Chapter 1
Introduction

1.1. Structure and anatomy of skeletal muscle

Skeletal muscle is a highly structurally organized organ present in the human body acquiring nearly 45% of the body mass. Each of the 600 skeletal muscles is connected to the skeletal system with the help of tendon (Figure 1.1A). Skeletal muscle tissue consists of a cylindrical elongated multinucleated cell called as myofiber (50-100 μm in diameter) or a muscle cell (Ostrovidov et al. 2014). Each of the muscle cells consists of cell membrane known as sarcolemma. A layer of endomysium connective tissue further surrounds the sarcolemma. Also, a bundle of myofibers forms a muscle fascicle. Muscle fascicles are covered with another layer of connective tissue called the perimysium. A bundle of such fascicles creates the skeletal muscle as a whole. Entire muscle is covered with connective tissue layer known as epimysium (J. B. Dixon, Drum, and Weatherwax 2016).

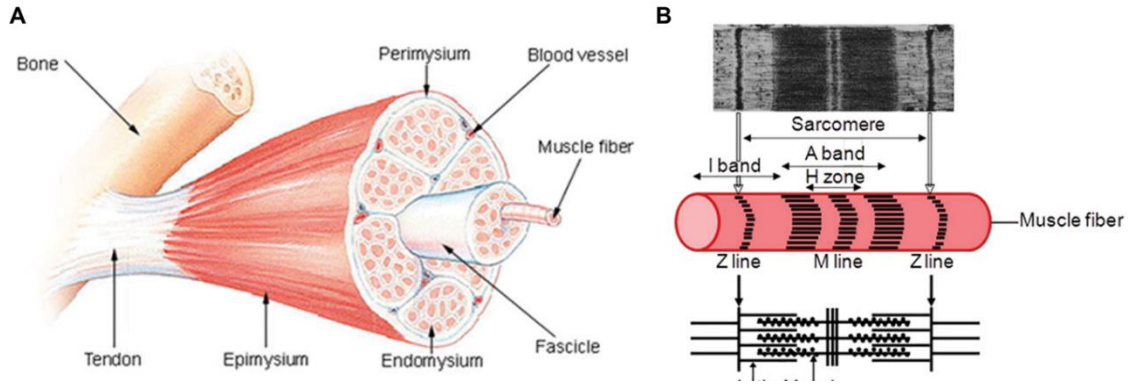

Figure 1.1 (A) Structural organization of a skeletal muscle starting from muscle fiber to a muscle as a whole attached to bones via tendons. (B) Structural organization of a sarcomere with the arrangement of thick myosin and thin actin myofilaments in the the form of protein rich bands (Ostrovidov et al. 2014).

A thick layer of connective tissue also known as deep fascia further envelops the epimysium. Each of these connective tissues consists of collagen, proteoglycans, and glycosaminoglycans. These layers together function to maintain and protect the cells from a range of forces acting on the tissue. The connective tissue layers continue beyond the fleshy part of the skeletal muscle to form tendon, which indirectly connects to the periosteum of the bone or other muscle tissues. Neuronal and blood vessel tissues form

connections at the epimysium level, which allows the execution of contractile functions of the muscle tissue (Gillies and Lieber 2011).

At the level of myofiber, each of the muscle fibers consists of a sarcolemma, cytoplasm and peripherally located flattened nuclei. The cytoplasm of the muscle cell consists of many myofibrils. Each of the myofibrils is inclusive of a considerable number of myofilaments. Two major types of myofilaments include thick myosin filaments (15 nm in diameter) and thin actin filaments (7 nm in diameter) (Figure 1.2A). Groups of organized myofilaments in regular patterns form sarcomeres. Sarcomeres also are known as structural and functional units of muscle, lie along the length of the muscle cell. They are basic units responsible for the contractility of the skeletal muscle. Based on electron microscopic observations, a sarcomere unit lies within two dense protein lines or Z lines. The structure of sarcomere shows various dark and light bands.

A pair of lightly stained bands or I bands lie at the outer ends of a sarcomere, within the boundaries of two I bands lies a densely stained A band, a H zone lies within the center of the A band. An M line with densely stained lies at the center of the H zone. The region represented by I band mainly consists of the thin actin filaments, the H zone region consists of only thick filament region, and the area of A band on either side of the H zone represents overlapping of the thin and thick filaments. When the sarcomere undergoes contraction, various changes are observed with respect to the position of the bands (Figure 1.2B). The length of A band remains unaltered. A decrease in the width of H zone and I band and subsequent disappearance of the bands is observed. As the contraction proceeds, the Z lines move inward to overlap with the outer ends of the A band (Sherwood 2011).

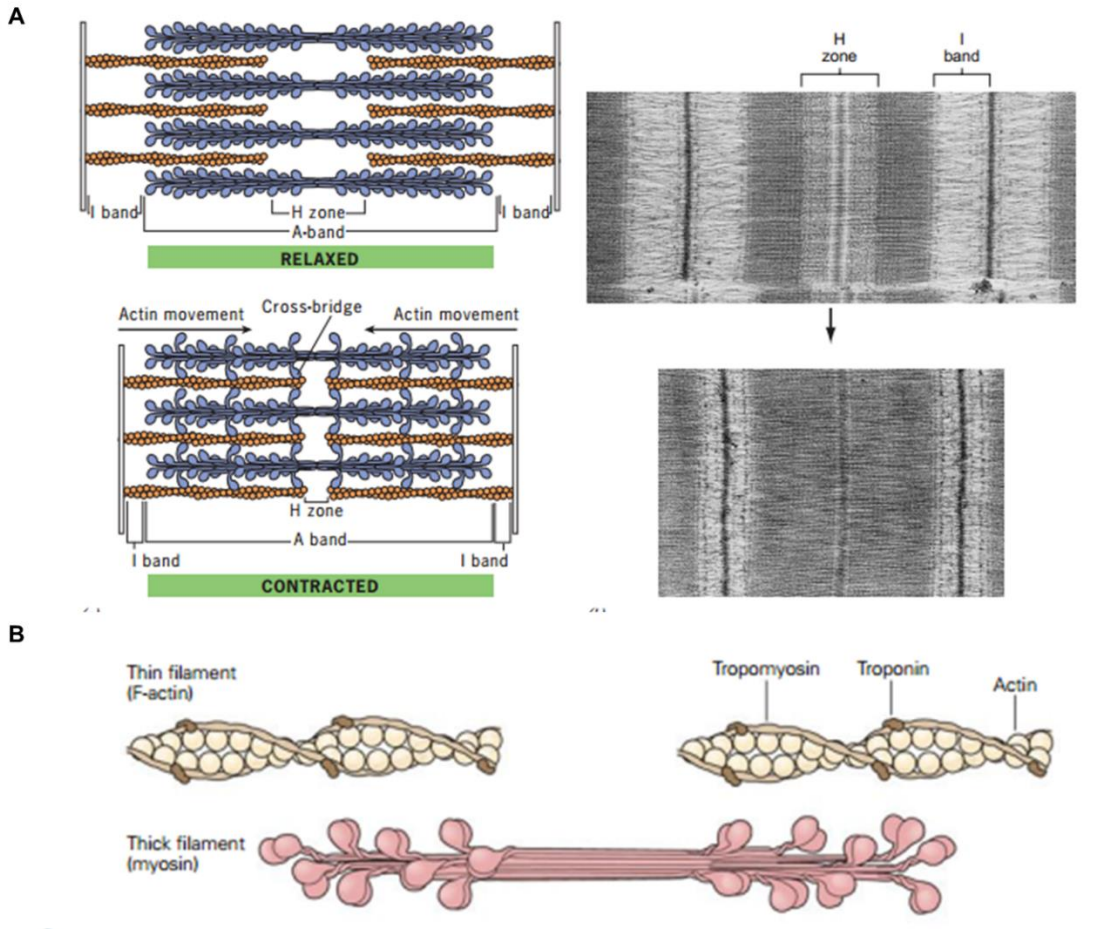

Figure 1.2 (A) Structure of thin actin filament and thick myosin filament. (B) Structural changes observed in sarcomeres during relaxation and contraction cycles of myofilaments actin and myosin (Karp 2009; Barrett et al. 2009).

