NOTTINGHAM TRENT UNIVERSITY

DEVELOPMENT OF SKELETAL MUSCLE MODELS TO STUDY AGEING AND ASSOCIATED CONDITIONS

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A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

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Declaration of Ownership

I confirm that this thesis is the result of my own work and that it has not been submitted, in whole or in part, in any previous application for a degree. All the help and assistance received have been acknowledged. Main sources of materials and information have been clearly attributed. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

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I dedicate my research to my father, my biggest fan and supporter, who sadly passed away during my doctoral studies. He is the reason I have continued to pursue my research journey.

List of publications

Tarum J, Degens H, Craig S, Turner M, Stewart C, Santos L. (2023). *Modelling skeletal muscle ageing and repair in vitro*. Journal of Tissue Engineering and Regenerative Medicine.

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Tarum, J, Turner, M, Sale, C, Stewart, C, Degens, H & Santos, L. (2022). *In Vitro Regeneration Capacity in Response to Chemical Injury Is Compromised in Aged Vs Young Muscle Cells*. Medicine & Science in Sports & Exercise. 54. 614-614.

Table of Contents

List of Figures

List of Tables

Abbreviations

#	
μm	Micrometres
μL	Microliters
2D	Two Dimensional
3D	Three Dimensional
4E-BP1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
A	
ADP	Adenosine diphosphate
AI	Artificial intelligence
Akt	Protein Kinase B
AMPK	5'adenosine monophosphate-activated protein kinase
ANNI	Artificial neural network inference
ATCC	American type culture collection
ATP	Adenosine triphosphate
ARHGAP11B	Rho GTPase activating protein 11B
B	
$BaCl_2$	Barium chloride
BIA	Bioelectrical impedance analysis
BMP-GDF	Bone morphogenetic protein-growth differentiation factor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	
cDNA	Complementary deoxyribonucleic acid
CDKN1A	Cyclin dependent kinase inhibitor 1A
Cdkn2a	Cyclin dependent kinase inhibitor 2A
CHAD	Chondroadherin
CO2	Carbon dioxide
CRP	C-reactive protein
D	
DAPI	4',6-diamidino-2-phenylindole
DGC	Dystrophin glycoprotein complex
DMEM	Dulbecco's modified eagle medium
DM	Differentiation medium
DNA	Deoxyribonucleic acid
DXA	Dual-energy X-ray absorptiometry
E	
EdU	5-ethynyl-2-deoxyuridine

EIF4A2	Eukaryotic translation initiation factor 4A2
ECM	Extracellular matrix
F	
FAP	Fibro/adipogenic progenitor
FBS	Foetal bovine serum
FGF2	Fibroblast growth factor 2
FOXO	Forkhead box O
FOXP2	Forkhead box protein P2
FST	Follistatin
FUSIP1	Serine/arginine rich splicing factor 10
G	
GM	Growth Medium
GO	Gene Ontology
GPa	Gigapascals
H	
hPSCs	Human pluripotent stem cells
I	
IFN-γ	Interferon γ
IGF-1	Insulin-like growth factor 1
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-15	Interleukin 15
IL-1β	Interleukin 1β
IMAT	Intramuscular adipose tissue
IPA	Isopropyl alcohol
J	
JAK2	Janus kinase 2
JAK/STAT	Janus kinase/signal transducers and activators of transcription
K	
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KDM5D	Lysine demethylase 5D
kPa	Kilopascals
M	
Mef2	Myocyte enhancer factor 2a
MEM	Minimum essential medium
MFN	Mitofusin

Mg	Milligrams
MHC	Myosin heavy chain
mL	Millilitres
MLP	Multilayer perceptron
MO25	Calcium binding protein 39 (aka CAB39)
MRFs	Myogenic regulatory factors
Mrf4	Myogenic regulatory factor 4
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING-finger protein-1
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein 1

N

NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B
NIPAL3	NIPA like domain containing 3
NMJ	Neuromuscular junction
NO	Nitric oxide
NTX	Phospholipase A2 peptide notexin
CTX	Protein kinase C inhibitor cardiotoxin

P

p70S6K	70kDa ribosomal S6 protein kinase
Pax3/7	Paired box 3/7
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator α
PI3K	Phosphoinositide 3-kinase

R

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-PCR	Real time polymerase chain reaction

S

SC	Satellite cell
SCFD1	Sec1 family domain containing 1
SD	Standard deviation
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SKAP2	Src kinase associated phosphoprotein 2

SPPB	Short physical performance battery
SR	Sarcoplasmic reticulum
Z	
ZDBF2	Zinc finger DBF-type containing 2
T	
TEM	Tissue engineered muscle
TGFβ-1	Transforming growth factor β-1
TNFα	Tumour necrosis factor α
U	
USP9Y	Ubiquitin specific peptidase 9 Y-linked
USP54	Ubiquitin specific peptidase 54
UV	Ultraviolet
V	
VEGF	Vascular endothelial growth factor

Abstract

Rapid ageing of the population together with raised prevalence of chronic metabolic diseases provokes huge economic burden on healthcare systems. Furthermore, age-related loss of skeletal muscle is associated with increase in disability, injuries, dependency and eventually mortality. Given the importance of skeletal muscle mass in both locomotion and homeostasis, developing countermeasures to hinder the raising prevalence of sarcopenia is critical.

Human skeletal muscle experiments *in vivo* are limited due to ethical and safety concerns, and animal models are poor predictors of clinical outcomes and drug responses in humans. Therefore, developing biologically advanced *in vitro* systems to investigate regulation of skeletal muscle mass and underlying mechanisms is pivotal. Hence, the aim of this project was to develop advanced models to study age related changes in skeletal muscle. This study focused on a) expanding our knowledge on biomarkers regulating muscle ageing and the effect of exercise on these markers; b) investigating the causes of impaired regeneration in aged muscle; and c) developing tissue engineered muscle model with structural and functional properties of native muscle.

The findings in this study indicate that using artificial neural network inference (ANNI) analysis we could identify novel age- and exercise related genes such as *USP54, JAK2, CHAD, ZDBF2, EIF4A2, NIPAL3, SCFD1 and KDM5D* previously not associated with skeletal muscle. Furthermore, we characterise an *in vitro* human muscle regeneration model and describe the development of novel non-custom-made inserts to fabricate high-throughput tissue engineered muscle. Together, the findings suggest that established models in this project have a potential to contribute to research on muscle ageing by providing high-fidelity platform to further examine regulation of muscle mass across the lifespan and to investigate potential effects of drugs and/or therapies.

Chapter 1: Main Introduction

Skeletal muscle comprises approximately 40% of the human body mass and its contraction (together with tendon and bone interaction) is important for voluntary movements, maintaining body posture and metabolic homeostasis (Wilkinson et al. 2018, Reid and Fielding 2012). Age-related decline in skeletal muscle mass and function (sarcopenia) is accompanied by physical disability, reduced independence, increased risk of falls and mortality (Cesari et al. 2014, McCormick and Vasilaki 2018, Cruz-Jentoft and Sayer 2019). The population aged >65 years in Europe has increased from 15% to 25% in the last 20 years and the prevalence of sarcopenia is expected to increase rapidly in the next decades forming 22.3% of population by 2045 (Ethgen et al. 2017, Petermann-Rocha et al. 2022, Cristea et al. 2020). Rapid demographic ageing of the population together with raised prevalence of metabolic diseases and sarcopenia is directly linked to huge economic burden on healthcare systems with staggering annual cost of $19.12 and £2.5 billion in 2019 in US and UK, respectively (Goates et al. 2019, Buch et al. 2016, Seals et al. 2016, Pinedo-Villanueva et al. 2019).

Since current animal models are poor predictors of clinical outcomes and drug responses in humans (Vignaud et al. 2005, Smith et al. 2012); and *in vivo* human skeletal muscle experiments are limited due to ethical and safety concerns (Hay et al. 2014), biologically advanced *in vitro* systems to investigate muscle mass regulation and associated mechanisms must be developed (Nikolaev et al. 2020, Carraro et al. 2022). A high-fidelity tissue-engineered human skeletal muscle system holds promise to be such a system.

Therefore, the aim of this PhD-project is to develop a physiologically relevant *in vitro* cell model of skeletal muscle with mature structural and functional properties that can be used to a) investigate age-associated changes in muscle; b) understand why muscle regeneration is impaired in older adults; and c) enhance our knowledge of biomarkers of muscle ageing and d) investigate the effect of exercise on skeletal muscle ageing.

To achieve this aim, the thesis addresses the following objectives:

- Identify the most influential and influenced genes and pathways related to muscle ageing and effects of long-term resistance training on age-associated processes using artificial neural network inference (ANNI) analysis of publicly available datasets.
- Develop and characterise an *in vitro* muscle regeneration model using human-derived myoblasts from young and aged individuals.
- Develop tissue engineered muscle using the Flexcell bioreactor and novel re-usable inserts to replicate the 3D structure and function of native muscle and enable physiologically relevant studies of muscle ageing, *in vitro*. Current methods only allow to generate tens of tissue engineered muscle at a time. The inserts are advantageous because they provide a high-throughput platform that enabling to generate hundreds of muscle constructs simultaneously rather than tens.

1.1 Development of skeletal muscle

Skeletal muscles originate from the somites that arise from paraxial mesoderm, which forms during gastrulation and embryonic axis elongation (Chal and Pourgie 2017, Tajbakhsh et al. 1997) (Figure 1.1). The somites are segmented into dermomyotomes that differentiate to dermatomes and myotomes which are the origin of the skeletal muscle (Buckingham et al. 2003, Maroto et al. 2012). Myogenesis in the somites starts with the activation of transcription factors *Pax3* (Paired Box 3) and *Pax7* (Paired Box 7) in the dorsal epithelial dermomyotome that initiates proliferation of muscle progenitors (Venters and Ordahl 2002, Pu et al. 2013). During embryonic myogenesis, primary muscle fibres are generated from the embryonic muscle cells derived from *Pax3* expressing dermomyotomal progenitors (Romero et al. 2013, Nassari et al. 2017). Myogenic fate requires the expression of myogenic regulatory factors (MRFs) *Myf5, Mrf4* and *MyoD* (Tedesco et al. 2010, Francetic and Li 2011). Myogenin and *Mef2* are responsible for terminal differentiation of myoblasts into myocytes by directing cell exit, differentiation, and fusion of multinucleated myotubes

(differentiated muscle cells) (Ganassi et al. 2020). Myocytes are the first postmitotic skeletal muscle cells in the myotome that express slow and embryonic myosin heavy chains (MHC), α-actin and desmin (Maltzahn et al. 2012, Lee et al. 2013).

During secondary myogenesis, a subset of myoblasts expressing transcription factor Pax7 become quiescent, termed satellite cells (SCs), that do not commit to differentiation, but instead form the pool of adult stem cells that proliferate in response to injury to generate new myoblasts for muscle regeneration (Chal and Pourgie 2017, Lee et al. 2013). While the expression of fast MHC (type IIa (MHC2), IIx (MHC1)) occurs during later myogenesis, the final fibre type of the muscle is also determined by neural input and physiological demands (Talbot 2016, Schiaffino et al. 2015).

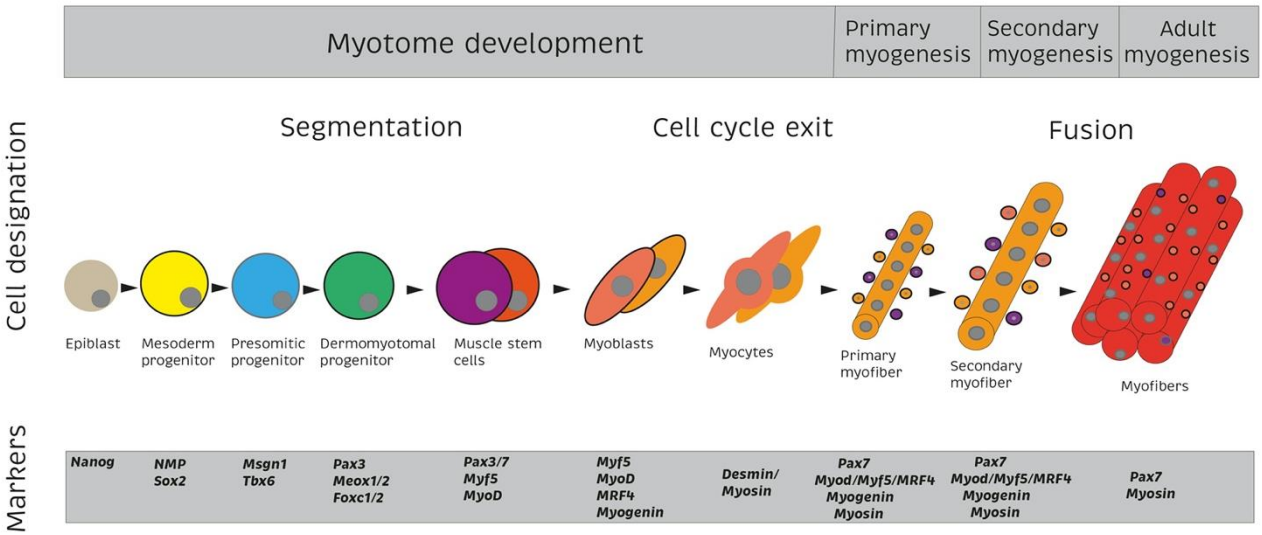

Figure 1.1: Schematic diagram of skeletal muscle development.
Image prepared by the author using Adobe Illustrator and BioRender. Adapted from Chal and Pourqie (2017), Tedesco et al (2010), Khodabukus et al (2018).

1.2 Skeletal muscle structure and function

Skeletal muscle is composed of bundles of myofibres in various length that are surrounded by basal lamina (Figure 1.2). Myofibers consist of highly organised myofibrils that are

divided into contractile units called sarcomeres, whose organization gives myofiber a striated pattern (Mukund and Subramaniam 2019, Frontera and Ochala 2014). Transverse tubules (T-tubules) are a branched network of membrane running along the myofiber at the junction of the dark A band and light I band that facilitate calcium delivery from the sarcoplasmic reticulum (SR) to the sarcomere for muscle contraction (Jaysinghe and Launikonis 2013). Skeletal muscle contraction is regulated by neural input through the neuromuscular junction (NMJ), synapses between pre-and postsynaptic muscle fibre. Nerve impulse triggers influx of calcium ions into the synapse in response to depolarization of presynaptic membrane. This in turn leads to the release of acetylcholine that diffuses over the synaptic cleft to bind to the acetylcholine receptor on the motor endplate that leads to sodium entry and muscle fibre depolarization (Rebbeck et al. 2013). The sarcolemmal membrane depolarization wave propagates into the T-tubuli where through dihydropyridine receptors on T-tubules the ryanodine receptors open and release calcium from SR into cytosol (Dulhunty et al. 2002). The calcium ions then bind to troponin C which releases actin from tropomyosin and enables the formation of cross-bridges between myosin and actin that result in force generation by and/or shortening of the sarcomere (Gehlert et al. 2015). When muscle stimulation terminates, acetylcholinesterase in the motor endplate breaks down acetylcholine to enable the muscle to respond to new impulses. Calcium is rapidly pumped back into SR by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and tropomyosin is positioned back to inhibitory state (Huang et al. 2020, Xu and van Remmen 2021).

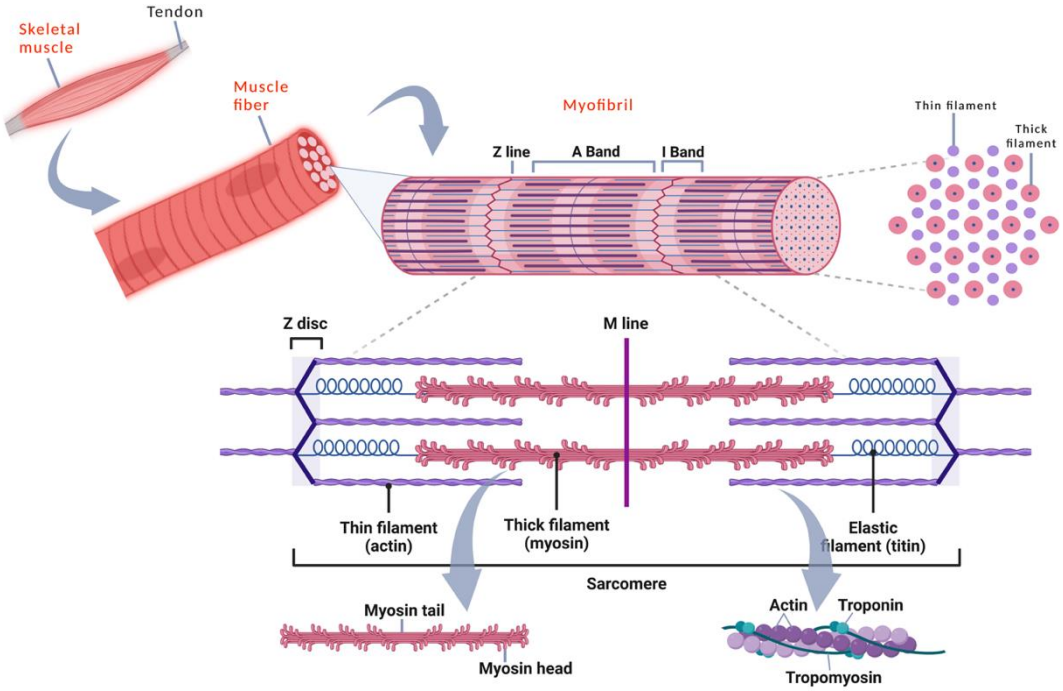

Figure 1.2: Skeletal muscle structure.
Image created by the author using Adobe Illustrator and BioRender. Adapted from Frontera and Ochala (2014), Rebbeck et al (2013).

The force output of skeletal muscle depends mainly on impulse frequency and motor unit recruitment, and ultimately determined by the number of cross-bridges formed (Huxley 2000, Sugi et al. 2018). Efficient force transmission from sarcomere to extracellular matrix (ECM), tendon and bone is a prerequisite for skeletal muscle function. Basal lamina is an ECM layer surrounding myofibers composed of collagen IV, laminin, proteoglycans and glycoproteins that contributes to SC quiescence, proliferation and differentiation (Schürer et al. 2022, Sinha et al. 2020). The sarcolemma is linked to basal lamina through integrins and dystrophin glycoprotein complex (DGC) which provides sites for myofiber attachment and connects intracellular cytoskeletal proteins to ECM. The intramuscular connective tissue composed of collagen I and III, is divided into endomysium surrounding basal lamina and perimysium that encloses fibre bundles and epimysium that surrounds the whole muscle and attaches it to tendon (Wilson et al. 2022, Purslow 2020).

1.3 Skeletal muscle regeneration

Healthy skeletal muscle is a very regenerative organ and has a capacity to recover itself upon ischaemic, mechanical and toxin-induced injuries and minor acute damage (Wang et al. 2022). The regenerative capacity of skeletal muscle cells is a prerequisite to inhibit skeletal muscle wasting during aging (Munoz-Canoves et al. 2019, Barberi et al. 2013) (Figure 1.3). Adequate regeneration of muscle tissue is a result of well-coordinated process between different cell types like SCs, fibroblast, inflammatory cells, and an optimal myofiber niche (Mackey et al. 2017, Scala et al. 2021).

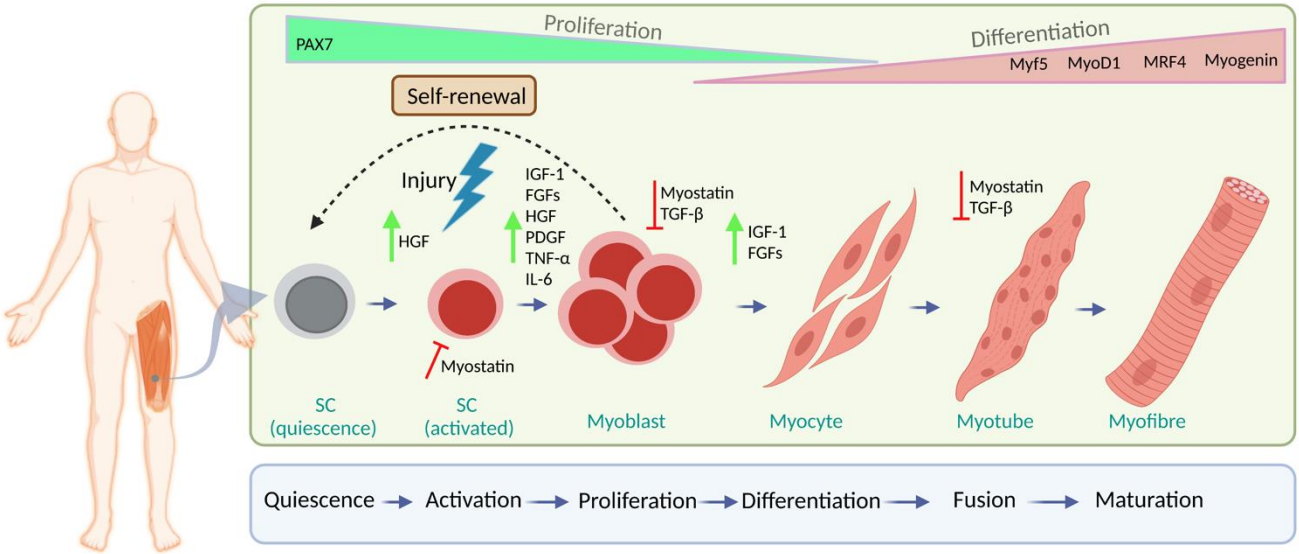

Figure 1.3: Schematic showing skeletal muscle regeneration.

Image created by the author using Adobe Illustrator and BioRender. Adapted from Barberi et al (2013), Scala et al (2021), Fukada et al (2020).

SCs are resident stem cells that reside as quiescent cells between myofibers and the basal lamina. They constitute 2-10% of total myonuclei while comprising the main population of muscle tissue-specific stem cells originating from *Pax7*+ embryonic progenitors of the dermomyotome central domain (Zammit et al. 2006, Sousa-Victor et al. 2022). The requirement for SC formation is Notch signalling which maintains the *Pax7*+ pool (Verma et al. 2018, Liu et al. 2018). During early stages of muscle growth, the contribution of muscle progenitors to the development of muscle mass is considerable, whereas in adult muscle, the

prevalence of *Pax7*+ mononucleated cells is trivial and the majority remain quiescent in SC pool (Fukada et al. 2020). Myogenic regulatory factors *Myf5* and *MyoD* are upregulated to stimulate SC proliferation by tissue damage or stretch. Activated SCs divide asymmetrically, being thus able to either self-renew or differentiate to form myofibers to contribute to muscle regeneration (Kuang et al. 2007, Wang et al. 2019). Further fusion of myotubes with each other or with existing myofibers is necessary to repair damaged fibres or to promote increases in muscle size and is marked by *Pax7* down-regulation and up-regulation of *Mrf4* and Myogenin (Hernandez et al. 2017, Bi et al. 2017, Singh and Dilworth 2013). Since the nuclei of permanently differentiated adult myofibres are mitotically inactive, additional new myonuclei from SCs are needed to assure postnatal growth of skeletal muscle (Cutler et al. 2022, Blaauw and Reggiani 2014, Hansson et al. 2020).

Mechanism of skeletal muscle regeneration includes the destruction phase (necrosis, hematoma formation, inflammatory cell influx), the repair phase (necrotic debris is phagocytosed, myofiber regeneration by SC activation) and the remodelling phase (the regenerated myofibers mature and contract) (Brigitte et al. 2010, Lauonier and Menetrey 2016). Macrophages, fibroblasts and fibro/adipogenic progenitor cells (FAPs) act co-ordinately to ensure complete regeneration and functional recovery of skeletal muscle (Murphy et al. 2011, Serrano et al. 2008). Cytokines released from damaged muscle attract pro-inflammatory M1 macrophages that play pivotal role in skeletal muscle regeneration process by secreting cytokines like tumour necrosis factor α (TNFα), interleukin-1β (IL-1β) and interferon γ (IFN-γ). Thereby, M1 macrophages clear muscle injury site from necrotic tissue, promote SC proliferation while inhibiting development of FAPs (Juban 2021, Koike et al. 2022, Teixeira et al. 2003). Following the removal of cell debris, a shift from pro-inflammatory M1 macrophages towards anti-inflammatory and pro-regenerative M2 macrophages takes place. M2 macrophages acquire restorative phenotype by expressing transforming growth factor factor β1 (TGF-β1) and IL-10. M2 macrophage stimulated TGF-

1β results in ECM formation by fibroblasts and SC differentiation (Zhang et al. 2019, Summan et al. 2006, Varga et al. 2016).

Muscle regeneration is impaired when transition between macrophages fails and/or when the activation of M2 macrophages is extended resulting in fibrosis. Supportive role of fibroblasts in SC activation and differentiation is compromised in severe injuries and muscle dystrophies when excessive ECM deposition and fibroblasts over-production occurs (Moyer and Wagner 2011, Lorts et al. 2012). Coordination between inflammatory cells, SCs and FAPs becomes hampered during ageing. Apoptosis of FAPs is dysregulated in response to elevated levels of macrophages producing *TGFβ* which results in fibrotic degeneration of muscle. To avoid fibrosis, FAPs must be activated briefly upon injury in order to exhibit successful eosinophils-derived IL-4 secretion for tissue debris removal and SC differentiation (Molina et al. 2021, Biferali et al. 2019, Gallardo et al. 2021). This highlights the impact of inflammation on the SC and regenerative capacity of skeletal muscle.

Myofiber self-repair as an alternative process to SC-driven muscle regeneration has been proposed (Roman et al. 2021). It is suggested that cell-intrinsic mechanisms based on existing myonuclei is activated to repair muscle fibre structure in response to localised injury (e.g., during physical exercise) where damage is not disastrous. It is unclear how such muscle self-repair is affected by ageing, but mediators of such a microdamage-induced repair process are suggested to be myonuclei that express sarcomeric mRNAs that reconstruct the sarcomere once delivered to injury site (Roman et al. 2021).

1.4 Skeletal muscle ageing

1.4.1 Sarcopenia

Ageing of the population is currently one of the main socio-economical global challenges. It is estimated that by 2050, 40% of the population will be older than 65 years (Seals et al. 2016, Harper 2014). Advanced longevity has many disadvantages as increased life-

expectancy allows to encounter several chronic degenerative disorders. As such, age-related loss of skeletal muscle mass and function is a widespread condition known as sarcopenia (Evans 1995, Wolfe 2016). The definition of sarcopenia provided by European Working Group on Sarcopenia in Older People (EWGSOP) is the most widely cited and approved by several international scientific societies. Accordingly, sarcopenia is defined as multifactorial syndrome characterized by progressive loss of muscle mass and function during ageing with a risk to cause frailty, physical disability, prevalence of weakness, fractures and eventually death (Dennison et al. 2017, Cruz-Jentoft et al. 2010, Crus-Jentoft et al. 2019, Cesari et al. 2004). Assessment of sarcopenia in clinical practice and research include muscle strength (grip strength, chair rise test), muscle mass (dual-energy X-ray absorptiometry (DXA), bioelectrical impedance analysis (BIA), magnetic resonance imaging (MRI)) and physical performance (400m walk test, short physical performance battery (SPPB)). The updated EWGSOP advises specific cut-off points for sarcopenia parameters which are set at -2SD (standard deviations) compared to reference (healthy young adults) values where possible (Dodds et al. 2014, Studenski et al. 2014, Distefano et al. 2018, Newman et al. 2006).

