinning of scaffolds provides them

with flexibility and the diameter of fibres can also be altered by changing the parameters,

which results in larger or smaller pores in scaffolds or fibre orientation.

5.1.2 Biopolymer matrix- Fibrin based

Synthetic polymers used in tissue engineered constructs have been discussed widely in the

literature for their possibility in wound healing and tissue replacement but their use in

skeletal muscle models is still very vague (Bian, Bursac 2008). Use of rigid synthetic

polymer scaffolds in modeling *in vivo* skeletal muscle tissue *in vitro* have several

drawbacks such as 1) Uneven cell seeding density throughout the scaffold due to its rigid

structure and the scaffold structure cannot be altered in order to vary the cell alignment

and tissue thickness, 2) Due to the rigid structure of scaffold it prevents the macroscopic

contraction of construct, therefore evaluation of construct cannot be performed in terms of

its functional ability, 3) In case of degradable scaffold constructs, it's unclear how much

the cellular alignment will be affected when the disintegration of polymer will occur.

Therefore in order to overcome these problems naturally derived polymers such as

collagen and fibrin hydrogels can be used to model *in vitro* skeletal muscle tissue.

Fibrin based hydrogels provide a matrix where tissue engineered skeletal muscle can be

constructed. The most widely used fibrin based model is based on the work of Strohman et

al. (1990), where the gel is cast in between fixed points. Strohman and his colleagues

experimented by coating saran wrap with type 1 rat tail collagen before pinning them on to

Sylgard coated 35 mm tissue culture dish followed by plating avian muscle derived cells

on the membrane (Strohman, Bayne et al. 1990). They observed that as the muscle cells

were growing they started remodelling the saran wrap layer as well by detaching it from

the edges of the dish and ended up with a tightly wrapped construct held in place by the

pins. Construct attachment to the pins allowed generation of tension within the constructs

as the seeded cells started pulling and got reorganized along the lines of strains, leading to

the formation of aligned myotubes in the construct. Immunostaining demonstrated the expression of mature myosin heavy chain proteins as compared to standard 2D cultures.

Dennis and colleagues (Dennis, Kosnik II 2000, Dennis, Kosnik et al. 2001) developed the model established by Strohman, developing a more accurate and biologically relevant model for the *in vitro* study of skeletal muscle. They replaced saran wrap with laminin and used two pinned sutures as anchor points rather than the 7 pins used in Strohman study. After these adjustments cells were plated and it was observed that the laminin sheet began to detach from the Sylgard coated plate due to the contracting cell and formed a cylindrical shape held between two anchor points (Dennis, Kosnik II 2000). Similar to the saran wrap construct tension generated by the cells, pulling against fixed points helped the alignment of muscle cells along the lines of strains. The presence of only two anchor points led to the formation of uniaxially aligned myotubes in culture between the two fixed points, and cross-sectional histology demonstrated the same after up to 50 days in culture.

Further Khodabukus and his colleagues developed this model by replacing laminin with fibrin, which helped to speed the maturation time of the constructs (Khodabukus, Paxton et al. 2007, Khodabukus, Baar 2009). These fibrin constructs developed in 7-10 days instead of 35 days previously, also didn't require supplementary fibroblasts to help in development and produce more specific force, therefore are termed as a better model for understanding muscle physiology (Khodabukus, Paxton et al. 2007, Khodabukus, Baar 2009).

5.1.3 Zebrafish skeletal muscle cells in 3D constructs

Zebrafish muscle cells cultured in 3 dimensional tissue engineered constructs will help us understand more about the muscle physiology and their development *in vitro*. Also, as previously described zebrafish have been reported as a good model to examine human muscular dystrophies, and this feature will be enhanced when cultured in 3 dimensional tissue engineered constructs. Until today there has been no literature reported for culturing zebrafish skeletal muscle cells in any three dimensional tissue engineered constructs, therefore it will be a novel approach.

5.1.4 Aims of the chapter

In this chapter, zebrafish skeletal muscle cells were isolated, cultured and optimised in monolayer cultures and cultured in a three dimensional fibrin based culture model and further optimisation will be performed using immunocytochemistry and PCR techniques. In order to achieve this aim, these following need to be achieved:

1) Optimise seeding density of zebrafish skeletal muscle cells on fibrin constructs.

2) Culture zebrafish skeletal muscle cells in fibrin based tissue engineered construct.

3) Immuno stain the constructs and characterisation will be performed.

4) RNA extraction and qRT-PCR across different time points, to access the expression of different markers of muscle cell differentiation.

5.2 Materials and methods

5.2.1 Cell culture

Zebrafish skeletal muscle cells were isolated as explained in chapter 3 and plated on already prepared fibrin based tissue engineered constructs (as explained in **Section 2.3.1.2**). Parallel cells from the same batch were plated as two dimensional controls on gelatin coated coverslips. Growth media was changed every day, whereas differentiation media was changed after every 2 days.

5.2.2 Seeding density for fibrin construct

Fibrin constructs were engineered as described previously in **section 2.3.1.2**. In order to obtain an optimal seeding density, constructs were seeded at 600k, 800k, 1 million and 1.5 million cells per fibrin plate initially and solely at 1 million cells per plate thereafter as required. Constructs were maintained in growth media for five to seven days until they reached confluency at which point they were switched to differentiation media for the remainder of the experiment. Upon the cessation of these experiments constructs were either fixed for immunocytochemistry (as explained in **Section 2.4.4**) or homogenised in TRIzol for RNA extraction (as explained in **Section 2.5.1**).

Parallel monolayer cultures were setup as a control for each n-number of fibrin experiment at 100k cells per cm^2, maintained in growth medium until they reached confluency at which point they were switched to differentiation media for the remainder of the experiment. Parallel to fibrin constructs, monolayer cells were also either fixed for immunohistochemistry or homogenised for RNA extraction.

5.2.3 Immunohistochemistry

Immunostaining was conducted as described in **section 2.4.4** and imaged using confocal microscopy. Constructs were stained for desmin which is a muscle specific cytoskeletal intermediate filamentous protein and were counterstained using 4',6-diamidino-2-phenylindole (DAPI) for nuclei, in order to assess if myotubes had formed and the general characteristics of the zebrafish skeletal muscle cells within the constructs.

5.2.4 qRT-PCR

qRT-PCR was conducted using Rotor gene instrumentation as described in **section 2.5.6**. The primer sequences for the genes of interest are shown in **table 2.1** and exhibited specificity according to **figure 2.8**. Data was analysed using two standard curve quantification methods as described in **section 2.5.7**. When analysing expression of genes in tissue engineered constructs, data were made relative to zebrafish muscle cells just after isolation and data were normalised to housekeeping genes EF1α and β-actin.

5.2.5 Statistical analysis

Shapiro-Wilk and Levene's tests were used to test for normality of distribution and homogeneity of variation respectively. Thereafter, one way ANOVA was used to determine differences between groups for total time in culture for normally distributed data. Differences within constructs for different gene expression data was analysed at 5 days, 10 days and 15 days at differentiation using one way ANOVA with repeated measures. Where significant main effects were found, pairwise comparisons using a bonferroni post-hoc test were carried out. Significance was taken at an alpha value of $p<0.05$.

5.3 Results

In this chapter zebrafish skeletal muscle cells were isolated from the dorsal muscle tissue of zebrafish and plated on pre-prepared fibrin constructs and cultured for different time points in order to find the optimum construct maturation time.

5.3.1 Optimum seeding density for fibrin constructs

Fibrin constructs were plated with zebrafish skeletal muscle cells at 600k, 800k, 1 million and 1.5 million density per gel. It was observed that fibrin gels seeded with 600k and 800k didn't roll up completely to form a whole three dimensional cylindrical structure and after five days in culture on fibrin gels, cell death was observed due to lack of cell interaction (as shown in **Figure 5.1**). Plating density of cells also affected the alignment and fusion of cells on fibrin gels as shown in **figure 5.1**, cells plated at optimum (one million cells per gel) density were more aligned and fused to form myotubes. Fibrin gels were observed to be disintegrated at the seeding density of 1.5 million cells per gel, whereas at one million cells per gel, the gel rolled up more firmly and completely to form a three dimensional cylindrical structure and the fusion of cells was also observed in the phase contrast images, which was later confirmed by immunostaining.
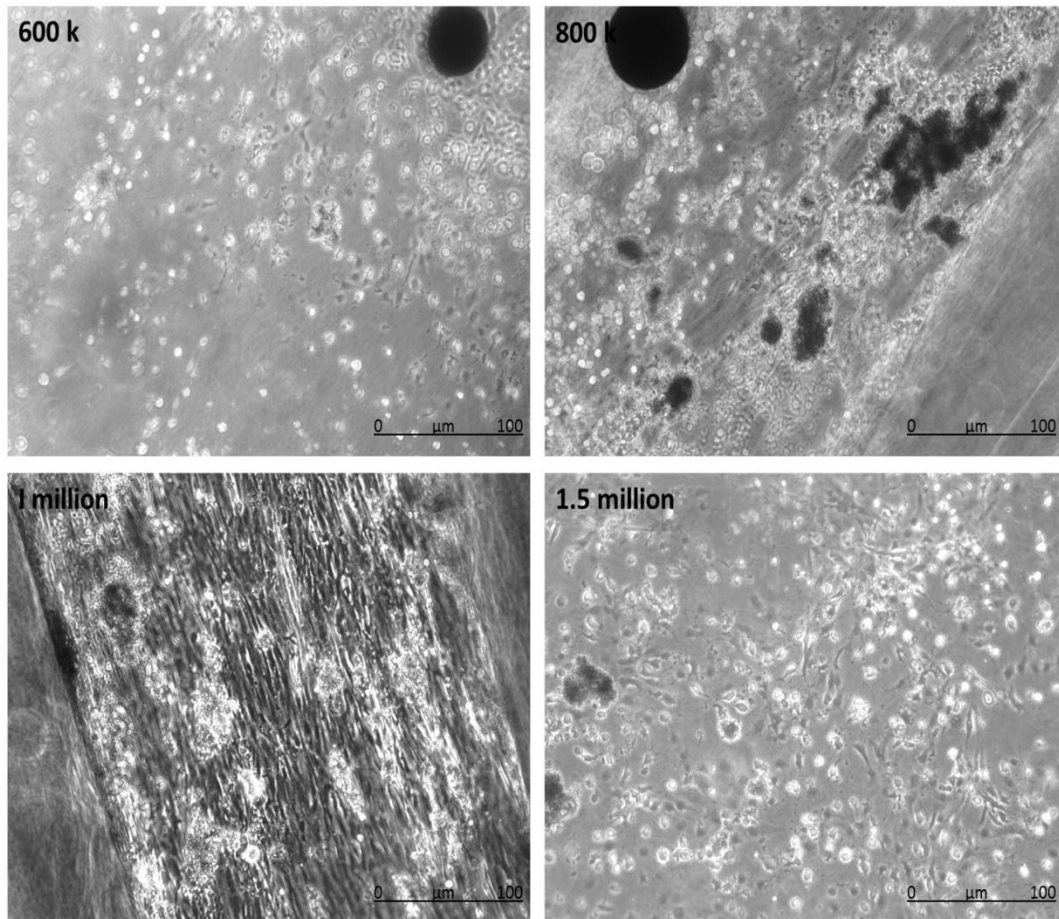
Figure 5.1: Impact of different plating densities of zebrafish skeletal muscle cells cultured on fibrin gels. Phase micrographs taken after six days in cultures at different plating density i.e. 600k, 800k, 1 million and 1.5 million cells per gel. Cells were observed to be more aligned along with initial myotube formation which was later confirmed by immuno staining at 1 million cells per gel. All images were captured at 100 X magnification.

The average number of cellular structures was counted after 7 days in culture before

switching them into differentiation media on fibrin based tissue engineered

constructs. Images were captured at 100X magnification for counting the number of

cellular structure from 20 images captured from each construct and n=3 samples.

Due to the fact that the focal plane differs along different surface of three dimensional

constructs; therefore the average number of cellular structures counted represents the

number of cells from one plane. Alignment of cells in the fibrin model was observed

using the similar images shown in figure 5.1 and the number of cells were counted in

those images similar to those presented in figure 5.2. Cellular structures were

confirmed to be muscle cells after immunohistochemistry in later part of chapter.
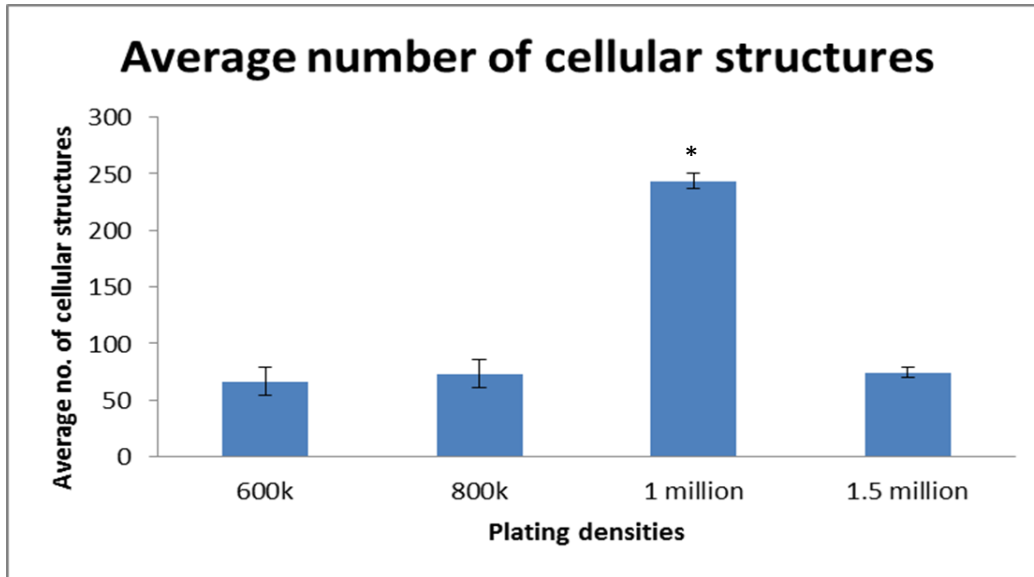
***Figure 5.2: Average number of cellular structures formed at different plating
densities in tissue engineered fibrin constructs.*** *Y-axis represents average number of
cellular structures per microscopic field whereas; X-axis represents different plating
densities per fibrin gel. Average number of cellular structures was found significantly
higher in cells plated at one million per gel. Error bars represents ± S.D. Statistical
difference among the samples is denoted by letters. N=60 images.*

5.3.2 Maturation of fibrin constructs

Maturation of the fibrin constructs was determined at different time points, considering the following aspects 1) Rolling up of the construct as shown in **figure 5.3**, 2) Alignment of muscle cells in between the line of strains as shown in **figure 5.1** via phase contrast images and confirmed by immunohistochemistry in **figure 5.3**, 3) Formation of maximum number of myotubes as confirmed in **figure 5.4** and in **graph 5.5**, 4) Markers for maturation via quantitative real time pcr shown in **section 5.3.4**.

Maximum macroscopic maturation (rolling-up) of fibrin constructs was observed when the cells had been cultured in differentiation media for 10 days. Constructs cultured for longer than 10 days in differentiation media appeared to revert back to a larger size and become less tightly rolled as shown in **figure 5.3** below.
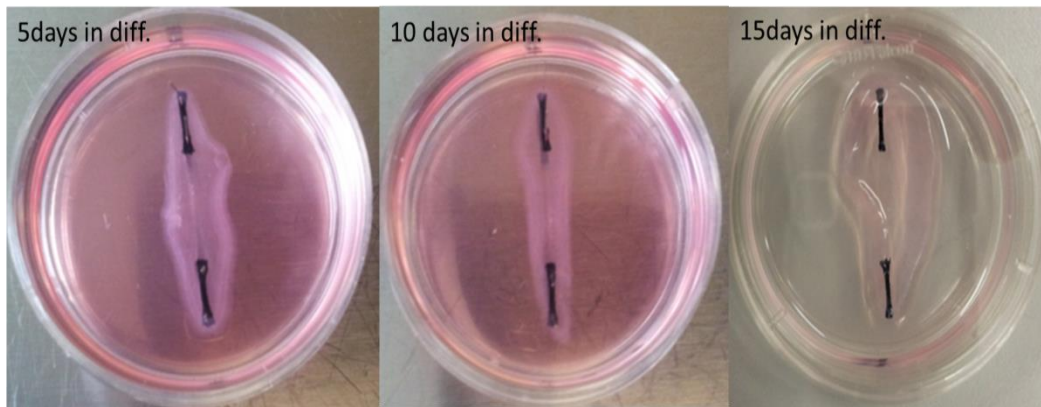
Figure 5.3: Macroscopic images of fibrin based tissue engineered constructs cultured at 5, 10 and 15 days in differentiation. Fibrin gels rolled tightly as a cigar shape up to 4 mm were considered as fully mature and functionally three dimensional tissue engineered model (Khodabukus, Baar 2009).