1.1.1. Excitation and contraction coupling

The contractile units of the muscle involve the action of T tubules (muscle cell membrane invaginations facilitating the transfer of depolarizing signals) and sarcoplasmic reticulum. Sarcoplasmic reticulum acts as a reservoir of calcium ions necessary for contraction of the sarcomere. The contractile machinery also consists of associated regulatory proteins of the actin filament, including troponin and tropomyosin. In a relaxed state of the muscle, the actin filament is covered by tropomyosin protein which does not allow binding of myosin head to actin (Figure 1.2B). Troponin protein complex consists of three subunits troponin I, troponin T and troponin C. Troponin I binds to actin, troponin T binds to tropomyosin, and troponin C binds to calcium. Release of calcium from sarcoplasmic

reticulum is followed by binding of calcium to troponin C, thus releasing the myosin heavy chain binding sites on the actin filament. The myosin head binds to the released actin filament and pulls it inwards, affecting a contraction at the level of a sarcomere (Figure 1.3). Multiple sarcomere contractions produce an overall muscle contraction (Karp 2009). First of the steps in muscle contraction involves the discharge of action potential from the nerve influencing a release of acetylcholine at the motor endplate. Binding of acetylcholine at nicotinic acetylcholine receptors results in the increased flow of Na^+ and K^+ ions in the endplate membrane. The flow of ions subsequently directs the depolarization of the muscle membrane, which spreads throughout the muscle with the help of T tubules. Dihydropyridine receptors present on the T tubules release Ca^{2+} ions, which activate the ryanodine receptors of the sarcoplasmic reticulum (Rebbeck et al. 2014). This results in the release of Ca^{2+} from the sarcoplasmic reticulum into the sarcoplasm. Ca^{2+} ions further bind to the troponin protein releasing actin filament from the regulatory protein tropomyosin that allows the myosin head to bind with actin

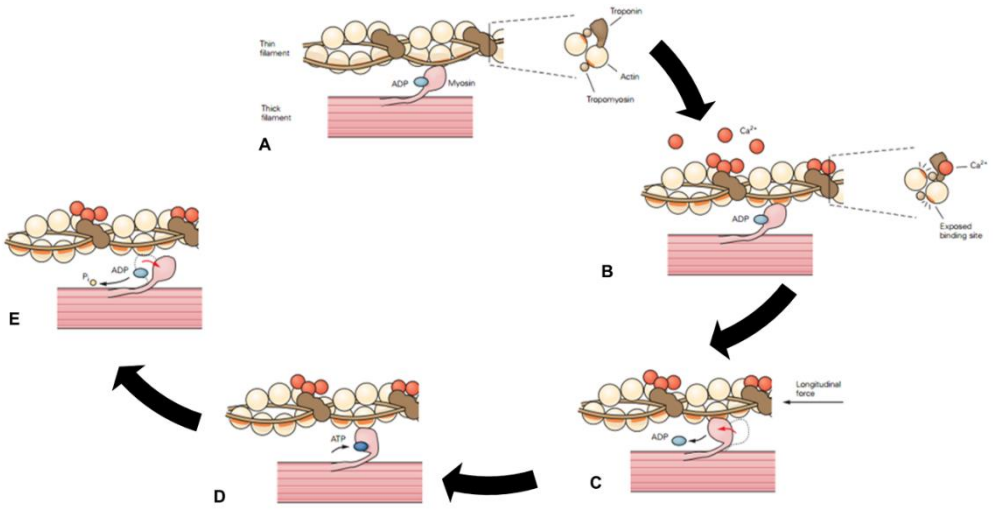

Figure 1.3 Power stroke of myosin on the actin filament. (A) In the resting state ADP bound myosin unable to bind with actin due to troponin and tropomyosin cover.(B) In the presence of Ca^{2+}ions structural changes in troponin tropomyosin complex allowing bond between myosin head and actin filament.(C) Myosin head rotates and moves the actin filament inwards releasing ADP. (D) Binding of ATP over the myosin head detaching it from actin filament. (E) Hydrolysis of ATP to ADP and Pi in order to reattach myosin head to actin (Barrett et al. 2009).

filament. The myosin head associated with ATP hydrolyses it to produce ADP and Pi. The energized myosin head bound to actin filament produces a power stroke, which slides the actin filament inwards and then contraction cycle, is complete. During the relaxation of the muscle, the Ca^{2+} ions are pumped back in to the sarcoplasmic reticulum. Subsequently, Ca^{2+} ions are released from the troponin protein and the interaction between myosin head and actin filament stops, serving the relaxation of the sarcomere and in turn, the whole muscle (Barrett et al. 2009).

1.2. Myogenesis and regeneration of skeletal muscle

1.2.1. In vivo myogenesis

Myogenesis in vivo involves two stages primary myogenesis and secondary myogenesis. Primary myogenesis involves the precursor cells of dermomyotome derived from somite part of the embryo. These precursor cells being Pax3 and Pax7 positive fuse to form primary myofibers. Primary myofibers, characterized by the expression of slow myosin heavy chain and myosin light chain 1, serve as a backbone for the formation of adult skeletal muscles (Messina and Cossu 2009). During secondary myogenesis, a subgroup of cells positive to Pax3 increase their Pax7 expression (along with a decrease in Pax3 expression) and form precursors known as fetal myoblasts. The fetal myoblasts fuse either with each other or with primary myofibers to form secondary myofibers. The expression of myosin light chain 3 and fast myosin heavy chain proteins characterize these myofibers (Matsakas et al. 2010). During this stage, some of the $Pax7^{+}$ cells undergo quiescence and form a pool of stem cells known as satellite cells. Post birth the percentage of satellite cells reaches to about 30% of the mononuclear cells, which reduces drastically to a few (1-5) percentages in a matter of two months (Cardasis and Cooper 1975).

1.2.2. In vitro myogenesis

Regeneration in skeletal muscles proceeds mainly in three phases, namely inflammatory phase, repair phase, and remodeling phase. During the inflammatory phase, the injured myofibers undergo necrosis, which leads to the secretion of factors that attract inflammatory cells in the blood to the site of injury. These factors include various growth factors and cytokines like tumor necrosis factor α, fibroblast growth factor, insulin like growth factor, interleukin-1β, and interleukin-6. Neutrophils are first of the cells to reach the site followed by macrophages. Repair phase involves the action of macrophages. Macrophages M1 clean the site of injury by phagocytosis of damaged fibers. They are also involved in the activation of satellite cells. Other factors influencing the activation include nitric oxide and hepatocyte growth factor secreted by the surrounding tissue (Tatsumi et al. 2006). Anti-inflammatory macrophages M2 contribute to proliferation and differentiation of satellite cells, which follows repair, and formation of connections with respect to nerves and blood vessels and finally deposition of fibrotic tissue. In the remodeling phase, the reorganization and fusion of newly formed myofibers with damaged myofibers occurs. Scar tissue is remodeled, and muscle starts to regain its contractile function.