While the prevalence of sarcopenia rises 14% between ages 65-70 years and 53% after 80 years, it is not the exclusive condition in aged adults as sarcopenia can be related to malnutrition, cachexia and other comorbidities like obesity, diabetes and osteoporosis (Santilli et al. 2014, Schnerider et al. 2008, Trovato et al. 2016b). Skeletal muscle mass declines are commonly observed beyond 50 years with roughly 0.5% and 1% a year in women and in men, respectively (Mitchell et al. 2012, Brook et al. 2016). The balance between muscle protein synthesis and breakdown is disturbed in older adults which progressively leads to decrease in muscle mass characterized by the reduced contractile proteins, particularly of type II fibers (Fry and Rasmussen 2011) (Figure 1.4).

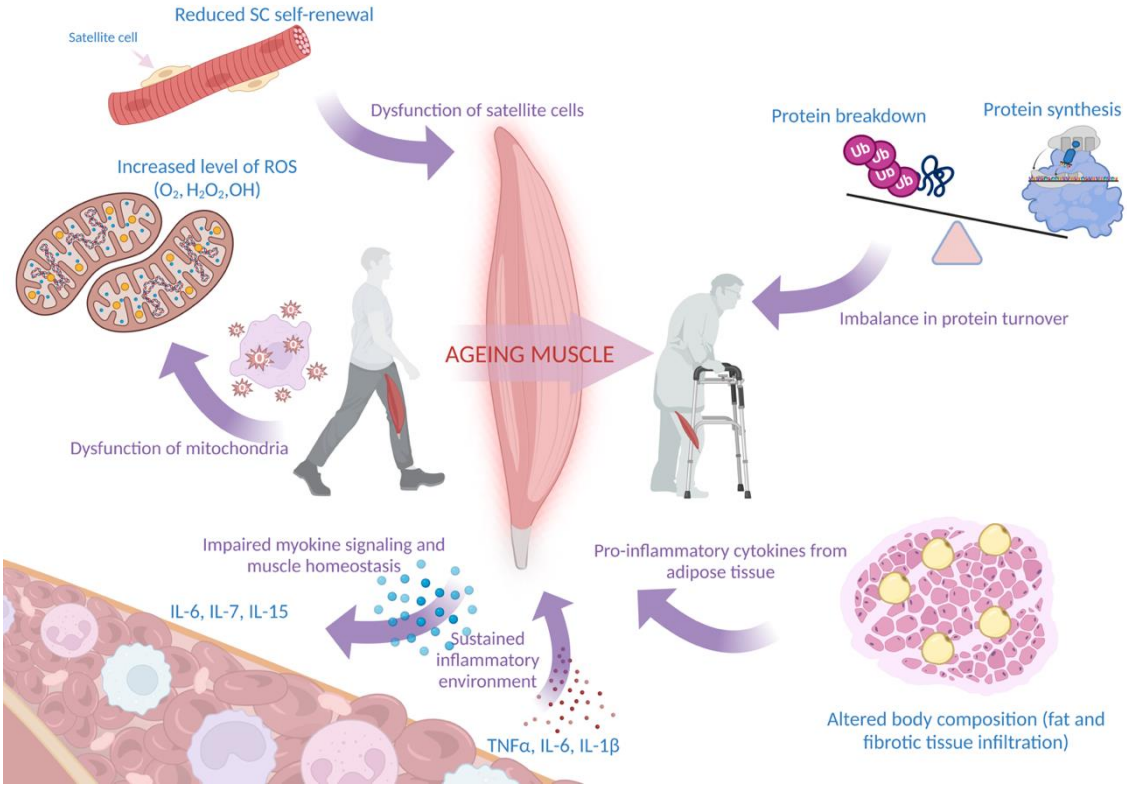

Figure 1.4: Illustrative schematics depicting development of sarcopenia.
Image created by the author using Adobe Illustrator and BioRender. Adapted from Thorley et al (2015), Bian et al (2017), Batsis et al (2016), McCromick and Vasilaki (2018).

The potential factors being associated with accelerated muscle protein breakdown and consequent development of sarcopenia include underlying acute/chronic diseases, decreased physical activity (Kortbein et al. 2008), ectopic fat deposition (Addison et al. 2014), hormonal imbalance and neuromuscular remodelling (Feldman et al. 2002, McNeil et al. 2005, Sheth et al. 2018), inadequate nutrition and chronic inflammation (Bian et al. 2017, Schaap et al. 2009), decreased regenerative capacity and SC function (Joanisse et al. 2016, Kadi et al. 2004, Thorley et al. 2015).

In addition to its role in locomotion, skeletal muscle contains huge amounts of amino acids and serves as the largest site for glucose disposal (Poortmans and Carpentier 2016, Ramos et al. 2021). It is therefore to be expected that maintenance of skeletal muscle throughout the lifetime prevents the development of metabolic morbidities and physical dependence (Wolfe et al. 2016). Several studies on skeletal muscle atrophy using animal models have been established (Kang et al. 2022, Brett et al. 2020, Joanisse et al. 2016, Clemens et al. 2021), but as the mechanistic processes mediating sarcopenia appear to be different, underpinning

pathways relevant to human sarcopenic skeletal muscle *in vivo* are incompletely understood. High prevalence and its profound clinical impact highlight the importance of ongoing research to identify effective therapies for sarcopenia.

1.4.2 Skeletal muscle as an endocrine organ

Biomedical engineering together with stem cell biology are currently leading fields in developing biomimetic models of human skeletal muscle where the presence of other cell types is a prerequisite for muscle repair (Rimington et al. 2021, Wragg et al. 2019, Juhas et al. 2018). Cross talk with other tissues and organs is of paramount importance as skeletal muscle is an endocrine organ secreting numerous myokines in response to exercise (Benatti and Pedersen 2014, Hoffman and Weigert 2017, Santos-Lozano et al. 2016). Such release of endocrines (e.g., interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 15 (IL-15), vascular endothelial growth factor (VEGF), myonectin) contributes to skeletal muscle adaptation to exercise, systemic inflammation and overall health (Zeng et al. 2010, Weigert et al. 2014, Kanzleiter et al. 2013, Broholm et al. 2008).

Along with sarcopenia, a subset of individuals with concomitant obesity classified as sarcopenic obesity exhibit accelerated adverse outcomes like reduced life expectancy, higher risk of disability and mortality which highlights the strong relationship between fat and muscle mass (Batsis and Villreal 2018, Petterson et al. 2004, Stoklossa et al. 2017, Baumgartner et al. 2008) (Figure 1.5).

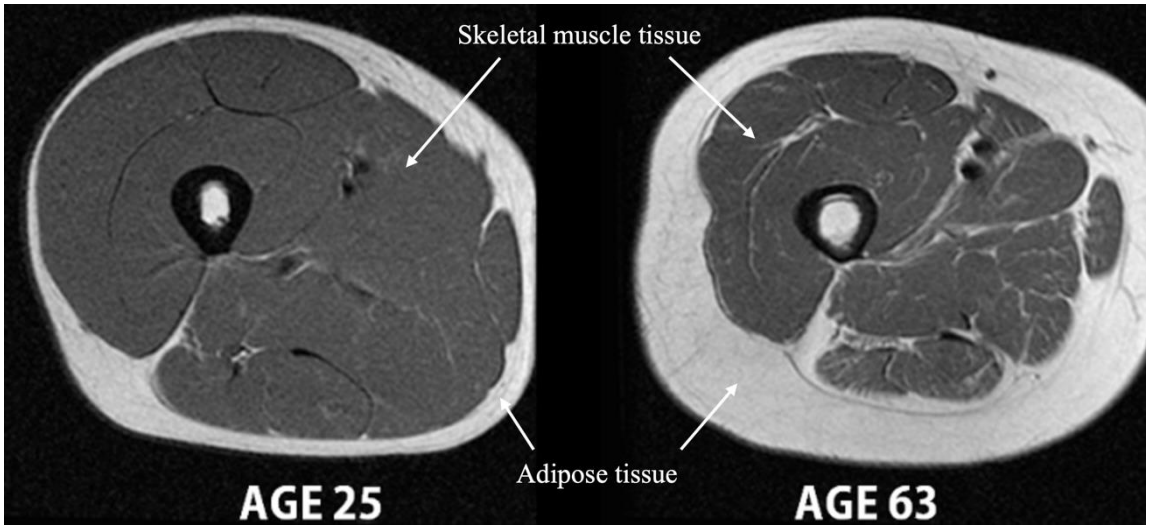

Figure 1.5: MRI images comparing m. quadriceps in two people (young and older adult) indicating reduced skeletal muscle mass and increased fat mass with ageing.
Modified image obtained from (Buck Institute for Research on Ageing)

The obesity-associated state of low-grade inflammation is due to the function of adipose tissue as an active endocrine organ secreting several bioactive proteins like IL-6, IL-8, TNFα, leptin and adiponectin (Beasley et al. 2009, Trayhurn et al. 2011, Lehr et al. 2012). Given the increased accumulation of intramyocellular triglycerides with aging, subclinical systemic inflammation is suggested to be strongly linked with intramuscular adipose tissue (IMAT) depots and thus loss of muscle quality and strength (Addison et al. 2014, Tunon-Suarez 2021, Konopka et al. 2018). Observational studies have reported that in comparison with lean persons, the inflammatory status is higher among overweight people and in fact, circulating levels of C-reactive protein (CRP) and IL-6 together with IMAT are reduced in response to weight-loss and exercise among older adults (Lambert et al. 2008, Rejeski et al. 2019, Nicklas et al. 2004, Marcus et al. 2010).

The role of exercise-induced myokines to regulate cross talk of myofibers with immune cells, fibroblasts, vasculature, liver, adipose and bone tissue is crucial to improve the function of the muscle, immunometabolism, glucose homeostasis and insulin sensitivity.

1.4.3 Regeneration of aged skeletal muscle

Despite the regenerative capability of healthy skeletal muscle, its function becomes compromised as a result of several conditions including ageing, chronic metabolic and neuromuscular diseases, and in response to acute injuries like volumetric muscle loss (Distefano and Goodpaster 2018, Domingues-Faria et al. 2015). Such impairment in skeletal muscle function leads to declined quality of life, increased falls, disabilities and mortality. Successful muscle regeneration following damage depends on several coordinated processes involving different cell types essential for optimal restoration of muscle microenvironment (McCromick and Vasilaki 2018, Chakkalakal et al. 2012, Wosczyna and Rando 2018). Such remodelling is dysregulated in aged muscle possibly resulting in accumulation of ECM and intramuscular fat due to altered fate of myogenic cells, Wnt signalling and inflammatory response (Brack et al. 2007, Bernet et al. 2014, Stearns-Reider 2017, Wosczyna et al. 2019) (Figure 1.6).

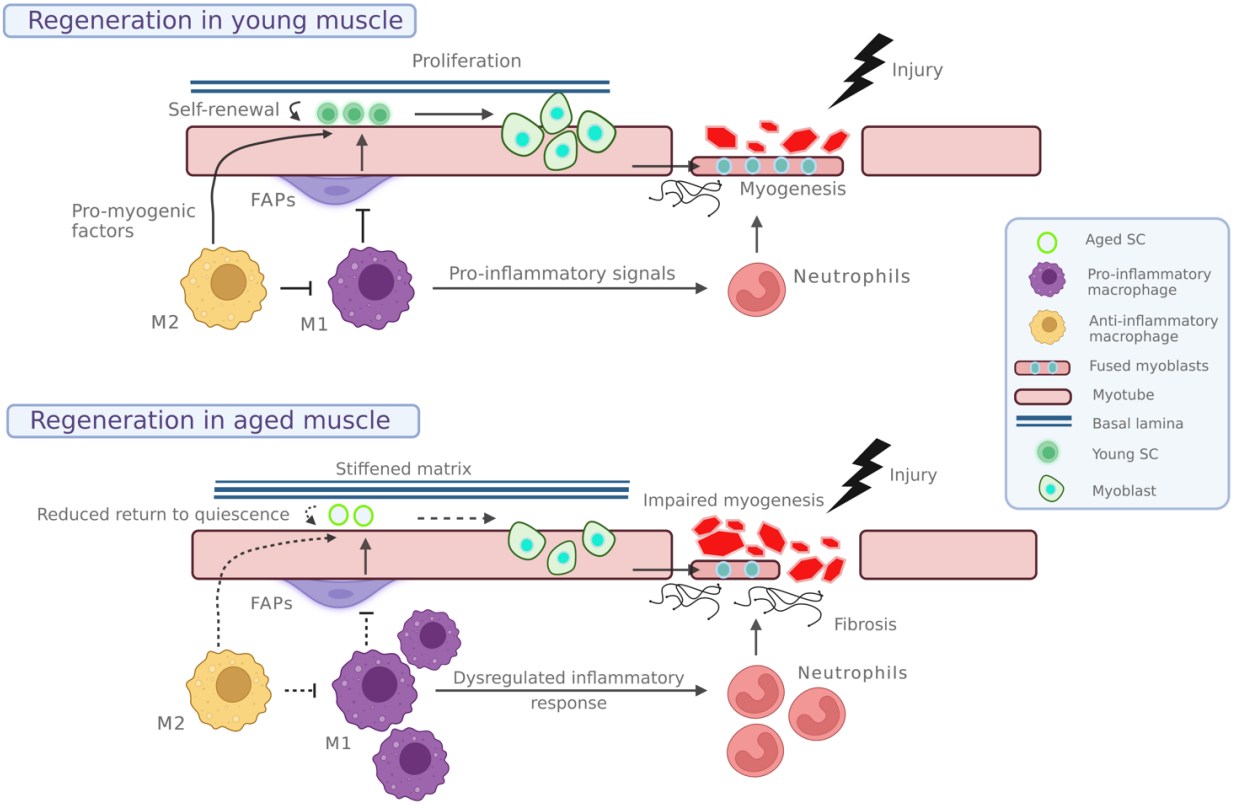

Figure 1.6: Differences in regeneration process in young and aged skeletal muscle.
Image created by the author using Adobe Illustrator and BioRender. Adapted from Wosczyna and Rando (2018), Farup et al (2015).

Insufficient presence of SC pool together with impaired function of SCs is partly responsible for the loss of muscle function and hampered regeneration capability in aged muscle (Snijders et al. 2015, Mackey et al. 2014, Suetta et al. 2013, Verdijk et al. 2013). The main feature of SCs is self-renewal and contribution to stem cell pool upon injury and thus, skeletal muscle regenerative response requires adequate stock of *Pax7*+ SCs with the potential to replenish in response to physiological injury like acute or chronic exercise (Olguin et al. 2007, Farup et al. 2015, Evano et al. 2020). However, adult muscle can adapt and be sustainable without the presence of SCs as the source of new myonuclei and the absolute requirement of SCs fusion for hypertrophy is being questioned (Roman et al. 2021, Borowik et al. 2022). The role of SCs in regenerative response is determined by the extent of muscle fiber damage when accumulating dysregulated muscle niche eventually leads to compromised potential to adaptively grow and function (Murphy et al. 2011, Yin et al. 2013, Haroon et al. 2020). For example, high volume eccentric resistance exercise or intense running require reconstruction of the large portion of muscle fibres when SCs are vital (Karlsen et al. 2020, Parise et al. 2008, Bansal et al. 2003, Sambasivan et al. 2011, Lepper et al. 2011, Fry et al. 2013). Nevertheless, the impaired function of SCs appears not to be the cause of sarcopenia (Fry et al. 2015) and furthermore, recent findings state that when the membrane damage is not disastrous like during daily wear and tear, cell intrinsic membrane repair mechanism is sufficient and SCs not required for regenerative adaptation (Bansal et al. 2003, Demonbreun et al. 2016, Barthelemy et al. 2018, Carmeille et al. 2016). Understanding the basis of skeletal muscle formation and function unravels underlying mechanisms of muscle pathologies and helps to elucidate treatments for muscle loss like sarcopenia.

1.4.4 Strategies to counteract muscle ageing and sarcopenia

Given the rapidly ageing population with high prevalence of sarcopenia, developing cost-effective interventions to preserve physiological function is vital. Decline in cardiorespiratory fitness, metabolic homeostasis, neuromuscular, cardiac, vascular and cognitive function with ageing have shown to be reduced among regularly active adults compared to sedentary peers (Booth et al. 2011, Zampieri et al. 2014, Kortebein et al. 2008). Alterations in skeletal muscle at cellular level during ageing are related to the loss of muscle mass, strength and endurance (Lang et al. 2010, Vina et al. 2016, Riuzzi et al. 2018). Several mechanisms responsible for sarcopenia have been suggested, though no reasonable approach to counteract skeletal muscle loss among elderly is elucidated. The mechanisms behind the decline in muscle mass and function and aerobic capacity with increasing age are multi-factorial involving metabolic dysregulation as inherent and physical activity together with nutrition as extrinsic inputs (Martone et al. 2015, Giresi et al. 2005, Han et al. 2021, Cleasby et al. 2016). Lifestyle changes such as optimized exercise paradigms and adequate nutrition are suggested as main strategies to confer benefits in sensitizing anabolic responsiveness in muscle to prevent the progression of sarcopenia (Law et al. 2016, Arnold and Bautmans 2014, Cruz-Jentoft et al. 2014, Pennings et al. 2012) (Figure 1.7).

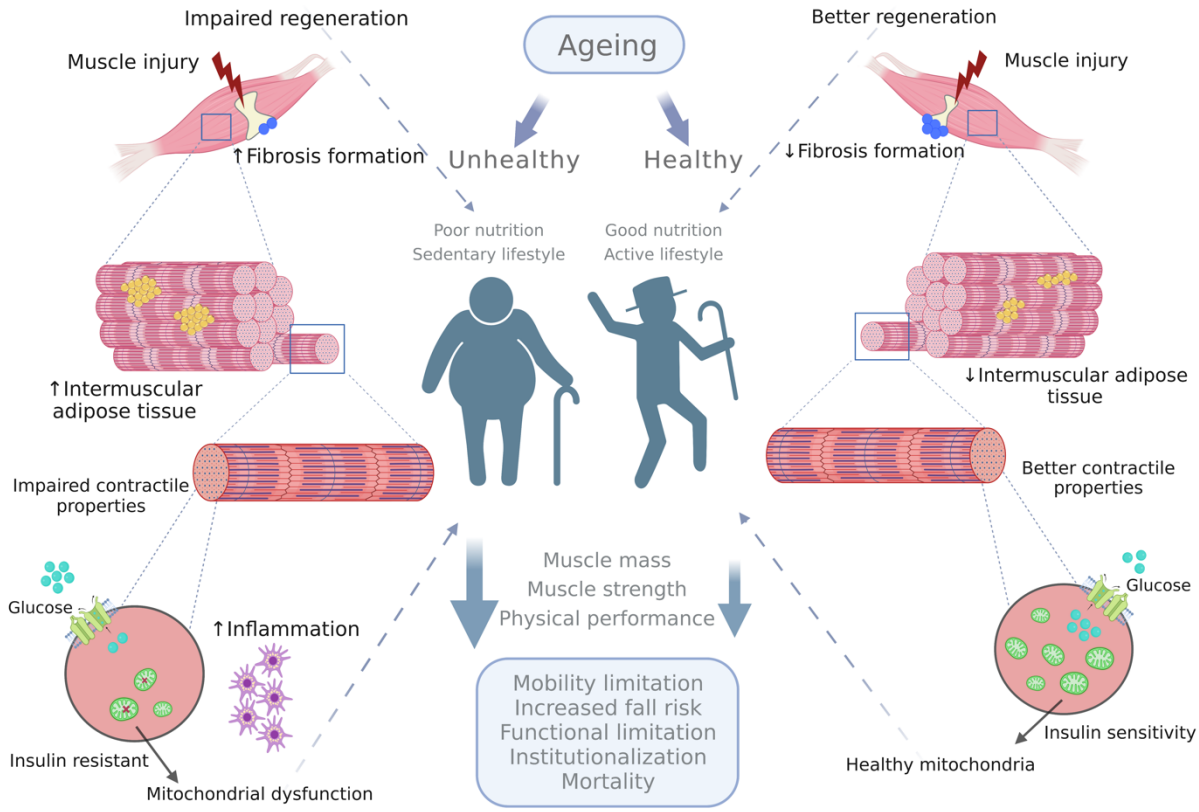

Figure 1.7: Schematic comparing quality of the skeletal muscle during ageing in different lifestyles.
Image created by the author using Adobe Illustrator and BioRender. Adapted from Brook et al (2016), Law et al (2016), Seals et al (2016).

1.4.3.1 Exercise training

Regular physical activity, even when performed at moderate level, have significant effect on greater life-expectancy, delaying the onset of morbidities and maintaining the function during ageing (Moore et al. 2012, Seals et al. 2019, Fiuca-Luces et al. 2013). Different modalities of resistance training have resulted in increases muscle strength (1RM of different muscle groups) and functional performance (6 minute walk test, chair rise, stair climbing, balance) in both older men and women (Henwood and Taaffee 2006, Holviala et al. 2012, Chen et al. 2017, Hamaguchi et al. 2017).

The cellular and molecular mechanisms underlying the impact of chronic exercise on physiological function are incompletely understood, but specific adaptations are likely determined by the activity performed (Mendondca et al. 2016, Da Boit et al. 2016, Safdar et al. 2010, Verdijk et al. 2016). Depending on the type of exercise, metabolic changes (such as increased oxidative stress, ATP/ADP ratio) lead to activation of several signalling pathways (Figure 1.8).

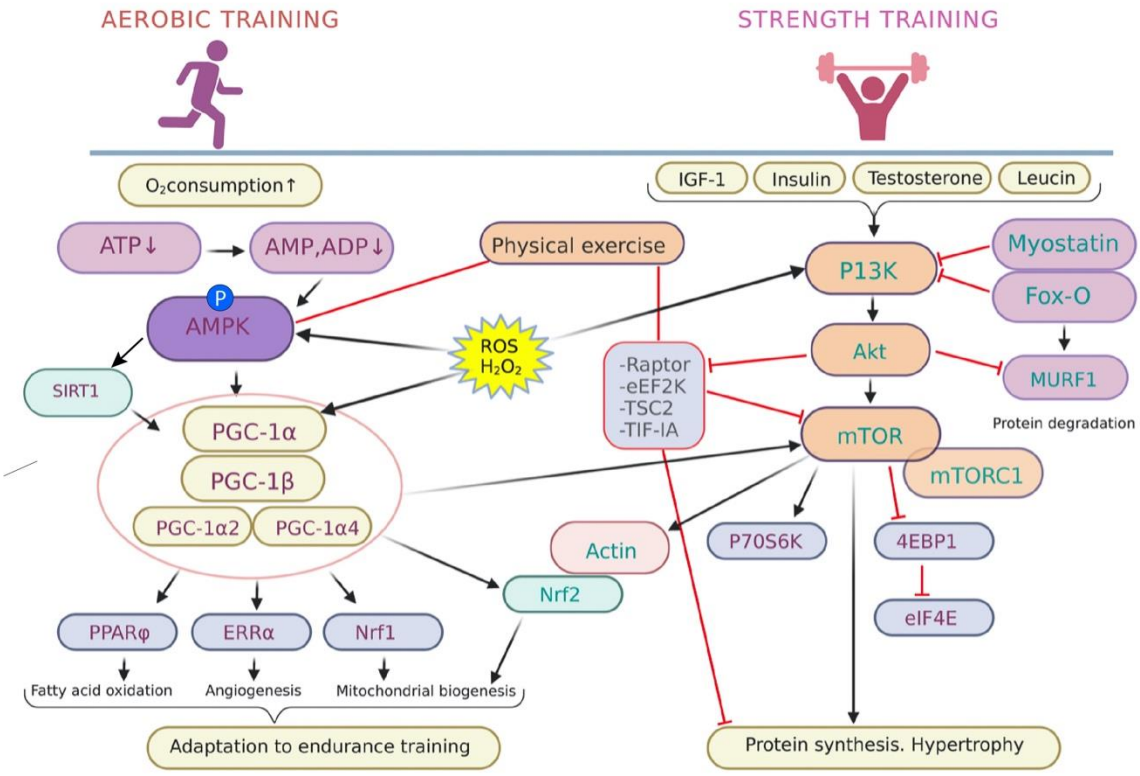

Figure 1.8: Illustrative schematic comparing signalling pathways regulated by strength and aerobic exercise.
Image created by the author using Adobe Illustrator and BioRender. Adapted from Brook et al (2016), Atherton et al (2005), Song et al (2017).

As such, decreased energy status during aerobic exercise inhibits mammalian target of rapamycin (mTOR) and activates MP-activated protein Kinase (AMPK) signalling pathway (Atherton et al. 2005, Hawley et al. 2014). While basal mitochondrial fractional synthesis rate together with mitochondrial enzyme activity (cytochrome c oxidase and citrate synthase) are reduced with ageing (Konopka et al. 2014, Gouspillou et al. 2014, Drummond et al. 2014), chronic endurance exercise training improves cardiovascular fitness and

remains effective to maintain muscle strength and function in older individuals (Bori et al. 2012, Konopka et al. 2010, Wroblewski et al. 2011, Short et al. 2005). Furthermore, long-term aerobic exercise regimen has shown to increase mitochondrial biogenesis in response to upregulated transcription factors and protein content of mitochondrial fusion factors mitofusin (*MFN*) 1 and 2, and peroxisome proliferator-activated receptor-gamma coactivator α (*PGC-1α)* (Zhang et al. 2020, Kang et al. 2013, Kim et al. 2017). By attenuating ROS production and oxidative stress, regular exercise prevents or reverses age-associated increases in superoxide production from several sources and increases the bioavailability of nitric oxide (NO) (Simioni et al. 2018, Gleeson et al. 2011, Seals 2014, Vina et al. 2013).

Resistance training is proven to be the most effective non-pharmacological strategy to mitigate muscle wasting (Walker et al. 2011, Tsuzuku et al. 2018, Hurst et al. 2022, Eckardt 2016). The cellular processes regulating muscle protein synthesis and hypertrophy are coordinated by mTORC1 signalling pathway (Sandri et al. 2013, Schiaffino and Mammucari 2011, Song et al. 2017). Increased protein synthesis via phosphorylation of mTOR through the insulin-like growth factor (*IGF-1*) and Protein Kinase B (*Akt*) further leads to the activation of 70kDa ribosomal S6 protein kinase (*p70S6K*) (Terzis et al. 2010, Ato et al. 2019). Skeletal muscle breakdown occurs via inactivation of Akt which leads to the expression of atrogenes ubiquitin E3, atrogin-1 and muscle RING-finger protein-1 (*MuRF1*) through dephosphorylation of Forkhead box O (*FOXO*) (Sandri et al. 2004, Senf et al. 2010). TNF-α which regulates downstream transcription factor nuclear factor kappa B (*NF-κB*) followed by upregulation of MuRF1 is an alternative Akt-independent atrophy-pathway (Jackman et al. 2013, McMahon et al. 2019).