5.3.3 Immunohistochemistry

Zebrafish skeletal muscle cells cultured in tissue engineered fibrin model were fixed and blocked as previously explained in **section 2.4.4**. Fibrin constructs were subsequently whole mount stained for muscle specific filamentous desmin protein and counterstained with DAPI for nuclei. Stained fibrin constructs were then mounted on poly lysine coated glass slides and viewed using Leica confocal microscope. Stained constructs after 5 days in differentiation were observed to be bigger in size i.e. not rolled completely, which was similar to constructs at 15 days in differentiation, whereas construct at 10 days in differentiation were observed to be completely rolled and more mature as determined by number of myotubes per field of view from n=3 samples (20 images per construct). There were statistically increased number of myotubes per field of view in constructs fixed and stained after 10 days in differentiation (approximately 12 myotubes per microscopic field of view) compare to 5 and 15 days in differentiation (Approximately 3 and 4 myotubes/ microscopic view respectively) as shown in **figure 5.5**. Fusion index of the constructs stained at 5, 10 and 15 days in differentiation was also discovered statistically higher at 10 days relative to 5 and 15 days in differentiation as shown in **figure 5.6**.
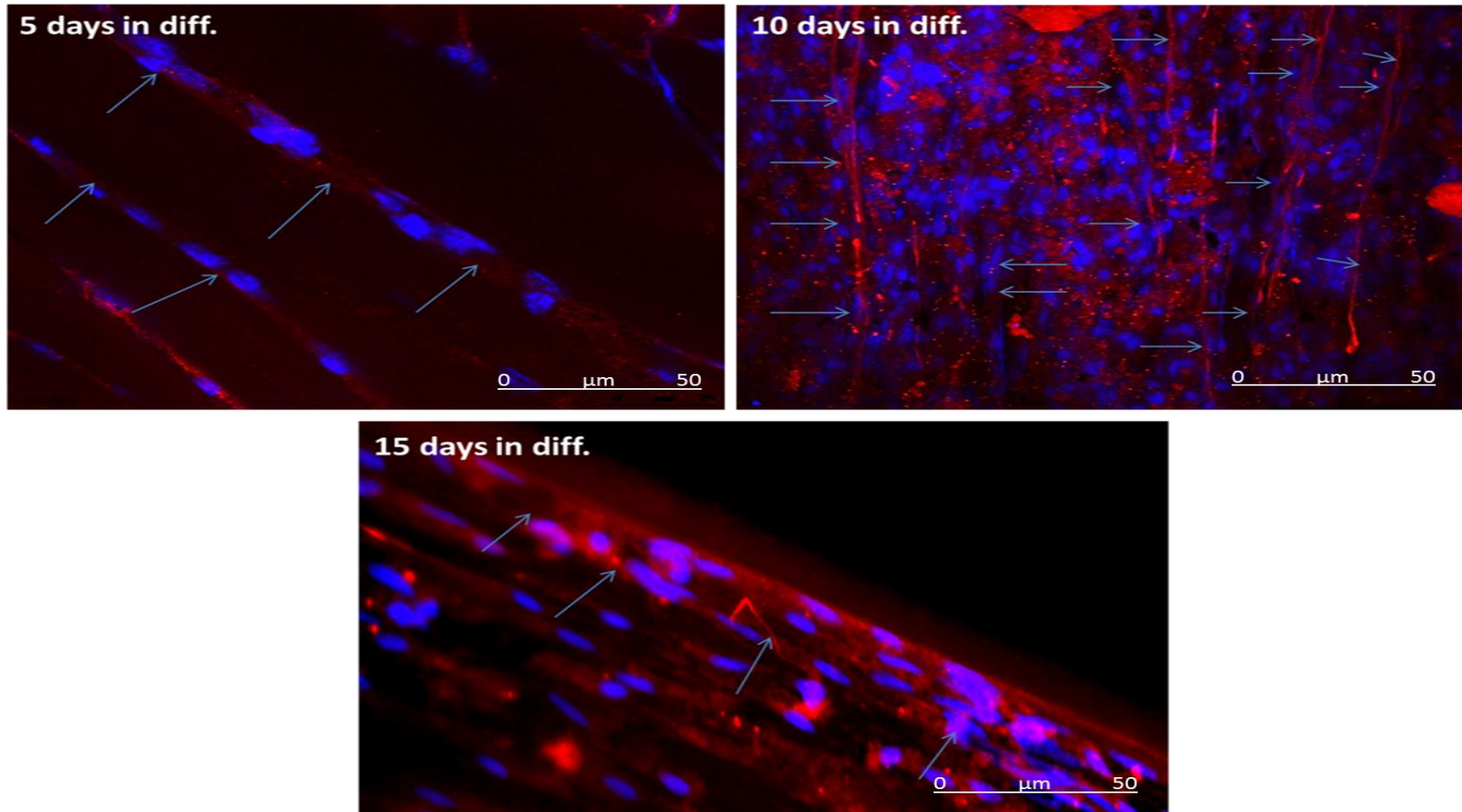
Figure 5.4: Zebrafish skeletal muscle cells cultured on fibrin based tissue engineered construct whole mount stained after 5, 10 and 15 days in differentiation. *Constructs were stained for desmin protein (shown in red) with desmin antibody and counterstained with DAPI (shown in blue) for nuclei. Myotubes are shown by the help of grey arrows in each image. Images were captured using Leica confocal microscope at 200X magnification.*

155
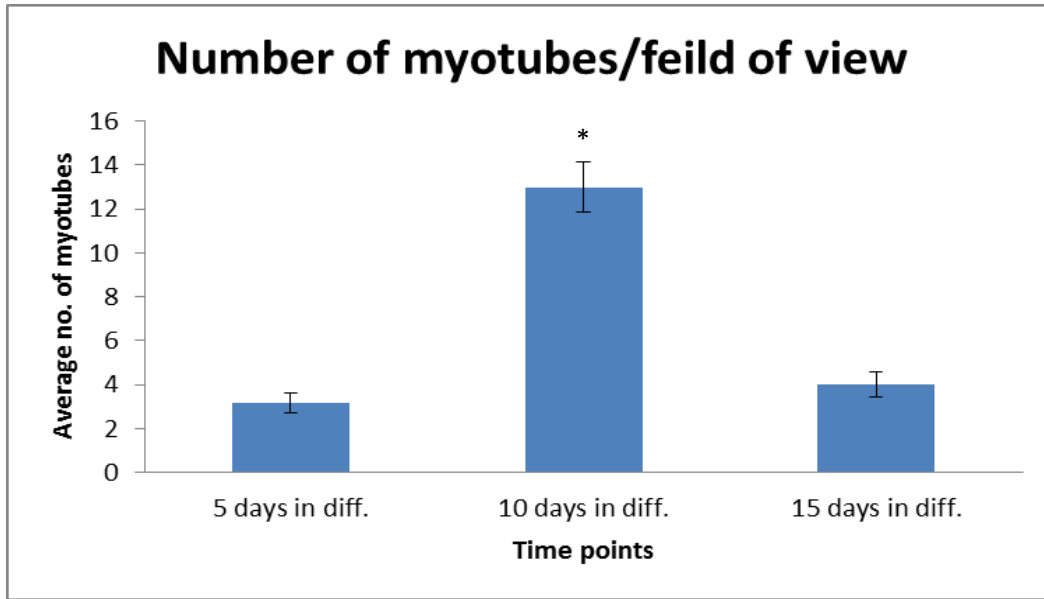
Figure 5.5: Average number of myotubes of zebrafish skeletal muscle cells in fibrin constructs at different time points. *Y-axis represents average number of myotubes per field of view (n=60 images) and X-axis represents different time points i.e. 5, 10 and 15 days in differentiation. Error Bars represents ± S.D. Statistical difference annoted by letters (p<0.05).*
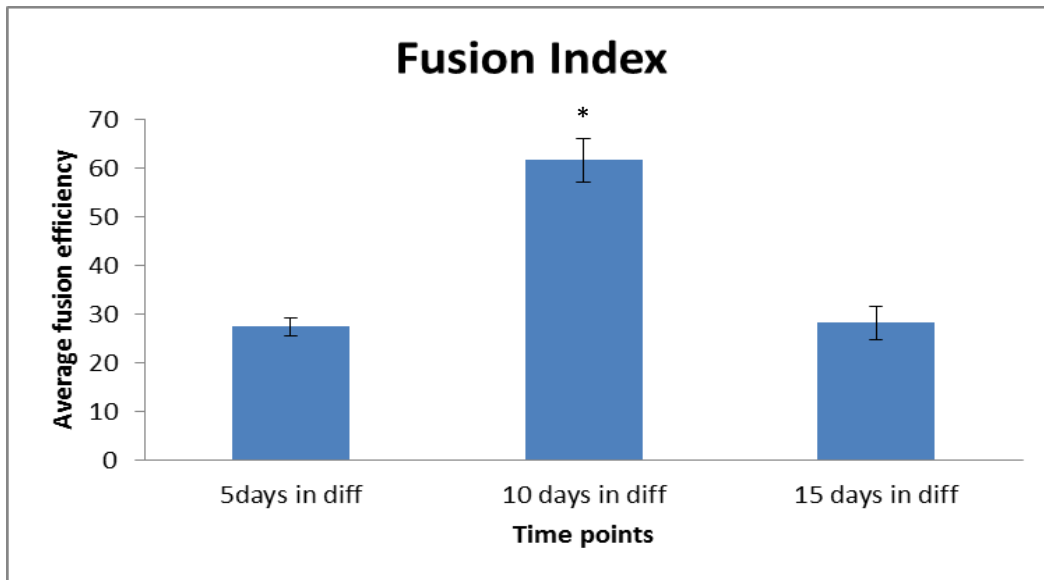
Figure 5.6: Average fusion index of zebrafish skeletal muscle cells in fibrin constructs at different time points (n=60 images). *Fusion index was noticed to be statistically higher at 10 days in differentiation time point relative to 5 and 15 days in differentiation. Error Bars represents ± S.D. Statistical difference annoted by letters (p<0.05).*

5.3.4 Expression of muscle specific markers in fibrin constructs

Zebrafish skeletal muscle cells were cultured on fibrin constructs initially for five to
seven days until they reached confluency and then differentiated for fusion and
forming myotubes in presence of low serum media. Subsequently these constructs
were homogenised in TRIzol at different time points as per the requirement of the
experiment for extraction of RNA (as explained in **section 2.5.1**) and converted into
cDNA for measuring the expression of different markers of myogenic regulatory
factors MyoD, myogenin, M-cadherin, MRF4 along with myogenic inhibitor
myostatin, hypertrophic gene insulin like growth factor-1 and slow and fast isoform
of myosin heavy chain.

All data was normalised against housekeeping genes EF1α and β-actin and expression
at after isolation was used as calibrator. One sample Kolmogorov-Smirnov statistical
test was used in order to determine the normality of data set. Expression pattern of
MyoD was observed to be increasing over the time points but statistically no
significant difference was observed with the p-value = 0.284. Expression of
myogenin was observed to be peaked at 10 days in differentiation and then dipped at
15 days in differentiation, which made the classic expression pattern of bell shaped
for myogenin as termed in the literature (Ytteborg, Vegusdal et al. 2010). But here
again similar to MyoD, there was no significant statistical difference in the relative
expression across different time points with the p-value of 0.079.
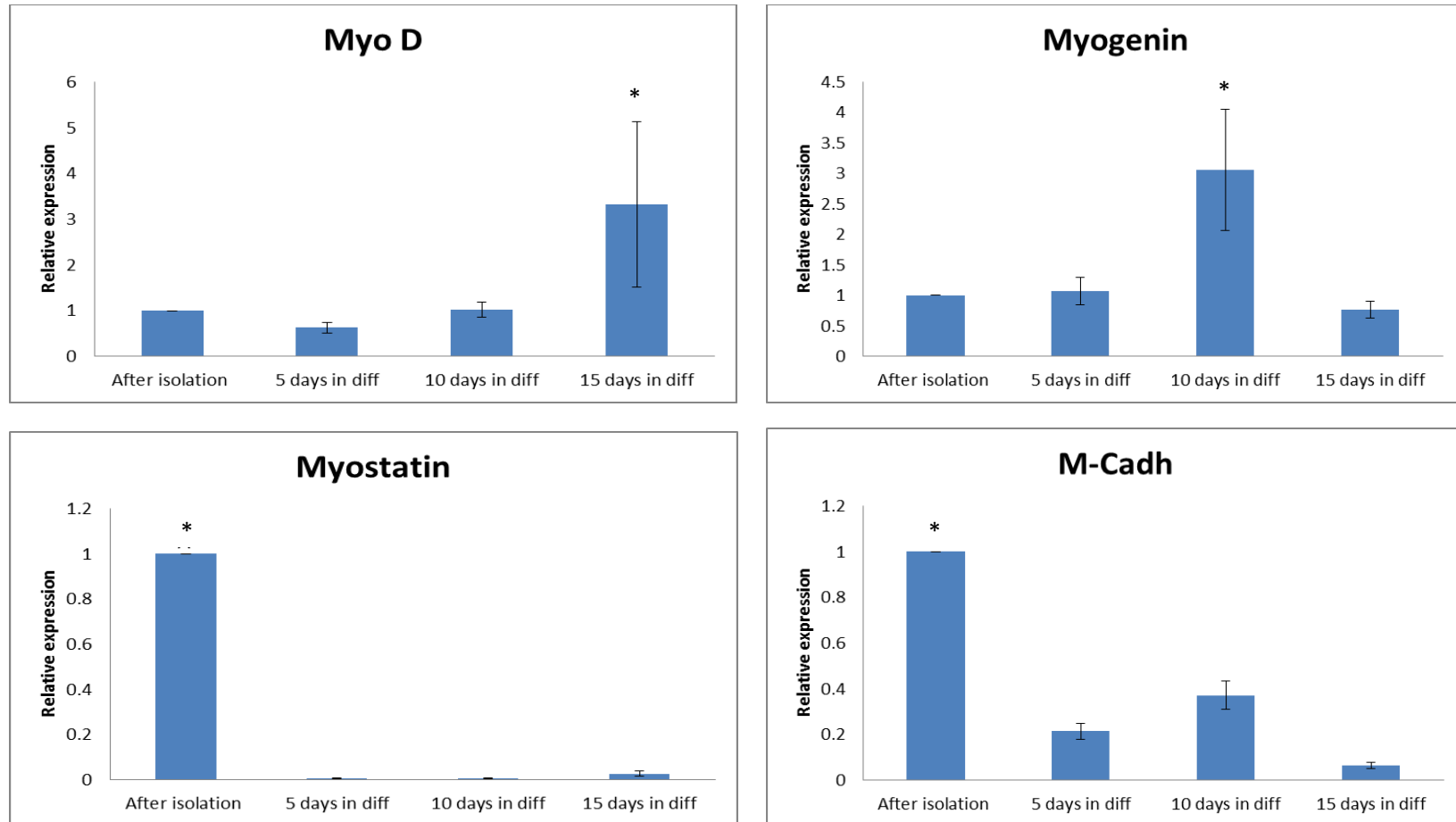
Figure 5.7: Relative expression of muscle lineage markers MyoD, Myogenin, M-cadherin and negative regulator myostatin in fibrin constructs (n=3). *On the graphs Y axis represents relative expression of proteins whereas X axis represents different time points where expression was measured in the fibrin constructs i.e. 5, 10 and 15 days in differentiation. All data was normalised against housekeeping genes EF1α and β-actin and calibrated against after isolation time point for all genes.*
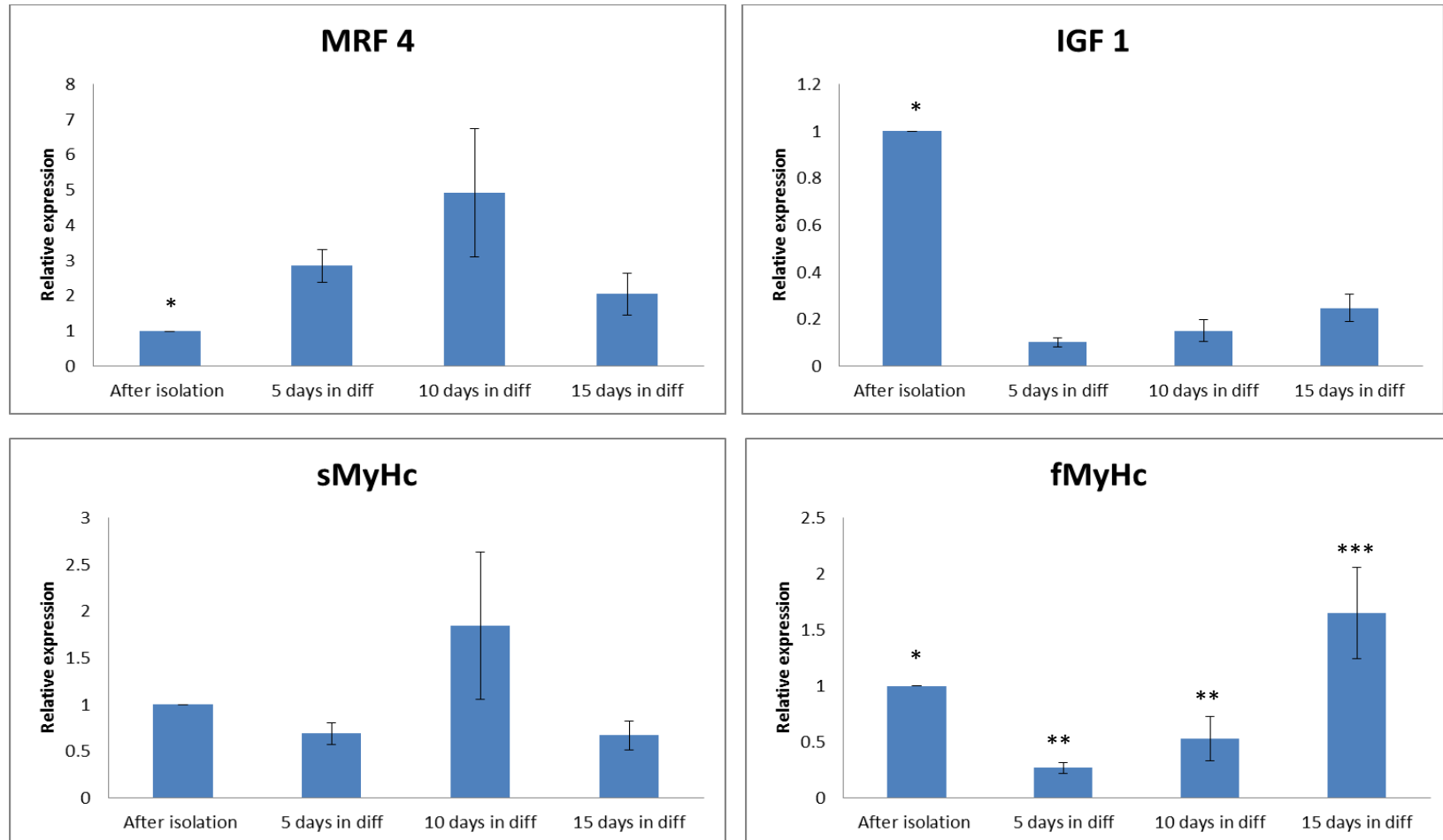
Figure 5.8: Relative expression of MRF4, slow and fast isoforms of myosin heavy chain and hypertrophic gene Insulin like growth factor-1 from fibrin constructs (n=3). *On the graphs Y axis represents relative expression of proteins whereas X axis represents different time points where expression was measured in the fibrin constructs i.e. 5, 10 and 15 days in differentiation. All data was normalised against housekeeping genes EF1α and β-actin and calibrated against after isolation time point for all genes.*