Remarkable regenerative capabilities of the adult skeletal muscles are due to the presence of satellite cells. Satellite cells are positioned between the basal membrane and sarcolemma of a myofiber (Mauro 1961). These mitotically quiescent cells are activated under a range of signals, including mechanical stretching, conditions of injury, etc. Upon activation, the cells proliferate, differentiate to form myoblasts which fuse with previously damaged muscle fibers or form new muscle fibers (Le Grand and Rudnicki 2007). During regeneration, some of the cells undergo asymmetric division to form Pax7 positive cells, which return to the anatomical position between basal membrane and

sarcolemma as shown in Figure 1.4; thus, illustrating the self-renewal behavior of the cells (Kuang et al. 2007).

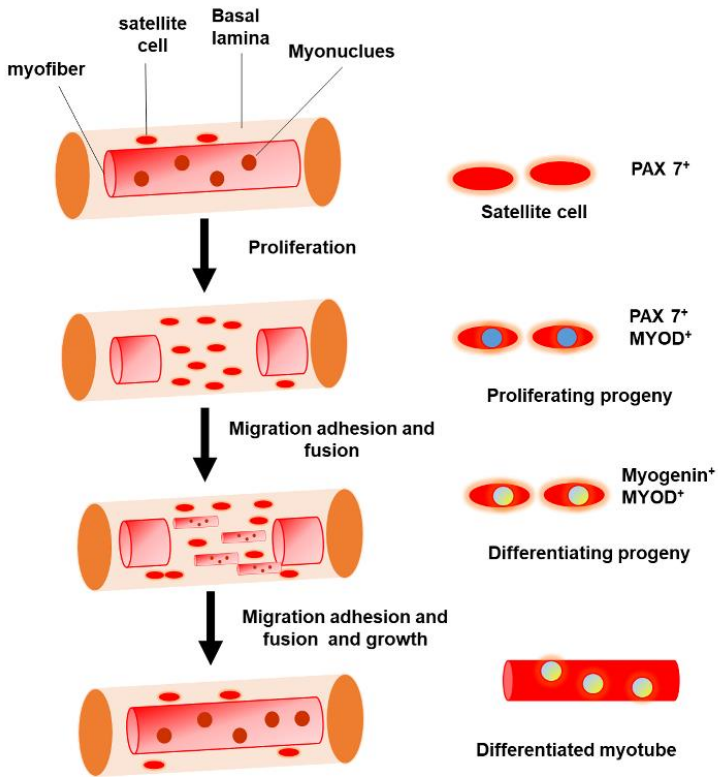

Figure 1.4 Adult muscle regeneration process (Abmayr and Pavlath 2012).

1.2.3. Regulation of in vitro myogenesis

Satellite cell regeneration and myogenesis mostly depend on the expression of paired box transcription factors (Pax7 and Pax3) and myogenic regulatory factors (MyoD, Myogenin, Myf5, MRF4) (Z. Yablonka-Reuveni et al. 2008). Myogenic regulatory factors consist of a basic helix loop helix responsible for DNA binding. A systematic activation of paired box transcription factors and myogenic regulatory factors is necessary for the progression of adult muscle myogenesis. The myogenesis proceeds from activated satellite cells, which show positive staining for Pax7. These cells next express myogenic regulatory factor MyoD and Myf 5 and are called as myoblasts. Further, under the influence of myogenin and MRFs cells differentiate and fuse to form myotubes, as shown in Figure 1.4. Myogenesis is the process of maturation of myotubes and ultimate

generation of contractile skeletal muscle. Myogenesis coincides with fibrosis and angiogenesis. The fibroblasts get attracted to the injured site due to secretion of TGF and produce a fibrotic scaffold, which acts as a temporary extracellular matrix. In addition to fibrosis, angiogenesis occurs due to secretion of vascular endothelial growth factor (VEGF) and angiopoietin. These processes of angiogenesis, fibrosis, and myogenesis finally result in appearance of functional contractile skeletal muscle (Juhas and Bursac 2013).

The expression of transcription factor Pax7 is indispensable in adult muscle regeneration as Pax7 mutant mice were found to have disturbed regeneration of muscles (Sambasivan et al. 2011). Pax3 and Pax7 transcriptional factors are responsible for the activation of satellite cells. Pax7 expression has additional roles like survival and protection from apoptosis of the satellite cells. Pax3 expression degradation is essential as it allows for the myoblast differentiation and further progression of myogenesis. Pax7 is mainly responsible for promotion of myoblast proliferation and delays the differentiation process by regulation of MyoD expression (P. S. Zammit 2006; Kuang et al. 2006). Each of the myogenic regulatory factors has specific roles. MyoD is responsible for myoblast differentiation. Myf5 is responsible for proliferation and homeostasis of the myoblasts. MyoD influences the expression of p21 protein (cell cycle inhibitor) subsequently, leading to cell cycle exit (Andres and Walsh 1996). Loss of expression of myf5 leads to a decrease in proliferating myoblast population whereas lack of MyoD expression results in deviation from myogenic differentiation to increased self-renewal of myoblasts. Myogenin is a negative regulator of Pax7 expression. Myogenin expression in the myoblasts starts before the p21 protein expression. The differentiation of myoblasts to form multinucleated myotubes involves enhanced expression of myogenin along with p21 protein expression. MRFs are also responsible for the differentiation of myoblasts into

myotubes and are expressed at a later stage of myotube formation (Le Grand and Rudnicki 2007).

Regulation of myogenesis involves intervention from various growth factors like fibroblast growth factor (FGF) and transforming growth factor β (TGF). These growth factors are involved in the inhibition of differentiation of myoblasts (S. S. Rao and Kohtz 1995). FGF inhibits myogenesis by phosphorylation of protein kinase C DNA binding domains of various myogenic regulatory factors (L. Li et al. 1992). TGF β negatively effects the activity of MyoD and myogenin without affecting their DNA binding domains (D. Liu, Black, and Derynck 2001).

1.3. Need for skeletal muscle tissues

Skeletal muscles may undergo damage or loss by congenital and acquired conditions, traumatic injury, or tumor ablations (Ostrovidov et al. 2014). Skeletal muscle loss due to congenital defects includes inherited myopathies and muscle dystrophies. These muscle related diseases are characterized by general signs and symptoms of muscle weakness, slow development of fine and gross motor signals, respiratory dysfunction, and dysfunction of the bulbar region of the brain. The diseases not only lead to a decrease in patient life quality but also prove to be fatal as there are no current treatment options for these diseases. The medical interventions are only successful in managing longevity of patient life and quality to a limited extent. Worldwide prevalence of inheritable myopathies is at alarming 16% (Cardamone, Darras, and Ryan 2008). Prevalence statistics for all the muscular dystrophies combined ranges from 19.8 - 25.1 for every 100,000 people (Theadom et al. 2014).

Table 1.1 shows the various inherited myopathies and muscular dystrophies, along with their incidence. Sarcopenia translated, as poverty of flesh, is a condition of muscle loss due to aging. After the age of 50, there is a muscle loss rate of 1-2% every year. Over the

age of 50, the muscle strength decreases at the rate of 1.5% per year between the ages of 50-60 and the rate decreases further to the extent of 3% per year after that (von Haehling, Morley, and Anker 2010). Age related muscle loss has a strong risk factor of disability, hospitalization, and death in older adults. World health organization predicts a doubling of the world population with age greater than 60 by 2050 ("Ageing and Health" 2018). These facts increase the magnitude of the age-related muscle loss. Muscle loss may also occur due to various endocrine diseases like diabetes, obesity, hypogonadism, growth hormone deficiency, hyperthyroidism, hypercortisolism, vitamin D deficiency, and osteoporosis.