Ageing is associated with increased expression of inflammatory cytokines like TNF-α and IL-6 in skeletal muscle that are stimulating muscle atrophy through ubiquitin-proteasome pathway (Zembron-Lancy et al. 2019, Bian et al. 2017, Aleman et al. 2011, Hangelbroek et

al. 2018). The benefits of regular exercise even at modest activity levels are implicated by declined levels of circulating inflammatory markers (Colbert et al. 2004, Sardeli et al. 2018). For instance, expression of IL-6 in response to regular exercise has demonstrated anti-inflammatory properties due to the capacity to inhibit the secretion of other pro-inflammatory mediator like TNFα in older adults (Brandt and Pedersen 2009; Kim and Yeun 2022, Sa Souza et al. 2022). Reduced ROS content in response to exercise-related activation of antioxidant defence system also results in reduced age-associated subclinical inflammatory state (Barbieri & Sestilli 2012). Long-term strength training program in older people have shown significant improvement in time in chair rise test and 4 minute walk test (Da Boit et al. 2016, Eckardt 2016), leg extension (Beneka et al. 2008, Kennis et al. 2013) and grip strength (Ramirez-Campillo et al. 2014, Timmons et al. 2018). Moreover, exercising elderly >65 years exhibit lower level of systemic inflammatory markers like IL-6, TNFα and CRP (Colbert et al. 2004, Kim and Yeun 2022).

Resistance exercise coupled with adequate protein intake remains the most effective methods to either maintain or increase muscle mass in older adults despite of a phenomenon called anabolic resistance (Burd et al. 2013, Moore et al. 2015, Aragon et al. 2022). Anabolic resistance is characterized by insensitivity to protein intake and exercise resulting in decreased muscle protein synthesis and is accompanied or caused by downregulation of *p38MAPK* and *mTOR/P70S6K/4E-BP1* pathway and associated proteins (Fry et al. 2011, Kumar et al. 2009, Katsanos et al. 2005). Optimal combination of exercise, nutrition and pharmacology is to be developed to promote anabolism and impair insulin-mediated suppression of proteolysis (Dickinson et al. 2014, Drummond et al. 2011, Moro et al. 2019). Due to reduced sensitivity, recent finding highlights the need of higher protein intake and greater exercise volume in older adults to generate rate of muscle protein synthesis and inhibition of muscle protein breakdown equivalent to younger individuals (Kumar et al. 2012, Szwiega et al. 2020, Borde et al. 2015). This suggests that preventing decline in muscle

mass could be achieved by low-load high-volume exercise supported by optimized protein intake.

Available data clearly indicate that regular physical activity as the main modifiable factor for sarcopenia show positive effect on physical function mediated by improved muscle strength, endurance and cardiorespiratory fitness. Accompanied downregulation of inflammatory state during exercise further inhibit prevalence of physical impairment and disabilities and has shown to be associated with improved longevity.

1.5 *In vivo* vs *in vitro* muscle models

The study of the ageing muscle and sarcopenia relies on *in vivo* and *in vitro* models. *In vivo* refers to the experiments on a living organism ("within the living" in Latin) and includes animal studies and clinical trials. Small animal models, mainly rat and mouse, have been widely used as a cost, and time-efficient, feasible alternative to human donors to study skeletal muscle function, diseases and ageing (Cecconi et al. 2022, Hoppeler 2016, Khodabukus et al. 2021). Different rodent models have been used to investigate biological mechanisms of muscle ageing including denervation, hindlimb suspension, immobilization and naturally aged animal (Baek et al. 2020, Schiaffino and Mammucari et al. 2011, Batt et al. 2006, Madaro et al. 2008). *In vivo* experiments are considered to be superior to *in vitro* testing (outside of the body, in the laboratory set-up) by providing a physiologically more relevant environment and allowing to observe the whole-body reaction to drugs and treatments (Yucel et al. 2018, Shen et al. 2011, Coley et al. 2016). Inaccuracy of current animal models to predict clinical outcomes in humans and to replicate human skeletal muscle diseases have challenged researchers to develop *in vitro* models to elucidate molecular mechanisms underlying regulation of skeletal muscle mass and function (Wang et al. 2019, Khodabukus et al. 2020, Rao et al. 2018).

1.5.1 Monolayer cell culture

In vitro models have aided to shed light on aspects of myoblasts fusion and SC differentiation as well as provide insights into disease modelling, drug screening and cell therapeutics (Uezumi et al. 2016, Serrano et al. 2008, Feng et al. 2015). Culturing myoblasts on typical monolayer culture (2D) has many advantages including high-throughput testing, easy-to-use with simple equipment, smaller cell amount requirement, easily reproducible gene expression and/or immunohistochemical analysis (Prüller et al. 2018, Li et al. 2018) and by passed ethical issues related to animal research. However, 2D culture models lack the functional and structural organization of native muscle tissue making its usefulness in biological studies and in tissue replacement limited (Cornelison 2018). Furthermore, flat 2D surface enables homogenous access to nutrients and growth factors to all cells which is not representative for native muscle (Li et al. 2018, Smith et al. 2012). Detachment of myotubes from tissue culture plastic during long-term experiments sets a limitation to drug testing and measuring force production and fatigue resistance. In addition, too stiff 2D culture substrate (1GPa) does not favour physiological myogenesis (12-18 kPa) leading to immature myotubes (Engler et al. 2004, Engler et al. 2006, Khodabukus et al. 2018). Given the limitations of the monolayer culture systems, development of bioengineered *in vitro* systems is crucial to effectively reduce the use of animals and to improve the identification of effective novel therapies and drug screening in human patients (Figure 1.9).

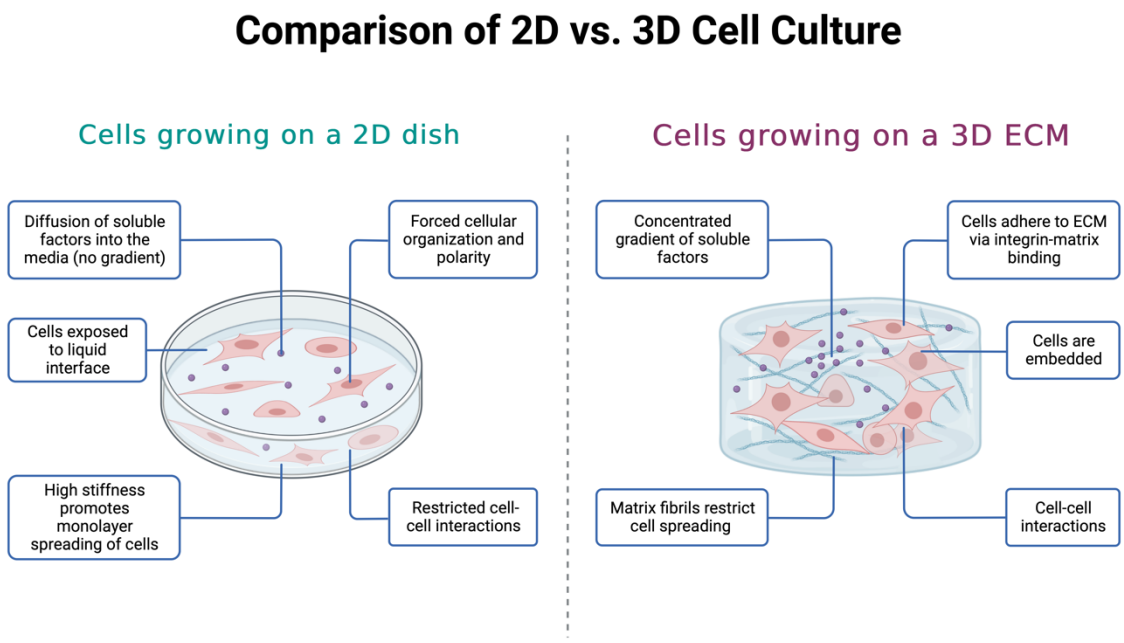

Figure 1.9: Representative illustration of monolayer and tissue engineered culture systems.
Image created using Adobe Illustrator and BioRender. Adapted from Engler et al (2004), Li et al (2018), Labro et al (2018).

1.5.2 Tissue engineered muscle cell culture

Tissue engineering is a subdiscipline of biomedical engineering with a goal to restore, replace or improve different types of damaged biological tissue by integrating progenitor cells, biomaterials and optimal biochemical cues (Langer and Vacanti 1993, Koning et al. 2000). Recent advances in bioengineering could provide biologically relevant tissue engineered muscle (TEM) systems to investigate adaptations to exercise and to assess the role of the *in vitro* niche for pharmacological and genetic modifications to improve our knowledge of the fundamental mechanisms of physiological responses observed *in vivo* skeletal muscle (Kasper et al. 2018, Player et al. 2014, Juhas and Bursac 2014). Important factors in tissue engineering include cell type (primary cells, cell lines, stem cells); biomaterials of the scaffold (natural or synthetic) to enhance the attachment, proliferation and differentiation of the cells and chemical and physical stimuli that determine optimal

micro-environment to initiate specific response (Labro et al. 2018). TEM systems using cells seeded in scaffolds under tension exhibit resemblance to structural organization and functional capacity of native skeletal muscle (Ikeda et al. 2016, Prüller et al. 2018, Fleming et al. 2019). TEM cultures have advantage to traditional monolayer models by allowing a prolonged culture period, improving maturation of contractile and metabolic function, and rapid drug screening and disease modelling (Wang et al. 2019, Bersini et al. 2018). As such, development of TEM organoids mimicking the structural and biological characteristics of native tissue has led to platforms that are used for preclinical drug discovery and to generate patient-specific disease models, which will inform treatment regimens (Agrawal et al. 2017, Capulli et al. 2014, Bhatia and Ingber 2014). While TEMs are promising muscle models for tissue regeneration, it remains a challenge in the field of musculoskeletal tissue engineering to generate *in vitro* TEM models that replicate native skeletal muscle niche and structure (Smith et al. 2016, Urciuolo et al. 2020, Martin et al. 2016, Heher et al. 2015).

1.5.2.1 Tissue engineered mouse skeletal muscle models

Cell types predominantly employed in muscle engineering include mouse C2C12 myoblast cell line, primary myoblasts from different species and mesenchymal stem cells (Khodabukus and Baar 2016, Carraro et al. 2022). C2C12 cells are mouse-derived immortalized myoblasts, and the most used cell type in skeletal muscle bioengineering, making comparative analysis possible with previous works (Agrawal et al. 2017, Aguliar-Agon et al. 2019, Hofemeier et al. 2021, Sharples et al. 2012). Despite its accessibility, cheap culturing and rapid proliferation compared to primary cells, metabolic characteristics of C2C12 cells remain distinct from primary muscle cells with respect to skeletal muscle physiology and the lack of inter-individual variability (Kim et al. 2019, Labro et al. 2018, Turner et al. 2021). Primary myoblasts as direct myogenic precursors are used in tissue engineering, although they are therapeutically limited with reduced availability for drug screening and for potential treatments testing due to reduced myoblast proliferation capacity

in vitro and lack of donor cell availability (Sicari et al. 2013, McCullagh and Pernigeiro 2015). Therefore, sustainable sources of cells alternative to primary myoblasts are required to provide physiological relevance and sufficient *in vitro* cell expansion (Dugan et al. 2014). Muscle progenitors derived from rat and mouse are widely used in tissue engineering with rat myoblasts exhibiting greater cell yield than mouse myoblasts. The myotubes or TEMs derived from these cells produce higher *in vitro* forces that are similar to that of native muscle (Juhas et al. 2018, Juhas et al. 2014, Kohabukus et al. 2021). Unlike rat cells, mouse muscle cells require ECM proteins such as collagen, fibrin, Matrigel and fibroblasts to achieve complete differentiation in co-culture, suggesting that rat myoblasts possess intrinsic factors that lead to improved muscle function of engineered muscle (Huang et al. 2006, Khodabukus and Baar 2015). Lastly, mesenchymal and pluripotent stem cells can be harvested in great amount from rats offering thus promising alternative to myogenic precursor cells (Witt et al. 2017, Farup et al. 2015, Bersini et al. 2018).

1.5.2.2 Tissue engineered human skeletal muscle models

Several TEMs known as myobundles derived from human myoblasts have been developed that display contractile, structural and metabolic properties similar to native skeletal muscle (Madden et al. 2015, Khodabukus et al. 2019, Wang et al. 2021). SCs isolated from their niche by enzymatic or mechanical dissociation induces a rapid activation of primary myogenic cells positive for *MyoD* and negative for *Pax7* gene expression (Uezumi et al. 2016, Maesner et al. 2016). Nevertheless, the considerably high seeding density of primary human cells, limited proliferation capacity with reduced myogenic potential, difficulties to obtain biopsies from aged or diseased human donors are limitations that suggest the need for an alternative myogenic cell source to biofabricate high-throughput *in vitro* TEM recapitulating features of human skeletal muscle (Machida et al. 2004, Cerletti et al. 2008, Day et al. 2010, Smith et al. 2012, Rimington et al. 2021). Recreating muscle tissue from

pluripotent stem cells is such an alternative and overcomes ethical concerns and senescence limitation observed with primary myoblasts (Dimos et al. 2008, Rao et al. 2018, Mazaleyrat et al. 2020). Using human induced pluripotent stem cells (hiPSCs) in cell therapy and in skeletal muscle regeneration research has become more common due to its limitless availability and myogenic differentiation capacity (Maffioletti et al. 2018, Bakooshli et al. 2019).

In contrast to hiPSCs stem cells that can differentiate into any given cell type, the differentiation capability of multipotent mesenchymal stem cells (MSCs) is somewhat limited. These cells are isolated from tissues, such as bone marrow or adipose tissue, from adult organisms (Witt et al. 2017, Dugan et al. 2014). In addition, their multipotency, high self-renewal ability and the ability to isolate large quantities make MSCs an appealing alternative for myogenic progenitor source (Koning et al. 2009, Pittenger et al. 1999). Several methods to induce myogenic differentiation of MSCs have been applied including DNA methylation using 5-azacytidine (Wakitani et al. 1995), upregulating of PAX3 expression via retroviral transfection to activate MRFs (Gang et al. 2008) and using induction media consisting of dexamethasone and hydrocortisone (Zuk et al. 2001, Gang et al. 2004, Lin et al. 2006). It has been shown that MSCs derived from either bone marrow or adipose tissue are capable of myogenic fusion with myoblasts and differentiation in co-culture to myotubes (Kulesza et al. 2016, Bitto et al. 2013), suggesting the myogenic potential of MSCs in tissue engineering.

1.5.2.3 Tissue engineered skeletal muscle methods

In vitro skeletal muscle engineering is imperative to generate constructs as a source of tissue for engraftment of bioengineered muscle following significant skeletal muscle loss (Mertens et al. 2014, Dugan et al. 2014). TEM is prepared using bioreactors, electrospun scaffolds, molds or microfluidic devices with or without muscle cells embedded in biomaterial-based

constructs (Elsaadany et al. 2017, Burkel et al. 2016, Capel et al. 2019, Gilbert-Honick et al. 2018). Scaffold-based TEM constructs aim to replicate the native muscle environment by embedding and culturing cells within ECM proteins like collagen and fibrin (Gholobova et al. 2015, Hinds et al. 2011, Madden et al. 2015). Hydrogels are water-absorbing polymer networks widely used in tissue engineered culture systems due to similar characteristics to native ECM, the ability to support cell adhesion and mimic *in vivo* tissue environment (Caliary and Burdick 2016, Jabbari 2019). Although, the hydrogel systems recreate complexity of native tissue more accurately than standard tissue culture plastic, there are some polymer properties to be considered such as mechanics, swelling, mesh size, and degradation. Hydrogels are divided into natural (collagen, fibrin, alginate), synthetic (polyacrylamide (PA), polyethylene glycol (PEG)) and hybrid (hyaluronic acid (HA), polypeptides) polymers (Wang et al 2019, Han et al 2017). Collagen and fibrin are widely used degradable and biocompatible (nontoxic or harmful to host environment in order to perform suitable biological response) organic compounds with a great structural semblance to native muscle shown to support and improve myogenesis (Heher et al 2015, Martin et al 2013, Somers et al 2017, Witt et al 2017).

Scaffold-free approaches promote the synthesis of ECM proteins to self-create TEM under favourable conditions by myoblasts seeded on a monolayer together with fibroblasts (Li et al. 2011, Takahashi et al. 2018, Nagamori et al. 2013). The first TEM tissue was engineered nearly 30 years ago using avian myotubes cultured in collagen hydrogels. Compared to 2D cultures, long-term culturing of myotubes under tension between nylon anchors resulted in enhanced maturation evidenced by increased protein and DNA content, and MHC expression (Vandenburgh et al. 1988). Cell alignment and advanced myogenic differentiation in response to mechanical tension and encapsulation of matrix protein have since become key aspects in muscle tissue engineering (Engler et al. 2004, Juhas et al. 2014, Hinds et al. 2011).

Complexity of skeletal muscle has also been attempted to recapitulate within a microfluidic device, a skeletal muscle-on-a-chip system used as screening platform and injury model of *in vitro* skeletal muscle (Bhatia and Ingber 2014). Organs on a chip comprise of microtissues of native tissue within a continuous perfusion device aiming to capture structural and biological features of *in vivo* muscle in a more reproducible and cost-effective manner (Cimetta et al. 2010, Tumarkin et al. 2011). Constant perfusion of the microdevices enables variation of timing and dosage of the drug applied similar to that in the human body and reduces the usage of culture reagents. Additionally, these functional engineered micro-fluidic systems are suited for pre-clinical drug and cell-based therapies screening (Agrawal et al. 2017, Capulli et al. 2014, Shimizu et al. 2017).

Electrospun hydrogel scaffolds provide an additional platform to regenerate the structure and function of skeletal muscle (Gilbert-Honick et al. 2018, Chen et al. 2013). Electrospun polymer nanofibers are produced through electrospinning process where synthetic polymer fibres are aligned using an electric field (Chemmarappally et al 2020, Doshi and Reneker 1995, Narayanan et al 2016). Natural ECM protein, fibrin, electrospinned within myofiber bundles, exhibit biocompatibility and bioactivity with modifiable chemical and mechanical properties in a pro-regenerative environment (Zhang et al. 2014, Page et al. 2011). Pro-myogenic alignment and substrate stiffness, hierarchical structure, and scale-up capabilities characterise the myoblast-seeded scaffolds that can regenerate muscle with high myofiber and vascular density (Rossi et al. 2011, Grasman et al. 2015, Sicari et al. 2014). Furthermore, microfibre bundles mimic the elastic modulus of native tissue that obtain mature levels of contractile and vascular properties of skeletal muscle (Engler et al. 2004, Choi et al. 2011).

One of the latest advances in skeletal muscle tissue engineering is the incorporation of dynamic cell culture systems to improve tissue maturation (Powell et al. 2002, Player et al. 2014). The use of bioreactors allows physiological stimulation of TEM cultures by means of electrical or mechanical loading over repetitive/sustained periods (Heher et al. 2015, Turner et al. 2021). TEM culture in conjunction with bioreactors allows controlled provision

of stimulation to induce early myogenesis and higher experimental throughput, aiding functional maturation of the *in vitro* skeletal muscle (Seaborne et al. 2019, Kasper et al. 2018, Maleiner et al. 2018).

The computer controlled dynamic cell stretching system Flexcell is used to apply mechanical load on monolayers and TEM cultures *in vitro* (Juffer et al. 2013, Chen et al. 2013, Hua et al. 2016). It is based on a vacuum technique to deform the flexible growth surface over a cylindrical post machined with truncations that results in a uniaxial strain field. The vacuum is modulated to give cyclic or static strain with a choice of waveforms. This unique bioreactor system enables the application of physiological strains and adjustable mechanical loads to cells in TEM scaffolds thereby creating an *in vivo*-like environment for cell proliferation and growth of cell constructs. TEM systems like bioreactors enable mechanistic molecular outputs to be coupled with morphological and functional measures in a highly controlled environment without the methodological and ethical constraints of repeated biopsy sampling or animal sacrifice (Aguilar-Agon et al. 2019, Tchao et al. 2013, Kook et al. 2008). This is therefore an appropriate model to explore regulatory pathways responsible for skeletal muscle adaptations to various exercise regimens through mechanical and/or electrical stimulation.

1.5.3 In vitro injury models

The regenerative capacity of skeletal muscle is highly efficient, but becomes compromised in certain pathologies like myopathies, traumas, infections and ageing (Owens et al. 2008, Grasman et al. 2015, Rahimov and Kunkel 2013). The prevailing goal of regenerative medicine is to recover functional tissues after injury, mainly focusing on the use of stem cells (Sicari et al. 2013, Zammit et al. 2006, McCullagh and Perlingeiro 2015). To understand the physiological processes regulating tissue homeostasis and regeneration, several injury models have been used (Hardy et al. 2015, Gayraud-Morel et al. 2009). The

most common injury models to characterize skeletal muscle regeneration and underlying cellular cascades include mechanical, chemical and myotoxin injury. As different mechanical destructions evoke different responses of the regenerative process, it is crucial to elucidate standardized injury models to appropriately investigate tissue regeneration in diseased and/or rejuvenating conditions.

Freeze injury induces cryolesions with a nonviable cell zone indicating necrosis of the tissue. The regeneration is initiated by viable part unaffected by the freezing cycle. Unlike other methods, freeze-injury does not affect the whole muscle therefore allowing to observe cell infiltration from live to dead section of the muscle (Le et al. 2016, Yoon et al. 2019). Phospholipase A2 peptide notexin (NTX) and a protein kinase C inhibitor cardiotoxin (CTX) are myotoxins isolated from snake venoms (Chang et al. 1972). NTX inhibits neuromuscular transmission through blocking acetylcholine breakdown thereby causing hypercontraction of the muscle (Dixon et al. 1996, Lee et al. 2022) and CTX destructs cell membranes leading to depolarization of the muscle cell (Guardiola et al. 2017). The scale of the damage and consequently regeneration depends on the dose of the intramuscular injection of the NTX or CTX (Wang et al. 2022, Plant et al. 2006). Barium chloride ($BaCl_2$) is the most used chemical injury method that similarly to myotoxins elicit a degree of muscle damage dependent on the chosen volume and muscle (Brett et al. 2020, Morton et al. 2019). Regardless of the injury methods, muscle regeneration process occurs in response to any damage (Fleming et al. 2019, Juhas et al. 2018, Buono et al. 2012). However, differences in the scale and timing of the events have been observed. For instance, SC count have shown to reach a pre-injury level after a few months in response to freeze and $BaCl_2$ injury but remain decreased following myotoxins provoked damage (Bjornson et al. 2012). In addition, the level of inflammation was observed for months in myotoxin injury while no significant number of macrophages could be detected in the muscle following freezing at same time-point (Hardy et al. 2015, Lee et al. 2013). Kinetics and molecular changes characterizing

skeletal muscle regeneration should be considered when choosing the method and the amount of the injury as these features have shown to differ between models.

1.6 Rationale and novelty of this thesis

The aim of the present Thesis is to develop new high-throughput tools to further gain understanding of mechanisms underlying muscle ageing and to test potential therapies. To achieve that, following objectives were devised:

1. For the first time we use artificial neural network inference analysis (ANN) to identify novel genes linked to age-related changes in human skeletal muscle.

2. We perform RNAseq analysis to generate a transcriptomic profile of aged and young human muscle cells in response to *in vitro* chemical injury.

3. Develop novel bioengineered re-usable inserts to generate TEM within ECM scaffold without supportive molds that resemble native muscle more closely.

Chapter 2: Materials and Methods

2.1 Cell culture

2.1.1 C2C12 cell culture

C2C12 murine myoblasts (LGC Standards/ATCC) were cultured in 6-well plates at the density of 3500 cell/cm^2 and incubated in a humidified 5% CO_2 atmosphere at 37°C in growth medium (GM) composed of high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 1% penicillin-streptomycin solution (Invitrogen) and 10% foetal bovine serum (FBS, Gibco). Myoblasts were cultured to ~90% confluence before GM was replaced with low-serum differentiation medium (DM), composed of low-glucose DMEM plus 2% horse serum and 1% penicillin-streptomycin solution. Horse serum was preferred to FBS due to its lower levels of growth factors, higher rate of immunoglobulins and proteins aiding to inhibit cell growth and induce differentiation (Chelladurai et al 2021). In addition, horse serum is widely used to induce myotube formation (Aguilar-Agon et al 2019, Agrawal et al 2017, Bakooshli et al 2019, Brett et al 2020). Total culture period was 2 days in GM followed by a further 4 days in DM for myotube formation. All experiments were performed at passage below 10 and 3 independent experiments were obtained from each cell line.

2.1.2 Human myoblasts cell culture

Human skeletal muscle cells (aged donor (68yrs, male) purchased from Promocell and young donor (20yrs, male), purchased from Lonza) were seeded at 3500 cell/cm^2 onto 35-mm 6-well plates and grown in GM (low-glucose DMEM with 20% FBS and 1% penicillin-streptomycin). At 90% confluence, GM was replaced with DMEM containing 2% horse serum and 10 µg/mL insulin (Sigma) to induce myogenic differentiation. The total culture period was 3 days in GM followed by a further 6 days in DM. All experiments were

performed at passage 3-5 and 3 independent experiments were obtained from each cell line. Myoblasts were expanded and differentiated following the manufacturer instructions.

2.1.3 Cell passage

At 80-90% confluence, cells were counted and passaged. After GM removal, cells were washed with phosphate buffered saline (PBS) (Fisher-Scientific, UK) and 0.25% Trypsin-EDTA (Gibco) was subsequently used to detach cells from the flask surface. An equal volume of GM was added to Trypsin to inhibit the action of trypsin and the resulting solution was placed into a falcon tube for cell counting.

Cells were counted using a haemocytometer (Neubauer) using the equation: total cells = (total no of cells/no of quadrants) X $1x10^4$ X cell suspension (mL). A volume of 10µL solution was placed into both chamber of haemocytometer and counted under light microscope. Cell count was determined by the number of cells within four quadrants (16 squares/quadrant).

2.1.4 Freezing and cryopreservation of cells

Surplus cells were suspended in freezing media (Recovery Cell culture Freezing Medium, Gibco) at $5x10^5$-$1x10^6$ in cryovials that were placed in Mr Frosty (Fisher-Scientific) and stored at -80ºC for no longer than 3 days before placed in liquid nitrogen for long term cryopreservation.

2.1.5 Resuscitation of cells

Cryopreserved vials were thawed in water bath at 37ºC until complete thawing of the cell solution. Cells were placed in cell culture flasks and grown as previously described (Sections 2.1.1 and 2.1.2).