Relative expression of negative regulator myostatin for myogenic lineage was observed as significantly lower in all the later time points compare to its expression at after isolation time point, which follows the previous literature as its expression peaks when the muscle precursor cells start differentiating and forming myotubes. Similar to myostatin the expression of m-cadherin was also observed to be highest after isolation time point but its expression in later time points during differentiation was also observed without any significant difference among them. The expression of MRF4, another gene from myogenic regulatory factors along with myoD and myogenin, increased along with time until 10 days in differentiation but then dipped at 15 days in differentiation. Statistically again there was no significant difference across the time points for MRF4 gene, with the p-value of 0.198.

No significant difference was observed across different time points for expression of slow myosin heavy chain with the p-value of 0.297, whereas the expression of fast myosin heavy chain was observed to be increasing significantly relative to the time points. Its expression was 4 fold lower at 5 days in differentiation compare to after isolation but after that it increased by 6 fold at 15 days in differentiation time point. Expression of hypertrophic gene IGF-1, decreased by 10 fold from after isolation time point to 5 days in differentiation and after that there was no significant increase observed in its expression across the later time points i.e. 10 and 15 days in differentiation. However, mean expression was observed to have an increasing trend from 5 days to 10 and 15 days in differentiation time points.

5.4 Discussion

In vitro tissue engineered models of skeletal muscle have been used in order to enhance mechanistic understanding for skeletal muscle formation, function and adaptation (Baar 2005) and have also been successfully used as model for drug testing and exercise related plasticity (Khodabukus, Paxton et al. 2007, Vandenburgh 2009). Due to the fact that these tissue engineered skeletal muscle models mimic the *in vivo* environment and to some extent functionality as well and they are well characterized *in vitro*, it is a possibility in future to transplant them *in vivo* and cure many muscle related diseases (Bian, Bursac 2008). Until now however, there has been no literature published on zebrafish skeletal muscle cells cultured *in vitro* on fibrin based tissue engineered constructs. Development of a fibrin based tissue engineered zebrafish skeletal muscle model would allow for high throughput testing due to its *in vivo* bio mimicity, and reduce the reliance on other animal cells (refinement, replacement and reduction) which have reduced homology with those of *homo sapiens*. In this chapter zebrafish skeletal muscle cells have been successfully cultured on fibrin based tissue engineered hydrogels and characterized using immunohistochemistry and qRT-PCR techniques.

5.4.1 Effect of seeding density on fibrin constructs

In the previous literature fibrin based self-assembling tissue engineered skeletal muscle constructs have been seeded with muscle cells from different species without performing any count on them due to presence of large amount of debris from isolation of muscle cells (Dennis, Kosnik II 2000, Dennis, Kosnik et al. 2001). However later isolation techniques were improved and muscle precursors were

seeded at a 100,000 cells per construct (Khodabukus, Baar 2009, Khodabukus, Baar 2011). In light of the finding from chapter 4; that plating density of zebrafish muscle cells in monolayer was very high compared to human or other cell lines used in our laboratory (unpublished data), zebrafish skeletal muscle cells were plated at higher plating densities of 600k, 800k, a million and 1.5 million per fibrin gel. It was observed from the phase-contrast images in **figure 5.1**, that as the plating density increased, rate of cells survival for zebrafish muscle cells after 5 days in culture also increased, confirmed later by the average cell counts using Image J software. A significant five-fold increase in average number of cells was observed from cells seeded at lower densities than one million per construct, which further dipped again at higher plating density i.e. 1.5 million per gel. The hypothesis was that at the lower plating densities the cell to cell interaction was less, therefore they were not able to produce sufficient amount of force to pull the construct from edges and roll completely. It was also supported with the fact that cells at lower plating density cells were more spread compare to when plated at 1 million or 1.5 million (as seen in **figure 5.1**). Constructs seeded with 1.5 million cells were also not able to firmly roll the hydrogels, due to the fact that cells started decomposing the fibrin gel itself at that plating density which is evident from **figure 5.3**.

Khodabukus and Baar, discovered that the diameter of fully formed tissue engineered fibrin construct should be <4mm (3.23 ± 0.83mm) (Khodabukus, Baar 2009). Constructs which reached this diameter at the end of the culture period (10 days in DM) were only from constructs seeded at 1 million per constructs, whereas other density plated constructs didn't reached the 4mm mark (as shown in **figure 5.3**).

Therefore in general the constructs seeded at 1 million cells per constructs appeared more suitable for future use, but still it was necessary to analyse the formation of myotubes in order to determine if a skeletal muscle construct has been formed.

5.4.2 Immunostaining

Fibrin constructs were whole mount immune-stained for desmin protein, expressed both in mononuclear muscle cells and multinuclear myotubes, which allowed for the determination of fusion index at different time points i.e. 5, 10 and 15 days in differentiation media. Fusion index was observed to significantly increase at 10 days in differentiation which was 61.66% compare to 5 and 10 days in differentiation which was 27.43 and 28.20% respectively. Fusion index at 5 and 10 days in differentiation indicated that only approximately one-quarter of cells were fused or incorporated into myotubes whereas the number increased to 61.66% at 10 days in differentiation which is similar to cells plated in monolayer which was 58%. The fusion index of tissue engineered fibrin construct is still on the lower side when comparing to *in vivo* muscle where approximately 95% of cells are incorporated in muscle fibres (Kadi, Charifi et al. 2005). The numbers of myotubes per microscope image were counted in order to confirm the maturity the construct at different time points in culture after immunostaining. It was further confirmed that constructs were more mature as well as bio mimic to *in vivo* muscle at 10 days in differentiation as the number of myotubes were significantly three fold higher with average of 13±1.15 myotubes. Myotubes at 5 and 15 days in differentiation were 3.16±0.44 and 4±0.57 respectively as shown in **figure 5.5**. This is consistent with the densely packed

muscle fibres as reported for muscle *in vivo,* making the model more representative than 2D monolayer culturing.

It was concluded from the previous experiments that the seeding density of 1 million cells per construct would be optimum for further use based on the fact that significantly higher number of cells were counted after considering the ratio of seeding to survival of cells for each density. Cells were more aligned and formed structurally biomimetic muscle and macroscopically the construct assembled to a diameter of less than 4mm at the end of experimentation. The plating density is 10 times higher compared to monolayer culturing of zebrafish skeletal muscle cells, but still it is significantly less when compared to reported densities used in the literature for tissue engineered skeletal muscle constructs for human muscle derived cells and other cell lines. In the literature tissue engineered skeletal muscle constructs are seeded with cells from a range of 2 million to 20 million cells and they have been stated to create most biomimetic constructs using collagen and fibrin based tissue engineered constructs (Mudera, Smith et al. 2010, Brady, Lewis et al. 2008, Martin, Passey et al. 2013, Smith, Passey et al. 2012).

5.4.3 mRNA expression of muscle markers in fibrin constructs

The expression patterns of myogenic regulatory factors MyoD, myogenin and mrf4 were observed to be following the classical pattern for skeletal muscle cells but there was no statistical significant difference observed across the different time points (Pownall, Gustafsson et al. 2002, Coutelle, Blagden et al. 2001, Lin, Yung et al. 2006). Expression of MyoD was increasing though out the time points whereas expression of myogenin peaked at 10 day in differentiation compare to 5 and 15 days

in differentiation (Wright, Sassoon et al. 1989). Expression of the negative regulator myostatin, was negligible in later time points compare to after isolation time point, which is in line with the study published by Vianello and colleagues on zebrafish, that expression of myostatin is observed mainly during the early onset of muscle precursor cells and later when the muscle cells starts differentiating (Vianello, Brazzoduro et al. 2003). M-cadherin which is an adhesion protein and mainly expressed when muscle precursor cells adhere to the substrate either *in vivo* or *in vitro* and later when they start differentiating (Cortes, Daggett et al. 2003). Similarly expression of M-cadh in fibrin constructs was significantly higher at after isolation and decreased thereafter that, but in the later time points as well it peaked at 10 days in differentiation suggesting it to be the best maturation time. Expression of igf-1 was significantly higher at after isolation and it decreased by 10 fold in 5 days in differentiation followed by no further significant increase in it expression at later time points as well, similar to results obtained for monolayer culturing of zebrafish muscle cells. Slow and fast isoforms of myosin heavy chain were analysed to find which phenotype is expressed higher, but no significant difference was observed in slow myosin heavy chain expression pattern across different time points. However expression of fast isoform of myosin heavy chain was increasing significantly during the differentiation course and it peaked highest at 15 days in differentiation surprisingly, as the fibrin constructs have been proved to mature at 10 days in differentiation.

To conclude the chapter, fibrin constructs have been successfully established to culture zebrafish skeletal muscle cells and characterized using phase contrast images, immunohistochemistry, qPCR and macroscopic images of constructs. Optimum

maturation time have been considered to be 10 days in differentiation media and the

optimum seeding density for zebrafish skeletal muscle cells for fibrin constructs has

been optimised to 1 million cells per gel. These fibrin constructs can be further

optimised for sport and exercise studies by mechanically or electrically stimulating

them for different time intervals. They can also be used as test beds for different

toxicological experimentation or for drug testing in order to reduce or replace the use

of animals in research.

6. Zebrafish skeletal muscle cells in collagen based three dimensional tissue engineered construct

6.1 Introduction

As discussed previously during introduction chapter, there is a vital role for the ECM for successful 3D *in vitro* culture of skeletal muscle, which supports the alignment and fusion of myoblasts (Martin, Passey et al. 2013). This has been achieved using both synthetic (Acarturk, Peel et al. 1999, Saxena, Marler et al. 1999) and naturally derived polymers as collagen (Mudera, Smith et al. 2010, Brady, Lewis et al. 2008, Smith, Passey et al. 2012, Vandenburgh, Karlisch et al. 1988, Cheema, Yang et al. 2003, Cheema, Brown et al. 2005, Khodabukus, Baar 2009, Khodabukus, Baar 2011, Dennis, Kosnik II 2000). Cheema and colleagues (2003) also discovered that it is not only the ECM environment important, but also the tension throughout the ECM which is of principal importance in the development and maturation of skeletal muscle *in vitro* (Cheema, Yang et al. 2003). Muscle cells when cultured with in these axes of strains results in differential activation of key signalling proteins responsible for fusion and maturation (Hornberger, Armstrong et al. 2005).

Vandenburgh and colleagues were the first group in 1988, to report tissue engineered collagen based skeletal muscle tissue (Vandenburgh, Karlisch et al. 1988). They cultured partially differentiated avian myotubes in collagen gels attached to a circular ring which increased the tension formation due to the contraction from seeded cells in the system. They observed that myotubes seeded in collagen gels can be maintained for three weeks, compare to monolayer where myotubes start detaching from the substrate after a week time in culture. The myotubes formed in the collagen gels

possessed a high concentration of myofibrillar myosin heavy chain proteins and nuclei on periphery (Vandenburgh, Karlisch et al. 1988).

Collagen matrix embedded with skeletal muscle cells and other cell lines have been further developed to a huge amount in order to provide uniaxial tension and support seeded cells alignment (Eastwood, Mudera et al. 1998, Eastwood, McGrouther et al. 1994, Vandenburgh, Tatto et al. 1996, Cheema, Yang et al. 2003, Vandenburgh, Shansky et al. 2008, Eastwood, Porter et al. 1996). Collagen gels embedded with skeletal muscle cells were set in between two fixed points and later floated in media, set-up derived from cytomechanics studies of fibroblasts in 3D collagen cultures (Eastwood, Porter et al. 1996). Cheema and colleagues performed a gene expression study on human skeletal muscle cells seeded with a 3D collagen matrix and discovered the expression of insulin like growth factor 1 (IGF-1) splice variants at different points, along with shift in myosin heavy chain isoform expression from fast to slow phenotype (Cheema, Yang et al. 2003).

In the same study (Cheema et al, 2003), they also observed the differences between seeding density and force characteristic response of C_2C_{12} cell line and primary isolated cells including human dermal fibroblasts and rabbit smooth muscle cells. The study suggested a vast difference in cell-matrix interaction and contraction between all cell types (Cheema, Yang et al. 2003), where C_2C_{12}'s where delayed by 8 hours. Additionally, these constructs were 5 ml collagen preparations with 20 million cells, requiring long monolayer sub-culturing. Recently, (Smith, Passey et al. 2012) has published a study where they have characterized and optimised this collagen model using 3 ml constructs, to enable the longer term culture of primary rat MDC's.

Constructs were cultured for long term which, enhanced maturation of the seeded MPC's, which was almost similar to *in vivo* tissue (Smith, Passey et al. 2012). Histological analysis of the collagen constructs further supported the improved bio-mimicity of 3D culture system compare to conventional monolayer culture.

The long term characterisation of the culture conditions required for zebrafish skeletal muscle cell culture within this system would combat experimental as well as ethical issues with the previous 5 ml constructs model and the high number of primary muscle cells required. As per previous literature in order to achieve fusion or differentiate skeletal muscle cells in this model a high seeding density is required (Smith, Passey et al. 2012, Cheema, Yang et al. 2003). To obtain this high number of primary skeletal muscle cells with current protocol for isolating and culturing zebrafish skeletal muscle cells is methodologically challenging, as explained earlier in chapter 4, zebrafish skeletal muscle cells were not be able to passage successfully. Increasing the number of fish per isolation to obtain such high number of primary cells will increase the chances of contamination in the culture. Therefore, there was a need for development or further miniaturization of this model to be used with a cell line or cells from primary sources which cannot be passaged such as zebrafish skeletal muscle cells in present study. Furthermore developing the miniaturized collagen construct within specific cell lines or primary cell source, will enhance the comprehensive and skilful exploration of key genes and proteins involved in skeletal muscle adaptation, regeneration and degeneration.

6.1.1 Collagen as matrix

Collagen is the most abundantly found protein in mammalian body's extra cellular
matrix as explained earlier and it is one of the oldest natural polymers to be used as
biomaterial (see Section 1.2.5) (Neel, Cheema et al. 2006). Therefore in order to
provide more biological relevant environment during *in vitro* culture of any
mammalian skeletal muscle cells, use of collagen as a scaffold is well justified.

Skeletal muscle cells can easily remodel the organization of collagen fibres. Muscle
cells *in vivo* are attached to collagen via integrins, and similarly they attach to
collagen *in vitro* and facilitate the transmission of mechanical signals to the cells
embedded in the matrix (Neel, Cheema et al. 2006). High biocompatibility along with
low immunogenicity and high level of conservation in different species, makes
collagen a good model to culture muscle derived cells from different species (Neel,
Cheema et al. 2006). As the cells grow in collagen gels, they exclude fluids which
improve the mechanical properties of collagen gel. Collagen gels are highly hydrated
in nature and therefore the gels are fundamentally weak and hard for any
manipulation (Neel, Cheema et al. 2006). However this cell compression in the gel
slow as it depends from different cell type as well, therefore there is very shy increase
in density of collagen (Neel, Cheema et al. 2006).