Muscle loss may occur in patients with diseases like rheumatoid arthritis, peripheral arterial disease, chronic obstructive pulmonary disease (COPD), congestive heart failure, advanced kidney disease, cirrhosis, and HIV AIDS (Kalyani, Corriere, and Ferrucci 2014). In addition to the diseases, critically ill patients develop a condition often referred to as intensive care-induced skeletal muscle weakness. Skeletal muscle loss may also occur due to traumatic injuries and accidents. Sportspersons and athletes experience contusions and strains during various sports related injuries. Out of all the sports injuries, 35-55% of the injuries are related to myofiber and connective tissue damage (Counsel and Breidahl 2010). Failure in the treatment of such damages drastically affects the sportspersons recovery and time away from the field (Järvinen, Järvinen, and Kalimo 2014). Armed personnel undergo high-energy combat injuries on the battlefield. Out of this, 53 % of the cases are related to soft tissue damage (Owens et al. 2007). Trauma due to road traffic accidents in most of the cases involves soft tissue injuries (Hussaini et al. 2007).

Table 1.1 Various prevailing myopathy types and their incidence as reported by (Cardamone, Darras, and Ryan 2008)

Myopathy type	Incidence rate
Inheritable myopathy	
Congenital myopathies	
Nemaline myopathy	1/500,000 live births
Central core disease	Rare
Myotubular myopathy	Rare
Congenital fiber type disproportion	Seen in 1% of all muscle biopsies
Multiminicore myopathy	Rare
Distal myopathies	Rare
Muscular dystrophies	
Congenital muscular dystrophies	1/21500
Dystrophinopathies	
Duchene`s muscular dystrophy	1/3300 males
Becker`s muscular dystrophy	1/36000 males
Myotonic dystrophy	5-20/100,000 live births
Fascioscapulohumeral muscular dystrophy	1/20,000 live births
Emery Dreifess muscular dystrophy	1/50,000 live births
Limb girdle muscular dystrophy	8.1-69/1000,000
Oculopharyngeal muscular dystrophy	1/8000

Cachexia is a condition of progressive skeletal muscle loss due to cancer. The syndrome is associated with functional impairment, which cannot recover by typical dietary supplements (Powers et al. 2016). The syndrome is prevalent in 80% of the patients with advanced stage cancers of gastrointestinal tract, breast, sarcoma, lung, prostrate, pancreas, and colon (Dewys et al. 1980). The condition not only reduces patient`s quality of life and efficacy of radio and chemotherapy but also increases the risk of death. The ever-growing cancer cases also magnify the importance of skeletal muscle loss due to cancer (K and Thomas 2018).

In cases of skeletal muscle loses of up to 20%, the tissue shows remarkable capabilities to regenerate. However, in cases of volumetric muscle loss (with muscle loss greater than 20%) and serious trauma the capacity to regenerate impedes leading to the formation of

fibrotic scar tissue (Ma et al. 2011). The fibrotic tissue formation can be attributed to the repeated activation and failure of regenerative pathway due to loss of the mediators of regeneration, including satellite cells and basement membrane. Fibrosis does not allow for proper angiogenesis, neurogenesis, and myogenesis. Moreover, the reparative stem cell population depletes as these cells go through repetitive wound healing pathway leading to shortening of the telomere (Juhas and Bursac 2013). The scar tissue formed has properties different from the surrounding muscle tissues leading to incomplete functional recovery of the final tissue. The current gold standard employed for the problem includes the replacement of the damaged tissue with autologous muscle tissue from a donor site. The autologous muscle tissue transferred along with blood vessels and nerve connections at the site of injury is popularly known as a muscle flap (C.H. Lin et al. 2007). Such surgical treatment of the damaged tissue results in decent functional recovery but often leaves the donor site morbid. Reports suggest that the procedure of transplant fails 10% of the time due to infections and necrosis (Bianchi et al. 2009). Another approach involves transplantation or delivery of myogenic origin cells at the site of injury to improve skeletal muscle regenerative capacity. The method failed due to poor retention and viability of delivered cells along with immunological reactions (Urish, Kanda, and Huard 2005).

1.4. Skeletal muscle tissue engineering

To overcome the problems of lack of donors, donor site morbidity and loss of functionality, skeletal muscle tissue engineering emerged as a technology to recreate, replace and regenerate damaged skeletal muscle tissue (Lanza, Langer, and Vacanti 2011). Tissue engineering employs the triads, including skeletal muscle progenitor cells, suitable scaffolds, and growth factors to fabricate engineered tissue substitutes (Klumpp et al. 2010; Bach et al. 2004). The approach has various distinct advantages including the

ability to engineer and control the architecture of the tissue substitutes according to the structure of injured site. Site-specific delivery of growth factors and drugs within the tissue substitutes promoting enhanced healing and implant acceptance (Stern-Straeter et al. 2007; Juhas and Bursac 2013; Ostrovidov et al. 2014). The possibility of preconditioning tissue substitutes with respect to the hostile environment of the injured site for their better integration. Thus, skeletal muscle tissue engineering provides ways to create physiological and pathological skeletal muscle models. Studies in these models are necessary for further understanding of skeletal muscle tissue in their diseased states as well as finding solutions for the problems.

Current skeletal muscle tissue engineering approaches are successful in the creation of tissue substitutes capable of expressing functional muscle proteins along with an ability to produce force upon application of electrical and neuronal stimulation (Cs et al. 2014). Given the progress, the fabricated tissue substitutes do not completely mimic the native skeletal muscle. The engineered substitutes lack with respect to structural organization, distribution, and the extent of differentiation of the consisting myotubes. Moreover, the myotubes are immature with respect to the dimensions as well as the expression of functional proteins. As a result, in vitro tissue substitutes produce very low forces compared to the in vivo counterparts (Bursac, Juhas, and Rando 2015). Thus, various challenges lie ahead in engineering functional skeletal muscle tissue. First of them includes the fabrication of tissue engineered substitute with uniform myotube density and alignment, which mimics the organized structure of the native tissue architecture. The choice of a biomaterial, which not only supports the myogenic cell proliferation, differentiation, and maturation but also allows for increased active force generation of the skeletal muscle tissue construct (Qazi et al. 2015). Another significant deficit in skeletal muscle tissue engineering is to mimic the vascularization and innervation of the native

skeletal muscle tissue, which ensures the integration of the artificial tissue upon implantation (Bian and Bursac 2008).

1.5. Literature review

An extensive review of literature has been carried out while keeping in mind the implemented strategies used for fabrication of skeletal muscle tissues.