2.2 Injury model

2.2.1 Experimental design

The experimental design is illustrated in Figure 2.1. The acute effects of $BaCl_2$ exposure on skeletal muscle regeneration were studied using a modified experimental design previously described by Fleming *et al*. (2019) (Fleming et al. 2019). After myotube differentiation and reaching myotube confluency, human and mouse muscle cell lines were injured by exposure to the toxin $BaCl_2$. After injury, cells underwent a proliferation and differentiation period that replicated the pre-injury protocol. Differences in exposure and culture times between cultures are explained in section 2.2.2. All experiments were performed at passage below 10, and 3 independent experiments were performed with each cell line.

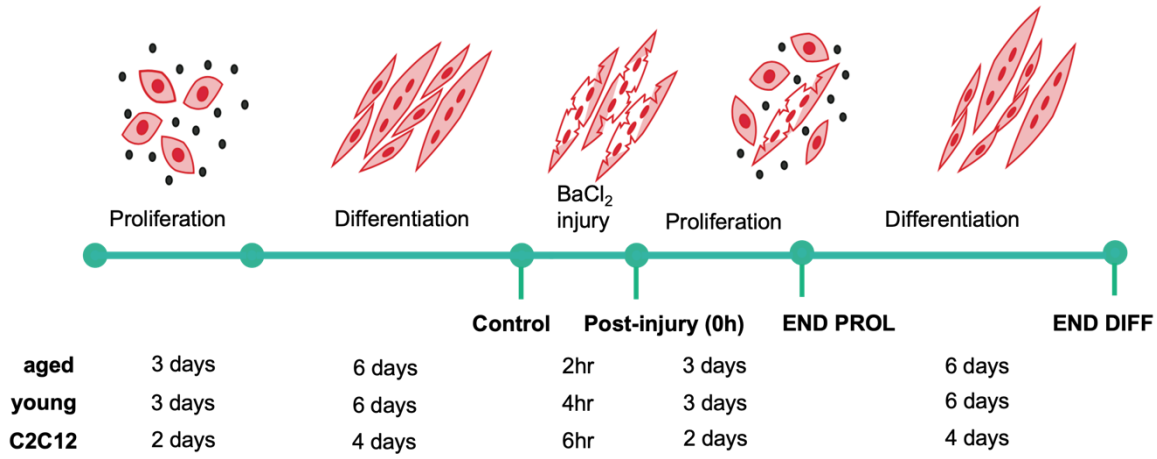

Figure 2.1: Schematic showing experimental design of the in vitro injury and regeneration model.

2.2.2 Cell culture for barium chloride induced injury

Confluent myotubes were differentiated from human and mouse myoblasts as detailed above (sections 2.1.1 and 2.1.2). Next, they were exposed to the toxin $BaCl_2$ to injure the cell's cytoskeleton and trigger the muscle repair process. Myotubes derived from the C2C12 cell line were injured for 6h as detailed in the literature (Fleming et al. 2019). Initially, we also exposed human myotubes for 6 h to $BaCl_2$, but this appeared to cause complete removal of

the nuclei, in addition to the cytoskeleton injury, and an inability to repair the injury (Figure 4.1). This prompted us to identify the optimal injury time, defined as the period of $BaCl_2$ exposure that causes damage to the cytoskeleton while maintaining the number of nuclei constant (Figure 4.2), for each cell line. To identify this optimal period, we stained the cytoskeleton with phalloidin and the nuclei with DAPI and quantified the nuclei number (Figure 4.1 and 4.2). We found that the optimal exposure duration was 6 h for C2C12, and 4 h and 2 h for young and aged human-derived myotubes, respectively.

Fresh DM was added to all cell lines before inducing injury. Next, 50 µL/mL of 12% w/w $BaCl_2$ solution was added to the medium, followed by a 2h, 4h, and 6h incubation for C2C12, young and aged human myotubes, respectively to cause injury. Cultures were subsequently washed with PBS to remove residual $BaCl_2$ containing media. Control (pre-injury) and 0h (directly post-injury) samples were collected at the end of $BaCl_2$ incubation. Injury was followed by a regenerative period that follows the exact protocol used to differentiate cultures. During regeneration, cells for morphological and transcriptome analysis were collected: on day 2 (END PROL) and day 7 (END DIFF) for C2C12, and on day 3 (END PROL) and day 9 (END DIFF) for human cells.

2.2.3 Cell proliferation assay

To assess S-phase entry of skeletal muscle cells exiting quiescence in culture, Click-IT®Plus EdU Imaging Kit (Invitrogen) was used. Stock solutions were prepared as indicated by the manufacturer. 2X working solution of 10 mM EdU was added to prewarmed culture medium (GM or DM, depending on the time-point) and incubated for 2h under optimal growth conditions (humidified incubator 37ºC, 5% CO_2). After incubation, cells were washed 2X with PBS followed by immunostaining protocol.

2.3 Tissue engineering skeletal muscle constructs

The formation of collagen:Matrigel tissue-engineered muscle (TEM) constructs using human skeletal muscle cells (hydrogel composition) was based on previous literature (Somers et al. 2019, Fleming et al. 2019, Madden et al. 2015, Khodabukus et al. 2019) with modifications. TEM was prepared using Flexcell bioreactor using muscle cells from C2C12, and old and young human donors. This is the first time that the Flexcell bioreactor was used to prepare bioengineered tissue construct of human muscle cells. Additionally, novel TEM inserts were developed to provide high-throughput, cost-effective, feasible and re-usable alternative to expensive bioreactors and/or custom-made TEM set-ups for fabricating aligned and functional TEM.

2.3.1 Hydrogel preparation

All plasticware used for making hydrogel was pre-frozen and preparation was performed on ice. Type I Rat Tail Collagen (Collagen) with concentrations ranging from 3.43-4.37 mg/mL was pre-mixed with 10xPBS to achieve a collagen working concentration of 3 mg/mL. Subsequently, 65% v/v freshly prepared collagen solution was mixed with 10% v/v MEM (Gibco) and 20% v/v Matrigel (Corning). After thorough mixing, the hydrogel solution was neutralised by dropwise addition of 1M NaOH (Fisher Bioreagents) until the colour turned pink (pH=7, checked with pH strips). Hydrogel was kept in ice for a maximum of 2h before further use.

2.3.2 Myoblasts/hydrogel mixture

Prior to hydrogel preparation, cells were cultured as described above (section 2.1.1 and 2.1.2). After cell detachment and counting (section 2.1.3), the cell suspension was centrifuged for 5 min at 1000 rpm. After discarding the supernatant, cells were resuspended in the hydrogel solution at the density of 3500 cells/μL. The cell/hydrogel suspension was mixed thoroughly while avoiding the formation of bubbles.

2.3.3 Preparation of Flexcell tissue train culture system

The schematics of the Flexcell bioreactor setup can be seen in figure 2.4. In brief, the linear Trough Loaders were placed beneath the Tissue Train culture plates in a way that the anchor stems are aligned with the long axis of the Trough Loaders. To facilitate uniform and unrestricted conformation of the membrane in the trough, a thin layer of lubricant (e.g., silicone) was applied to the top surface of the Trough Loader prior to the placement of the Tissue Train culture plates on top. Subsequently, Tissue Train culture plates were placed on a BioFlex baseplate with gaskets and connected to the Flexcell-5000 vacuum source. The longer tubes were connected to the rear connection of the FlexLink ports FLEX IN and FLEX OUT (vacuum source) and to the BioFlex baseplate. A steady "hold" mode of vacuum was then applied to the baseplate so that the flexible membrane is deformed and held in place in the Trough Loader. Maximum elongation of 20% was used with the Tissue Train Loading Station (equal to -90 kPa). The baseplate was sterilised (70% methylated spirits/1% bleach) prior placing in the cell culture hood.

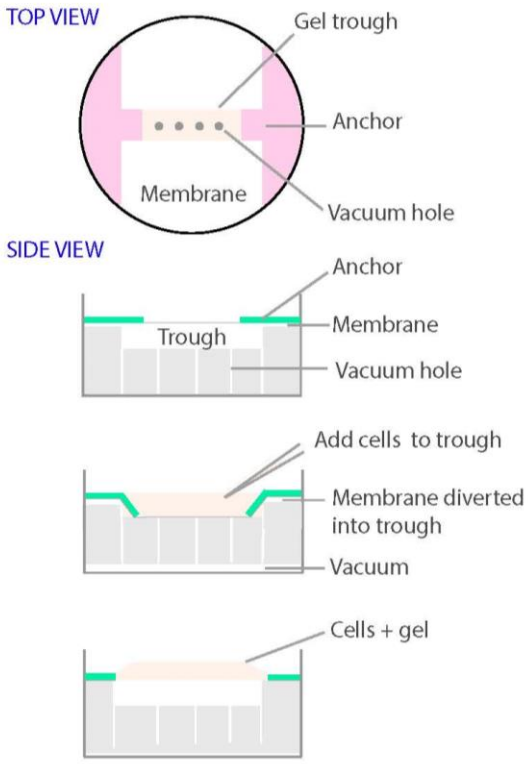

Figure 2.4: Schematics of Flexcell Tissue Train Culture System used to prepare TEM. *Image adapted from Flexcell International Corporation*

2.3.4 Loading the hydrogels in Flexcell Tissue Train culture plates

The cell-hydrogel solution that was kept in ice while setting up the Flexcell culture system was mixed once again thoroughly before pipetting 200µL of the solution into the "trough" in each Tissue Train well. First, a small drop of gel was pipetted under the anchor stems at each end of the trough. The anchors were then pressed and released several times into the trough to ensure they are covered with the gel. Finally, the middle of the trough was filled with gel by moving the pipette back and forth to create a uniform strip of the gel in the well. The baseplate with culture plates was placed in the incubator (37°C, 5% CO_2) for 2h to allow the gel to polymerise. Just before releasing the vacuum, 3mL of GM was carefully added to each well. Culture plates were then removed from the BioFlex baseplate. Mouse C2C12

myoblasts were proliferated in GM (as in section 2.1.1) for 4 days prior switching to DM (as in section 2.1.2) for further 3 weeks to allow myotube formation.

2.3.5 Preparation of TEM insert culture system

TEM inserts were made using AutoCAD 3D printing software. TEM inserts and associated anchors were prepared from biocompatible materials to ensure *in vitro* compatibility. To remove resin residues after printing, inserts were washed for 3x5min on the rocker with 100% IPA (isopropyl alcohol) followed by 5min rinse under running tap water. Anti-bacterial treatment was performed with an autoclave and TEM inserts were kept in sterile conditions until use in the cell culture hood. TEM inserts were placed in the 6-well tissue culture plates for further hydrogel loading.

2.3.6 Preparation of hydrogels in TEM inserts

The cell-hydrogel solution used for TEM was identical to the one prepared for bioreactor-based TEM constructs (sections 2.3.1 and 2.3.2). The gel solution was mixed thoroughly before pipetting 200µL of the solution into the "trough" in each insert as described in section 2.3.4. The inserts within 6-well culture plates were placed in the incubator (37°C, 5% CO_2) for 2h to allow gel to polymerise. Thereafter, 3mL of GM was carefully added to each well. Mouse C2C12 myoblasts were proliferated in GM (as in section 2.1.1) for 4 days prior switching to DM (as in section 2.1.2) for further 3 weeks to allow myotube formation.

2.4 Transcriptome analysis

2.4.1 RNA extraction of monolayer lysates

Myoblasts/myotubes were collected at three time-points across the experiment: before injury (CTRL), end of proliferation (END PROL) and end of differentiation (END DIFF) during the regenerative period; and transferred to low binding/RNase free microcentrifuge tubes containing 350µL Rneasy lysis Buffer (Qiagen) supplemented with ß-Mercaptoethanol (Sigma-Aldrich) to isolate total RNA using Rneasy Mini Kit (Qiagen). Following lysis, samples were homogenised and loaded onto Rneasy silica membrane for RNA binding. Concentrated and pure RNA was eluted in Rnase-Free water. Concentrations were quantified using spectrophotometry (NanoDrop™ 2000; Thermo Fisher Scientific).

2.4.2 RNA sequencing

RNA was sequenced and library made by Novogene Co, Ltd (Cambridge, UK). In brief, RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 370~420 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Then PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was

performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Reference genome and gene model annotation files were downloaded from the genome website. Index of the reference genome was built and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. Feature Counts v1.5.0-p3 was used to count the reads numbers mapped to each gene. Differential expression analysis of two groups was performed using the DESeq2 R package (1.20.0). The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P* value < 0.05 found by DESeq2 were assigned as differentially expressed. Four independent experiments were performed.

2.4.3 Pathway analysis for injury model

Pathway analysis was performed by Novogene Co, Ltd (Cambridge, UK). In brief, GO enrichment analysis was calculated using the GOseq R package. KOBAS software was implemented for enrichment analysis in KEGG pathways. Terms with corrected *P* values of less than 0.05 were considered significantly enriched by differentially expressed genes. The database generated is available in the ArrayExpress repository, E-MTAB-12248, permanent link:https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-12248?key=4c57e37a-23c7- 4c81-8d59-1f8c7e6c9373.

2.4.4 Publicly available datasets for ANNI analysis

Six datasets were selected from the Gene Expression Omnibus data repository. Inclusion criteria were transcriptome profiled by RNA-seq, data from the skeletal muscle of young (21-43yrs) and older healthy males (63-79yrs) on exercise training with a duration of 6 months minimum. All time points of isolated RNA from biopsies taken after 24h of intervention were included, samples taken immediately post exercise and up to 24h were

excluded. Other exclusion criteria included: evidence of coronary heart disease (by history and exercise test); congestive heart failure; hypertension; chronic obstructive pulmonary disease; diabetes mellitus; renal failure; orthopaedic disability precluding exercise training; and smoking. Datasets with less than 10,000 gene transcripts were excluded for accurate assessment and comparison of data sets. In total, 2 datasets of young vs older adults and 2 datasets of long-term resistance exercise in older adults were included in analysis, all deposited and searchable prior to 2020.

2.4.5 Bioinformatics analysis

Enriched gene transcripts were fed into an Artificial Neural Network inference (ANNi) algorithm (Intelligent Omics) to identify the top driver and target genes in ageing and exercise (see Figure 2.5 representing schematics of the interaction algorithm). More specifically, a backpropagation neural network was used to model the potential interaction between genes. The underlying structure of the multilayer perceptron (MLP) is a weighted, directed graph, interconnecting artificial neurons (i.e., nodes) organized in layers with artificial synapses (i.e., links) which carry a value (i.e., weight), transmitting data (i.e., signals) from one node to the other nodes. All incoming signals from the input layer were processed based upon a set of defined parameters (i.e., error computation function, acceleration measure, input weights) by the nodes in the intermediate layer (i.e., hidden layer) and an activation function is applied to the resulting sum. This sum is then used to determine the output result (i.e., predicted value) generated by the nodes in the output layer. Due to the connectionist computation in ANNs, the architecture of the ANN can be easily modified to address different questions and compose complex hypotheses that can explain a high degree of correlation between features without any prior information from the datasets. Hence, a backpropagation MLP was chosen as ANN to model the gene-gene interaction. The principle of the algorithm is to show the relationship between genes from the same pool, to shed light on how these molecules interact with each other and to identify new

relationships between these molecules by iteratively calculating the influence that multiple variables may have upon a single gene. The prediction weights and signal directions were used to model the strengths of the interaction signals and the direction of the interaction link between genes. The ANN model was validated using Monte Carlo cross-validation to minimize the risk of over-fitting and to optimize generalization ability of the model.

The interactome maps of the top 200 driver and target genes previously identified by ANN were generated using Cytoscape and STRING software. Cytoscape is an open-source bioinformatics software used for visualizing the networks of molecular interactions together with any integrated data like gene expression profiles. MetaCore and Metascape platforms were used to detect significantly enriched signalling pathways for top 200 genes. MetaCore is an online tool to perform functional network analysis on any type of multi-omics data. Metascape is another bioinformatics resource based on meta-analysis that provides gene list annotation and analysis to explain new and common pathways and networks. In addition, to examine the presence of the exercise-responsive genes in published studies, selected genes were inserted to MetaMEx online database which is the largest dataset on exercised skeletal muscle transcriptome.

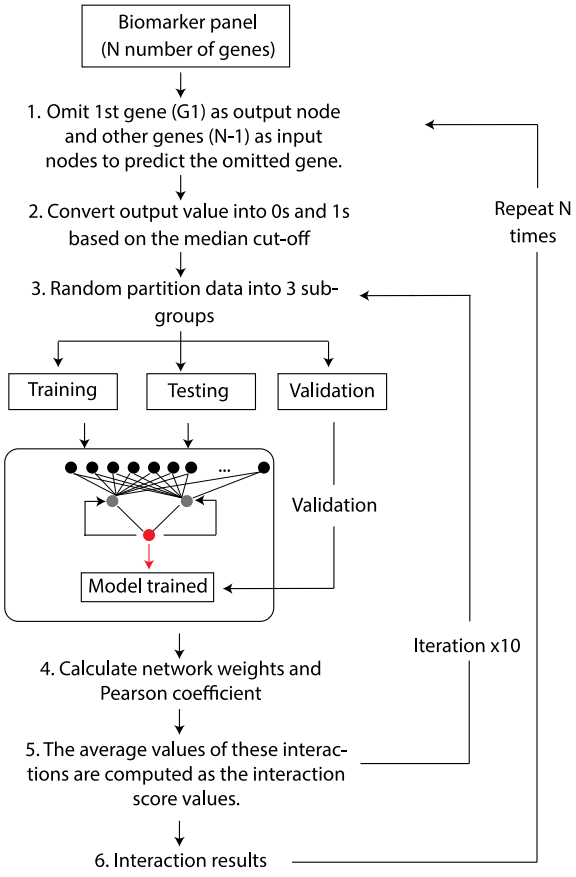

Figure 2.5: Overview of the interaction algorithm
Data mining for ANNi analysis was performed by Professor Graham Ball (Medical Technology Research Centre, Anglia Ruskin University).

2.4.7 Study design and subject characteristics of muscle biopsies used for RT-PCR

Muscle biopsies for the PCR were obtained from 8 young (26±5yrs) and 8 aged (75±9yrs) men. Aged participants completed a 12-week resistance training program with 3 sessions/week with 100% compliance. Biopsies from *m. quadriceps* were obtained before the training program (baseline) and 24h after last training session (for aged). For analysis comparisons were made between young and aged baseline and aged baseline vs aged exercised. Subjects were informed of the potential risks and discomforts prior to signing an informed consent form. The study protocol was approved by the Regional Ethical Review Board and conducted according to the to the Declaration of Helsinki.

2.4.8 RNA isolation, reverse transcription, and real-time PCR

To assess differential expression of the top drivers and target genes, RT-PCR was employed on muscle biopsies from 8 young and 8 aged male donors. One aliquot of ~10mg frozen muscle tissue was homogenized using TRIzol (Invitrogen Life Technologies Carlsbad, CA) and total RNA was extracted. RNA concentration and purity were obtained by UV spectroscopy (Nanodrop 2000, Thermo Scientific). 300ng of total mRNA was subsequently reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit Kit (Biorad) in a total volume of 10µL. Real-time PCR was performed on CFX96 Touch Real-Time PCR Detection System (Biorad). The reaction mix consisted of 2.5µL of the diluted cDNA template, 6.25µL of the SYBR Green PCR Mastermix (Biorad) and 0.5µL gene specific primers. The cycling procedures were 20 sec at 95°C and 1 min at 95°C followed by 40 cycles at 95°C for 20 s and 60°C for 1 min. Primers were purchased from Sigma Aldrich. A complete list of primers used for RT-qPCR, including names and sequences, is provided in Table 2.1

Each individual sample was assayed on the same plate. *GAPDH* (Hs00172113_m1) was used as the housekeeping gene. For further control, b-actin (Hs01375212_g1) was analysed as an additional reference gene. The results were almost identical with *b-actin* or *GAPDH* as housekeeping genes. Hence the *GAPDH/b-actin* ratio did not change across time points. Target gene expression was subsequently reported as a ratio relative to the respective reference genes by the $2^{-\Delta\Delta CT}$ formula.

Table 2.1 List of primers used for RT-qPCR.

Gene Name	Sequence 5'to 3'	NCBI Gene ID
NIPAL3	Fw-TGTTCGTGTGCATGGTGGCAAC Rv-CAACGGTGGTACGTGTGCTTGT	57185
SCFD1	Fw- CAGAGCACAGGAAGATGAGGTC Rv- CTGGAGTAGAAGGACACGAGAC	23256
ZDBF2	Fw- ACAGGATGTACTGCAGCACCAC	57683

	Rv- CACCACGACGTCATGTAGGACA	
USP54	Fw- GAGTTAGAGGCAGCGAAAGGGT Rv- TGGGAAAGCGACGGAGATTGAG	159195
CHAD	Fw-CCTTTGGCAGATACCTGGAGAC Rv- CAGAGGTCCATAGACGGTTTCC	1101
EIF4A2	Fw-CTCTCCTTCGTGGCATCTATGC Rv- CGTATCTACGGTGCTTCCTCTC	1974
JAK2	Fw-CCAGATGGAAACTGTTCGCTCAG Rv- GACTCGCTTGTCAAAGGTAGACC	3717
KDM5D	Fw-GGCTGAGTCTTTTGACACCTGG Rv- GGTCCACAGTTTTCTGAGTCGG	8284

2.5 Immunohistochemical analysis

2.5.1 Monolayer Immunofluorescence Staining

Cells were fixed using 10% formaldehyde and permeabilised with 0.25% Triton X-100 (Sigma Aldrich) solution. The actin cytoskeleton of cells was identified using Alexa Fluor 568 phalloidin conjugate (1:40, Invitrogen) and Prolong Gold Antifade mountant with DAPI (Invitrogen) was used to counterstain myonuclei. To further assess the cell dynamics post-injury, EdU assay (Click-iT Plus EdU Imaging kit, Invitrogen) was applied to cultures marking the proliferative cells. In brief, 30 min incubation with Click-It Plus reaction cocktail consisting of 1X reaction buffer, copper protectant, reaction buffer additive and Alexa Fluor picolyl azide was performed at room temperature in the dark. Cells were then washed with PBS. Myonuclei were visualised by Hoechst staining for 30 min.

All fluorescence staining was performed before injury (CTRL), post-injury (0h), end of proliferation (END PROL) and end of differentiation (END DIFF) time-points during the regenerative period.

2.5.2 TEM immunofluorescence staining

Hydrogels were washed with 2x1mL PBS before fixation with 10% neutral buffered formalin for 10min at room temperature. Samples were permeabilised with 0.25% Triton X-100 in PBS after 2x5min wash with 1mL 0.1% Tween-20 in PBS. Hydrogels were subsequently blocked for 1h at room temperature in blocking buffer (3% BSA and 0.25% Triton X-100 in PBS) with gentle agitation. The actin cytoskeleton of cells was identified using overnight Alexa Fluor 568 phalloidin conjugate (Invitrogen) antibody incubation at 4ºC diluted in blocking buffer. Similarly, anti-α-actinin (Santa-Cruz) primary antibody incubation was performed with gentle agitation in blocking buffer overnight at 4ºC. Samples were washed again with 0.1% Tween-20 in PBS for 3x5 min. Secondary antibody incubation for anti-α-actinin with Goat Anti-Mouse Alexa Fluor 488 was then carried out at room temperature in dark for 1h. Nuclei were identified using a Hoechst dye and TEM were subsequently mounted with mounting medium (Invitrogen). TEM were left to dry overnight at room temperature before being imaged by confocal microscope.

2.5.3. Image collection and analysis

Cells were visualized with a fluorescence microscope (Olympus) and image acquisition was performed using Leica Microscope software. At least 10 fields within each well were captured and approximately 180 myotubes from each condition were included in the analysis. A myogenic fusion index was calculated as the ratio between the number of nuclei within and outside of myotubes in each microscopic field, and myotube width was defined as the mean of three measurements along the length of the myotube. To quantify the percentage of cells synthesizing DNA, the ratio between total EdU+ cells and myonuclei per well was calculated. Morphological analyses were performed using ImageJ Software (NIH, U.S). TEM constructs (both bioreactor- and insert-based) were observed with Leica confocal

camera system at excitation/emission 579/603 nm for F-actin and at 495/519 nm for α-actinin, and at 350/415 nm for nuclei labelling.

2.6 Statistical analysis

The data were generally presented as mean ± the standard error of the mean (SEM). Statistical analysis was conducted using Graph Pad Prism for Mac Version 9.04. *P*-values <0.05 were considered statistically significant and were indicated within figures as * $P<0.05$. Normal distribution of the obtained data on myonuclei number, EdU+ cells, fusion index and myotube diameter was confirmed by the Kolmogorov-Smirnov Test for Normality. The intention to test statistical difference between control and a certain test variable determined the choice of unpaired two-tailed T-test. To test if the mean value of the sample is either significantly greater or significantly smaller compared to mean of the control, two tailed test was chosen. For RNA sequencing, differential expression analysis of two groups was performed using the DESeq2 R package (1.20.0). The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P* value < 0.05 found by DESeq2 were assigned as differentially expressed.

2.7 Data availability

The publicly available data of skeletal muscle transcriptome analysed in chapter 3 can be found at GSE8479, GSE9419, GSE117525.

The datasets generated and/or analyzed in chapter 4 are available in the ArrayExpress repository, E-MTAB-12248, permanent link:

https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-12248?key=4c57e37a-23c7-4c81-8d59-1f8c7e6c9373.

Chapter 3: Using artificial intelligence analysis to identify novel genes linked to age-related changes in human skeletal muscle

3.1 Introduction

Skeletal muscle is the most abundant tissue in the body with the greatest protein reservoir and main glucose uptake and storage site (Argilés et al. 2016, Baskin et al. 2015, Brook et al. 2016). Ageing is accompanied by sarcopenia, defined as a gradual loss in lean skeletal muscle mass, strength and endurance (Cruz-Jentoft & Sayer, 2019, Dennison et al. 2017, Larsson et al. 2019), and is correlated with impaired physical functioning and increased susceptibility to several metabolic disorders (Distefano & Goodpaster, 2018, Landi et al. 2015, Zane et al. 2017) making it a major public health concern (Lang et al. 2010). Although the exact underlying mechanism of muscle wasting is still unknown, a decreased level of physical activity, increased systemic inflammation and elevated oxidative stress have been recognized as important risk factors behind the loss of muscle mass (Chabi et al. 2008, Marzetti et al. 2013, Tchkonia et al. 2010, Tezze et al. 2017). Thus, understanding the molecular mechanisms responsible for the regulation of muscle mass is crucial in the prevention of sarcopenia and associated dependency and frailty among older people (Giresi et al. 2005, Singh & Newman, 2011, Ziaaldini et al. 2017).