Fibrin gels are more characterized in the literature in terms of their contractile ability
(Khodabukus, Paxton et al. 2007, Khodabukus, Baar 2009), but it is still under
experimentation, in terms of their development maturation of seeded cells. Whereas
use of collagen gels as a scaffold for muscle derived cells has been studied thoroughly
and its ability to promote differentiation (Cheema, Yang et al. 2003, Cheema, Brown

et al. 2005, Mudera, Smith et al. 2010, Smith, Passey et al. 2012). But still these models are far from their destination i.e. *in vivo* transplantation because muscle derived cells in these constructs do not generate force equal to that they produce *in vivo*. After their ability to generate controlled contraction been documented, it will be more important to consider their maturation of a phenotypic characteristic of adult tissue (Hinds, Bian et al. 2011). In the current work collagen model will be further miniaturized and used to culture zebrafish skeletal muscle cells *in vitro,* which further can be used to study different dystrophies and myopathies and also will open a window in human physiology to study important pathways.

6.1.2 Aim of chapter

In the context of the aims of this chapter, the further development and characterisation of this model will provide a foundation from which the effects of mechanical signals on cell response can be investigated. Experiments designed in this chapter are to culture zebrafish skeletal muscle cells in collagen models along with further miniaturization of collagen gels and their characterisation. Expression pattern for muscle markers will be observed to find their similarities with differentiation pattern with *in vivo* muscle or with fibrin model in thesis.

1) Culture zebrafish skeletal muscle cells at different plating densities in further miniaturized collagen gels.

2) Immunostain for muscle specific proteins at different time points and characterize the zebrafish muscle cells in collagen gels.

3) Extract RNA at different time points and observe the expression pattern of different markers of muscle lineage in order to observe fusion, maturation and its physiological similarity with *in vivo*.

6.2 Materials and Methods

6.2.1 Cell culture

Zebrafish skeletal muscle cells were isolated using the protocol developed and
optimised as explained in chapter 3 and 4. Isolated ZMC's were counted using a
haemocytometer (as explained in **Section 2.2.1**) and embedded in 0.75ml collagen
gels (formation of collagen constructs explained in **Section 2.3.2.2**), parallel to some
cells plated as control on gelatin coated coverslips. Growth media was changed every
day, whereas differentiation media was changed on alternate days.

6.2.2 Seeding density for collagen constructs

Collagen gels and the flotation bars for the anchor points were engineered as
described in **section 2.3.2.2**. Plating density plays a crucial role in any three
dimensional constructs due to the fact that if the plating density is too high then the
collagen gels might pull off from the anchor points due to the contractile force
produced by the cells. However, if the plating density is lower than optimum then
cells will not survive due to the lack of cell- cell interaction. Therefore in order to
obtain an optimal seeding density, collagen constructs were seeded at 4, 6, 8, 10 and
12 million cells initially and solely at 10 million cells per construct thereafter as
required. Constructs were maintained in growth media for five to seven days until
they reached confluency, at which point they were switched to low serum
differentiation media for the remainder of the experiment. Upon the cessation of these
experiments constructs were either fixed for immunocytochemistry (as explained in
Section 2.4.4) or homogenised in TRIzol for RNA extraction (as explained in **Section
2.5.1**).

Parallel monolayer cultures were setup as controls for each n-number of collagen experiment at 100 thousand cells per cm^2, maintained in growth medium until they reached confluency at which point they were switched to differentiation media for the remainder of the experiment. Parallel to collagen constructs, monolayer cells were also either fixed for immunohistochemistry or homogenised for RNA extraction.

6.2.3 Immunohistochemistry

Collagen constructs were fixed, blocked immunostained and mounted as described in **section 2.4.4** and stained constructs were imaged using confocal microscopy. Constructs were stained for desmin which is a muscle specific cytoskeletal intermediate filamentous protein and were counterstained using 4',6-diamidino-2-phenylindole (DAPI) for nuclei, in order to assess whether myotubes had formed and the general characteristics of the zebrafish skeletal muscle cells within the constructs.

6.2.4 Quantitative real time-PCR

qPCR was conducted using Rotor Gene instrumentation as described in **section 2.5.6**. The primer sequences for the genes of interest are shown in **table 2.1** and exhibited specificity according to **figure 2.8**. Data was analysed using two standard quantification method using equation mentioned in **section 2.5.7**. When analysing expression of genes in tissue engineered constructs, data was made relative to zebrafish muscle cells just after isolation and data was normalised to housekeeping genes EF1α and β-actin.

6.2.5 Statistical analysis

Shapiro-Wilk and Levene's tests were used to test for normality of distribution and homogeneity of variation respectively. Thereafter, one way ANOVA was used to

determine differences between groups for total time in culture for normally

distributed data. Differences within constructs for different gene expression data was

analysed at after isolation, before differentiation and 5 days at differentiation stages

using one way ANOVA with repeated measures. Where significant main effects were

found, pairwise comparisons using a bonferroni post-hoc test were carried out.

Significance was taken at an alpha value of $p<0.05$.

6.3 Results

Zebrafish skeletal muscle cells were isolated from the dorsal muscle tissue as explained in earlier chapter 3 and embedded in tissue engineered collagen gels and cultured for different time points in order to find optimum construct maturation time.

6.3.1 Cells seeding density for collagen gels

Seeding density plays a crucial role in collagen constructs as, the gels and the cells embedded will not be able to survive for longer time points at any end point either higher or lower plating density. Collagen gels were embedded with zebrafish skeletal muscle cells at different seeding densities ranging from 4 million/ml to 12 million cells/ml. It was observed that collagen gels embedded with 4, 6, 8 million cells/ml, there was no attachment of cells to the matrix as shown in **figure 6.1** and eventually after 2-3 days they died likely, due to lack of cell-cell interaction. However when cells were seeded at 12 million cells/ml, cells attached to the matrix but due the matrix remodelling by the cells in first 2-3 days they pull off from the gel from the anchor points or flotation bars. Cells seeded at 10 million cells/ ml were found to be attached to the matrix as shown in **figure 6.1** and did not pulled off the collagen gel from the flotation bars. Therefore for further experiments cells were seeded in collagen gels at 10 million cells/ml plating density. In the phase contrast micrographs, alignment and fusion of cells was observed which was later confirmed by immunohistochemistry.

Figure 6.1: Impact of different plating densities of zebrafish skeletal muscle cells cultured in tissue engineered collagen gels. Images captured after six days in culture at different plating densities i.e. 4, 6, 8 and 10 million cells/ml. Cells were observed to be more aligned and forming myotubes at 10 million/ml seeding density, whereas at lower densities it was observed only few cells survived with random alignment. Arrows denotes the cellular structures on the phase micrograph images. Images were captured at 100X magnification.

6.3.2 Cell attachment

In order to find the number of cells attached to the collagen matrix after 3 days in culture, the average number of cellular structures was counted from the phase contrast images of collagen based tissue engineered constructs at different seeding densities. Due to the fact that, focal plane differs along different surfaces of three dimensional collagen gel, the average number of cellular structures counted represents the mean number of cells from one plane. 20 images at 10X magnification were captured from each plane of collagen gels seeded with different plating densities ranging from 4 million cells/ml to 10 million cells/ml as shown by in figure 6.1 using arrows and the experiment was repeated three times. Alignment of cells in collagen gels was observed using the images from figure 6.1, shown by dark arrows in gel seeded at 10 million cells/ml and the number of cells was counted in similar images from different seeding densities, presented in figure 6.2. It was observed that there was no significant difference in the number of cellular structures at 4, 6 and 8 million cells/ml plating densities but there was significant increase in the number of cellular structures at 10 million cells/ml seeding density. Cellular structures were confirmed to be muscle cells and myotubes in later time points using immunohistochemistry in later part of chapter.

Average number of cellular structure/image
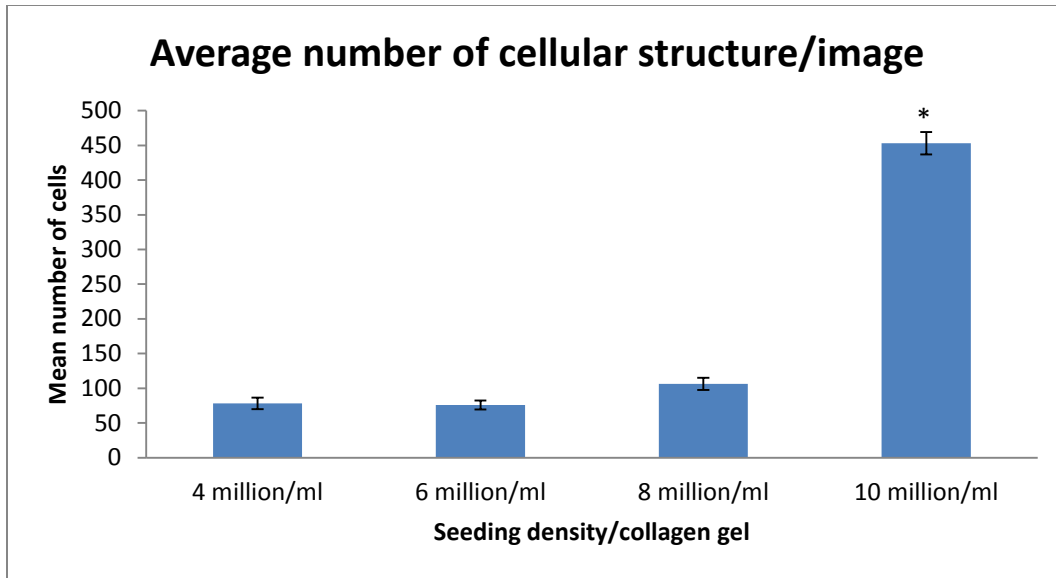
Figure 6.2: Impact of different plating densities on average number of cells survived in tissue engineered collagen constructs. Data obtained from images of zebrafish skeletal muscle cells cultured for seven days, imaged captured at 100X magnification (n=3 for each plating density). Y-axis represents average number of cellular structures whereas, X-axis represents different plating densities. Average number of cellular structures was found significantly higher (p=0.03) in cells plated at one million per gel. Error bars represent ± S.D.

6.3.3 Gel contraction

Zebrafish skeletal muscle cells were cultured at optimum seeding density (10 million cells/ml) in tissue engineered collagen based three dimensional constructs, adherence of cells to the matrix was shown in **figure 6.2** at same plating density. After a few days in culture cells in collagen constructs started growing and remodelling the matrix, resulting in production of a passive force. Since the gel is held by two anchor points as shown in **figure 6.3**, the gel started contracting from its width. It was observed that this reduction in width of gel is a continuous process as shown in graph in **figure 6.4**, where initially gels started at 12mm width and at the end of 12 days in culture reduced to 4mm. Due to this reduction of width of gels the myotubes forming

on the edges of gels came closer to each other, forming bundles of myotubes similar

to the basic structure of muscle cells *in vivo*. Gel reduction was measured every day

from the macroscopic images using Image J software. Formation of myotube bundles

around the edges of gel was confirmed later with the help of immunohistochemistry.
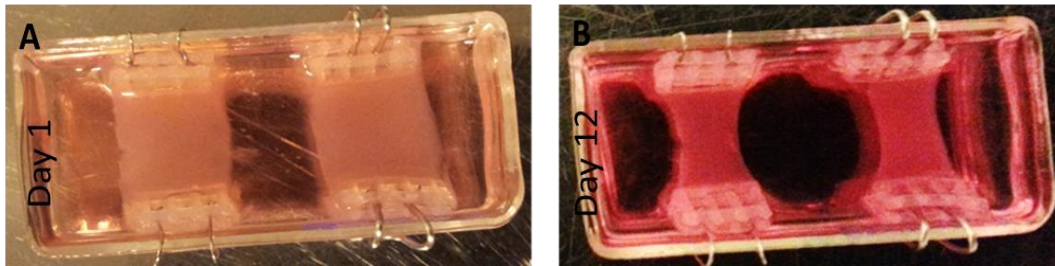
Figure 6.3: Macroscopic images of collagen gels at two different time points embedded by zebrafish muscle cells. A) Collagen gels at day 1, B) Collagen gel after 12 days in culture showing the reduction of width from its edges.
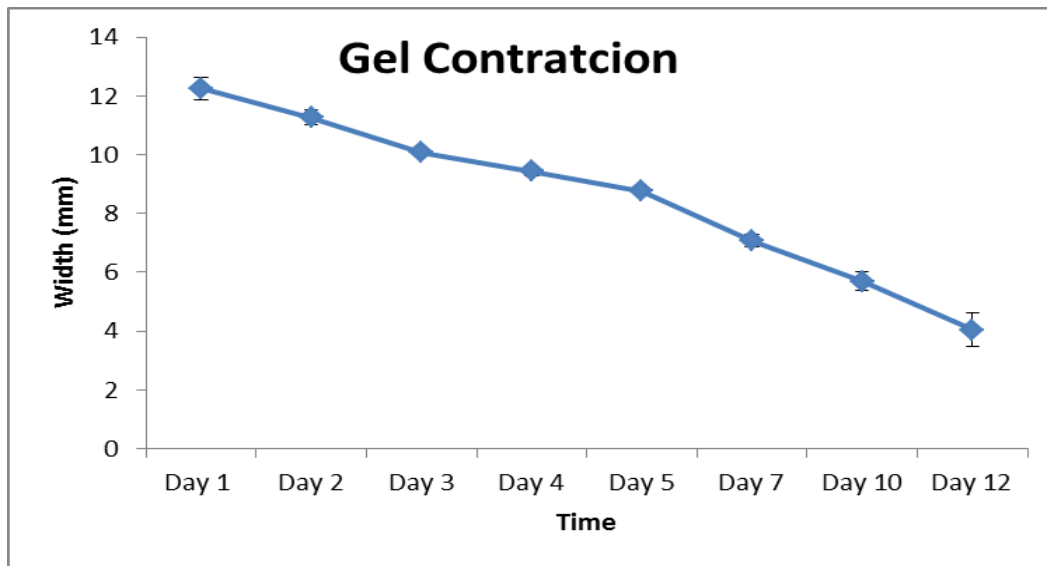
Figure 6.4: Gel contraction data collected over a time period of 12 days from macroscopic images of collagen gels embedded with zebrafish muscle cells (n=6). Error bars represents ± S.D.