Over the years, conventional tissue engineering strategies have been reinforced by the emergence of enabling technologies in order to create more physiologically relevant tissue substitutes. Figure 1.5 shows the conventional tissue engineering process, along with the modifications being carried out to enhance the process. Successful fabrication of skeletal muscle substitute greatly depends on proper cellular functions like adhesion,

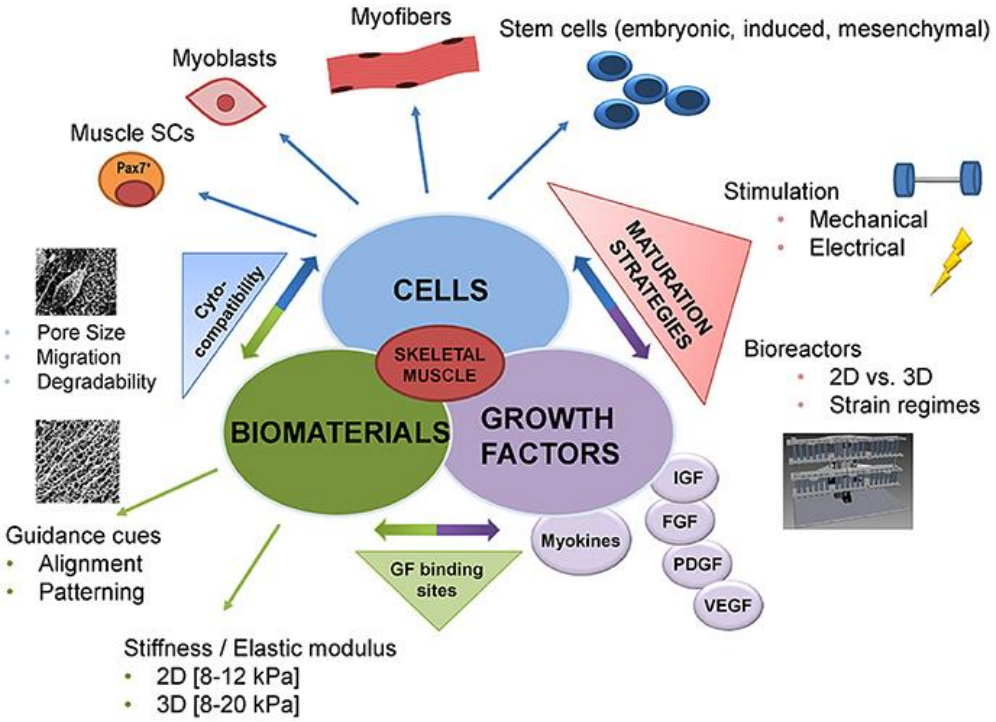

Figure 1.5 The conventional tissue engineering process, along with the modifications being carried out to enhance the process (Maleiner et al. 2018).

proliferation, differentiation, and maturation. Tailoring of the biomaterials with respect to surface properties (topography geometry), mechanical properties have been one of the excellent strategies to influence the cell functions.

Various biomaterials have been processed through techniques like electrospinning and microfabrication-based techniques to include biochemical and physical cues, which help in recapitulating organized structure of the skeletal muscle microenvironment (Scott et al. 2017; Bajaj et al. 2014; Vajanthri et al. 2019; Bettadapur et al. 2016; Luo et al. 2018; Langhammer et al. 2013; Jana and Zhang 2013). Recreation of skeletal muscle environment by modulating mechanical properties of the biomaterials has also been seen as an essential strategy to form in vitro skeletal muscle model (Engler et al. 2004, 2006; Gilbert et al. 2010). Similarly, owing to the greater relevance of 3D microenvironment, biomaterials of synthetic and natural origin have been used to create 3D skeletal muscle substitutes (Jones et al. 2018; Smith et al. 2012; Matsumoto et al. 2007).

In some cases, the 3D microenvironment created is embedded with growth factors to better mimic the muscle microenvironment (Hill, Boontheekul, and Mooney, 2006.; Kuraitis et al. 2011; Borselli et al. 2010; Baker et al. 2017). Recently, 3D bioprinting has emerged as a technique to fabricate complex skeletal muscle architecture along with control over spatial positioning of the cells (J. H. Kim et al. 2018; Kang et al. 2016; Pati et al. 2015; Costantini et al. 2017; Mozetic et al. 2017). In order to mimic the dynamic environment of the skeletal muscle mechanical, electrical and light-based stimuli have been employed. Mechanical conditioning protocol have been effective in enhancing the myogenic gene regulation, protein expression and cell proliferation (Powell et al. 2002; H. Vandenburgh and Kaufman 1979; Ma et al. 2011; Bt et al. 2012; Heher et al. 2015; Goldspink et al. 1995; Passey et al. 2011). Shear stress based mechanical stimulation has been studied. Electrical stimulation of the cellular constructs has also been associated with increased cell differentiation and subsequently lead to an enhanced contractility (Ahadian et al. 2013; 2012; Y.-C. Huang, Dennis, and Baar 2006; Khodabukus et al. 2015; Khodabukus and Baar 2012). More recently, use of optogenetic tools like

channelrhodopsin has allowed optical stimulation of skeletal muscle constructs in a spatio-temporally controlled manner resulting in the maturation of myotubes (Asano, Ishizua, and Yawo 2012; Asano et al. 2015). Furthermore, these optically sensitive skeletal muscle cells have been used to create biological robots. The current thesis presents the development of multiple strategies towards the fabrication of skeletal muscle tissues in vitro using the enabling technologies such as three-dimensional (3D) bioprinting, micropatterning, and optogenetics.

1.5.1. 3D bioprinting strategy

3D bioprinting has emerged as an excellent technique to fabricate three-dimensional tissue constructs. 3D printing technologies like inkjet printing and extrusion-based printing have been explored for skeletal muscle tissue fabrication. Cui et al. utilized the flexibility of inkjet-based 3D bioprinting technique to combine biological, microelectro-mechanical system (Bio-MEMS) with a bioprinted tissue construct for biosensing applications. The group demonstrated the inkjet deposition of C2C12 cells on a cantilever. The bioprinted cells remained live (>90% viability) and were able to proliferate, differentiate on the cantilever surface. The differentiated cells could form myotubes and respond to electrical stimulus by showing contractility. The bioprinted myotubes responded to external stimulus like toxins and behaved as functional biosensors (Cui, Gao, and Qiu 2013).

Cvetkovic et al. utilized for the first time stereolithography based 3D printing technology to create micropillars of poly ethylene glycol diacrylate. Further, they seeded C2C12 cells with appropriate extracellular matrix components of fibrinogen and collagen type I between the pillars. The cells utilized ECM and matured to form skeletal muscle biological robots. The response of biological robots in terms of force production was characterized by electrical stimulation. Force produced was quantified by the extent of

bending in the micropillars (Cvetkovic et al. 2014). The same group demonstrated the advancement of work when they went one-step ahead to incorporate optically sensitive C2C12 cells within ECM components between two 3D printed micropillars of poly ethylene glycol diacrylate. Formation of light sensitive skeletal muscle overcomes various problems of electrolysis-based damage to the skeletal muscle and allows spatial and temporal stimulation of skeletal muscle. The skeletal muscle developed maximum force of 300 μN in response optical stimulation (Raman et al. 2016). The group further went forward to create optically sensitive biological robots and studied the ability of a skeletal muscle to regenerate under conditions of mechanical damages like lacerations. The group tried to recreate an in vivo like healing strategy involving the addition of new myoblasts, addition of ECM components, optical stimulation and release of insulin like growth factors (Raman et al. 2017).

Stereolithography based 3D printing was recently used to fabricate a biohybrid robot which is actuated by electrical stimulation of a pair of antagonistic skeletal muscles. Electrical stimulation of the muscle leads to a rotational motion of flexible joint leading to actuation of the robot. The robot solves the problem of spontaneous shrinkage of skeletal muscle tissue when cultured on flexible, stretchable substrates (Morimoto, Onoe, and Takeuchi 2018). To mimic the organized alignment and 3D organization of skeletal muscle, Yeo et al. utilized a multi-technique approach involving extrusion-based printing, electrospinning, and bioprinting. The foundation layer of the scaffold consists of a perpendicular polycaprolactone struts which are further deposited with a second layer of electrospun polycaprolactone fibers (for cellular alignment) and a third layer of alginate-poly ethylene bioink consisting of C2C12 cells. This combined approach led to enhanced expression of myosin heavy chain and formation of myotube due to the influence of aligned electrospun fibers (Yeo, Lee, and Kim 2016).