Physical exercise is recommended as the most effective nonpharmacological strategy to maintain muscle mass, improve life expectancy and delay the onset of age-associated disorders like osteoporosis, diabetes, atherosclerosis and cardiovascular diseases (Distefano & Goodpaster, 2018, Peterson et al. 2010, Robinson et al. 2017). Long-term strength training has been shown to activate muscle protein synthesis which induces increase in muscle size and strength that reverse sarcopenia among elderly (Hurst et al. 2022, Joanisse et al. 2020, Snijders et al. 2017). Resistance training is known to increase protein synthesis via phosphorylation of *mTOR* through *IGF-1* and *Akt*, which further leads to activation of

p70S6K (Franchi et al. 2014, Mazo et al. 2021, Song et al. 2017). Skeletal muscle atrophy *e.g.*, during ageing occurs via inactivation of Akt that mediates relocation of FOXOs to the nucleus leading to expression of atrogenes ubiquitin E3 ligase *atrogin-1* and *MuRF1*. Activation of TNF-α which regulates downstream transcription factor *NF-κB* that in turn leads to upregulation of *MuRF1* is another catabolic pathway independent of *Akt* (Mammucari et al. 2007, Sanchez et al. 2014). Ageing is associated with increased expression of inflammatory cytokines like TNF-α and IL-6 in skeletal muscle that are stimulating muscle atrophy through ubiquitin-proteasome pathway (Bonaldo & Sandri, 2013, Dalle et al. 2017, Nelke et al. 2019). Although mTOR and inflammatory pathways play an important role in muscle ageing and adaptation to exercise, other genes and pathways are likely to be involved given the complexity and scale of such events. An example is apelin, a small peptide hormone recently identified as key molecular promotor of muscle regeneration and repair that decreases in expression during muscle ageing. Of note, the biotechnology company BioAge is testing BGE-105, an oral agonist of the apelin receptor APJ, in older adults. Clinical work conducted by BioAge has revealed positive effects of BGE-105 such as reduced symptoms of frailty and decreased muscle loss (Re Cecconi et al. 2022, Vinel et al. 2018).

Deep learning (DL) as a subdiscipline of artificial intelligence (AI) is a rapidly evolving field emerging across the healthcare sector with promising integration to develop tools for diagnosis, prognosis and treatment management (Tran et al. 2021). DL functions similar to the human brain by using multi-layered neural network algorithms to make predictions that enable it to solve complex problems thanks to an exponential increase of the data within models (Lecun et al. 2015). Artificial neural network (ANN) models, a type of DL technique, have discovered new genes with a role in the regulation of sarcomas (Tong et al. 2014); are used to develop more precise classification model to predict hypertension (López-Martínez

et al. 2020); to predict responsiveness of hepatitis C treatment (Lin et al. 2006) and clinical scores for the assessment of Alzheimer's disease severity (Bhagwat et al. 2019).

Investigations of the ageing skeletal muscle have provided important insights into the differentially expressed genes involved in mitochondrial protein synthesis, energy metabolism, cytokine signalling, cellular senescence, apoptosis and myognesis (Robinson et al. 2017, Tumasian et al. 2021, Welle et al. 2003). However, the vast majority of the transcriptome studies on ageing and exercise employ conventional bioinformatics methods (*e.g.,* linear and multiple regression analysis, R and Python programming) to detect differentially regulated genes (Kulkarni et al. 2020, Mahmassani et al. 2019, Robinson et al. 2017, Tumasian et al. 2021) and do not provide further information on the nature, status and chemical changes of the selected genes. As such, identifying the biomarkers of ageing using ANN has the potential to improve the understanding of human muscle ageing by clinical evaluation of possible rejuvenating interventions without undertaking costly longitudinal studies (Mamoshina et al. 2018). Biomarker is an indicator of biological, pharmacological and/or pathogenic processes in response to intervention assessed for example as DNA or RNA characteristic (Strimbu and Tavel 2010, Mamoshina et al 2018, Tran et al 2021). Therefore, the aim of this study is to use the ANN model to predict for the first time the most influential (drivers) and influenced (target) genes associated with ageing muscle and adaptions to exercise interventions. Using ANN to identify novel muscle ageing-related biomarkers allows us to identify new targets for future research to develop more feasible and presumably cost-effective lifestyle interventions capable of sustaining muscle health across the lifespan and prevent age-related muscle diseases like sarcopenia.

3.2 Results

3.2.1 ANNI analysis identified *USP54, JAK2, CHAD* and *ZDBF2* as the top predictor genes for ageing skeletal muscle

Network inference analysis of differentially expressed transcripts, pathway analysis and Gene Ontology (GO) were performed to characterize the pathways affected by ageing or training in human skeletal muscle (grouping with the pooled data following exercise training in older adults and in aged vs young adults). A subset of publicly available datasets on transcriptomic changes during ageing in muscle and in response to chronic exercise among older subjects was chosen to model the relationships between the genes from the pool using ANNI. The most influenced (drivers) and influential (targets) genes with centralization and weighted interactions are shown in the interactomes created by Cytoscape software. We identified ubiquitin specific peptidase 54 (*USP54*), janus kinase 2 (*JAK2*), follistatin (*FST*) and src kinase associated phosphoprotein 2 (*SKAP2*) as main drivers in older adults whereas the main target genes were chondroadherin (*CHAD*), zinc finger DBF-type containing 2 (*ZDBF2*), cyclin dependent kinase inhibitor 1A (*CDKN1A*) and rho GTPase activating protein 11B (*ARHGAP11B*) (Figure 3.1 and 3.2). Metacore software was used to perform pathway analysis on the top 200 genes with strongest interactions in the ANNI analysis. TRAIL-activated apoptotic signaling pathway and pathways involved in sucrose processing were the main GO processes in ageing muscle (Figure 3.3a and 3.4a). For the networks, p53 signaling and androgen receptor signaling were identified as the most significant ones (Figure 3.3b and 3.4b), while *BMP-GDF* and IL5 signaling via *JAK/STAT* were found as the main pathway maps (Figure 3.3c and 3.4c). Interestingly, extrinsic apoptotic signaling pathway was found among top10 GO processes in both age-related datasets (Figure 3.3b and 3.4b).

Online resource Metascape was then used (Zhou et al. 2019) to create common and unique pathway and network maps based on the given list of genes. Top level enriched biological

processes with the strongest interactions in the ANNI analysis clustered into growth, signaling, metabolic and cellular processes (Figure 3.5a and 3.6a). Regulation of growth, apoptotic signaling pathways and mitochondrion organization were found as the most enriched network clusters in ageing predicting datasets (Figure 3.5b and 3.6b).

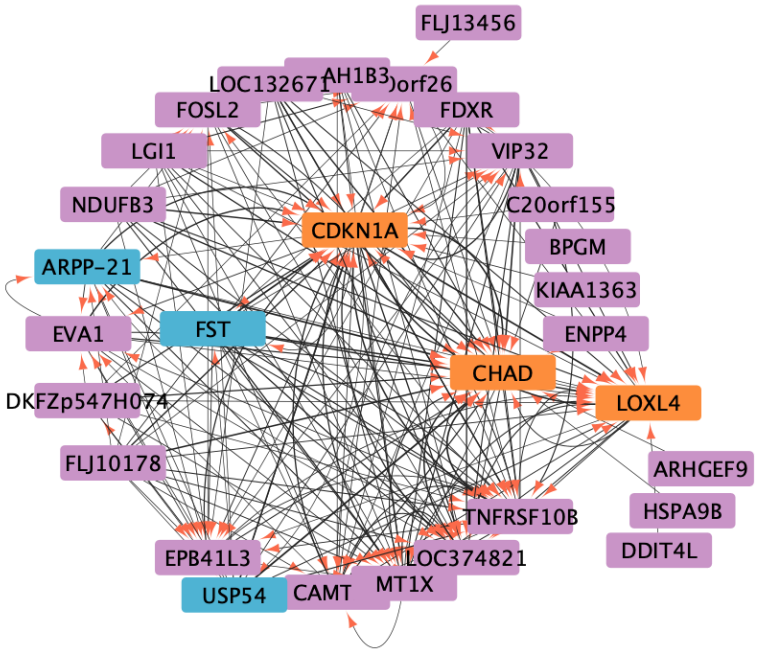

Figure 3.1: The interactome map based on GEO dataset GSE8479 on muscle ageing. *USP54 and FST are displayed as the main drivers (i.e., influencing genes in blue); and CHAD and CDKN1A main targets (i.e., the influenced genes in orange) in aged muscle. Thickness represents the interaction strength and arrows the directionality.*

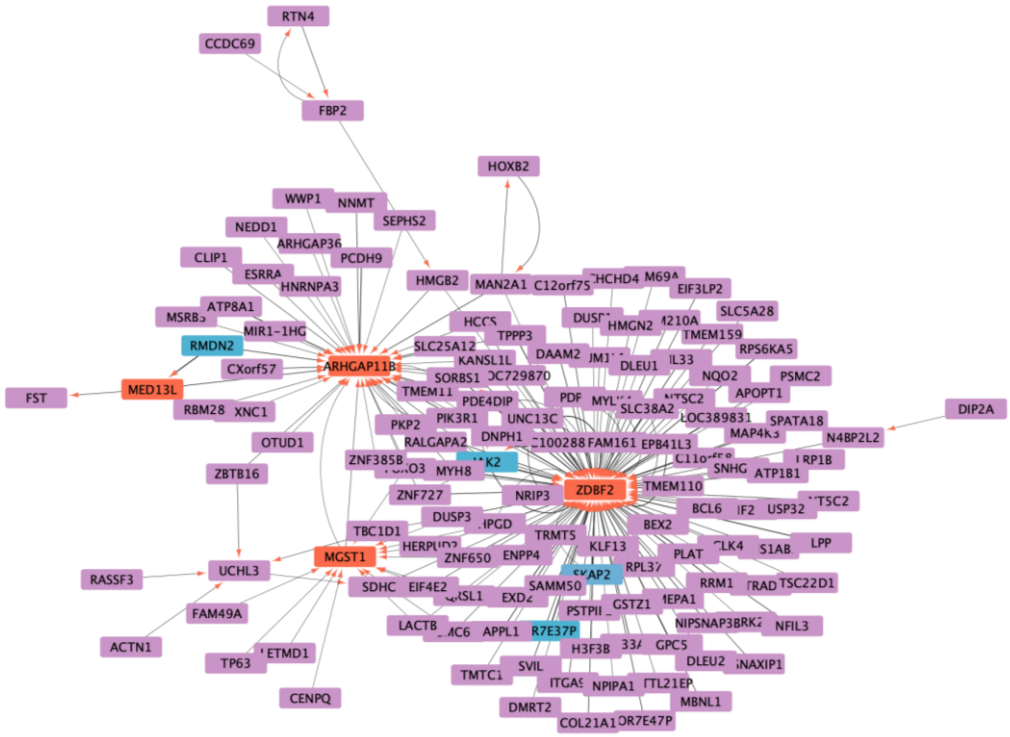

Figure 3.2: The interactome map based on GEA dataset GSE9419 on muscle ageing. *JAK2 and SKAP2 were the main drivers (blue); and ZDBF2 and ARHGAP11B as top targets (orange) in aged muscle. Thickness represents the interaction strength and arrows the directionality.*

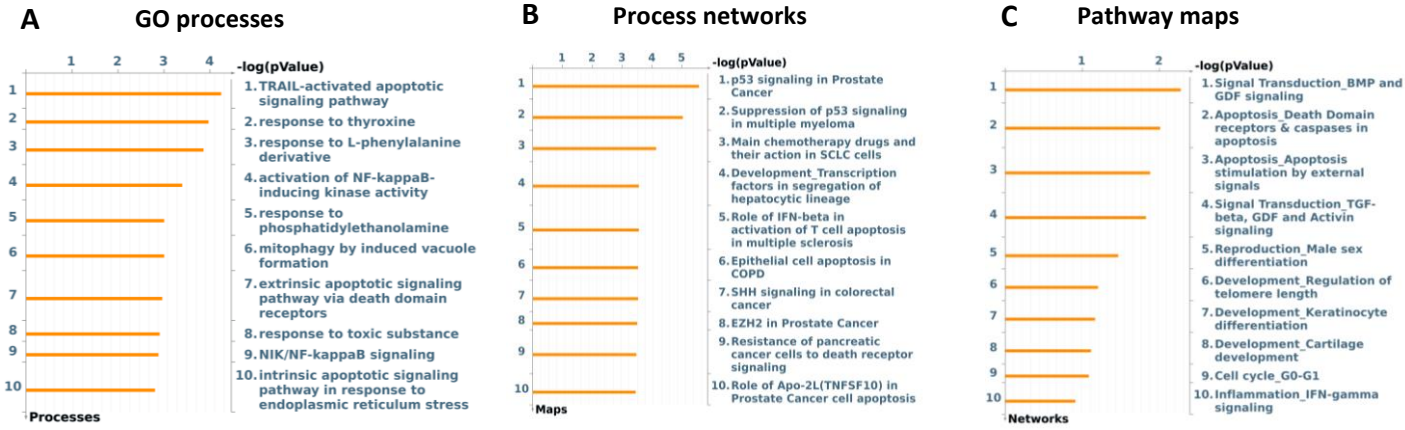

Figure 3.3: Charts depicting top ten overrepresented Gene Ontology cellular processes (a), process networks (b) and pathway maps (c) in aged vs young adults based on GEO dataset GSE8479.

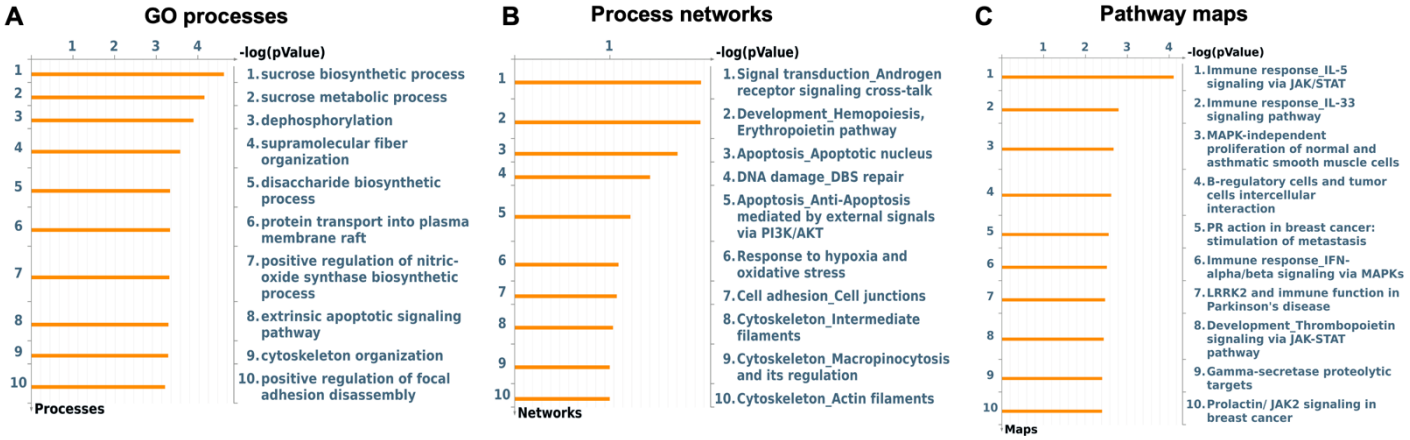

Figure 3.4: Charts depicting top ten overrepresented Gene Ontology cellular processes (a), process networks (b) and pathway maps (c) in aged vs young adults based on GEO dataset GSE9419.

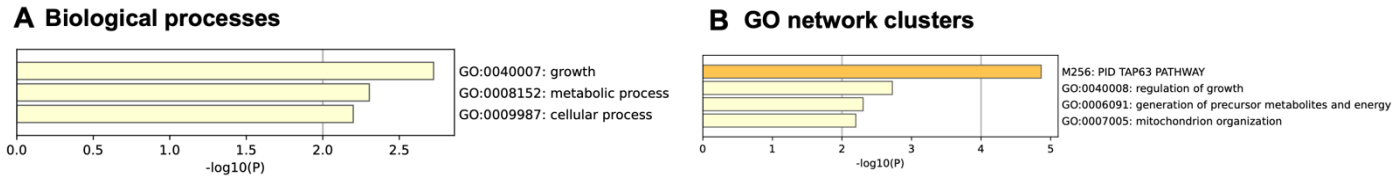

Figure 3.5: Bar graphs indicating most significantly enriched biological processes (a) and networks clusters of GO terms (b) in GEO dataset GSE8479.
Metascape was used to create bar graphs indicating age responsive genes with the strongest interactions as a result of ANNI analysis.

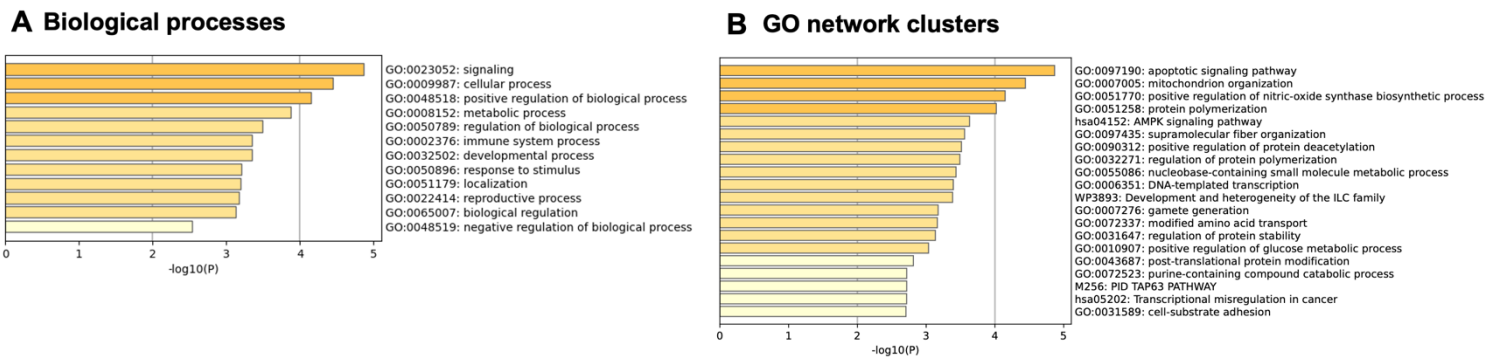

Figure 3.6: Bar graphs indicating most significantly enriched biological processes (a) and networks clusters of GO terms (b) in GEO dataset GSE9419.
Metascape was used to create bar graphs indicating age responsive genes with the strongest interactions as a result of ANNI analysis.

3.2.2 EIF4A2, NIPAL3, SCFD1 and KDM5D are the genes with strongest interactions in response to exercise

The top drivers among exercise-responsive genes in the context of skeletal muscle ageing were eukaryotic translation initiation factor 4A2 (*EIF4A2*), calcium binding protein (*MO25*), NIPA like domain containing 3 (*NIPAL3*) and forkhead box protein P2 (*FOXP2*) while sec1 family domain containing 1 (*SCFD1*), serine/arginine rich splicing factor 10 (*FUSIP1*), lysine demethylase 5D (*KDM5D*) and ubiquitin specific peptidase 9 Y-linked (*USP9Y*) were identified as the top target genes in the respective interactome maps in Figure 3.7 and 3.8. According to Metacore pathway analysis, cellular and primary metabolic processes and the apoptotic process of luteolysis were the main GO processes (Figure 3.9a and 3.10a) while protein folding and proteolysis were identified as the top process networks (Figure 3.9b and 3.10b). With regards to pathway maps, Th2-cytokine-induced mucous metaplasia in asthma and ECM remodelling prevailed among training studies (Figure 3.9c and 3.10c).

Interestingly, network processes of actin filaments within the cytoskeleton were present in the top for both aged and exercised older skeletal muscle (Figure 3.9b and 3.10b).

Exercise-responsive genes with the strongest interaction (Top200) that were fed into the Metascape database showed metabolic and developmental processes, and biological regulation as the most enriched GOs (Figure 3.11a and 3.12a). Translation initiation, histone lysine demethylation and protein localization within chromosome were the top clusters with most significantly enriched GO networks (Figure 3.11b and 3.12b).

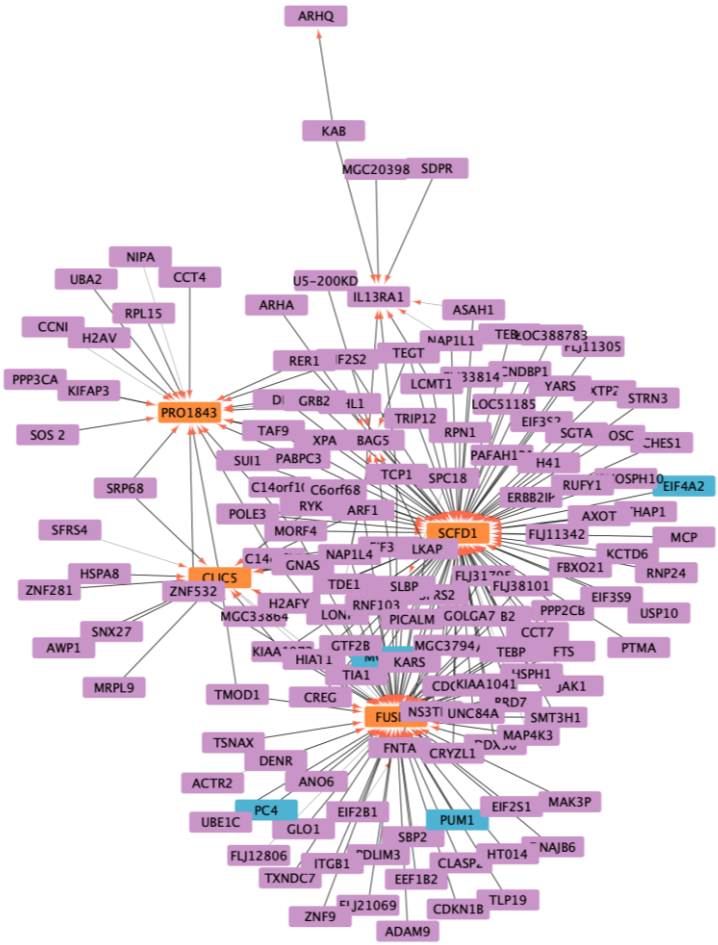

Figure 3.7: The interactome map based on GEO dataset GSE8479 on exercised aged muscle. *EIF4A2 and MO25 were detected as the main drivers (blue); and SCFD1 and FUSIP1 as targets (orange) in exercised aged muscle. Thickness represents the interaction strength and arrows the directionality.*

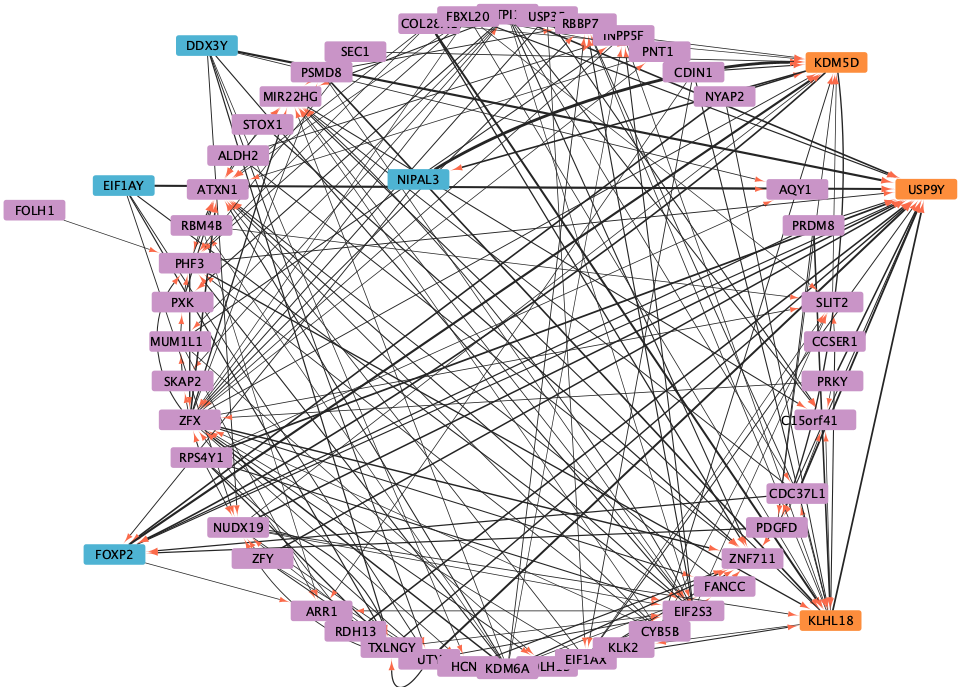

Figure 3.8: The interactome map based on GEO dataset GSE117525 on exercised aged muscle. *NIPAL3 and FOXP2 were the main drivers (blue); and KDM5D and USP9Y as top targets (orange) in exercised aged muscle. Thickness represents the interaction strength and arrows the directionality.*

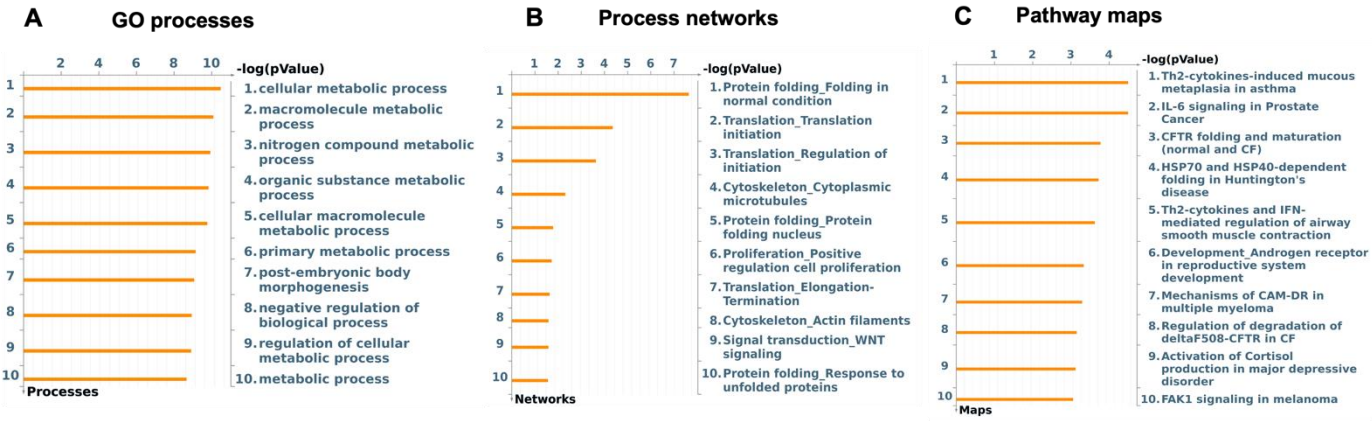

Figure 3.9: Charts depicting top ten overrepresented GO cellular processes (a), process networks (b) and pathway maps (c) in response to exercise in older adults based on GEO dataset GSE8479

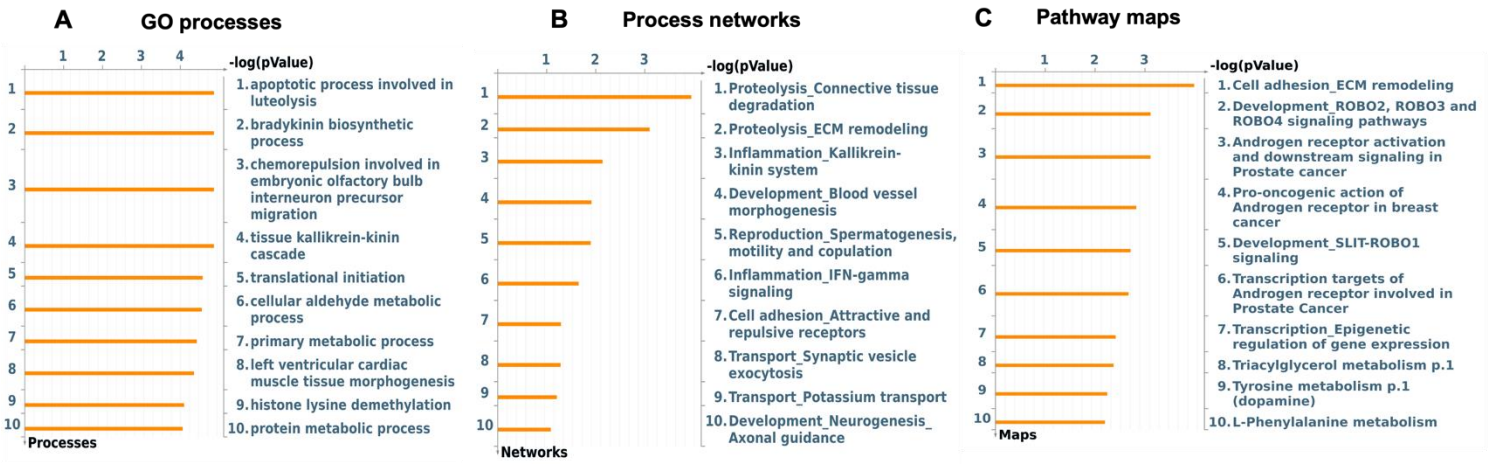

Figure 3.10: Charts depicting top ten overrepresented GO cellular processes (a), process networks (b) and pathway maps (c) in response to exercise in older adults based on GEO dataset GSE117525.