6.3.4 Immunohistochemistry

Zebrafish muscle cells cultured in three dimensional collagen constructs were fixed and blocked as previously described in **section 2.4.4**. Further whole collagen gels were stained along with the flotation bars in order to retain the symmetry of cells in the gel and after mounted on the poly lysine coated glass slides after staining. Once the gel was mounted on the glass slides the floatation bars were removed from the gel. Collagen gels were stained for muscle specific filamentous desmin protein and counterstained with DAPI for nuclei (as shown in **Figure 6.5**) and viewed using Leica confocal microscope. Time points were selected due to their interest in terms of pre and post differentiation activity (before differentiation and 5 days after differentiation) and further three replicates were carried out. Immunostained images captured at 100X magnification for further characterisation in terms of number of myotubes observed per microscopic frame at both the time points. A significant increase in the number of myotubes per microscopic frame was observed from before differentiation to 5 days in differentiation time points.
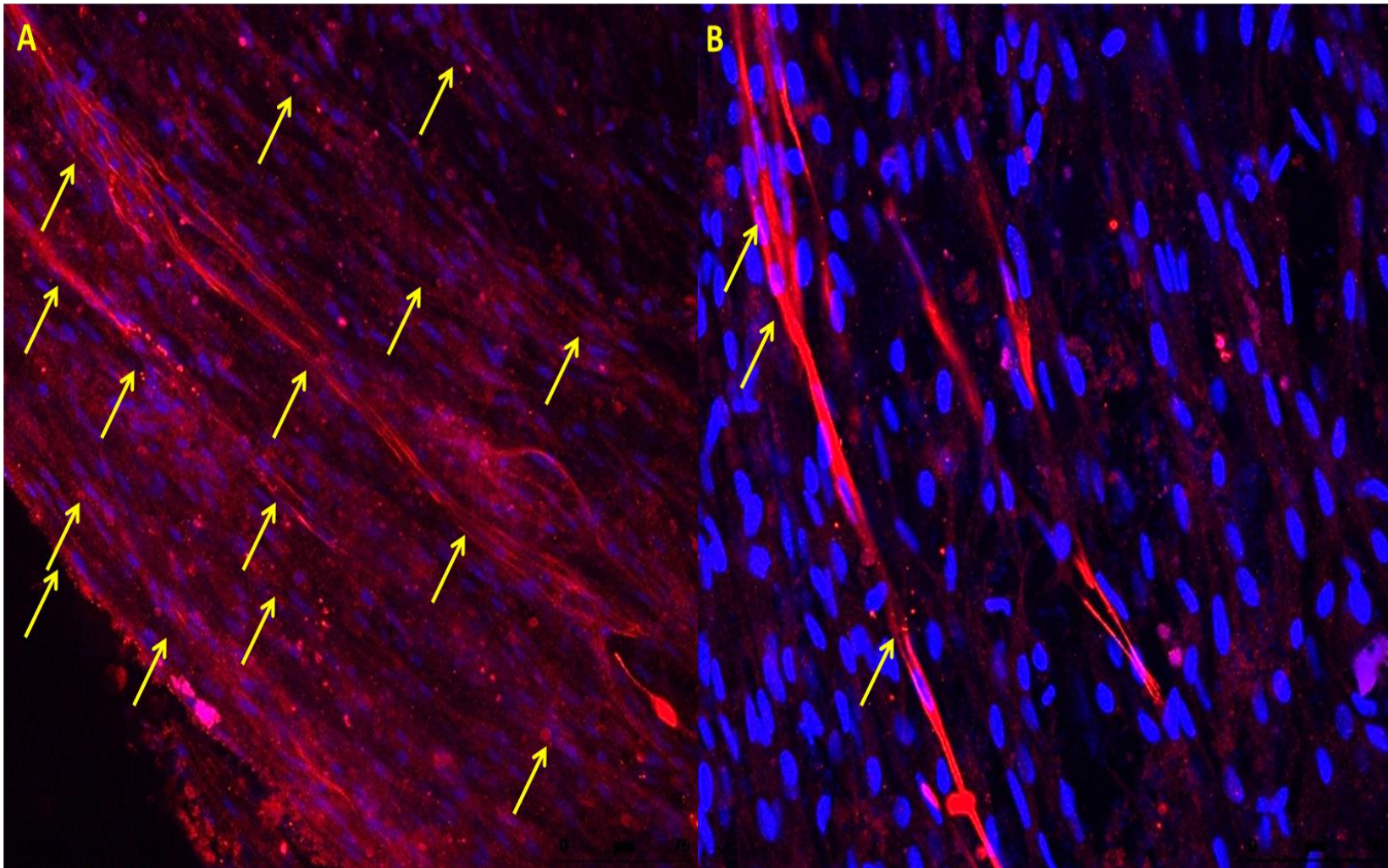
Figure 6.5: Immuno-stained images of collagen gels embedded with zebrafish muscle cells at different time points taken by Leica confocal microscope at 200X magnification. A) Stained image of collagen gel captured after 5 days in differentiation time point, B) stained image of collagen gel captured before differentiation time point. Red shows muscle specific protein desmin, whereas blue show nuclei stained with DAPI. Stained myotubes are shown on the images using yellow arrows.
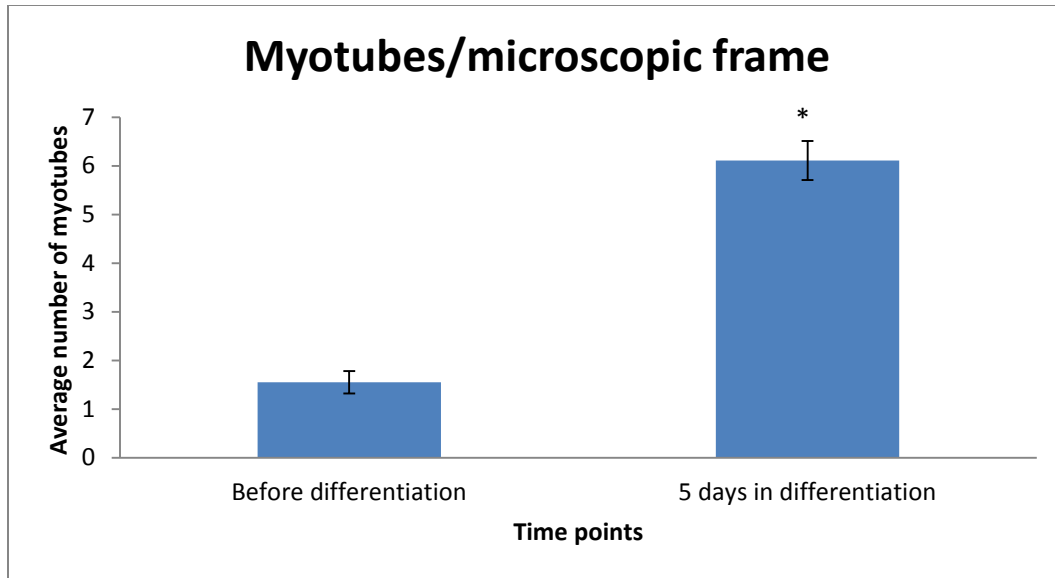
Figure 6.6: Average number of myotubes per microscopic image from immuno-stained collagen gels embedded with zebrafish muscle cells (n=20 images per time point). Statistically higher (P=0.04) numbers of myotubes were found at 5 days in differentiation time point compared to before differentiation time point. Star in the graph denotes significant difference in number of myotubes. Error bars represent ± S.D.

6.3.5 qRT-PCR analysis

Expression of different markers of myogenic regulatory factors MyoD, myogenin, M-cadherin and MRF4 along with myogenic inhibitor myostatin, hypertrophic gene insulin like growth factor-1 and slow and fast isoform of myosin heavy chain was measured at different time points in collagen model.

One sample Kolmogorov-Smirnov statistical test was used to determine the normality of data and further one way anova to find any statistical differences among the time points. Expression of myogenic determination factor myoD was observed to be significantly higher (p=0.00) at 5 days in differentiation time point compared to before differentiation time point, however still the relative expression was

significantly lower compared to after isolation. Myogenin, a gene specifically expressed in the differentiated muscle cells was observed to be significantly higher (p=0.02) after 5 days in differentiation time point compare to both other time points, where there was no statistical difference in relative expression among the early time points i.e. after isolation and before differentiation. Myostatin, a negative regulator for myogenic transcription factors which helps in initiation of differentiation was observed to be significantly higher at 5 days in differentiation time point compare to before differentiation time point. However expression at 5 days in differentiation time point was still 5 fold less compare to after isolation (p=0.00).
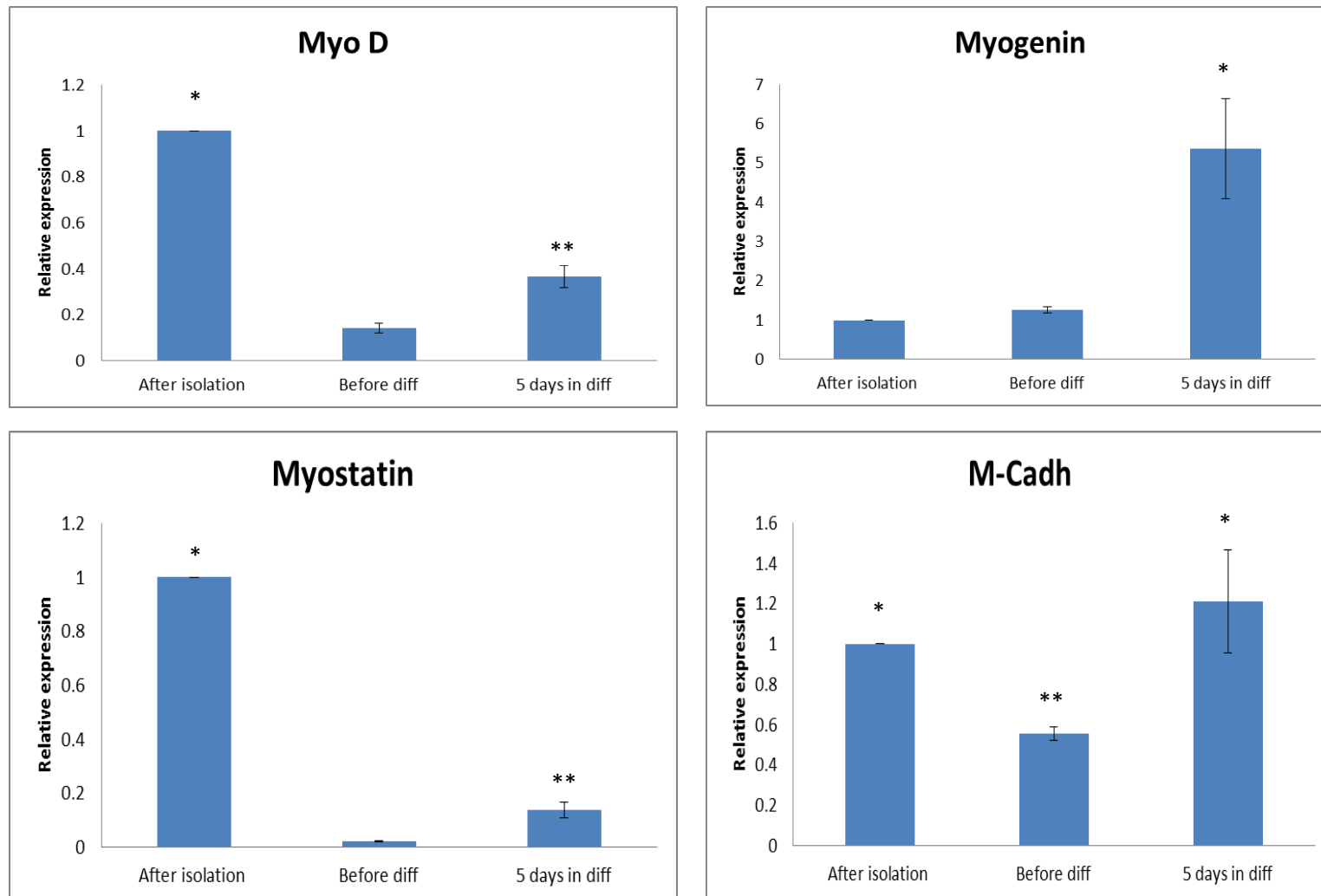
Figure 6.7: Relative expression of muscle lineage markers MyoD, Myogenin, M-cadherin and negative regulator myostatin (***n=3***). *On the graphs Y axis represents relative expression of proteins whereas X axis represents different time points where expression was measured in the collagen gels i.e. before (5 days in growth media) and after 5 days in differentiation. All data was normalised against housekeeping genes EF1α and β-actin and calibrated against after isolation time point for all genes. Error bars represents ± S.D and statistical differences among samples are denoted by different alphabets.*

185
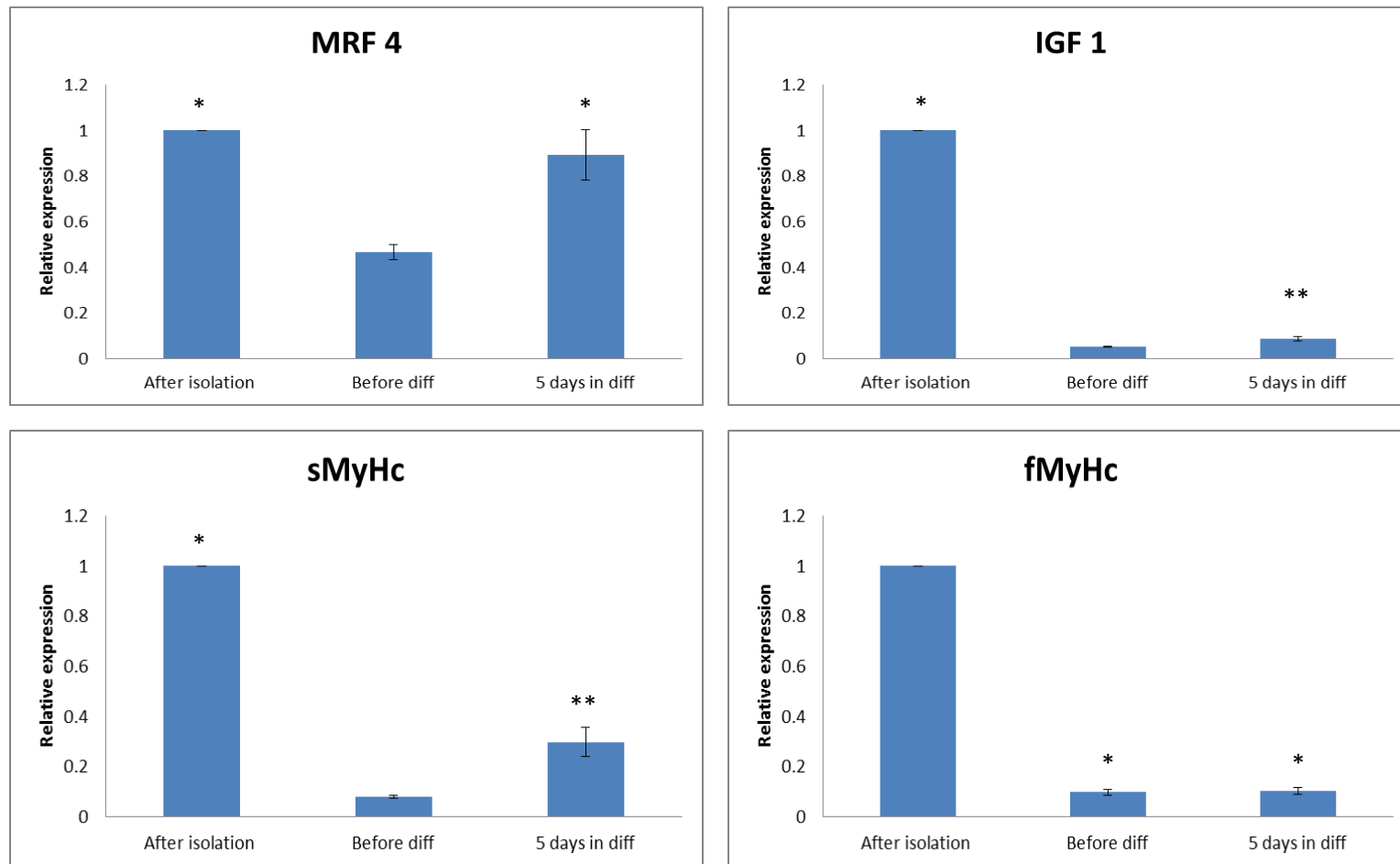
Figure 6.8: Relative expression of MRF4, slow and fast isoforms of myosin heavy chain and hypertrophic gene Insulin like growth factor-1 (n=3). On the graphs Y axis represents relative expression of proteins whereas X axis represents different time points where expression was measured in the collagen gels i.e. before and after 5 days in differentiation. All data was normalised against housekeeping genes EF1α and β-actin and calibrated against after isolation time point for all genes. Error bars represents ± S.D and statistical differences among samples are denoted by different alphabets.

186

M-cadherin, the gene expressing the adhesive proteins of the cells was observed to have significantly higher relative expression at after 5 days in differentiation (p=0.01) and after isolation time point (p=0.00) compare to before differentiation, but no statistical difference was observed in the relative expression between after isolation and after 5 days in differentiation time points (p=0.588). MRF-4, another myogenic transcription factor, that also plays a role in differentiation was observed to be expressed highly at after isolation (p=0.00) and after 5 days in differentiation (p=0.01) compare to before differentiation time point, but without any significant difference among them (p=0.501).

Igf-1, gene expressed highly during the hypertrophic growth of mature myotubes was expressed significantly higher in after 5 days in differentiation time point compare to before differentiation time point (p=0.00). However the relative expression was almost 5 fold lower compare to after isolation time point. Expression for both the isoforms of myosin heavy chains were significantly lower from after isolation in before differentiation and 5 days in differentiation time points. However there was no significant difference in fMyHc expression at before differentiation and after 5 days in differentiation, whereas sMyHc was expression increased at 5 days in differentiation time point compare to before differentiation (p=0.01).

6.4 Discussion

The aim of the current investigation was to develop and characterize the use of an established tissue engineered collagen based 3D culture model of zebrafish skeletal muscle *in vitro*. The requirement to characterize the use of zebrafish muscle cells in this system, will allow for the investigation of a homogenous primary muscle cell population in terms of their adaptability and remodelling of matrix *in vitro*. Moreover, the use of a primary muscle cell from zebrafish will combat experimental issues surrounding the inherent variability such as in other primary muscle derived cells from other organisms, particularly human cells. This system will provide a model to analyze the molecular mechanisms that govern *in vivo* skeletal muscle and particularly myogenic cell physiology.

Data have been presented here for the use of 10 million cells/ml in 0.75ml collagen constructs, which is a reduction in volume of gels from previous published data, where gel volumes were 5ml and 3ml (Mudera, Smith et al. 2010, Cheema, Yang et al. 2003, Smith, Passey et al. 2012). It has previously been established the importance of the aspect of long and short axis of collagen gels in determining behaviour of cells within collagen matrix (Eastwood, Mudera et al. 1998). The length and width aspect ratio of collagen constructs seeded in 5 ml and 3 ml were kept similar in 0.75 ml constructs demonstrated in the present study, which supports the concept of aspect ratio as a vital factor in cell behaviour, instead of the volume of construct (Mudera, Smith et al. 2010, Smith, Passey et al. 2012). In previous data, a culture force monitor was used in order to determine the optimum plating density, related to the peak force achieved reflecting the complex nature of cell matrix and cell-cell interaction

(Mudera, Smith et al. 2010). The relative peak force produced was defined as a consequence of remodelling of the collagen matrix by cells (Mudera, Smith et al. 2010). Therefore here as well the optimum seeding density was defined by two basic parameters initially by the attachment of cells to the matrix and secondly by remodelling of matrix over time period. Cells seeded at different plating densities were observed over time periods for attachment of cells to the collagen matrix as to remodel the matrix or to produce and force initially they need to attach or survive in the matrix (as shown in **Figure 6.1**). Number of cellular structures counted after 3 days in culture at different seeding densities demonstrated that constructs seeded at 10 million cells/ml survived for a longer time period along with significantly higher number of cellular structures attached (as shown in **Figure 6.2**). Adhesion of cells to the matrix was also confirmed by the expression of m-cadherin, which was observed to be significantly higher at after isolation and 5 days in differentiation. Expression pattern of m-cadherin suggests that cells initially take time but once they find the appropriate environment, attachment integrins increases over time and are expressed in high numbers.