Another study carried out intending to mimic the organized structure of skeletal muscle involved utilizing a hybrid scaffold of polycaprolactone with collagen coating. Kim et al. developed micropatterned polycaprolactone struts by 3D printing mixture of polycaprolactone and fibrillated polyvinyl alcohol (PVA) followed by washing with water. The PCL struts were further treated and cross-linked with collagen. Culture of C2C12 cells over the collagen coated substrates showed enhanced cellular alignment, proliferation, and differentiation compared to control PCL surfaces (W. Kim, Kim, and Kim 2018).

Kang et al. developed an integrated tissue-organ printer (ITOP) capable of overcoming the diffusion limit of 100-200 μm for cell survival in human scale tissue constructs; as already known that cells require a steady supply of metabolites, growth factors, oxygen, and other nutrients to survive an in vitro environment. Fabricating constructs not relative to physiological proportions may lead to the necrosis of the cells within the constructs when compared to the cells on the periphery of the constructs. Such constructs must cater to the needs of cells for persistent diffusion of nutrients and oxygen. This complication was sorted out by fabrication of microchannels within the tissue constructs. The system has been used to optimize and successfully print: human scale bone, cartilage, and skeletal muscle constructs (Kang et al. 2016).

Choi et al. hypothesized the use of decellularized muscle derived extracellular matrix (mdECM) with 3D printing technology to provide a myogenic environment and appropriate architecture of native tissue. They used decellularized muscle derived ECM (mdECM) and 3D printing technology to create 3D functional muscle constructs with capabilities of mimicking native muscle tissue. Decellularized tibialis anterior muscle from porcine source (mdECM) with C2C12 cells was formulated as bioink. The mdECM was characterized for its composition and mechanical properties. The bioink was printed

with their custom-built integrated composite tissue/organ building system (ICBS) to control cellular alignment. The mdECM constructs were compared with collagen printed constructs for various characteristics like cell viability, myotube formation, and myogenic differentiation. The mdECM constructs not only provided a myogenic environment but, also retained natural muscle ECM components of agrin and expressed the acetylcholine receptor clusters (Choi et al. 2016).

Along the same lines, Costantini et al. have very recently fabricated a macroscopic artificial muscle flap with the integration of innovative 3D bioprinting approach. The highlights of this research include the formulation of a customized bioink with a photocurable semi-synthetic biopolymer (PEG-fibrinogen) for the encapsulation of C2C12 muscle precursor cells. For a high-resolution printing of a cell-laden bioink, the system has been integrated with a microfluidic printing head along with a co-axial needle extruder. The cells were found to attain functional maturity and a high degree of alignment parallel to the hydrogel fiber deposition within 3-5 days of culture. Implantation of the construct into an immunocompromised mouse showed promising results with the generation of artificial muscle tissue (Costantini et al. 2017).

Recently Kim et al. successfully bioprinted an implantable skeletal muscle substitute consisting of primary human muscle precursor cells. An Integrated Tissue Organ Printing (ITOP) system was used to develop a three-layered implantable skeletal muscle substitute. The bioprinted skeletal muscle construct consisted of a foundational base layer of 3D printed PCL. A sacrificial gelatin layer that serves to create microchannel for transportation of media and waste products was co-printed with a layer of human muscle precursor cell laden bioink consisting of gelatin, hyaluronic acid, fibrinogen, and glycerol. The in vitro evaluation of the bioprinted constructs showed the importance of sacrificial gelatin microchannels in maintaining the viability of the constructs. The in vivo

implantation of the bioprinted constructs in muscle defects not only resulted in neural and vascular integration with the host tissue but also recovered the force production by 85% (J. H. Kim et al. 2018).

3D printing has also paved ways into studies related to the creation of in vitro models for neuromuscular junctions. Cvetkovic and coworker's, 3D printed rectangular moulds using stereolithography based technique. The moulds were used to create C2C12 cell-based muscular ring and embryoid body-based rings consisting of extracellular components fibrinogen and matrigel. Each of the ECM-cell based rings differentiated and formed a combined ring of tissue consisting of myotubes and motor neurons. The combined rings were next placed on previously 3D printed platform for biorobots (Cvetkovic et al. 2014) and cocultured to obtain neuromuscular junctions. The neuromuscular junctions` functionality was evaluated by chemical stimulation of motor neurons leading to contraction of myotubes (Cvetkovic et al. 2017). Another study by Kaplan and colleagues used extrusion-based extrusion-based printing technology to create silk fibroin-based T shaped cantilever pillars. These cantilever pillars printed in normal cell culture platforms were further seeded with human myoblast cells within a 3D matrix, consisting of collagen/matrigel and silk fibroin and further cultured to form myotubes for 14 days. Further human induced neural stem cells (hiNSCs) were seeded and differentiated to form motor neurons on the differentiated myotubes. The differentiation subsequently led to innervation of the myotubes and formation of functional 3D neuromuscular junctions, which showed positive results for acetylcholine staining, choline acetyltransferase staining and calcium imaging (T. A. Dixon et al. 2018).

1.5.2. Optogenetics strategy

Asano et al. first demonstrated the development of optogenetic skeletal muscle cell using channel rhodopsin. In this study, C2C12 cells were rendered optically sensitive transfecting a murine leukemia viral vector SRa-chop2-Venus with a gene responsible for expression channelrhodopsin (ChR) opsin. The C2C12 cells differentiated to form myotubes. The myotubes were found to be sensitive to blue light; as a result, they were able to be controlled in a time and space dependent manner (Asano, Ishizua, and Yawo 2012). The same group further utilized the previously generated optogenetic C2C12 cells to enhance the contractility and internal sarcomere organization of the myotubes. In order to do so, they genetically engineered C2C12 cells to express channel rhodopsin- green receiver a variant of ChR. The C2C12 cells were differentiated and provided with optical stimulation training, which significantly increased the contractile nature of myotubes along with sarcomeric patterning, that was not visible in control cells (Asano et al. 2015). Sakar et al. went a step further by incorporating ChR2 transfected C2C12 in a 3D gel to form optically sensitive 3D skeletal muscle constructs. Further, to mimic the in vivo tendons the cells along with the 3D gel consisting of collagen and matrigel were added onto and between two Poly dimethyl siloxane (PDMS) cantilevers. The optogenetic nature of the 3D skeletal muscle tissue thus allowed a spatial and temporal control over the tissue. The cantilever supports acted as sensors for characterizing the force and contraction of the optogenetic skeletal muscle tissue. The engineered skeletal muscle tissue formed between the two pillars showed characteristics of aligned, striated myotubes along with capabilities for force production (Sakar et al. 2012). The same research group headed by Rashid Bashir has explored the possibility of fabrication of biobots using the combination of optogenetic skeletal muscle tissue and 3D printed flexible hydrogel skeletons. These biobots have been further utilized to understand and develop healing

strategies for damaged skeletal muscle tissues. These studies have been reviewed in the previous section for 3D bioprinting (Raman et al. 2016; 2017). Recently, Osaki et al. studied the synergistic effects of co-culturing optogenetic C2C12 skeletal muscle myoblasts, and cadherin-GFP tagged human umbilical vascular vein (HUVEC) endothelial cells. The co-culture was setup in a rectangular 3D collagen gel. Microglass capillary was used to create cylindrical microchannel in gelatin (600 μm diameter) by sequential molding technique. One of the cylindrical compartments was filled with fibrin/collagen/ matrigel and optogenetic C2C12 leading to the formation of cylindrical 3D skeletal muscle tissue. Further two microchannels were created on either side of muscle tissue after addition of collagen by sequential molding technique. These microchannels on either side of the muscle construct were seeded with HUVEC cells; thus, creating a platform for testing the synergy between vascularization and myogenesis. During the co-culture it was observed that the muscle tissue enhanced the secretion of angiopoietin 1 in the presence of endothelial cells. A synergistic enhancement in contractile properties was also observed in the case of skeletal muscles due to the presence of endothelial cells. Optical stimulation-based training of skeletal muscle cells enhanced vascular sprouting from the endothelial compartment. Thus, creation of such an in vitro model permitted to understand the relation between vascularization and its effects on skeletal muscle regeneration (Osaki, Sivathanu, and Kamm 2018).