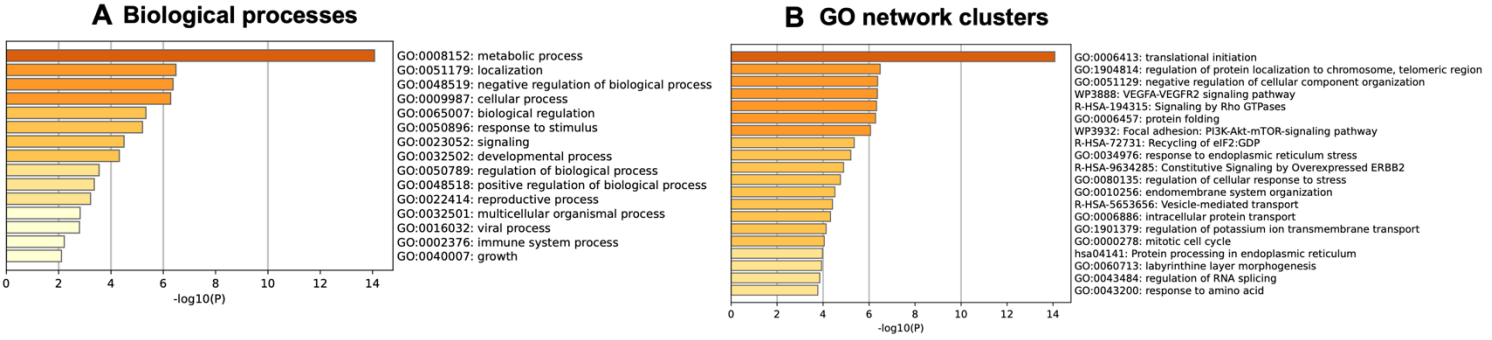

Figure 3.11: Bar graphs indicating most significantly enriched biological processes (a) and networks clusters of GO terms (b) in exercised muscle in GEO dataset GSE8479*. Metascape was used to create bar graphs indicating exercise responsive genes with the strongest interactions as a result of ANNI analysis.*

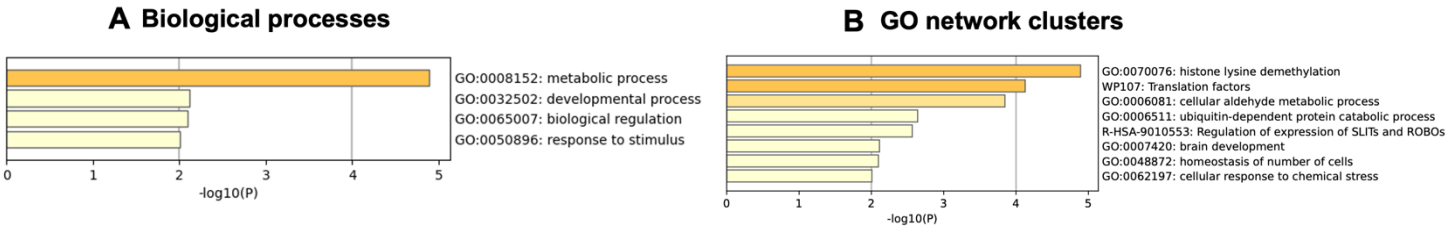

Figure 3.12: Bar graphs indicating most significantly enriched biological processes (a) and networks clusters of GO terms (b) in exercised muscle in GEO dataset GSE117525. *Metascape was used to create bar graphs indicating exercise responsive genes with the strongest interactions as a result of ANNI analysis.*

3.2.3 RT-PCR confirmed significant differential expression of age-related driver and target genes predicted by ANNI

Differential expression of the top genes assessed by real time PCR showed significant upregulation of *USP54, JAK2* and *ZDBF2* in ageing muscle (Figure 3.13) while exercise related driver and target genes were not differentially expressed (Figure 3.14). MetaMEx is an integrated skeletal muscle transcriptome database that uses gene ontology and pathway analysis to reveal exercise-responsive pathways (Pillon et al. 2020). The MetaMEx database provides an extensive dataset on the transcriptomic response of skeletal muscle to inactivity as well as acute and chronic exercise training. When inserting the top 4 genes with the strongest interactions in exercised muscle into the MetaMEx database, we observed that *EIF4A2, NIPAL3, SCFD1* and *KDM5D* genes are equally elevated in chronic resistance and aerobic training, but when the search was adjusted for healthy elderly subjects and filtered by 24-week training duration, more studies were detected to be associated with resistance than aerobic exercise training (Figure 3.15a-d). This suggests that despite non-differential expression in RT-PCR, the main target and driver genes found in ANNI analysis are exercise-activated in aged skeletal muscle discovered by MetaMEx meta-analysis. Moreover, there were differences in training program duration among datasets: muscle biopsies for PCR were collected from participants after 3 months strength training while

publicly available transcriptome data for ANNI came from older adults undergoing a 6-month exercise regimen. This could possibly explain non-differential expression of top predictor genes found in ANNi analysis when assessed by RT-PCR.

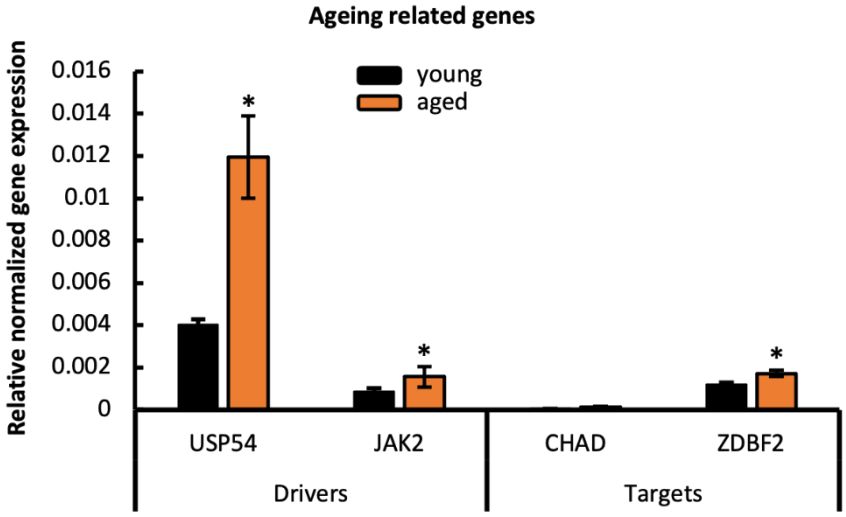

Figure 3.13: Differential expression of main age-related target and driver genes.
*Most influenced (CHAD and ZDBF2) and influencing (USP54 and JAK2) genes were confirmed by RT- PCR of young and aged muscle biopsies. *P<0.05.*

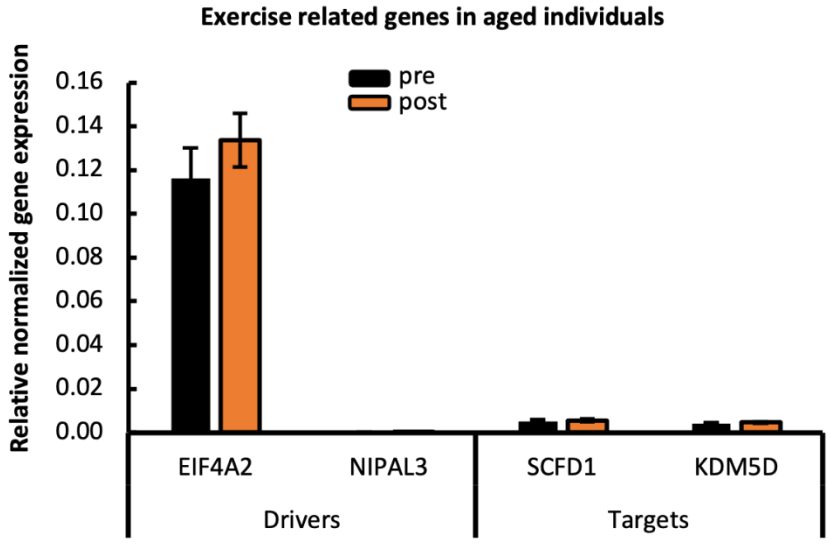

Figure 3.14: Differential expression of exercise-related target and driver genes.
*RT-PCR revealed that most influenced (SCFD1 and KDM5D) and influencing (EIF4A2 and NIPAL3) genes in older adults remained unchanged. *P<0.05.*

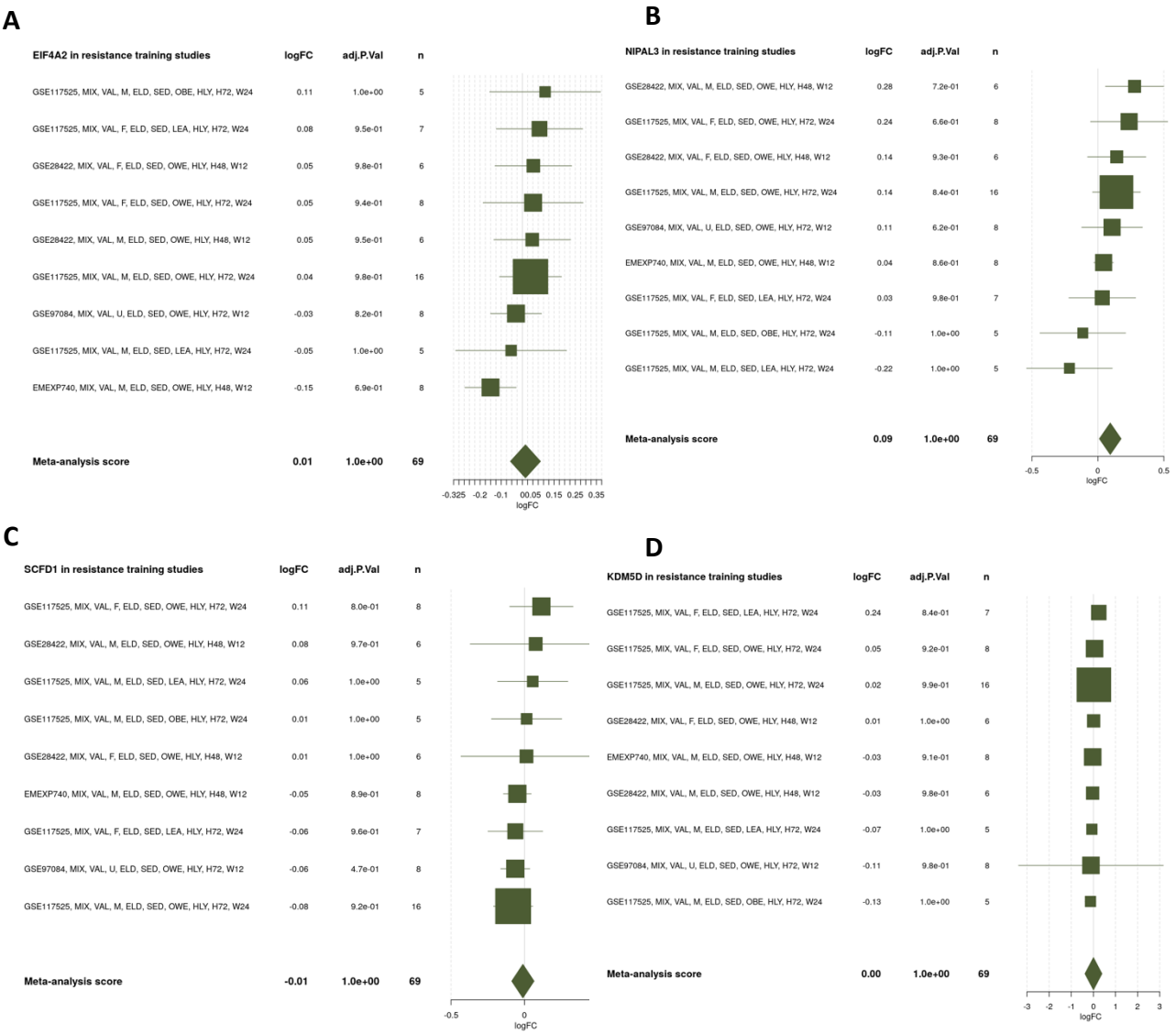

Figure 3.15: Forest-plots of exercise-related genes reported in different studies. *Online database MetaMEx provides list of published studies on skeletal muscle response to exercise for a single gene with forest plots of individual statistics (fold-change, FDR, 95% confidence interval) and meta-analysis score (a-d).*

3.3 Discussion

Age-related changes in gene expression may contribute to the loss of skeletal muscle mass during ageing (Han et al. 2021, Kang et al. 2022, Tumasian et al. 2021, Zeng et al. 2020) and may also play a role in the response to exercise training (Bolotta et al. 2020, Chapman et al. 2020, Rubenstein et al. 2020). Nevertheless, research to identify genes associated with skeletal muscle atrophy during ageing continues (Khanal et al. 2021) and recent advances in the field of AI provide new avenues to assess the role of certain genes in growth in response

to training and wasting of skeletal muscle during ageing at transcriptome level. Indeed, ANN has allowed to discover novel biomarkers of cancer (Tong et al. 2014, Tran et al. 2021) and is used in clinical practice to predict diseases like Alzheimer (Bhagwat et al. 2019). In this study we applied ANN to discover novel genes related with ageing and resistance training that could be used as biomarkers for sarcopenia or targets for interventions, respectively.

Notably, ANNI revealed age-related gene sets quite distinct from what was previously identified with traditional methods, that were confirmed by RT-qPCR. We believe that such findings significantly add to the area of muscle ageing and sarcopenia research. For instance, *CHAD, ZDBF2, USP54* and *JAK2* were identified as genes with strongest interactions and predicting ageing. CHAD is a member of leucine rich proteoglycan gene family, that is expressed in cartilage, as well as in bone, tendon, and human skeletal muscle (Tasheva et al. 2004). While *CHAD* deficient mice show altered cartilage and bone structural and functional development (Hessle et al. 2013), downregulation of *CHAD* has been observed in exercised muscle transcripts (Hjorth et al. 2015) indicating its involvement in skeletal muscle homeostasis as extracellular matrix component. The gene expression of *CHAD* in both aged and young skeletal muscle biopsies were almost undetectable in our RT-qPCR analysis which could indicate the importance of its low abundance in muscle regulation. *ZDBF2* gene is known to be involved in DNA methylation during embryo development and genomic imprinting (Gaudet et al. 2011) with favoured expression in the brain (Glaser et al. 2022). Identifying *ZDBF2* as one of top target genes by ANNI analysis is very likely the first time as no previous studies associate *ZDBF2* with skeletal muscle and/or ageing. Furthermore, significant upregulation in older adults seen in our analysis could indicate the *ZDBF2* involvement in epigenetic changes of ageing skeletal muscle. Owing to its involvement in the ubiquitin-proteasome system (*UPS*), *USP54* has known function in several tissue regulations such as tumour (Wang et al. 2021), but also in skeletal muscle (Kitajima et al. 2020) via ubiquitin-proteasome dependent proteolysis, which is a major protein degradation system with a crucial role in skeletal muscle homeostasis (Bilodeau et al. 2016) and therefore

muscle ageing. Significant upregulation of *USP54* gene expression in older vs young adults identified in RT-PCR analysis further highlights the role in ageing muscle mass regulation. Another age-related gene with the strongest interactions in the network found by ANN was *JAK2*, which similarly to *USP54* showed elevated gene expression in older adults. *JAK2* mediates several signalling actions in cell growth, development and differentiation and is also associated with cytokine receptors (Jäkel et al. 2011, Shahi et al. 2022). Expectedly, being multifunctional in various processes, high levels of *JAK2* mRNA have previously been reported in aged skeletal muscle (Saltzman et al. 1998, Zanders et al. 2022). The most enriched pathways and network maps we found based on ANN top genes were related to growth, apoptotic signalling and mitochondrial organization. So, although we identified new gene related with muscle ageing, the pathways in which these genes are involved were similar to previous transcriptome studies addressing muscle ageing (Giresi et al. 2005, Mahmassani et al. 2019, Zeng et al. 2020).

Similar to ageing, ANNI predicted exercise-related gene sets quite distinct from previously identified. Our analysis showed that *SCFD1, KDM5D, EIF4A2* and *NIPAL3* were the main genes interacting within the network in response to long-term exercise in older adults. The original studies of the datasets used for ANNI discovered the most prevalent differences in transcriptome profiles were related to mitochondrial regulation, extracellular matrix, glucose metabolism and vascularization in response to chronic resistance training in older adults (Hangelbroek et al. 2016, Melov et al. 2007). ANN findings provide new insight by highlighting the role of transport, binding and anti-cancerous related genes. Namely, *NIPAL3* is an integral component of the cell membrane and is predicted to be involved in magnesium ion transport (Grzmil et al. 2009) while *EIF4F* has a crucial role in muscle cell differentiation via the *PI3K/mTOR* signalling pathway (Galicia-Vázquez et al. 2014). While the expression of *EIF4A2* was not elevated in older skeletal muscle after exercise in our PCR analysis, its importance in muscle adaptation to resistance exercise is well known (Bodine et al. 2001). Furthermore, the *SCFD1* gene has been implicated in the pathogenesis of

amyotrophic lateral sclerosis (ALS) (Chen et al. 2018, Van Rheenen et al. 2016) while downregulation of histone *KDM5D* has been associated with poor prognosis of several tumours (Komura et al. 2018, Shen et al. 2019). Although, we could not detect differential expression of *KDM5D, SCFD1* and *NIPAL3* in exercised aged muscle, MetaMex software identified transcriptome data on these genes in long-term resistance training studies on older adults. Such a discrepancy could possibly be explained by the shorter training protocol used for our sample donors (3 vs 6 months) that might not have been sufficient for mentioned genes to be significantly upregulated from baseline. Interestingly, similar to original training studies that report differential expression of collagen and signal transduction related genes, we found ECM remodelling, proteolysis and protein folding as the most enriched pathways. Furthermore, dominance of the metabolic and developmental processes confirm the exercise-induced changes in the expression of related transcripts (Bolotta et al. 2020, Chapman et al. 2020).

3.4 Conclusion

Using ANN, in this chapter we identify genes related with ageing and exercise that have remained undiscovered until now. This impacts our understanding of muscle ageing and exercise but also suggests new targets for sarcopenia drug development. Our findings also confirmed the known impact of exercise in the cell cycle, apoptosis and cancer-related pathways. In addition, as the current data is based on males only, further experiments ought to be carried out for female participants to ascertain whether the findings are not gender neutral.

Chapter 4: Modelling skeletal muscle ageing and repair *in vitro*

4.1 Introduction

Skeletal muscle regeneration is a complex and finely regulated biological process that shares molecular and cellular aspects with embryonic development (Chargé & Rudnicki, 2004, Musarò, 2014, Oprescu et al. 2020). This homeostatic process is made possible by the coordinated cooperation between different cell populations, such as satellite cells (mitotically quiescent stem cell population residing between the sarcolemma and basal lamina), fibroblasts, inflammatory cells and to the optimal myofiber microenvironment, all decisive to the adequate regeneration of muscle tissue (Mackey et al. 2017, Mouly et al. 2005, Scala et al. 2021, Ten Broek et al. 2010). The role of satellite cells in muscle regeneration has been extensively studied – upon injury, they exit their quiescent state and initiate proliferation. This is mediated by a rapid activation of the Notch signalling pathway, upregulation of cyclin D1 and suppression of the *TGF-β-Smad3* signalling pathway (Brett et al. 2020). After generating enough progeny, the cells exit the proliferative state to initiate differentiation where they fuse into the injury site (Sousa-Victor et al. 2022).

Although muscle exhibits a robust regeneration capacity, this becomes impaired with ageing contributing to the decline of musculoskeletal health, which, in turn, is a contributory factor in the development of sarcopenia (Brzeszczyńska et al. 2018). This impairment is partially due to the limited proliferative capacity of satellite cells on account of Notch and Cyclin D1 downregulation and a suboptimal myofiber niche created by chronic inflammation - a hallmark of ageing muscle (Conboy et al. 2005, Farup et al. 2015, Fry et al. 2011).

It is vital to understand the muscle regenerative response in the context of ageing, since it has been suggested that an accumulation of contraction-induced microdamage that is not adequately repaired may be one of the causes of muscle wasting, sarcopenia and anabolic resistance typically observed in older people (Faulkner et al. 1995). Despite this, however,

no in vitro human model exists to enable such investigations. Therefore, this study aimed to develop an in vitro human muscle regeneration model and characterise the changes in morphology, cell cycle and mRNA transcription during recovery from damage via this process.

To generate the repair model, we will derive myotubes from myoblasts isolated from young and older individuals and provoke an injury using a muscle toxin. Muscle toxin barium chloride ($BaCl_2$) is commonly used to induce injury in studies examining muscle repair and regeneration models (Sicherer et al. 2020). $BaCl_2$ destroys the cytoskeleton without abrogating the cell's regeneration capacity (Hardy et al. 2016, Jung et al. 2019, Morton et al. 2019). Importantly, a $BaCl_2$-induced injury will enable to further understand myotube regeneration and profile nuclear messaging occurring during this process.

The proposed muscle regeneration model could be easily transformed into a reproducible high-throughput application. If successful, it will have significant application in studies investigating muscle regeneration and ageing, sarcopenia, metabolic and genetic diseases, as well as for initial drug discoveries.

4.2 Results

4.2.1 Human muscle cells are more sensitive to barium chloride-induced injury than mouse muscle cells

Previous studies reported the development of injury models using 12% $BaCl_2$ for 6 h in myotubes derived from the C2C12 mouse cell line. Since it is well established (Fleming et al. 2019, Juhas et al. 2018, Morton et al. 2019), we decided to utilise the C2C12 cell line in our experiments for comparative purposes. We were able to confirm that 12% $BaCl_2$ for 6 h adequately removed the cytoskeleton without affecting the number of nuclei in the myotubes (see Figure 4.1 and 4.2). However, when we incubated human myotubes under the same conditions, we discovered that such exposure time was causing excessive destruction of the cell structure and cell death (see Figure 4.1). Reducing the exposure time, we identified the

optimal incubation period – 2 h for myotubes derived from old and 4 h for cells derived from young myoblasts. These exposure times were deemed optimal as they caused cytoskeletal removal without significantly changing the total myonuclei number (see Figure 4.2).

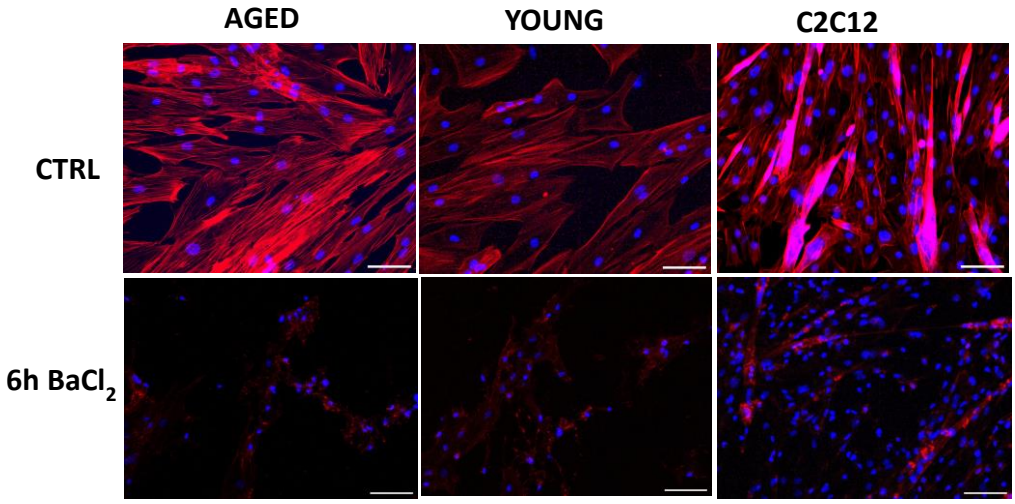

Figure 4.1: Immunofluorescence images showing F-actin and nuclei labelling with phalloidin and DAPI of C2C12, young and aged human donors incubated 6h with $BaCl_2$. *Scale bars represent 100µm.*

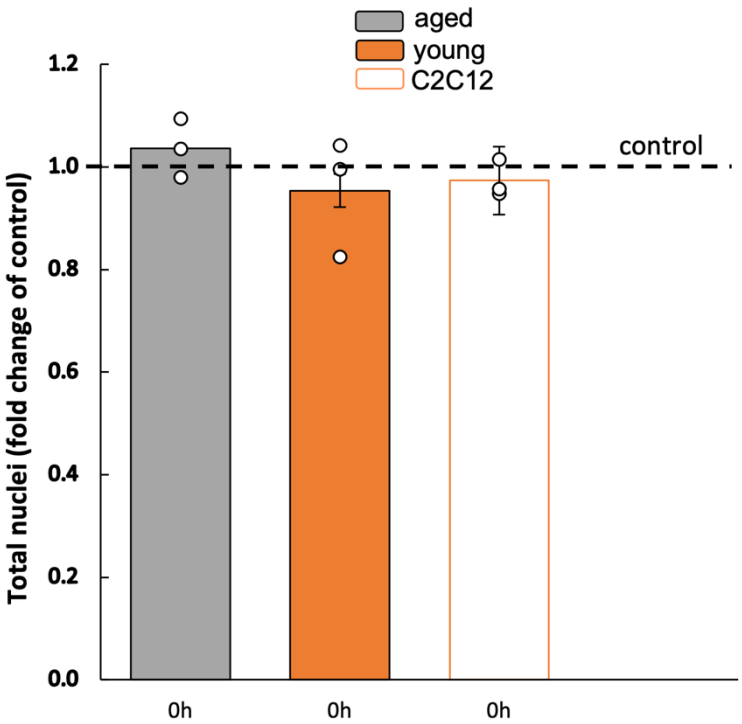

Figure 4.2: Number of total nuclei in C2C12, young- and aged- derived human muscle cells before and after injury.
No significant difference in total nuclei number before (CTRL) and after 2, 4 or 6h injury (0hr) in all cell lines show the ability of $BaCl_2$ to retain mononuclear cell population. n=3, all error bars represent standard error of the mean (SEM).