Remodelling of the matrix is in line with previous data that more the collagen matrix remodels or contracts results in increment of force produced, which is shown here in **figure 6.3 and 6.4**. It has previously being established that a significant reduction in the gels surface area during the time course of culture (Smith, Passey et al. 2012), data presented here also demonstrates the reduction of collagen gel surface area over time as shown in **figure 6.3 and 6.4**, which is in line with the previous data shown by Smith et al., (2012), that the cells remodel the matrix. However it has been suggested

that this phenomenon is mainly driven by the non-myogenic cells from the primary cell population (Darren Player, thesis, UoB, 2013). The magnitude of reduction of gel width was significantly lower when constructs were seeded with homogenous C_2C_{12} cell lines (Darren Player, thesis, UoB, 2013) compare to data published using primary muscle cells by Smith et al., (2012).

Further to confirmation of remodelling of the collagen matrix in **figure 6.3 and 6.4**, a second goal was to investigate fusion of single seeded zebrafish skeletal muscle cells into post mitotic multi nucleated myotubes. Expression of myogenin and mrf4 were observed to be significantly higher during the differentiation time point compare to before differentiation time point suggesting the fusion or differentiation of single zebrafish myoblasts as shown in **Figure 6.7 and 6.8** (Hawke, Garry 2001). Expression of myostatin also supported the hypothesis, as in the literature it has been suggested as a negative regulator for myogenic transcription factors and helps in initiation of differentiation, therefore it's expressed highly during that time period (Garikipati, Rodgers 2012). It is evident from **figure 6.7,** where expression of myostatin is significantly higher at 5 days in differentiation time point, suggesting differentiation of zebrafish skeletal muscle cells and forming myotubes. Data presented in this thesis nicely describes that the collagen model developed embedded with zebrafish skeletal muscle cells recapitulates the developmental and regenerative processes occur within skeletal muscle *in vivo*.

Expression of maturation and hypertrophic genes including Igf-1, slow and fast isoforms of myosin heavy chain showed that the construct needed more time to achieve maturation. However there was small but significant increase observed in the

expression of igf-1 and slow myosin heavy chains at 5 days in differentiation time point compare to before differentiation as shown in **figure 6.8**. Data suggests that myotubes forming in the three dimensional tissue engineered collagen constructs had a higher profile of slow myosin heavy chain. Partial maturation of collagen construct can also be demonstrated from the expression of myogenin again where according to previous literature in collagen model itself, after maturation or complete differentiation its expression should fall down making the classical bell shape as discussed in chapter 4 as well (Smith, Passey et al. 2012). Therefore the collagen constructs with zebrafish skeletal muscle cells needs to be cultured for longer time points as well for complete maturation of the constructs.

It was also necessary to confirm the formation of multi nucleated differentiated myotubes in this system at differentiation time point. Previously in the literature, it has been demonstrated the alignment of muscle cells over predictable line of strains all over the matrix from different cell types (Smith, Passey et al. 2012, Cheema, Yang et al. 2003, Eastwood, Porter et al. 1996). During the current investigation it was confirmed using immunohistochemistry, the presence of highly oriented myotubes, morphologically similar to *in vivo* muscle fibre as shown in **figure 6.5**. Number of myotubes were also counted and found significantly higher during differentiation time point (shown in **Figure 6.6**), confirming the fusion of myoblasts when switched to low serum differentiation media. Furthermore, the 3D environment and the mechanical tension progresses the cellular structure over monolayer cultures (shown in **figure 4.3**), where there were more branched and multidirectional myotubes from zebrafish skeletal muscle cells.

6.4 Conclusion of Chapter

Data presented here demonstrate use of a collagen based three dimensional model for zebrafish skeletal muscle cells at the seeding density of 10 million cells/ml in 0.75 ml constructs. These constructs allowed/ facilitated formation of multi nucleated myotubes displaying morphology representative of *in vivo* skeletal muscle. Expression patterns of specific genes also confirmed the model as recapitulating the *in vivo* bio-physiologically relevant environment for zebrafish skeletal muscle cells. Immunohistochemistry of the constructs also confirmed the presence of unidirectional, non-branched multi nucleated myotubes similar to *in vivo* muscle. It was also evident that that system needed more time to mature, therefore later time points in the system with zebrafish skeletal muscle cells can be included in future studies.

7. General Discussion

The data collected and presented in this thesis represents an attempt to develop, optimise and characterize a three dimensional tissue engineered culture model for zebrafish skeletal muscle cells, which is similar in structure and function to *in vivo* skeletal muscle. The main goal was to develop a model which can be used in future studies to investigate skeletal muscle adaptation to a variety of stimuli and in particular for environmental studies and drug testing. The work undertaken during the course of the present study has developed a novel protocol for isolation of zebrafish skeletal muscle cells (See Chapter 3 and appendix II). The isolated cells were characterized initially on conventional monolayer cultures (See chapter 4) and subsequently used to develop and characterize zebrafish muscle cell collagen (See chapter 6) and fibrin (See chapter 5) based tissue engineered three dimensional models.

Zebrafish have been used as a model organism in different studies to investigate human myopathies, dystrophies and other diseases, current work suggests that it has been performed *in vivo* (Bassett, Currie 2003, Chambers, Dodd et al. 2001, Guyon, Steffen et al. 2007). *In vivo* experimentation on zebrafish or any other species involves sacrifice of multiple organisms which has been viewed poorly. Many of the major companies test their products using LD50 test, whereby animals are tested for a lethal dose of particular chemical to find its toxicity. Companies such as Shell along with other ones are looking for an alternative method for their testing in order to reduce the number animals sacrificed (http://www.shell.com/global/environment-society/environment/product-stewardship/animal-testing.html), and specifically the

number of fish used compare to other organisms is observed to be very high as shown

in the **table 7.1** below. Whilst the number of fish used has been decreased every year,

but relatively still it is been a huge number of fish sacrificed every year compared to

other species as documented in their annual report for animal testing review report-

2103. Data obtained from page 12 in report from UK home office website on annual

statistics of scientific procedures on living animals in Great Britain-2012, shows the

increase in number of rat and mice used in research slightly but the number of fish

used in research has been increased almost five times during the same time period

(https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/21261

0/spanimals12.pdf).

Table 7.1: Data illustrating number of laboratory animals used in research from year 2008 till 2013. *Data used in table is collected from UK home office stats from page 12 of annual statics of scientific procedures in living animals in Great Britain-2012 and page 5 on annual report for animal testing review-2013 on Shell global website.*

Tests commissioned	Animals used	Number of animals or procedures					Source
		2008	**2009**	**2010**	**2011**	**2012**	*Animal testing review, Shell,2013*
Shell	Rodents	592	64	2,501	2,497	150	
	Rabbits	6	21	9	6	9	
	Fish	54,986	43,093	38,524	33,753	30,832	
	Birds	0	0	0	90	0	
UK Home office data	Rats			271,500		278,400	*Annual report of scientific procedure, Home office, Grea Britain,2012*
	Mice		2,630,000			3,060,000	
	Fish		131,100			500,800	

Skeletal muscles compromise the 70% body composition of fish demonstrating the importance for *in vitro* culturing of fish skeletal muscle cells. *In vitro* cell culturing has emerged as more popular method for studying different cellular and molecular pathways in skeletal muscle (Hinds, Bian et al. 2011, Khodabukus, Baar 2011, Khodabukus, Paxton et al. 2007). Though, conventional monolayer culture conditions do not accurately mimic the *in vivo* skeletal muscle niche, thus tissue engineering of skeletal muscle cells has been increasingly used in recent years (Smith, Passey et al. 2012, Khodabukus, Paxton et al. 2007, Brady, Lewis et al. 2008). There has been no repeatable and consistent published literature available with regards the culture of zebrafish skeletal muscle cells *in vitro,* either in monolayer or in biomimetic tissue engineered constructs. Therefore development of such a model would help us to study and understand the molecular and cellular adaptations in zebrafish muscle cells in contained conditions that precisely mimic skeletal muscle *in vivo* and have comparatively very few ethical issues. This model would also be able to help us understand the effects of different exercise regimes in a controlled environment (generally very similar to *in vivo* fish muscle) in order to increase the muscle mass and potentially been applied on the whole fish in order to improve the quality and quantity of fish in the food industry. These models could also be used to reduce, refine or replace the use of model organisms in drugs, toxicological and environmental testing.

The protocol developed by Alexander et al (2011) was not ideal because it lacked consistency, was not reproducible, was time consuming and costly due to the use of protease as well as collagenase for the digestion of zebrafish muscle tissue.

Furthermore, myotubes obtained were not of a great number (only one myotube or cluster of cells was shown) and there was a lack of quantitative data in the published paper relating to parameters such as desmin positivity, fusion index and number of myotubes (Alexander, Kawahara et al. 2011).

The isolation protocol developed in the current study uses only collagenase for enzymatic digestion of the dorsal muscle tissue of zebrafish, making it more time and cost effective. Skeletal muscle cells after isolation were cultured directly on the gelatin coated plates, instead of pre plating them on plastic plates, making the current protocol further less time consuming. Comparable results were repeatedly obtained from every culture in terms of multinucleated myotubes stained for muscle specific desmin protein along with DAPI for nuclei. A great number of myotubes was obtained in every culture compared to Alexander et al (2011) paper which showed only one image of myotube (cluster of cells). Quantitative results in terms of desmin positivity, fusion index, number of myotubes, nuclei per myotube, length and width of myotubes were obtained in order to maintain the homogeneity of zebrafish skeletal muscle cells. The current protocol therefore provides better results, despite being less time consuming and cost effective.

7.1 Key findings

Plenty of literature is available demonstrating the use of zebrafish as a model organism to study the muscular development along with their relation human diseases and cures (Alexander, Kawahara et al. 2011, Barbazuk, Korf et al. 2000, Bassett, Currie 2003, Bower, Johnston 2009, Chambers, Dodd et al. 2001, Cortes, Daggett et al. 2003, Coutelle, Blagden et al. 2001, Dodd, Curtis et al. 2000, Driever, Rangini 1993, Guyon, Steffen et al. 2007). Although, being used as a model organism for the past two decades to study human myopathies and dystrophies, for environmental studies, drug testing and for developmental studies, there was no published protocol for isolation and culture of zebrafish skeletal muscle cells *in vitro*. Therefore, in a present study a novel protocol for isolation and culturing zebrafish skeletal muscle cells was developed and optimised, followed by intensive characterisation in order to ensure their potential and ability of cells to form zebrafish muscle fibres *in vitro* (myotubes). In the present study zebrafish skeletal muscle cells were successfully cultured for the first time *in vitro* and formed myotubes as demonstrated by **Figure 4.3**, similar to *in vitro* cultured human skeletal muscle cells (Blau, Webster 1981, Martin, Passey et al. 2013) or C_2C_{12} cell line (Tanaka, Sato et al. 2011, Tanaka, Sato et al. 2011, Cheema, Yang et al. 2003, Khodabukus, Baar 2009).

From data presented in this study, it was observed that myotubes formed by zebrafish skeletal muscle cells *in vitro* were thinner, spindle shaped and the nuclei in the myotubes were also observed to be at the periphery of myotubes. However, the literature suggests that myotubes formed *in vitro* by human or C_2C_{12} cell lines, nuclei are completely wrapped in the myotubes and myotube morphology is also thicker,

compare to myotubes formed by zebrafish skeletal muscle cells. Data also suggested that the size of nuclei of zebrafish skeletal muscle cells was three times smaller than human or C_2C_{12} cell line explaining the need for much high plating density for zebrafish skeletal muscle cells (Froehlich, Fowler et al. 2013).

In order to assess zebrafish skeletal muscle cells behaviour in tissue engineered constructs, fibrin (Khodabukus, Baar 2011, Khodabukus, Baar 2009, Khodabukus, Paxton et al. 2007, Martin, Passey et al. 2013) and collagen (Vandenburgh, Karlisch et al. 1988, Vandenburgh, Tatto et al. 1996, Vandenburgh 2009, Cheema, Yang et al. 2003, Brady, Lewis et al. 2008, Mudera, Smith et al. 2010, Smith, Shah et al. 2010, Smith, Passey et al. 2012) based models which have been previously been established for culturing muscle cells from different species or cell lines were adapted. Plating densities are crucial in growth and development of cells in these models (Demonstrated in chapter 5 and 6), therefore different plating densities were optimised for both tissue engineered models to culture zebrafish skeletal muscle cells in order to achieve tightly contracted fascicular structure with maximum number of myotubes. Fibrin and collagen constructs seeded with zebrafish skeletal muscle cells were observed to have matured to a point where the expression levels of myogenin and both isoforms of myosin heavy chains were found almost similar to *in vivo* (Palstra, Beltran et al. 2013, Siegel, Gurevich et al. 2013, Alexander, Kawahara et al. 2011, Myhre, Pilgrim 2010). Expression of different muscle specific proteins for zebrafish skeletal muscle cells in 2 dimensional monolayer and both 3 dimensional tissue engineered constructs were compared at a specific time point and it was

observed that the expression pattern was similar in both tissue engineered constructs with the monolayer expression pattern was lagging behind (As shown in **Figure 7.1**).
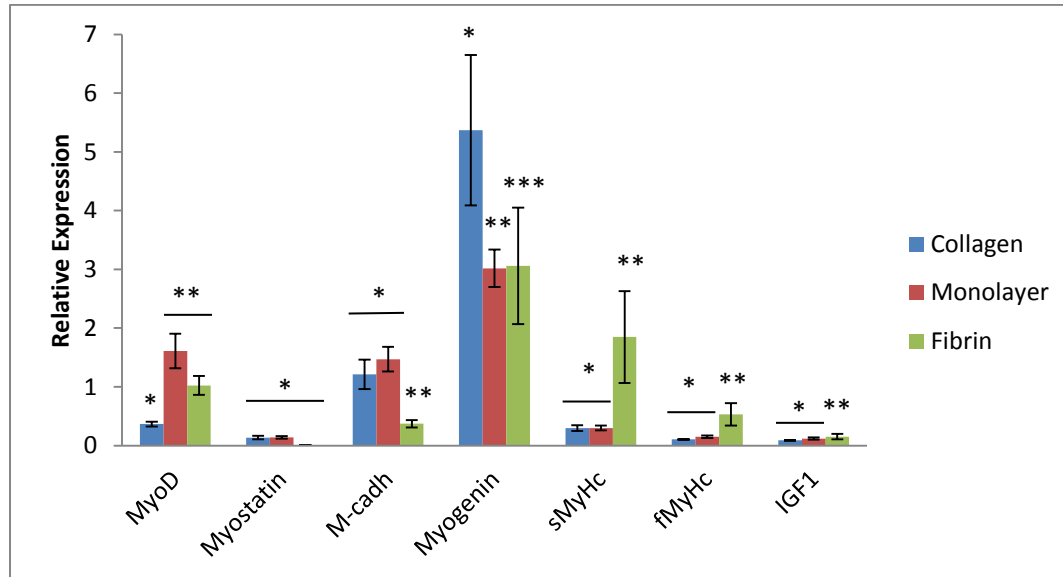
Figure 7.1: Relative expression of different genes in collagen, monolayer and fibrin after 5 days in differentiation, compared against each other in the same gene. Letters denote the significant differences among collagen, monolayer and fibrin cultures cells in the same genes. Significance was measured at p<0.05.

Immuno-histochemical analysis of zebrafish skeletal muscle cells in monolayer culture showed that the myotubes formed were branched and mutli-directional compared to fibrin and collagen based 3 dimensional tissue engineered constructs were observed to be aligned uni-directionally without any branching as found *in vivo* (Alberts, Johnson et al. 2007), shown in figure 7.2 below. Immuno-stained images were further analysed in order to find fusion index, myotubes per microscopic frame, nature of myotubes and nuclei per myotube and compared in different matrix (**See Table 7.2**).

Table 7.2: Comparison of different characteristics / parameters from immuno-histochemical analysis compared in monolayer, collagen and fibrin models.