1.5.3. Micropatterning strategy

Recently, fabrication of micropatterned substrates with multifunctional roles is gaining importance in the field of skeletal muscle tissue engineering. Park et al. fabricated a graphene oxide embedded in polyacrylamide hydrogel, which was micropatterned by using femto laser. Femto laser based micropatterning leads to the reduction of graphene oxide. The graphene oxide and polyacrylamide components in the substrate provided

electrical conductance and mechanical properties similar to the skeletal muscle tissue respectively. Femto laser-based patterning was done to fabricate micropatterns in the range of 20-80 μm. The micropatterning mimics the alignment and organization of skeletal muscle tissue. C2C12 cells were cultured on these substrates, and electrical stimulation was applied to recapitulate the neuronal stimulation. In vitro evaluation of the substrates showed that 50 μm substrates showed positive implications towards alignment and myotubes formation. Electrical stimulation of non-patterned and patterned substrates resulted in an enhanced expression of myogenic genes like MHC, myogenin and Myo D. A synergistic influence of electrical stimulus and micropatterning was observed in the myogenesis of C2C12 myotubes. In vivo studies confirmed the compatibility of the conductive hydrogels under both micropatterned and non-patterned cases (Park et al. 2019). Another study reported the fabrication of micropatterned poly (vinylidene fluoride-co-trifluoroethylene) scaffold for electromechanical stimulation of myoblasts and preosteoblasts. The micropatterned surfaces were patterned to include hexagonal and line shapes in the dimension range of 25-150 μm. Cell adhesion assays were carried out on porous and non-porous micropatterned substrates. The results indicated that the poly (vinylidene fluoride-co-trifluoroethylene) scaffold with linear patterns supported better myoblast adhesion, alignment, and elongation which are essential for myotubes formation. For preosteoblast cells anisotropic micropatterns proved to be showing potential as scaffolds for regeneration (Marques-Almeida et al. 2019).

Jiwlawat et al. report the fabrication of pompe disease model by using myogenic cells derived from human induced pluripotent stem cells. Micropatterned substrates with a width range of 15-105 μm were fabricated by using cell adherent matrigel and cell repelling pluronic F-127. These micropatterns were fabricated on rigid and soft substrates. EZ sphere cells were trypsinized and seeded on to micropatterned and non-

patterned substrates. The cells were further subjected to differentiation media. The human induced pluripotent stem cells on micropatterned substrates differentiated to form well-oriented bundles of myotubes with contractile behavior. Myotubes formed in patterned condition had a larger diameter and a higher number of nuclei along with expression of myogenin expression compared to the non-patterned state. Soft substrates with muscle like stiffness lead to early appearance of sarcomeric patterning. Pompe disease induced pluripotent stem cells formed myotubes with disease specific gene expression (Jiwlawat et al. 2019). Ko et al. demonstrated the importance of substrate topography in determining the orientation of innervation and synapse formation between neuron and muscle cells. For this purpose, micropatterned substrates were engineered to have groove widths ranging between 200-1600 nm. These C2C12 myoblasts were seeded on microgrooved substrates. The 1600 nm microgrooved substrate showed well-aligned mature myotubes. Further, neural stem cells were seeded and differentiated to form neural progenitor cells on the myotubes. These neural progenitor cells formed functional neuromuscular junctions following the previously aligned myotubes (Ko et al. 2019).

In this way, fabrication of in vitro skeletal muscle models has progressed a long way. Simple 2D cell culture systems are replaced by the application of passive mechanical forces in the form of micropatterning techniques. Micropatterning techniques have provided micrometer scale geometrical and mechanical constraints to skeletal muscle cells in order to mimic the structurally organized and aligned environment of the skeletal muscle tissue invivo. In addition, incorporation of electrically conductive biomaterials in micropatterns has enabled their usage as platform for maturation of the skeletal muscle tissue. Furthermore, application of micropatterned environments with neural stem cells and induced pluripotent stem cells has led to the fabrication of physiological and pathological relevant in vitro skeletal muscle models. Another way of mimicking the

structural architecture of skeletal muscle tissue is the formation of 3D skeletal muscle constructs by 3D bioprinting. The technique of 3D bioprinting has not only allowed the fabrication of anatomical scale constructs but also included microchannels to overcome the problem of limited diffusion of oxygen and nutrients. Furthermore, 3D bioprinting of decellularized ECM based bioinks has led to fabrication more physiologically relevant models. The 3D printing technique has also permitted the fabrication and functional force evaluation of skeletal muscle models on 3D printed cantilevers mimicking tendons. In continuation with 3D bioprinting technology, optogenetically modified skeletal muscle cells were bioprinted in the form of skeletal muscle biobots which could be stimulated in a spatial and temporal manner. These light sensitive biobots were applied to study regeneration of mechanical damaged skeletal muscle. Furthermore, coculture in vitro model involving optogenetic skeletal muscle cells and endothelial cells allows to study the synergy between vascularization and skeletal muscle regeneration. Thus, in this way micropatterning, 3D bioprinting and optogenetics based strategies have helped to develop in vitro skeletal muscle tissues.

1.6. Research objectives

The objective of this thesis is to develop strategies to mimic the structural organization and alignment of the skeletal muscle along with a prospect to control the cells by using light as stimulus.

In order to achieve the thesis objectives, some of the advanced technologies have been used as strategies that may help in fabrication of in vitro skeletal muscle tissue. The fabrication techniques utilized include three-dimensional (3D) bioprinting, microfabrication based micropatterning, and optogenetics.

With the aim of developing a 3D bioprinting based strategy which helps in fabrication of in vitro skeletal muscle substitute, a custom-made bioprinting system was utilized to print

C2C12 cell laden 3D constructs. For this purpose, an optimized bioink composition consisting of gelatin, sodium alginate and collagen type I was used. Inherent properties of the bioink components and the sequence of processing during printing not only allowed for printability of 3D constructs but also maintained the viability and functionality of the skeletal myoblast cells for extended periods. Further, another strategy was elucidated to develop optically sensitive skeletal muscle cells by using optogenetics technology. The optogenetic tool employed is a plasmid DNA consisting of a gene responsible for co-expression of a non-selective cationic channel channelrhodopsin-2 (ChR2) along with green fluorescent protein. C2C12, NIH3T3, and HepG2 cells were transfected with the plasmid DNA by using liposomal transfection method. Green fluorescent protein expression confirmed the expression of ChR2 in each of the cells. The novelty of the study is given by the comparative analysis of transfection efficiency of AAV-CAG-ChR2-GFP plasmid among the three cell lines. The thesis also reports for the first-time development of optically sensitive HepG2 cells. These optically sensitive cells hold great potential for creation of in vitro models.

To understand the satellite cell dynamics, myogenic cells were isolated from rat hind limb muscles and devised methods to characterise them morphometrically and functionally. Furthermore, efforts were made to understand the various events taking place during the in vitro culture of satellite cell origin myoblasts. Satellite cell origin myoblasts showed morphology starting from attainment of bipolar morphology to a fused myotube morphology with multinucleated nature. Myotubes formed due to the fusion of myoblasts showed positive functional behaviour concerning calcium dynamics as well as spontaneous twitch behaviour.