4.2.2 Young and aged muscle cells show a similar number of EdU+ cells during the proliferation phase

The proliferation activity of muscle cells was assessed by EdU (5-ethynyl-2'-deoxyuridine) incorporation, which detects the cells entering the S-phase (see Figure 4.3). The number of EdU+ cells increased significantly during the proliferation phase in muscle cells derived from both young and aged donors, referred to henceforth as young and aged muscle cells (Figure 4.5). By the end of the regenerative phase, the number of EdU+ cells declined in young and aged cells.

Total myonuclei number increased in human cells during proliferation and remained significantly elevated in young cells by the end of the differentiation period in relation to pre-injury (Figure 4.5).

Mouse myotubes derived from C2C12 showed significant increase in EdU+ cells during the proliferation phase (Figure 4.4) from baseline, and a significant increase in myonuclei number during proliferation and after the differentiation phase (Figure 4.5).

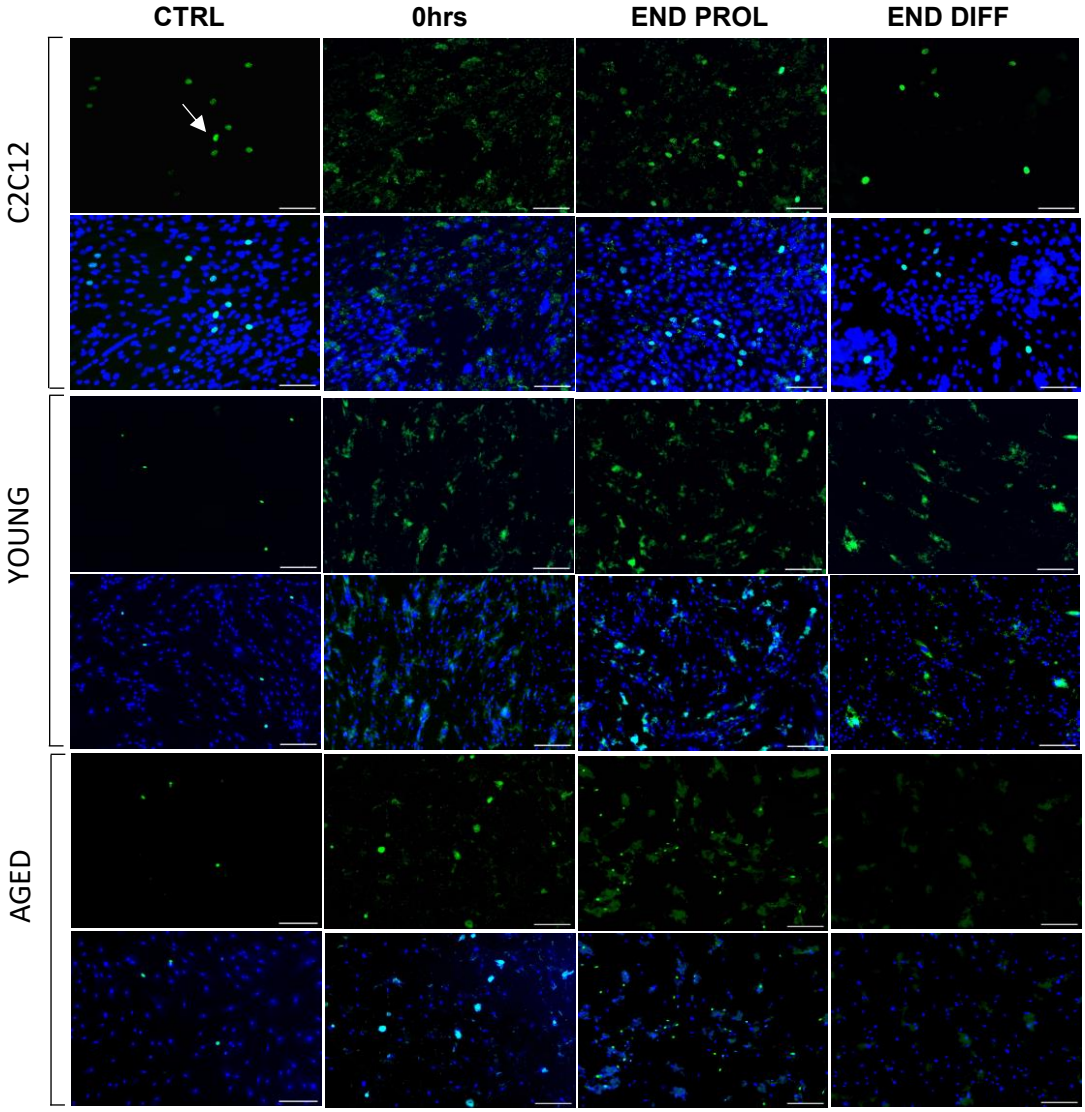

Figure 4.3: Immunofluorescence images depicting similar proliferation response in young and aged muscle cells.

EdU incorporation in mouse C2C12 and human skeletal muscle cells (EdU+ cells in green (arrow) and Hoechst+ cells in blue) indicating DNA synthesis in S-phase of cell cycle before (CTRL) and after $BaCl_2$ induced injury. No EdU+ cells were detected in old myotubes at the end of regeneration period (END DIFF). Scale bars 100µm.

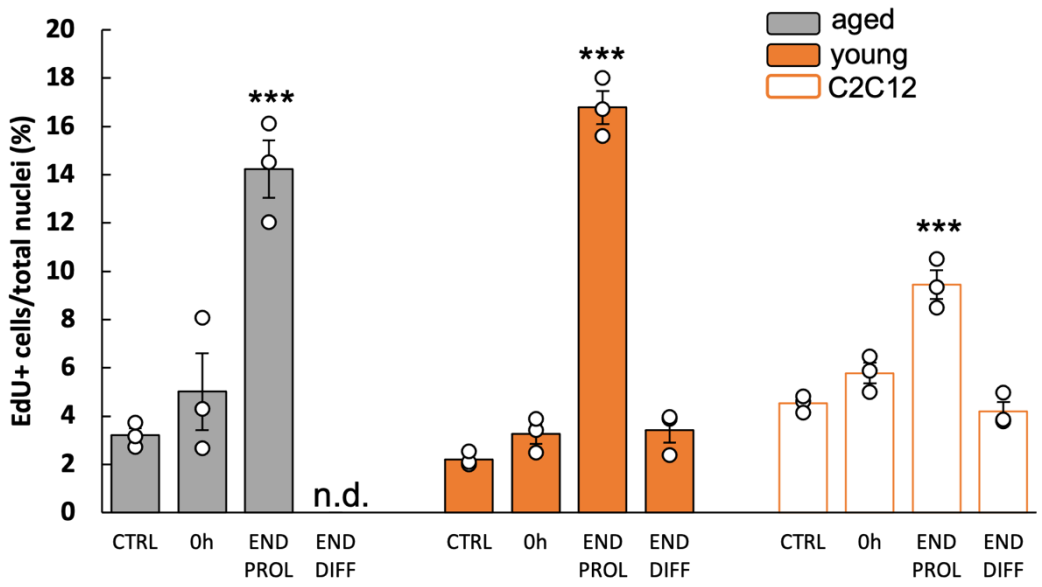

Figure 4.4: Ratio of Edu+ cells in each cell line before and after injury. *Significant increase in EdU+ cells in culture was reached by the end of proliferation of the regeneration period (END PROL) in each cell line (n=3, ***P<0.001).*

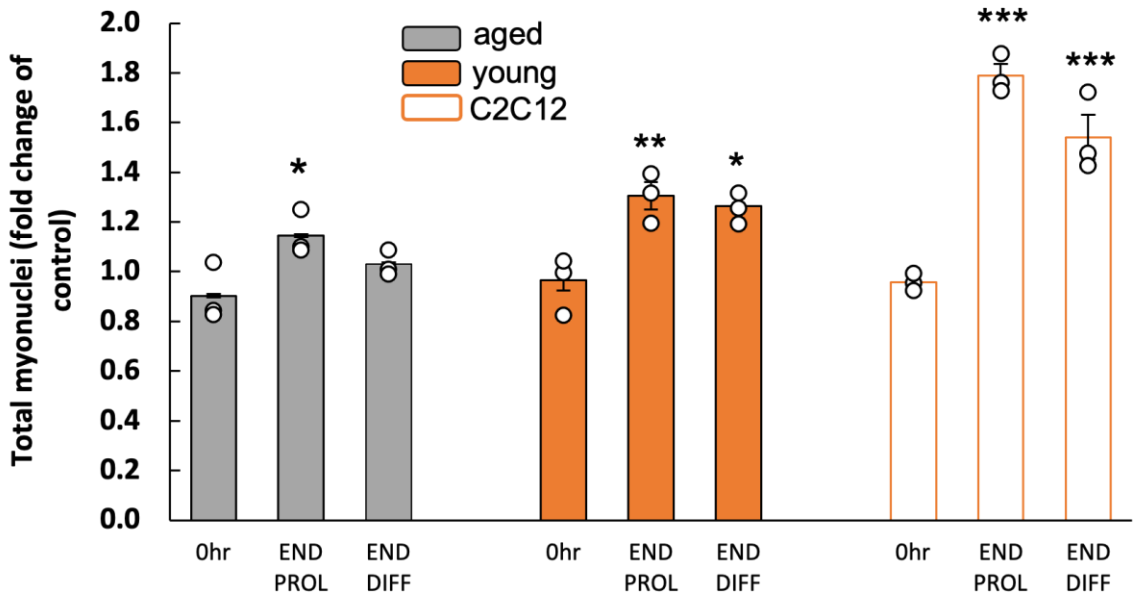

Figure 4.5: Number of total nuclei in C2C12, young- and aged- derived human muscle cells during regenerative period after injury. *Treatment with $BaCl_2$ retains precursor cells and initiates regenerative response in cultures. Significant increase in total nuclei number was reached by the end of proliferation of the regeneration period (END PROL) in each cell line (n=3; *P<0.05, **P<0.01, ***P<0.005).*

4.2.3 Aged human cells show impaired differentiation during regeneration

Next, we assessed myotube width and fusion which are important functional indicators of muscle cell differentiation (Figure 4.6). We showed that young muscle cells recovered

myotube width (Figure 4.7) and fusion index (Figure 4.8) to pre-injury values. In contrast, aged cells did not exhibit the same levels of recovery, as evidenced by smaller myotube diameter (Figure 4.7) and fusion index (Figure 4.8). Mouse muscle cells showed a slight increase in myotube width and fusion index by the end of the regeneration process compared to pre-injury (Figure 4.7 and 4.8).

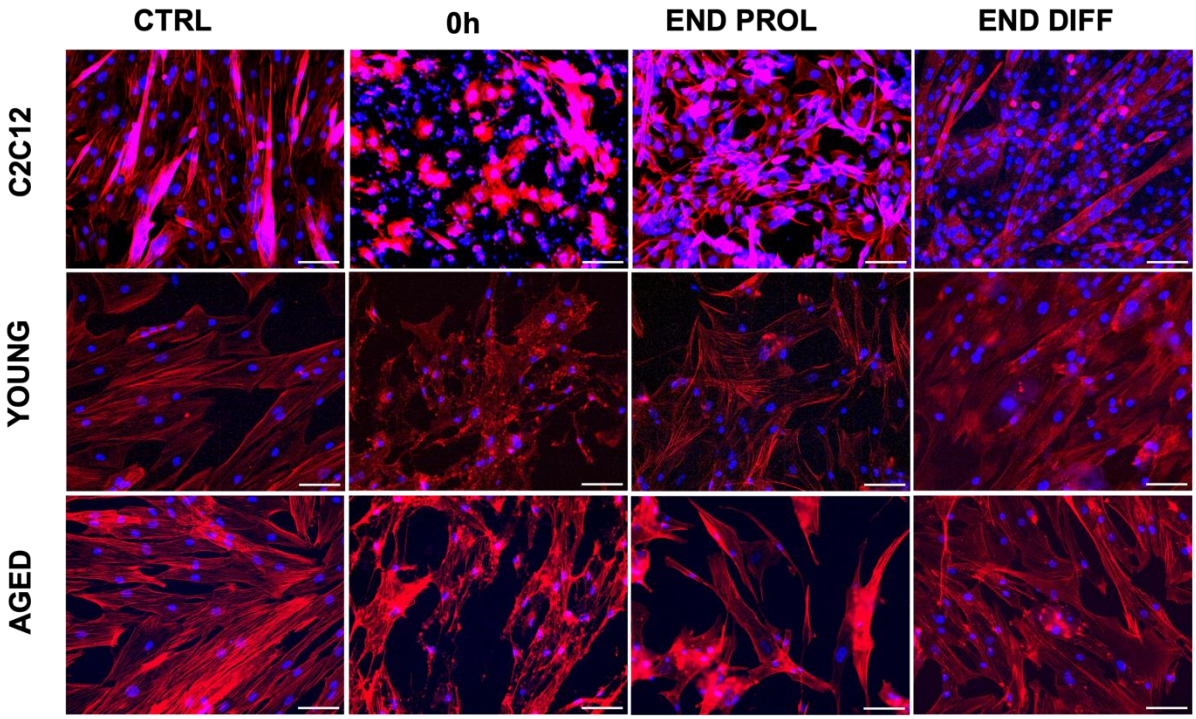

Figure 4.6: Immunofluorescence images showing recovery time points after $BaCl_2$-induced injury in mouse C2C12, young and old-derived human myoblasts.
Aged muscle cells show reduced myotube diameter and fusion index after chemical insult with $BaCl_2$. *Immunostaining for F-actin (red, phalloidin) and nuclei (blue, DAPI) show removal of myotubes, but preserved precursor cells in response to chemical injury. Scale bars 100µm.*

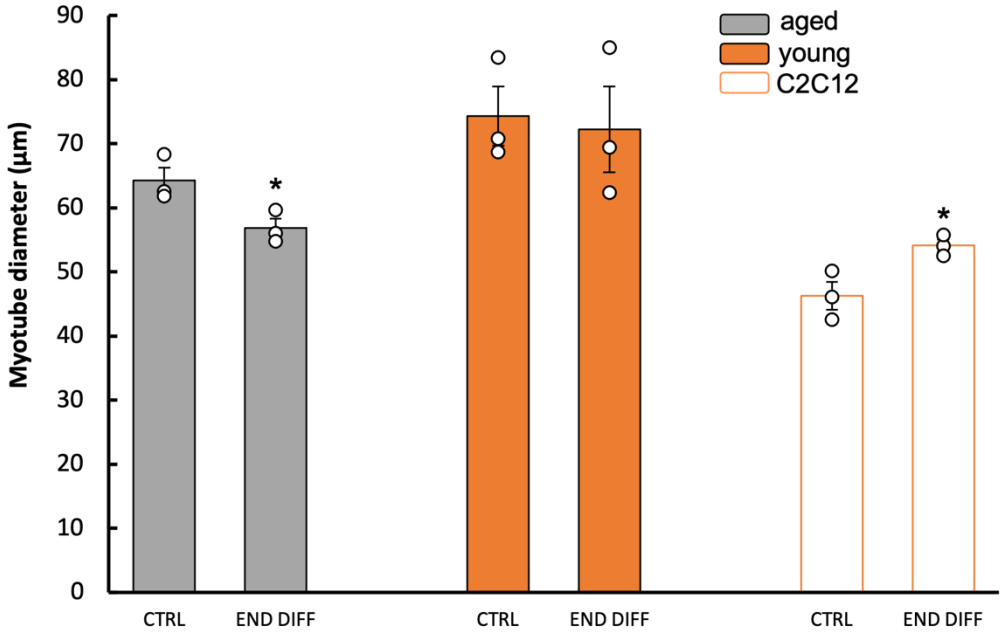

Figure 4.7: Myotube width in mouse and human cells before and after injury.
*Myotube diameter recovered to pre-injury (CTRL) size in young and mouse C2C12, but remained significantly smaller in myotubes from older donors (n=3, *P<0.05). Error bars represent standard error of the mean (SEM).*

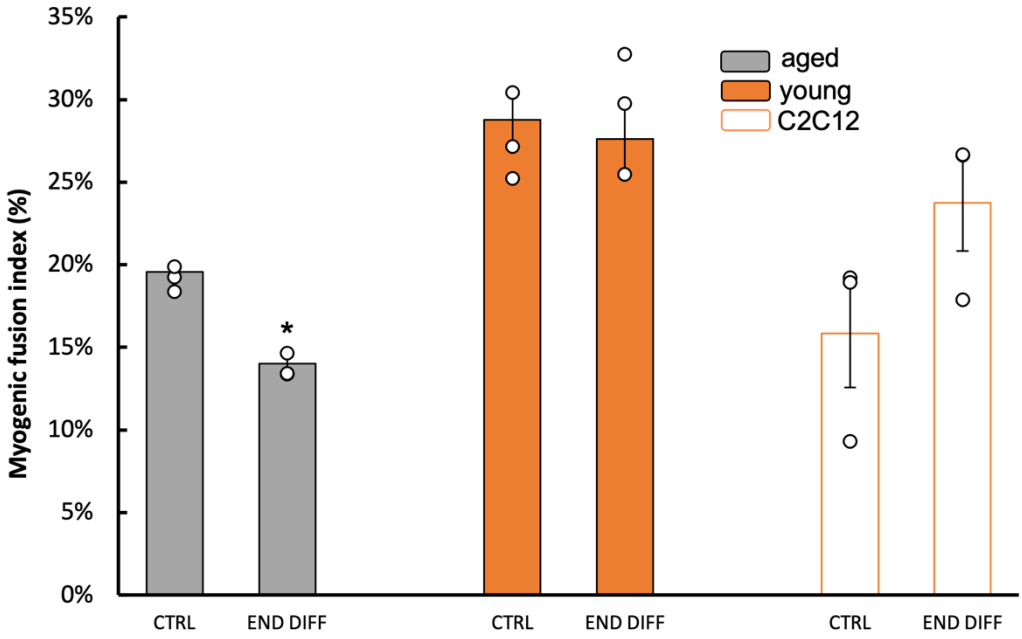

Figure 4.8: Myogenic fusion index of mouse and human cells before and after injury.
*Myogenic fusion index was significantly smaller in aged myotubes, but not in mouse C2C12 and young cells at the end of the regeneration period from the baseline (n=3, *P<0.05). Error bars represent standard error of the mean (SEM).*

4.2.4 Cell cycle and PI3k-Akt signalling pathways are significantly enriched in both young and aged muscle cells during the proliferative stage of regeneration

RNA-sequencing (RNA-seq) data were obtained from young and aged human muscle cells at pre-injury (control), and during the proliferation and differentiation phase. Data obtained during proliferation or differentiation were compared against the baseline. Volcano plots were generated for the young and aged muscle cells. Young cells displayed 895 upregulated and 1187 downregulated genes during proliferation (Figure 4.9a), while 1447 transcripts were identified as upregulated and 2284 downregulated in aged cells (Figure 4.9b). At the end of the regeneration phase, 253 genes were significantly up- and 257 downregulated in young muscle cells (Figure 4.9c) and 853 up- and 1326 downregulated in aged cells muscle cells (Figure 4.9d) when compared with pre-injury. The Venn diagram of young muscle cells during proliferation shows 10,715 gene transcripts common to baseline and 529 exclusive to proliferation (Figure 4.10a), while old muscle cells exhibit 10,643 common transcripts and 611 exclusive to proliferation (Figure 4.10b).

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes (DEGs) identified PI3-Akt signalling, cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction and cell cycle pathways as the most enriched in both young and older muscle cells at the end of the proliferation compared with baseline (Figure 4.11a and 4.11b).

The Venn diagram of young muscle cells during differentiation revealed 11,041 gene transcripts common between baseline and differentiation and 328 gene transcripts exclusive to differentiation, whereas the old cells exhibited 10,807 common gene transcripts and 449 exclusive to differentiation (Figure 4.12a and 4.12b).

Cytokine receptor interaction, protein digestion and absorption together with pathways related to immune response were the most overrepresented KEGG pathways in young at the end of the differentiation period (Figure 4.13a). Aged muscle cells continued to show

significant enrichment of PI3-Akt signalling pathway as well as cytokine receptor interaction and focal adhesion pathways (Figure 4.13b).

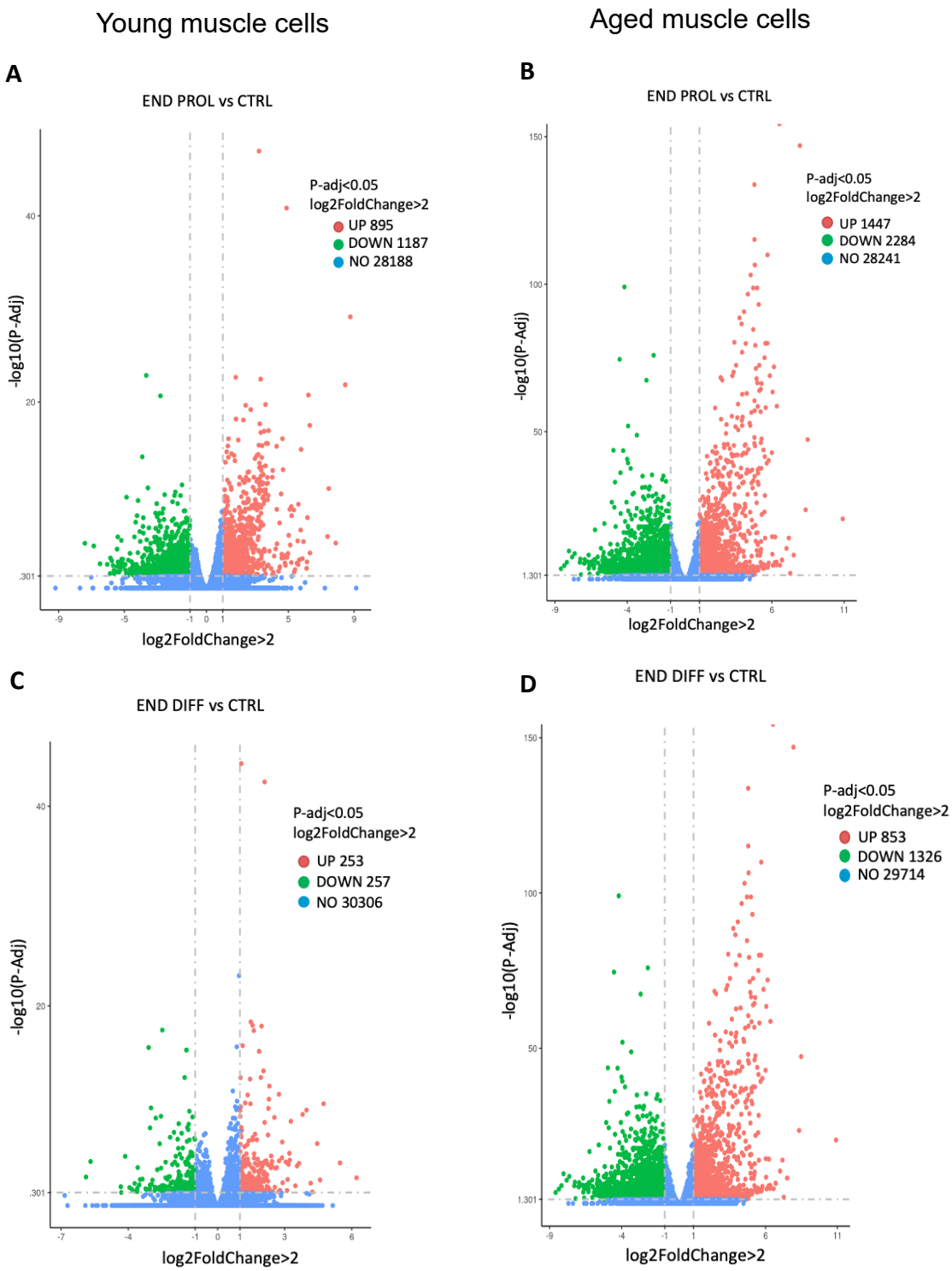

Figure 4.9: Volcano plots of young and aged muscle cells.
Volcano plots capturing the number of significantly up and downregulated gene transcripts at end of proliferation in regeneration period (END PROL) compared to baseline (A and B), and at the end of regenerative process in young (C) and aged (D) muscle cells vs control.

Red and green dots indicate significantly up and downregulated genes between timepoints. respectively. Blue dots denote genes not differentially expressed genes in END PROL or END DIFF vs control. n=4.