	Monolayer ZSMC's	Collagen constructs ZSMc's	Fibrin constructs ZSMc's
Fusion Index	59%	71%	62%
Myotubes per microscopic frame	4	9	12
Myotube nature	Branched and multi-directional	Unidirectional and un-branched	Unidirectional and un-branched
Nuclei/myotube	4.5	9	9

Immuno-histochemical analysis along with gene expression data illustrated that culturing zebrafish skeletal muscle cells in tissue engineered models based on collagen and fibrin provide more bio-physiological relevant environment to the cells.
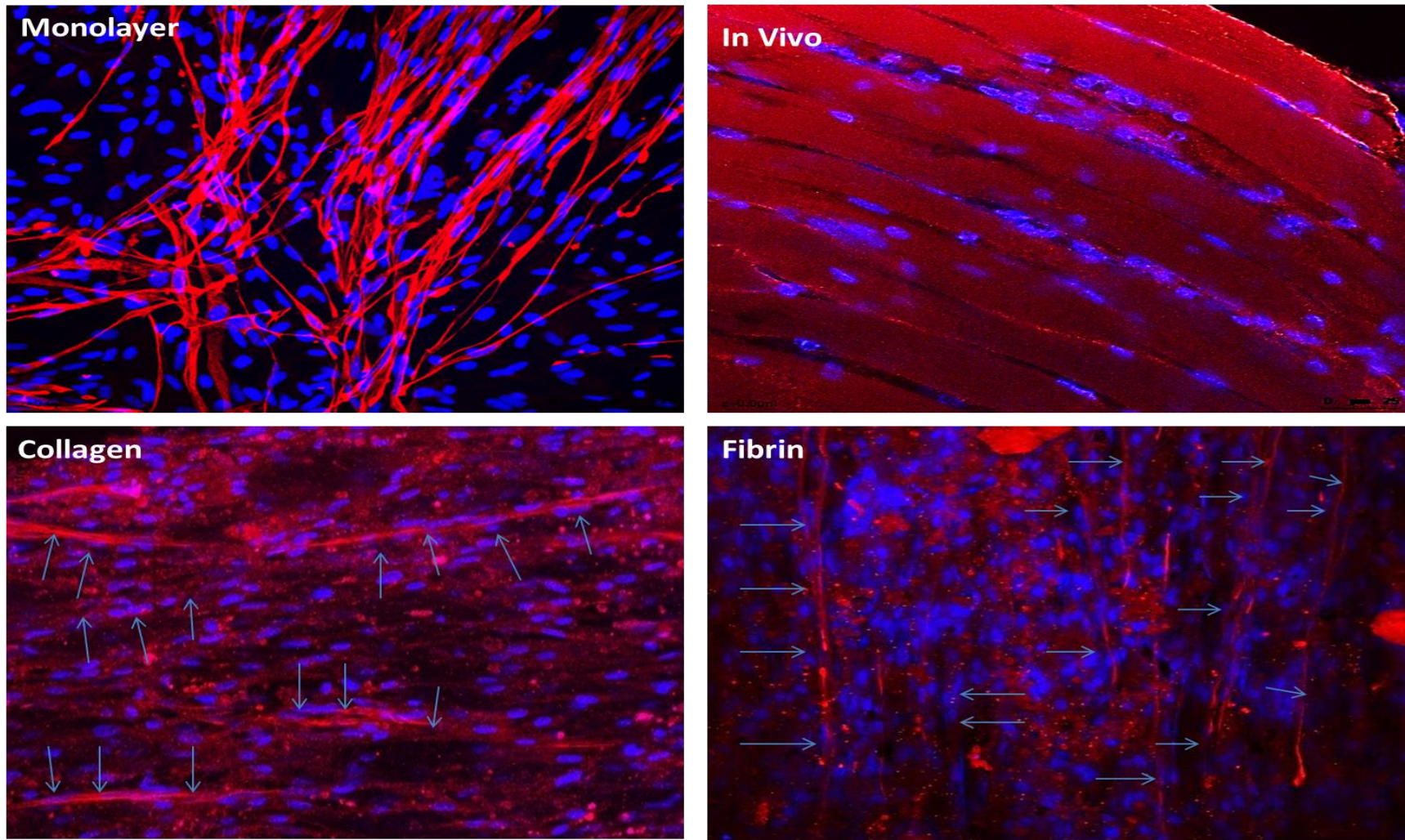
Figure 7.2: Immuno-stained images of zebrafish skeletal muscle cells in monolayer, in vivo zebrafish muscle, collagen and fibrin based tissue engineered constructs. Arrows in collagen and fibrin images are showing myotubes formed. Images captured at different magnifications and scale bars mentioned on right hand bottom corner of each image.

7.2 Future Directions

Data presented in this thesis has shown the novel development of a protocol for isolation and culturing zebrafish skeletal muscle cells. Zebrafish muscle cells were further cultured in tissue engineered constructs based on fibrin and collagen, in order to achieve more bio-mimetic *in vitro*. However, there is plenty work needs to be undertaken before using these models for further studies.

Firstly, as discussed in chapter 4, the effects of IGF on fish species need to be further investigated. In the present study, we have demonstrated that zebrafish skeletal muscle cells follow the hypertrophic growth. We also documented that, IGF was toxic during the differentiation, which needs to be further investigated, if zebrafish skeletal muscle cells are following different molecular pathway during differentiation compare to other fish species. Further investigation need to be under taken to find the consistency of hypertrophic growth of zebrafish skeletal muscle cells *in vivo,* and compare the results with zebrafish skeletal muscle cells cultured *in vitro*. This method of hypertrophic growth of fish muscle cells can be implemented on a large scale in fish food industries.

Zebrafish skeletal muscle cells need to be cultured for longer time periods in collagen based construct in order to achieve complete maturation. Further, the collagen model could potentially be used to investigate the effects of any mechanical stretch or strains on the zebrafish skeletal muscle cells (Palstra, Planas 2011), along with considering the intrinsic excitability which stimulates contraction before mechanical response.

Similarly, zebrafish muscle cells cultured in fibrin based models need to be investigated for their functionality. Constructs could be used to determine the

electrical or mechanical excitability of myotubes formed in the fully matured models. In this model, it is explained in the literature about measurements such as peak force, peak time and relaxation time provides information which can be useful to design further experiments or studies (Lamb 2002). This work of electrically stimulating the fibrin models is undergoing in our lab, using different cell type.

It would be very useful to test these models for different environmental conditions in order to find the switch of myosin heavy chains, as suggested in the literature about fish species. Also, it would have been useful to compare those results with *in vivo* fish, which would lead us to the interpretations and answer the question how bio-physiological these models are functionally (Palstra, Tudorache et al. 2010, Palstra, Beltran et al. 2013, Palstra, Planas 2011).

Further to the functionality of zebrafish skeletal muscle cells in tissue engineered constructs, it would be useful model to use as a test bed for drug testing in pharmaceutical companies, resulting in refinement, reduction or replacement of living organisms in research (Gibert, Trengove et al. 2013). These models would also provide us a window to study human diseases, due to the fact that zebrafish have similar gene orthologous responsible for human diseases (Guyon, Steffen et al. 2007, Howe, Clark et al. 2013) and it has already been used in the literature to study and investigate a cure human myopathies and dystrophies (Chambers, Dodd et al. 2001).

7.3 Conclusion

The work in this thesis describes the development of a protocol for isolating and culturing zebrafish skeletal muscle cells, followed by culturing them in 3D tissue engineered fibrin and collagen based constructs. Immuno-histochemical analysis and protein expression of specific muscle markers confirmed the enhancement of bio-mimicity of tissue engineered constructs. These models provide an ideal basis for molecular adaptation and translational studies from *in vivo* to *in vitro*, as well as having the potential in future to provide test beds for drug screening and environmental studies to reduce the number or use of living organisms in research.

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Appendix I

Quantitative real time pcr data calculations

All real time runs were conducted on RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor. Triplicates of each biological sample were prepared and each reaction details were explained in section 2.5.6. Average mean of raw threshold values were obtained after the run completed for both housekeeping genes (EF1α and β-actin) and gene of interest (MyoD in this example) shown in table I-1.

Table I-1: Raw threshold values obtained after quantitative real time pcr for MyoD as gene of interest and EF1α and β-actin as housekeeping genes from zebrafish skeletal muscle cells cultured in vitro at different time points.

Raw threshold CT values						
Time points	**β-actin**		**MyoD**		**EF-1 α**	
After Isolation 1	13.13	Mean	27.21	Mean	21.9	Mean
After Isolation 1	13.24		27.3		21.85	
After Isolation 1	13.24	13.20	27.22	27.24	21.8	21.85
After Isolation 2	13.5		27.54		21.98	
After Isolation 2	13.54		27.45		21.93	
After Isolation 2	13.61	13.55	27.33	27.44	21.95	21.95
After Isolation 3	12.9		27.09		21.65	
After Isolation 3	13.01		27.11		21.77	
After Isolation 3	13	12.97	27.07	27.09	21.75	21.72
Sub-Confluent 1	18.94		32.41		24.45	
Sub-Confluent 1	18.92		32.52		24.51	
Sub-Confluent 1	18.93	18.93	32.21	32.38	24.53	24.49

Sub-Confluent 2	18.6		32.33		24.22	
Sub-Confluent 2	18.65		31.52		24.16	
Sub-Confluent 2	18.7	18.65	32.08	31.97	24.3	24.22
Sub-Confluent 3	18.49		31.87		24.01	
Sub-Confluent 3	18.42		32.12		24.06	
Sub-Confluent 3	18.53	18.48	32.15	32.04	24.11	24.06
Confluent cells 1	16.85		29.84		22.76	
Confluent cells 1	16.84		29.81		22.77	
Confluent cells 1	16.86	16.85	30.34	29.99	22.79	22.77
Confluent cells 2	17.39		29.8		23.04	
Confluent cells 2	17.31		30.02		23.08	
Confluent cells 2	17.3	17.33	30.12	29.98	23.11	23.07
Confluent cells 3	15.93		28.71		22.05	
Confluent cells 3	16.03		28.81		22.16	
Confluent cells 3	16.36	16.10	28.74	28.75	22.42	22.21
2 Days in diff 1	15.57		24.82		20.27	
2 Days in diff 1	15.51		24.89		20.35	
2 Days in diff 1	15.43	15.50	24.79	24.83	20.31	20.31
2 Days in diff 2	15.08		25.09		20.13	
2 Days in diff 2	15		24.94		20.16	
2 Days in diff 2	15.08	15.05	25.07	25.03	20.36	20.21
2 Days in diff 3	15.58		25.49		20.78	
2 Days in diff 3	15.74		25.52		20.77	
2 Days in diff 3	15.49	15.60	25.47	25.49	20.84	20.79
5 Days in diff 1	18.38		29.98		23.54	
5 Days in diff 1	18.38		29.92		23.55	
5 Days in diff 1	18.52	18.42	30.1	30	23.53	23.54

5 Days in diff 2	18.26		29.75		23.06	
5 Days in diff 2	17.96		29.98		23.15	
5 Days in diff 2	18.21	18.14	29.81	29.84	23.17	23.12
5 Days in diff 3	18.23		29.66		23.17	
5 Days in diff 3	17.92		29.83		23.2	
5 Days in diff 3	18.18	18.11	29.84	29.77	23.18	23.18

Relative expression calculated using two standard curve quantification method following Pfaffl equation (Pfaffl 2001). Gene expression as normalised against two housekeeping genes EF1 α and β-actin as shown in table I.2 below.

$$\text{Relative quantification} = \frac{(E_{\text{target}})^{\Delta CP (\textbf{target}) \; (control - sample)}}{(E_{\text{ref}})^{\Delta CP (ref) \; (control - sample)}}$$

Where E = real time PCR efficiency (2 in this example)

ΔCP = Difference in threshold cycle (CT) values between unknown sample and reference sample (housekeeping genes)

Table I-2: qRT-PCR data for zebrafish skeletal muscle cells at different time points cultured in vitro for MyoD gene normalised against housekeeping genes and quantitative expression calculated taking pcr efficiency in consideration.

	GOI : MyoD		HKG -EF1α		ΔCt	ΔΔCt	2^-ΔΔCt
	Ct	Mean	Ct	Mean			
After Isolation	27.24	27.25	21.85	21.84	5.41	0	1
After Isolation	27.44		21.95				
After Isolation	27.09		21.72				
Sub-Confluent	32.38	32.13	24.49	24.25	7.87	2.45	0.18
Sub-Confluent	31.97		24.22				
Sub-Confluent	32.04		24.06				
Confluent cells	29.99	29.57	22.77	22.68	6.89	1.47	0.36
Confluent cells	29.98		23.07				
Confluent cells	28.75		22.21				
2 Days in diff	24.83	25.11	20.31	20.43	4.68	-0.73	1.66
2 Days in diff	25.03		20.21				
2 Days in diff	25.49		20.79				
5 Days in diff	30	29.87	23.54	23.28	6.59	1.17	0.44
5 Days in diff	29.84		23.12				
5 Days in diff	29.77		23.18				

HKG -βActin							
After Isolation	27.24	27.25	13.2	13.24	14.01	0	1
After Isolation	27.44		13.55				
After Isolation	27.09		12.97				
Sub-Confluent	32.38	32.13	18.93	18.68	13.44	-0.57	1.48
Sub-Confluent	31.97		18.65				
Sub-Confluent	32.04		18.48				
Confluent cells	29.99	29.57	16.65	16.69	12.88	-1.13	2.19
Confluent cells	29.98		17.33				
Confluent cells	28.75		16.1				
2 Days in diff	24.83	25.12	15.5	15.38	9.732	-4.284	19.48
2 Days in diff	25.03		15.05				
2 Days in diff	25.49		15.6				
5 Days in diff	30	29.87	18.42	18.22	11.64	-2.37	5.16
5 Days in diff	29.84		18.14				
5 Days in diff	29.77		18.11				

Average of relative expression values against both housekeeping genes were taken along with standard error of deviation was calculated using the formula in excel sheet i.e. = Stdev (range of values)/sqrt(number of values) as mentioned in table I.3 below.
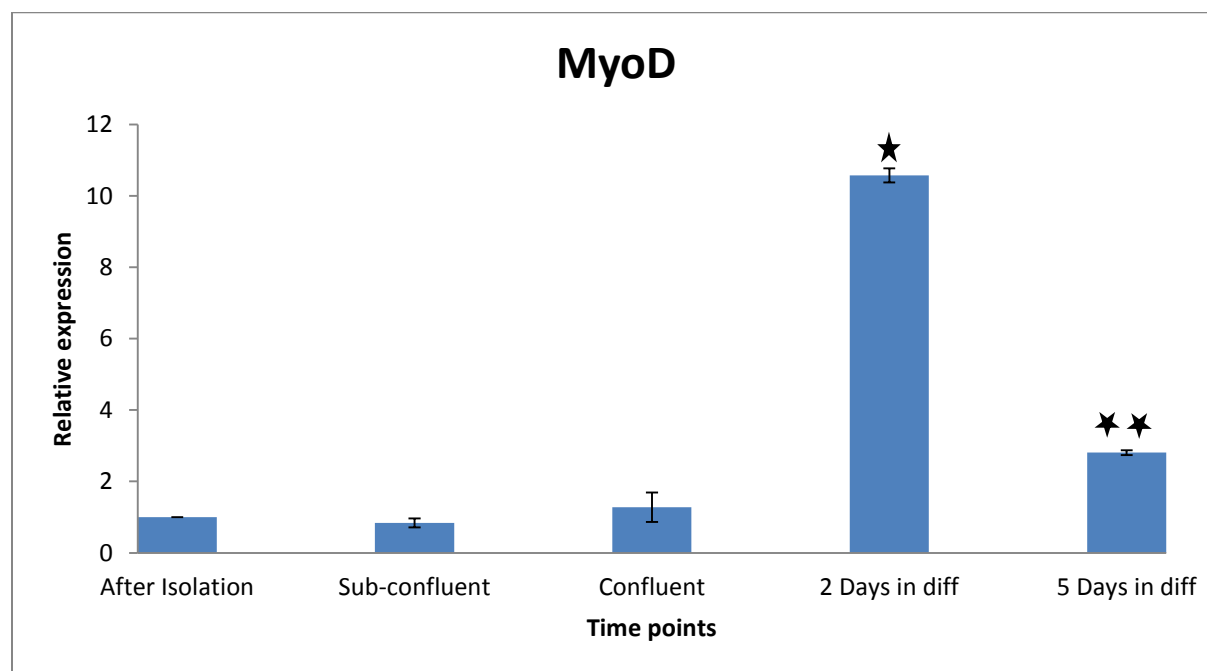
Expression pattern for after isolation time point is irrelevant to compare with all other time points, it has only been used in all the experiments for the consistency and calibrator. In order to calibrate the data, sacrifice of one sample has to be done therefore to avoid that after isolation has been used as calibrator in all the experiments throughout the thesis. Also expression of After isolation is irrelevant because the RNA extracted for this time point was from the cells of whole isolation (10-14 million cells along with debris), whereas once the cells were plated the number of cells attached on the surface is only half and the debris also get washed away by the media change.

Table I-3: Average values of relative expression for MyoD gene normalised against housekeeping genes and their standard error of deviation.

	MyoD (EF1-α)	MyoD (β-Actin)	Mean	Standard error deviation
After Isolation	1	1	1	0
Sub-confluent	0.18	1.48	0.83	0.12
Confluent	0.36	2.19	1.27	0.41
2 Days in diff	1.67	19.48	10.57	0.19
5 Days in diff	0.44	5.16	2.80	0.07

Graph was plotted in excel sheet using mean of relative expression calculated again both housekeeping genes and standard error bars were plotted in the graphs as shown in figure I.1 below. Statistical analysis was performed using SPSS V.19 (IBM, USA) and Microsoft Excel

(Microsoft, USA). One-sample Kolmogorov-Smirnov test was conducted to find the normal distribution of data and further data was analysed using one-way ANOVA followed by Tukey's and bonferroni post hoc tests.

Figure I.1: Relative expression pattern of MyoD against tow housekeeping genes EF1α and β-actin at different time points. After isolation time point has been used as calibrator only, therefore expression of after isolation time point is irrelevant to compare with the expression pattern of other time points. Star sigs on the graph represents the significant difference in the expression pattern among different time points.