To mimic the organized alignment and cellular organisation of the skeletal muscle tissue a microchannel flowed plasma based micropatterning procedure was applied. The

micropatterning process was used successfully to create micropatterns with dimensions comparable to myofiber and muscle fascicle. Further, in vitro culture of C2C12 cells on micropatterns lead to a transfer of alignment information from periphery to centre of the micropattern. The micropatterned environment also aligned the actin cytoskeleton of the cells. Thus, the micropatterning technique provides a controlled in vitro environment to explore alignment of C2C12 myoblasts.

Further, owing to the greater physiological relevance of primary cells, advances were made to study and understand the influence of substrate micropatterning over alignment and differentiation of primary myoblasts of satellite cell origin. In this study, the micropatterned substrates led to the formation of differentiated myotubes with better alignment compared to standard cultures owing to the contact guidance-based cues. The contact guidance cues arising due to micropatterning of the substrates were found to be a key regulator for controlling the size and degree of alignment of myotubes during the myogenesis process.

1.7. Thesis outline

The thesis is organized into seven chapters. Chapter 1 provides a general overview of skeletal muscle tissues including the anatomy and structure of skeletal muscle tissue, myogenesis, and regeneration of the tissue, need for skeletal muscle tissues and skeletal muscle tissue engineering as a solution for the fabrication of in vitro skeletal muscle tissue. The chapter also presents a detailed literature review of the advanced techniques applied to develop engineered skeletal muscle tissues. The research objectives of this thesis towards the goal of developing strategies for the fabrication of skeletal muscle tissue are also explained in this chapter. The second chapter of the thesis, utilizes 3D bioprinting technology to fabricate secondary myoblast C2C12 cells laden three-dimensional constructs. An inexpensive customized bioprinting system in combination

with a printable bioink blend comprising of gelatin, sodium alginate, and hydrolyzed collagen type-I allows the fabrication of C2C12 cells laden skeletal muscle constructs. The sequence of processing steps utilizes the thermoreversible property of gelatin, rapid crosslinking capabilities of alginate and cell adhesive nature of collagen; that altogether makes the printability of viable tissue constructs possible. The bioink is optimized for its mechanical and rheological properties along with the swelling and porosity-based properties. Live dead staining of the printed constructs shows a gradual increase in percentage of live cells from 80% post printing stage to above 90% after 14 days of culture. Fluorescence staining of the actin cytoskeleton with nuclei confirms cellular networking and communication. In total, the chapter developed a cost-effective and straightforward strategy by combining inexpensive bioprinting system and novel bioink composition for the potential development of skeletal muscle like tissue models.

The third chapter elucidates the strategy of creating optically sensitive cells by utilizing optogenetics based technology to control the cells. The optogenetic tool employed is a plasmid DNA (AAV-CAG-ChR2-GFP) present in E. Coli XL-10 bacteria. The plasmid DNA consists of a gene responsible for co-expression of a non-selective cationic channel channelrhodopsin-2 (ChR2) along with green fluorescent protein. A single isolated bacterial colony is grown in Luria bertani broth, and alkaline lysis method is followed to isolate the plasmid. Further, liposomal-based transfection method is employed to transfect the isolated plasmid DNA in three secondary cell lines, namely C2C12, HepG2, and NIH-3T3 cells. A significant difference in fluorescence intensity (GFP) is observed between transfected cells and control cells for each of the cell lines. The transfection of C2C12, HepG2, and NIH-3T3 cells was found significantly noticeable after a period of nine, six, and three days, respectively. The percentage of transfected cells is found to be 58.16%, 41.26%, and 46.04% for C2C12, HepG2, and NIH 3T3 cells, respectively. The study

delivers methodology to produce optically controllable C2C12, HepG2, and NIH-3T3 cells.

The fourth chapter demonstrates a plasma based micropattering technique known as microchannel flowed plasma process (μCFP). The micropatterning procedure involves the application of organosilanes octadecyltrichlorosilane (OTS) and 3-aminopropyltriethoxysilane (APTES) to create alternative hydrophobic and hydrophilic domains. Applying the μCFP, micropatterns of different hydrophilic widths, including 20 μm, 200 μm, and 1000 μm were created. Incubation of the micropatterned surfaces with 5-fluorescein isothiocyanate (FITC) solution followed by fluorescence microscopy reveals the development of micropatterned substrates. Further, culturing of C2C12 secondary myoblasts cells led to their gradual alignment in all the micropatterns. The alignment of myoblasts follows a trend where the cells align first in 20 μm followed by 200 μm and finally 1000 μm when observed until eight days of culture.

The fifth chapter explores the isolation, culture, and functional and morphometric characterisation of primary myoblasts from rat hind limb muscles. Isolation of cells from rat hind limbs involved dissection of muscles, enzymatic digestion, mechanical trituration followed by plating of cells. Characterization of isolated cells revealed the satellite cell origin of the cells. Nearly 65% of the cells stained positive for Pax7, which is a biomarker for satellite cells. The isolated cells are cultured on gelatin coated Petri plates to explore the events and progression of myogenesis. The isolated cells during culture show polygonal, bipolar, and elongated morphologies. The dynamics of these morphologies are studied and revealed that the isolated cells first start growing with polygonal morphology, differentiate to bipolar morphology, and then subsequently fuse to form elongated morphology. The live cell imaging of the isolated cells shows the proliferative and fusion nature of the cells. Fluorescence staining of the actin cytoskeleton and nuclei also show

that the cells form groups of aligned myoblasts with actin patterning indicating cells undergoing differentiation.

Similarly, when the cells with elongated morphology are stained and observed, the formation of multinucleated myotubes suggests the fusion of differentiated myoblasts. Besides, the observation of sarcomeric actin patterning also indicates the formation of myotubes with mature contractile proteins. The observation of spontaneous twitching in myotubes also reiterates the maturity of the formed myotubes. Calcium dynamics studies show an increase in the peak with the differentiated status of the cells. All these observations show that the isolated cells form rat hind limb muscles are of satellite cell origin, and they show highly dynamic behavior as the myogenesis proceeds from the stages of adhesion and proliferation to the appearance of bipolar myoblasts and finally differentiate into myotubes through alignment and fusion of the myoblasts.

The chapter 6 evaluates the influence of micropatterning based contact guidance cues over primary myoblast alignment, differentiation, and myotube formation. For this purpose, primary myoblast cells (i.e., satellite cells) isolated from rat hind limb muscle were characterized and cultured for 14 days on micropatterned surfaces having varying widths (20, 200 and 1000 μm) of hydrophilic regimes developed by microchannnel flowed plasma process (μCFP) using octadecyltrichlorosilane (OTS) and 3-aminopropyltriethoxysilane (APTES). Several characteristic parameters of muscle differentiation, including the fusion index, maturation index, and average width of the myotubes were quantified. In addition, analysis of nuclear parameters such as area and variation in the number of nuclei concerning a change in myotube width is done. The functional behaviour of cultured myotubes exhibiting spontaneous contractions is assessed through kymograph to determine the twitch frequency. It also evaluates the degree of alignment of myotubes on micropatterned substrates through examining the

orientation order parameter and two-dimensional fast Fourier transform analysis. Altogether, the studies reveal that the μCFP based micropatterned surfaces not only support differentiation and formation of myotubes but also lead to contact guidance cues, which act as an important regulator for controlling the size and degree of alignment of myotubes during the myogenesis process. Overall, the chapter provides a set of morphological and image processing-based methods and identification of specific parameters to characterize the in vitro satellite cell myogenesis. Finally, the key conclusions and future scope of work of the thesis is summarized in Chapter 7.