Appendix II

Summary of isolations performed during protocol optimisation for zebrafish skeletal muscle cells.

When present study was started there was no published protocol for isolation and culturing of zebrafish skeletal muscle cells, therefore after vast literature review from different fish species a protocol was developed. Further this protocol was optimised and developed into a robust protocol of isolation and culturing zebrafish skeletal muscle cells. Table II-1 below is a summary of isolations performed during the initial time period when the protocol was step by step optimised. 45 isolations mentioned below in the table are representative from all isolations performed during the study for different experiments.

Data was recorded from all isolations during the study for example the date when isolation was performed, number of fish used in isolation, serum used, media used and outcome of the culture was also recorded every day, until the isolation was sacrificed for an experiment or cells got infected. Phase contrast images of the cells were also taken and stored every day and according to the culture conditions the coverslip were blocked from isolation for staining the cells for desmin protein and nuclei. Single issue was taken in consideration at a time in these first 45 isolation shown in table II-1 below, for example initially different culture medias were tested followed by different concentration of fetal calf serum, sterilisation of fish, number of fish per isolation, collagenase concentration, use of Ficoll gradient, coating with laminin or gelatin or fish gelatin. At the end when desired cells were obtained in continuously from isolations, the protocol was fixed for further use in the study.

Table II-1: Summary of isolations performed for the development of a protocol for isolation and culture conditions for zebrafish skeletal muscle cells.

Isolation number and Date		Serum	DMEM used	No. Of Fish	Outcome
23-06-10	#001	10% FCS	D6421 Low Glucose	12 or 13 fish	• No Adherence after 2 days • After 6 days - cells stuck down • Coverslip out stained desmin 1st July • 1well trypsenised and plated in 1well of a 24 well plate- no adherence 6th July. • 9th July took coverslips from well 2 &3 • 27 July contaminated & DISCARDED
12-07-10	#002	20% FCS	D6421 Low Glucose	12 or 13 fish	• 14/7 After 2 days cells stuck down • 15/7 saw division in the cells • coverslips taken on 21/7 & 26/7 • contamination seen on 26/7 • DISCARDED on 27/7
13-07-10	#003	20% FCS	High Glucose D6429-Bedford, no bicarbonate, no Hepes	12-13 fish	• 14/7 No cells in any well • Till 27/7 checked occasionally for cells • No cells on 27/7 – DISCARDED
03-08-10	#004	20% FCS	D6421 Low Glucose	10-12 fish	• 10/08 Cells growing • 11/08 c/slips taken for staining • Trypsenized whole plate & transferred in new 6 well plate 12/08 • After that no cells were found in the wells • 25/08 DISCARDED

06-08-10	#005	20% FCS	D6421 Low Glucose	14-15 fish	• 10/08 No cells visible • 19/08 found dirt floating in media • 20/08 got pictures, looks contaminated & cloudy • 23/08 DISCARDED
17-08-10	#006	20% FCS	D6421 Low Glucose	17 fish	• 20/08 No cells seen • dirt & some stringy stuff was in media • 23/08 some cells were found in 1 well • coverslips from that well were stained • rest of the plate was contaminated • 23/08 DISCARDED
18-08-10	#007	20% FCS	D7777 High-Glucose From St. Andrews	16 fish	• 19/08 white stuff floating in media • 20/08 cells found in all wells pictures available • well 4 got contaminated • 25/08 cells fed & washed with PBS • Number of cells decreased after wash • Contamination is spreading slowly. • Media changed 26/08 but very less number of cells were seen • 31/08 DISCARDED : contaminated very badly
31-08-10	#008	10% FCS 20% FCS	D7777 High-Glucose From St. Andrews	20 fish	• 1/09 Media was changed, but lots of cell debris in media, very few cells. • 2/09 attached properly. Less amount of cell debris • 3/09 media changed, found more cells in 20% • 6/09 cells found in both concentration, but more & bigger in 20% • 8/09 some contamination found. Scratched it using scalpel specifically. • cells are dying may be due to contamination

					• 10/09 all coverslips blocked and the culture was DISCARDED.
08-09-10	#009	20% FCS	D7777 H-G From St. Andrews	18 fish	• 9/09 media changed. • 10/09 cells found in wells attached to coverslips and plate. • 13/09 cells were properly attached and growing • Cells were fed with 20% FCS media and also washed with media with FCS 20%. • 15/09 Cells were fed, but some white stuff was floating in the media • 17/09 Media was changed, but white stuff was still floating • 20/09 DISCARDED
16-09-10	#010	20% FCS	D7777 High-Glucose From St. Andrews	21fish	• 17/09 media was changed. • 1.6 millions/ml was the density when cells were plated. • 20/09 DISCARDED contaminated
22-09-10	#011	20% FCS	D7777 High -Glucose From St. Andrews	23fish	• 23/09 media was changed • 0.8 million cells/ ml density when plated • 24/09 cells were smaller in size and less in number. • 27/09 number of cells was not increasing as normal. • 1/10 got contaminated badly, so DISCARDED.
27-09-10	#012	20% FCS	D7777 High -Glucose From St. Andrews	23fish	• Cell density was 1.2 million cells/ml • 28/09 media was changed to remove cell debris. • 1/10 some white stuff was floating, but when washed with pbs lot of cells were there under it in groups. • 4/10 Got contaminated badly with bacteria • 6/10 DISCARED after plating agar plate's from contaminate

248

29-09-10	###	20% FCS	D7777 High -Glucose From St. Andrews	1 fish per well	• 1/10 changed media. • 01/10 prepared slides to see the contamination • 4/10 Found some white stuff floating in it. Contamination. • 6/10 DISCARDED.
11-10-10	#013 (Ethanol wash)	20% FCS	D7777 High –Glucose From St. Andrews	19 fish	• 12/10 changed media • 14/10 & 15/10 media changed but there was some contamination. • 18/10 DISCARDED
13-10-10	#014 (Ethanol wash)	20% FCS	D7777 High-Glucose From St. Andrews	19 fish	• 14/10 changed media • 15/10 changed media again but saw some white stuff floating • 18/ 10 got contaminated badly DISCARDED
14-10-10	#015 (Ethanol wash)	20% FCS	D7777 High-Glucose From St. Andrews	19 fish	• 15/10 media changed • 18/10 got contaminated. Pictures available • DISCARDED
19-10-10	#016 (Ethanol wash)	20% FCS	D7777 High -Glucose From St. Andrews	20 fish	• 20/10 changed the media • Some white stuff floating. • 22/10 no cells found. • 25/10 no cells • 28/10 no cells still. So, DISCARDED
21-10-10	#017 (Ethanol wash)	20% FCS Hepes negative bicarbonate negative	D7777 High -Glucose From St. Andrews 3 well with pen/strep	17 fish	• 22/10 media changed, no cells • Very few cells were seen in antimitotic after media change. • 28/10 antimitotic well got contaminated badly, pen/strep ones are also getting the contamination slowly. • But no cells are found until in pen/strep. Pictures taken.

			3 wells with antimitotic		• 1/11 DISCARDED as all the plate got contaminated badly
26-10-10	#018	20% FCS 3 wells- 20 minute collagenase 3 wells- 70 minute collagenase	D7777 High -Glucose From St. Andrews pen/strep	18 fish	• 27/10 media changed, cell debris reduced. Some cells seen attached. • Pictures taken step by step • 28/10 NO contamination in any time period. • Higher number of cells was found attached in 20 minutes collagenase activity. • 1/11 70 minutes collagenase got contaminated • Cells are seen in 20 minutes activity. • 3/11 DISCARDED
26-10-10	#019	20% FCS 3 wells- 20 min collagenase 3 wells- 70 minute collagenase	D7777 High -Glucose From St. Andrews	18 fish	• 27/10 media changed, cell debris reduced. Some cells seen attached. • 28/10 no cells found in both time scale isolations. • 20 minutes- one well more was contaminated then 70 minutes ones. • 1/11 got contaminated badly, so DISCARDED
02-11-10	#020	20% FCS 3 wells- 20 min collagenase 3 wells- 70 min collagenase	D7777 High -Glucose From St. Andrews	24 fish	• 4/11 cells attached in both time scales more in 20 minutes than in 70. • Cell debris was more in 70 minutes than 20 minutes. • 5/11 changed the media. • 8/11 no of cells got decreased during the weekend (dead cells floating in the media). • 9/11 DISCARDED contaminated.

04-11-10	#021	20% FCS only with 20 minutes collagenase	D7777 High -Glucose From St. Andrews	27 fish	• 5/11 changed the media. Hope they will survive on weekend. • 8/11 no cells found attached may be 1or 2 cells in plate. • 9/11 DISCARDED contaminated.
11-09-10	#022	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews pen/strep	28 fish	• 9/11 cell plating density was a bit high. Plated in 4 wells instead of 6. • 11/11 few cells found attached along with a bit of contamination. • 12/11 contamination increased (white stuff floating in media) • 15/11 contaminated badly, so DISCARDED.
11-10-10	#023	20% FCS hepes negative bicarbonate added 20 minutes collagenase	D7777 High -Glucose From St. Andrews pen/strep	24 fish	• 12/11 media changed • Cells found attached in this plate with a bit of contamination. • 15/11 cells found in this plate • 6 c/slips taken out for staining. • Remaining wells were trypsenized n cells transferred in 2 wells of a new plate. • 16/11 very few cells were seen in both wells. • 17/11 CONTAMINATED badly, so DISCARDED.

02-01-11	#024 No serum after trypsin activity **Protocol updated**	20% FCS 20 minutes collagenase	D7777 H-G From St. Andrews pen/strep	30 fish	• 1st Feb cells were plated at 1.4 million cells/ml. • 2 Feb media changed. Pictures taken, there were cells attached but with cell debris. • 4 Feb Media changed, cells increased in number and size. • 7 Feb media changed, number and size of cells increased. • 8 Feb one well got contaminated, so trypsenized the whole plate & plated cells in two wells of new plate. • 10 Feb cells growing and getting confluent. • 11 Feb media changed, confluent in one well but cells are dying in other well as lot of cell debris was seen. • 14 Feb DISCARDED.
03-02-11	#025 No serum after trypsin activity	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews pen/strep	30 fish	• 3 Feb cells were plated at the density of 1.6 million/ml (3ml cell suspension per well) • 4 Feb Cells were attached on the plate with less cell debris, pictures taken. • 7 Feb media changed, only 1-2 cells/well were found. • 10 Feb did not had much cells had sum traces of contamination in it. DISCARDED
09-02-11	#026 No serum after trypsin activity	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• 9 Feb cells were plated at 1.5 million /ml (5 ml in each well). • 10 Feb lot of debris was there, cells in small size. • 14 Feb media changed, cells are growing in size but not in number. • 16 Feb cells died in this culture, contamination was also seen in two wells. • DISCARDED.

15-02-11	#027 (Bedford)	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• 15 Feb cells were plated and cultured at Bedford. • During centrifugation instead of 1300 rpm, By mistake I did 1300 g. Cells were damaged totally. • 18 Feb No cells seen at all. So DISCARDED.
17-02-11	#028 (LIRANS) No serum after trypsin activity	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• 17 Feb Cells plated at the density of 1.6 million cells/ ml. • 21 Feb Very few cells were found (e.g. 1-2 cells per well). • 23 Feb media changed, number of cells is still the same i.e. 1-2 cells per well. • 24 Feb Trypsenized all wells and plated cells in 1 well of the same plate. • 25 Feb got contaminated (not fungus this time, but Bacteria) DISCARDED
21-02-11	#029 (Bedford)	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• 21 Feb Cells plated at 1.7 million cells /ml (5 ml each well) • 22Feb Media changed; no cells at all. • 25 Feb No cells still, lot of debris, so DISCARDED
23-02-11	#030 LIRANS No serum after trypsin activity	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• Repeat of #028, cells plated at the density of 1.6 million cells/ ml. • 24 Feb Media changed, no cells found. • 28 Feb 3-4 cells /well were seen attached, so trypsenised them and plated in one well of the same plate. • 28 Feb blocked 2 coverslips also after 4 days before trypsenisation. • 1 March cells were seen in the wells before media change, but not after the media change. • 2 March no cells seen • 4 March No cells seen, DISCARDED.

02-03-11	#031 LIRANS With serum after trypsin activity	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• 3 March media changed, layer of cells was seen attached • 4 March media changed, cells were attached • Coverslip blocked from each well. • 7 March media changed, almost 60-70% confluent, cells were small in size in the gills one with more debris comparatively. • 14 March No cells were seen. DISCARDED
02-03-11	#032 LIRANS Old Laminin	20% FCS 20 minutes collagenase	D7777 High -Glucose From St. Andrews	30 fish	• 3 March media changed, no cells were found • 4 March media changed, No cells were seen in any well. Coverslip blocked from each well. • Cells were found attached with lot of debris. • 8 March cells were not myogenic, • 14 March DISCARDED,
14-03-11	#033 New Laminin	20% FCS 20 + 15 minutes collagenase	D7777 High -Glucose From St. Andrews	15 fish	• 14 March cells plated • Each step was monitored by staining the cells with DAPI, to check which step cells are lost. Pictures taken. • Some cell suspension from each step plated in a 24 well plate on gelatin. • 16 March cells were seen • 21 March DISCARDED
14-03-11	#034 Gelatin No trypsin	20% FCS 2 mg/ml collagenase 5 mg/ml collagenase	D7777 High -Glucose From St. Andrews 30, 45 minutes	6 fish	• 14 March Cells were plated just after mincing step on gelatin coated plate. • 15 March supernatant from the culture was plated on another gelatin coated plate, as it had all the tissue which didn't stuck down. • 16 March No cells seen in both plates from this culture. • 17 March no cells yet. DISCARDED

		8 mg/ml collagenase 10 mg/ml collagenase			
16-03-11	#035 Gelatin No trypsin activity	3% FCS 4 mg/ml collagenase 5mg/ml collagenase	L15 Media Fish media 15, 30, 45 minutes 15, 30, 45 minutes	3 fish each concentration	• 16 March Cells were plated accordingly • 18 March two cells were seen growing in same well i.e. 5 mg/ml for 45 minutes. Rest of the wells didn't have any cells. • 21 March No cells were there so, DISCARDED
17-03-11	#036 Fish gelatin NO trypsin activity	3% FCS 5mg/ml collagenase for 45 minutes	L15 media Fish Media	10 fish	•17 March cells were plated after ficoll gradient spinning at 1.077g/ml density. •21 March cells were doubled in number. •22 March cells were growing fine •23 March cells decreased in number, more debris was floating, cells switched to 20 % FCS. •25 March cells were dying and one well also got contaminated, so trypsenized the rest plate and plated the cells in one well of new plate. •28 March cells died, DISCARDED.

21-03-11	#037 Fish gelatin 0.1% No trypsin activity	20% FCS (switched after 2 days) 5mg/ml collagenase for 45 minutes	L15 media Fish media	15 fish	• 21 March cells were plated after Ficoll gradient spinning at 1.077g/ml density. • 22 March media changed • 23 March switched to 20% FCS • 28 March, media changed, cells are growing normally and dividing. Coverslip blocked. • 1 April blocked 2 c/slips, tried to passage cells but they didn't pulled off at all even after 15 minutes incubation time in incubator. • 4 April tried to passage rest of wells using fresh trypsin, but didn't work.
09-05-11	#038 No trypsin activity	20% FCS 5mg/ml collagenase for 45 minutes	L15 media	15 fish	• 9 May cells plated at the density of 1.2 million cells per ml • 10 May media changed; cells were attached properly and growing well • 11 May unfortunately the isolation got contaminated, so DISCARDED.
11-05-11	#039 six isolations were done	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• Cells plated at 1.2 million cells per ml • In order to keep the conditions similar, all isolations were done on one day, replica of each other. • 12 May media changed, two wells per plate got contaminated • 14 May found more wells contaminated • 16 May half of the remaining wells were contaminated and the rest didn't have any cells in them. So, DISCARDED

17-05-11	#040	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• Fresh media prepared • 17 May Cells were plated at the density of 1 million cells per ml • Changed the incubator as well • 18 May media was changed; cells were attached and growing well in each well. • 20 May RNA extraction but didn't work.
18-05-11	#041	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• 18 May replica of #040, plating density was 2 million cells per well. • 19 May media changed, cells were attached and growing properly. • 20 May changed the media. • 23 May contaminated badly, DISCARDED.
19-05-11	#042	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• 19 May replica to #040 and #041, plating density 2 million cells per well. • 20 May media changed. Cells were growing. • 23 May got contaminated badly, so DISCARDED.
24-05-11	#043	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• 24 May In this isolation extra washing with NaOCl (bleach) was done, in order to remove the oils from the surface of fish. • Cells plated at 2 million per well • 25 May media changed; cells were attached and growing well. • 27 May Did the RNA extraction after 3 days and saved it.
31-05-11	#044	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• 31 May 2 million cells per well were plated. • 1 June got contaminated, DISCARDED

06-06-11	#045	20% FCS 5mg/ml collagenase	L15 media	15 fish each isolation	• Fresh media prepared and filtered. Used DPBS from sigma • 2 million cells per well were plated. • 7 June media changed, cells were growing fine and attached. • 10 June RNA extracted after 5 days i.e. confluent cells and saved at -80 degrees.