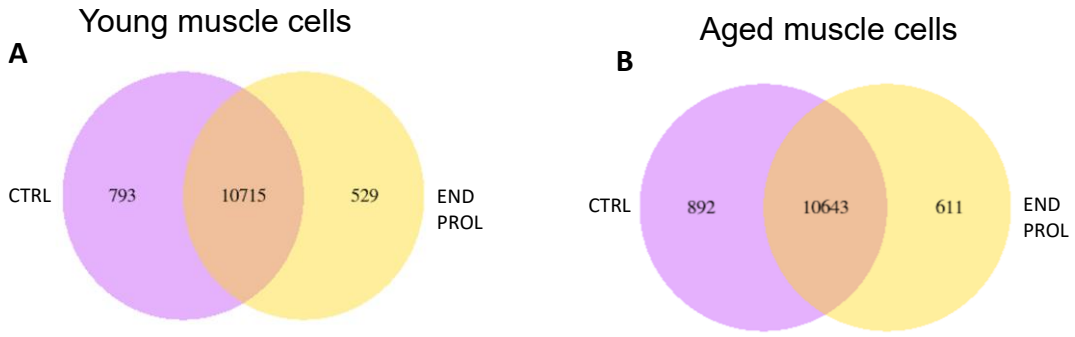

Figure 4.10: Venn diagram of gene transcripts at baseline and at the end of proliferation. *Young (A) and aged (B) muscle cells showing gene transcripts exclusive to baseline (CTRL) and end of proliferation in regeneration period (END PROL). n=4.*

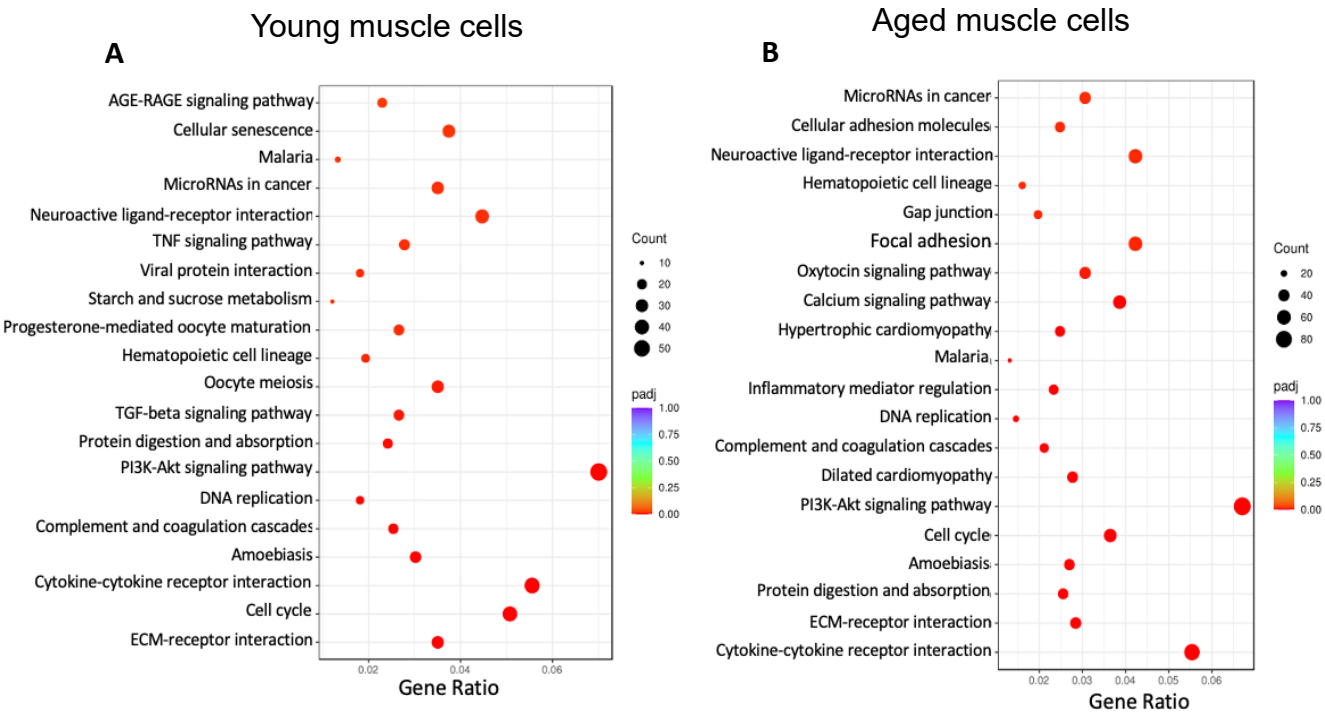

Figure 4.11: KEGG pathways analysis at the end of proliferation vs baseline.
Dot plot shows KEGG analysis of DEGs for different pathways in young (A) and aged (B) muscle cells at the end of the proliferation period vs CTRL. The size of the dot is based on the gene count enriched in the pathway. and the color of the dot denotes pathway enrichment significance. n=4.

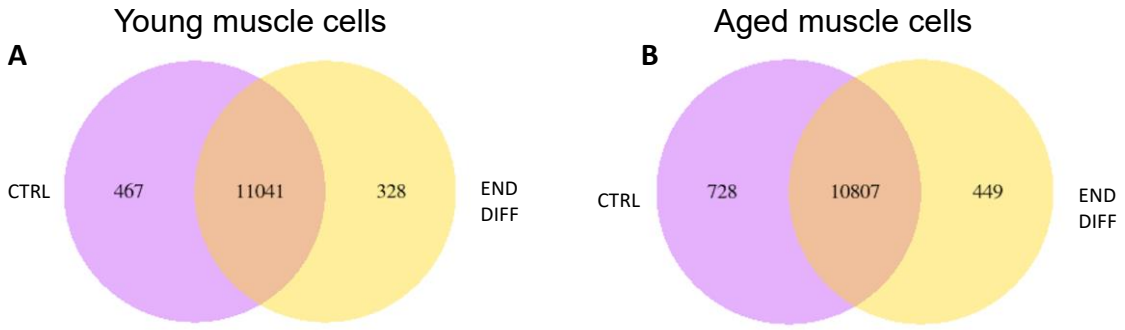

Figure 4.12: Venn diagram of gene transcripts at baseline and at the end of regeneration. *Venn diagram of young (A) and aged (B) muscle cells showing gene transcripts exclusive to baseline (CTRL) and end of differentiation in regeneration period (END DIFF). n=4.*

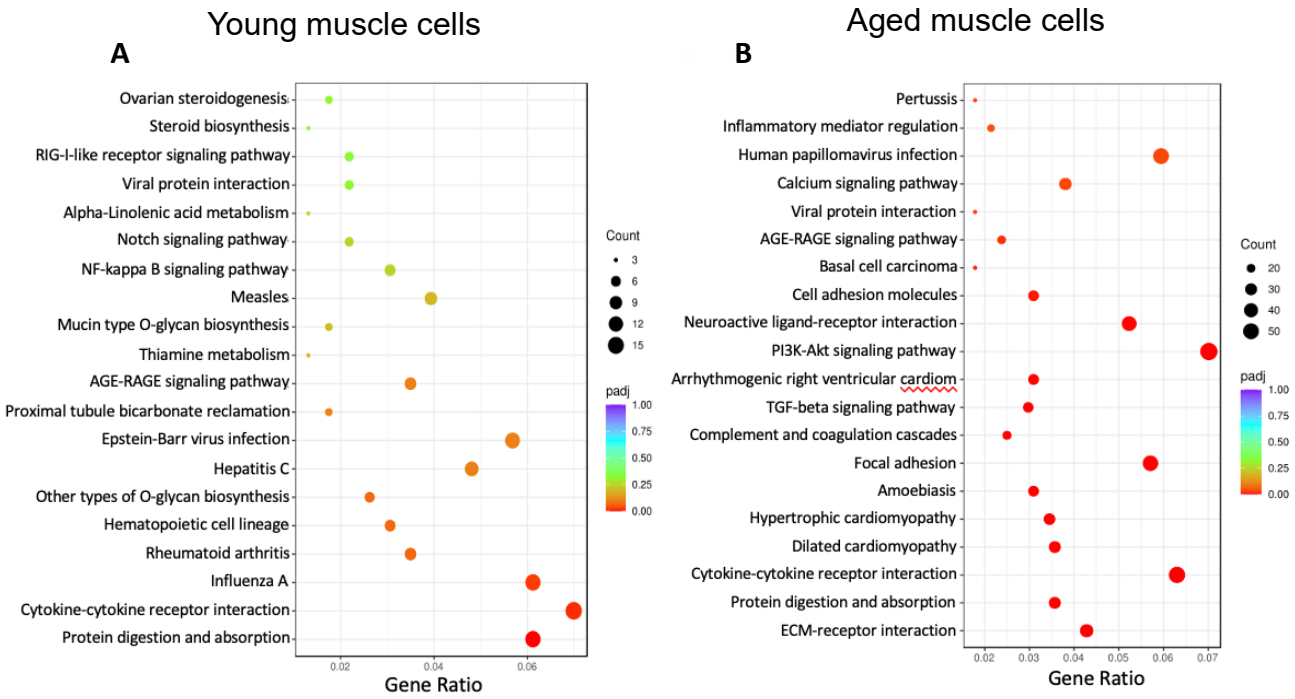

Figure 4.13: KEGG pathways analysis at the end of regeneration vs baseline. *Enrichment of different pathway in young (A) and aged (B) human muscle cells at the end of regeneration vs baseline. The size of the dot is based on the gene count enriched in the pathway, and the color of the dot denotes pathway enrichment significance. n=4*

4.2.5 Aged muscle cells depict downregulation of muscle cell differentiation and development-related processes

GO analysis of DEGs in young muscle cells identified the cell cycle as the most overrepresented molecular function during the proliferative period (Figure 4.12a). In aged

ones, genes involved in muscle circulation and muscle system processes showed the highest enrichment (Figure 4.12b). At the end of the regeneration, however, extracellular matrix-related processes were the most enriched in young cells (Figure 4.12c) whereas skeletal muscle processes (i.e., muscle adaptation, contraction, hypertrophy and relaxation) remained overrepresented in aged cells (Figure 4.12d). A closer analysis of GO enrichment demonstrates the downregulation of these biological processes in aged (Figure 4.13a) but not in young cells (Figure 4.13b), which could explain the impaired muscle cell regeneration in aged muscle shown in morphological analysis.

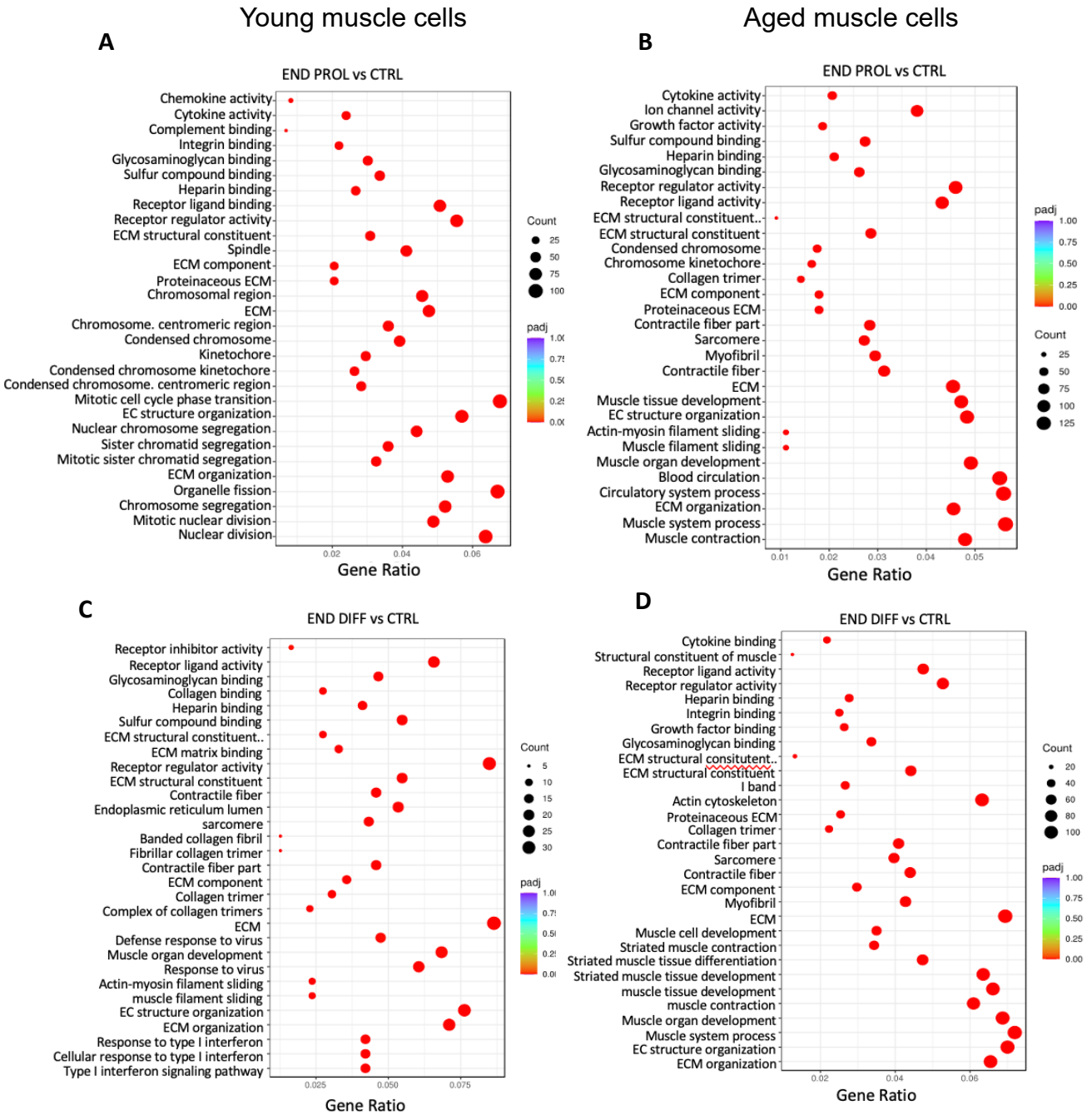

Figure 4.12: GO enrichment analysis of aged and young muscle cells.
Overrepresented GO terms of biological processes, molecular functions and cellular locations at the baseline vs in the end of the proliferation during regeneration period (END PROL) in young (A) and aged (B) and baseline vs at the end of the regeneration (END DIFF) in young (C) and aged (D) muscle cells. The size of the dot is based on the gene count enriched in the pathway, and the color of the dot denotes pathway enrichment significance. n=4.

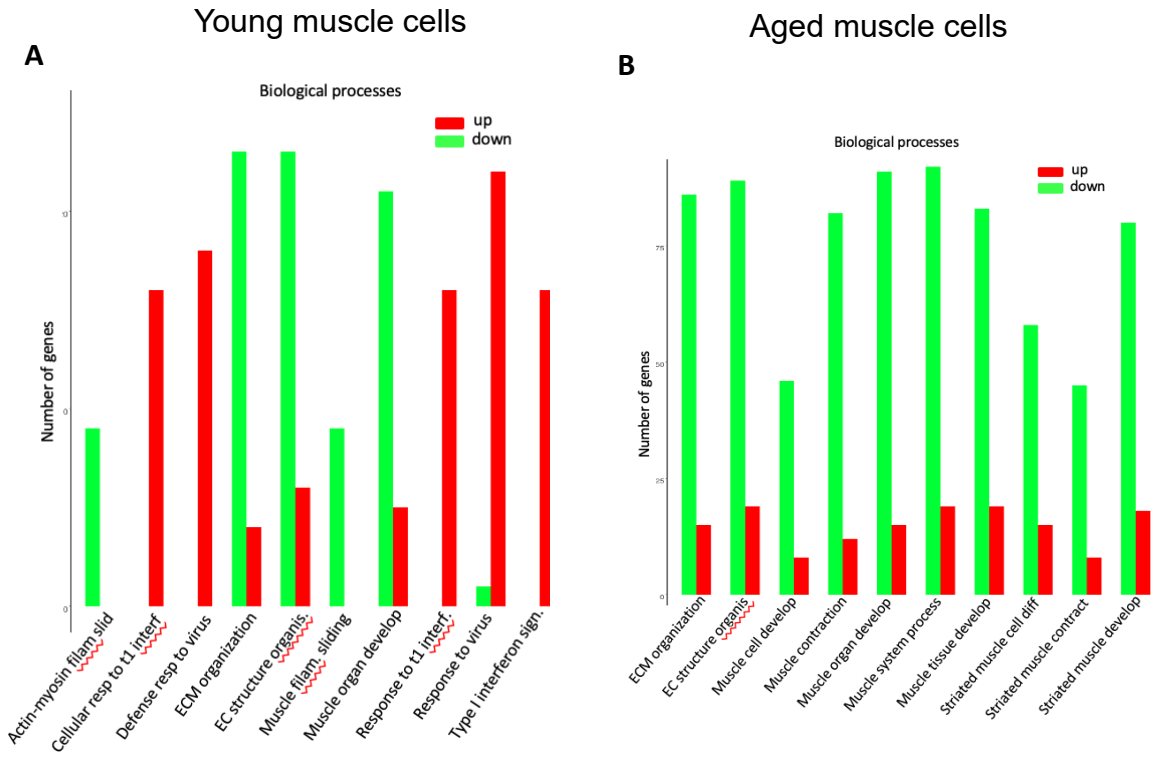

Figure 4.13: GO enrichment bar chart of biological processes. *Most enriched GO terms of young (A) and aged (B) muscle cells at the END DIFF. n=4.*

4.3 Discussion

Herein we report the successful development of a human *in vitro* model with which to investigate the muscle regeneration response across the lifespan. This was achieved using myoblasts from both young and old donors which were then differentiated into myotubes and injured. For injury, we exposed cells to $BaCl_2$ and characterised the follow-on repair process with a particular focus on nuclear proliferation, muscle morphology and the

transcriptome. Transcriptome analysis was of pivotal importance since muscle regeneration relies on specific mRNA profiles for muscle reconstruction (Roman et al. 2021).

$BaCl_2$ is a skeletal muscle toxin previously used to study muscle injury and regeneration in vivo (Fry et al. 2015, Jung et al. 2019, Lee et al. 2013, Morton et al. 2019) and in vitro in monolayer (Fleming et al. 2019) or tissue-engineered muscle (Agrawal et al. 2017, Juhas et al. 2018). While the majority of the studies use animal models (Bjornson et al. 2012, Brett et al. 2020, Stanley et al. 2022), only a few have used human-derived muscle cells (Fleming et al. 2020, Khodabukus et al. 2018), and none has investigated the transcriptome which is fundamental to further understand muscle regeneration.

The most common method to quantify cell proliferation in response to stimuli is the staining-based nuclear tracing of BrdU labelled cells (Abdelmoez et al. 2020, Kneppers et al. 2018). We employed EdU incorporation since in contrast to BrdU it does not require DNA denaturation which could disrupt the integrity of DNA, morphology and antigen recognition sites. The EdU incorporation assay has been successfully used to directly measure S-phase progression in the cell cycle during the repair period following injury (Brett et al. 2020, Juhas et al. 2018). We discovered that our muscle model retains the ability to re-enter the S-phase (proliferative) of the cell cycle as demonstrated by EdU incorporation in the repaired myotubes. Although the traditional view has been that post-mitotic nuclei - like those in differentiated myocytes - do not proliferate, recent findings show otherwise. For example, a recent study where mice were labelled with green fluorescent protein and deuterium water provided evidence for proliferative activity of myocyte (Borowik et al. 2022). Another mouse study utilising a ^{15N}thymidine stable isotope tracer, showed that cardiomyocyte nuclei proliferated in both normal and injured heart (Vujic et al. 2018). The same study confirmed that the microRNA, miR-222 was involved in the myocyte nuclei proliferation capability. Our findings are in line with these studies, but we appreciate that non-fused muscle cells may also have contributed to the observed proliferation and repair. This is an inherent limitation of cellular models – the maturation and hence fusion index is never absolute.

Despite this limitation, we feel that a cell model with human genetic makeup (unlike animal models) and compatible with high-throughput testing - paramount e.g., in nutrient and drug testing - offsets such limitation.

Aged muscle cells, however, were unable to rescue the diameter and fusion index to pre-injury levels, which contrasts with young cells where full recovery was shown. These findings are in agreement with human studies examining muscle growth capacity post-exercise (as a form of injury) showing an impaired hypertrophic responses in aged vs. young adults (Snijders et al. 2014, Suetta et al. 2013). Such impairment can be partly attributed to a pro-inflammatory systemic environment (Conboy et al. 2005, Visser et al. 2002) that may cause dysregulation of signalling pathway cascades like FGF2, Notch, Cdkn2a in muscle progenitor cells (Bjornson et al. 2012, Chakkalakal et al. 2012, Sousa-Victor et al. 2014), resulting in a diminished differentiation capacity, evidenced herein by thinner and less fused myotubes.

We successfully mapped gene transcripts before the injury and during regeneration in young and aged human muscle cells using RNA-seq. This enabled us to identify significantly enriched KEGG signalling pathways, and GO processes evoked during muscle repair. KEGG analysis revealed that the cell cycle signalling pathway was significantly enriched in both young and aged muscle cells during the proliferative phase which appears to have prompted both young and aged cells to enter the proliferative S-phase after injury as discussed above.

PI3k-Akt and downstream mTOR regulate muscle cell proliferation, survival and differentiation (Bodine et al. 2001, Briata et al. 2012, Khodabukus et al. 2018). *PI3k-Akt* pathway was significantly enriched in young and aged cells during the proliferation phase. When cells exited the S-phase and entered differentiation, *PI3k-Akt* remained enriched in aged, but not in young cells. The role of *PI3k-Akt* in aged cells during the differentiation phase remains somehow unclear since this pathway has many downstream effectors leading

to different cell fates (Briata et al. 2012, Rafalski & Brunet, 2011, Yazid & Hung-Chih, 2021, Zhang et al. 2018).

This notion is further supported by GO analysis, which suggests that aged cells during the differentiation and proliferation exhibit significant downregulation of processes involved in muscle tissue development indicating diminished maturation capacity. Interestingly, the Venn diagram of aged muscle cells revealed that 10,807 gene transcripts overlapped between differentiation and baseline, whereas in young muscle cells shared 11,041 gene transcripts between timepoints, further highlighting the gap between aged and young cell in their ability recover to pre-injury levels.

The cytokine-cytokine signalling pathway remained enriched in both young and aged cells during the proliferation and differentiation phase. Cytokines constitute a major class of regulators of skeletal myogenesis (O'Leary et al. 2017, Waldemer-Streyer et al. 2017) so its permanent overrepresentation during the recovery from injury is not surprising. Taken together, KEGG analysis strongly suggests that factors that stimulate cell cycling played an important role during proliferation while cytokines were central across the regeneration process.

Here we successfully developed an *in vitro* model that provides a high-throughput platform enabling cellular and molecular investigations of muscle regeneration across the life course. As expected, aged myotubes showed impaired regeneration as evidenced by reduced myofusion index and myotube width after repair. We postulate that this is due to down-regulation of genes involved in muscle development and function. We anticipate the use of our model as a high-throughput platform to further examine regeneration across the lifespan, as well as to investigate potential drugs or metabolic/genetic diseases.

4.4 Conclusion

The purpose of this chapter was to investigate the *in vitro* regeneration capacity in young and aged muscle cells in response to injury. These experiments allowed successful development of a high-throughput *in vitro* model enabling cellular and molecular investigations of muscle regeneration across the life course. Obtained data suggest that repair in response to *in vitro* injury is impaired in aged *vs.* young muscle cells. This study establishes a framework that enables further understanding of muscle regeneration across ageing.

Chapter 5: Development of a high throughput method to generate TEM

5.1 Introduction

The prevalence of a wide range of skeletal muscle related metabolic, neuromuscular and dystrophic disorders in human populations require the development of a functional tissue engineered muscle (TEM) model that could provide a biologically accurate system to study basic muscle physiology, disease progression and drug efficacy and toxicity without the need of animals (Wang et al. 2021, Smith et al. 2012, Gilbert-Honick et al. 2018). In addition, lack of pharmacological therapies to counteract muscle atrophy - mainly due to ethical consideration and inadequate animal models - highlights the need to generate accurate preclinical models (Wragg et al. 2019).

Pre-clinical dose-dependent responses to drugs can readily be assessed in TEM with structurally and functionally mature properties such as contractility, responses to stimulation, striated myofibers and a SC pool (Gholobova et al. 2018, Vandeburgh 2010, Madden et al. 2015). These findings suggest that TEM is a promising alternative to animal models. Advances in cell biology diminished the often-occurring de-differentiation of cell types used in disease modelling and made it possible to utilise patient-specific stems cells iPSC-derived myogenic precursors instead of embryonic stem cells in adequate culture conditions (Maffioletti et al. 2018, Takahashi et al. 2018, Egawa et al. 2012). Furthermore, improvements to replicate composition and architecture of SC niches will further advance cell therapy (Rao et al. 2018, Prüller et al. 2018). In addition, co-culturing different cell types and pro-myogenic factors in the muscle construct together with innervation and/or pre-vascularisation enhances the functional properties of engineered muscle (Gholobova et al. 2020, Juhas et al. 2018, Rimington et al. 2021, Borselli et al. 2011, Syverud et al. 2016).

These novel approaches need to be implemented to upgrade skeletal muscle tissue engineering to physiological and pathophysiological states resembling native tissue.

Most approaches to prepare TEM include complex and expensive bioreactors/devices and produce a small amount of TEMs thereby limiting high-throughput analysis (Clause et al. 2010, Elsaadany et al. 2017, Heher et al. 2015, Kondash et al. 2020). In addition, the small size of the muscle constructs (up to cm) limits *in vitro* testing for function and metabolism (Gholobova et al. 2020, Capel et al. 2019, Khodabukus et al. 2019, Maffioletti et al. 2018). To engineer standardized dynamic TEM-system, detailed consideration of fabrication method, biomaterials, cell sources, experimental design and analytical methods would accelerate the development of new therapeutic strategies, advance the field of skeletal muscle engineering and make results between groups comparable (Smith et al. 2012, Antoine et al. 2014, Engler et al. 2006, Gholobova et al. 2018, Juhas and Bursac 2014). To achieve this, the aim of the study was to develop high throughput technology that enables to generate simultaneously multiple highly reproducible TEMs with morphological and functional properties comparable to established TEM models. A greater access to high-throughput advanced cell culture systems will facilitate research on *in vitro* drug screening of potential treatment options for muscle wasting and disease modelling.

5.2 Results

5.2.1 Optimisation of collagen-based hydrogel to fabricate TEM

An appropriate hydrogel formulation is crucial for viable cell culture application. More specifically, mechanical features of the hydrogels influence the stability of the culture material, mechanotransduction within the myobundle as well as cellular proliferation and differentiation. Taking this into account, we tested a variety of type I collagen, Matrigel, MEM and NaOH ratios with different cell densities to achieve a hydrogel composition durable enough to ensure cell viability for extended culture periods (at least 4 weeks), to

remain intact during initial polymerisation and culture medium exchange, histochemical analysis and manipulation during re-positioning between anchor-posts in the insert. Table 5.1 summarizes hydrogel and bioreactor optimisation process.

5.2.2 Optimisation of the Flexcell bioreactor to prepare TEM

To obtain a suitable cell-hydrogel solution for fabricating TEMs on a bioreactor, several features of the Flexcell culture system were tested (see Table 5.2). Firstly, the amount of hydrogel loaded in the Tissue Train well ranged from 100-300µL with an amount of 200µL being optimal. Secondly, the recommended hydrogel polymerisation time by the manufacturer (30min) was not sufficient for established hydrogel composition and it appeared 2h was adequate for the gel to polymerise prior to vacuum release and growth medium addition. Mouse myoblasts C2C12, as well as muscle cells from young and aged human donors were used to fabricate TEMs on the Flexcell bioreactor. Images obtained with a confocal microscope indicating immunofluorescence labelling of cytoskeleton and nuclei are shown on Figure 5.2. Importantly, this was the first time a Flexcell bioreactor was used to prepare bioengineered tissue constructs of human muscle cells. Despite successful optimisation of the hydrogel-bioreactor system to generate TEMs with aligned myotubes, the set-up has many limitations. For instance, the price of the bioreactor and accompanied add-ins (gaskets, specific disposable cell-culture plates, tubes, generator etc.) is relatively high and unaffordable for many labs. Furthermore, the BioFlex baseplate fits four 6-well culture plate, which limits the number of TEMs that can be prepared making it time-inefficient. Given these restrictions, we aimed to develop re-usable inserts to make structurally and functionally mature TEMs that are environmental-friendly and enable high-throughput analysis while being affordable.

Table 5.1. Optimisation of hydrogel formula

	Composition/protocol	Results
1	Collagen concentration ~2.5mg/ml 200µl/construct. 30 min in 37°C vacuum (According to Corning protocol)	detached, hydrogel dissolved in medium
2	2.5mg/ml and 5mg/ml collagen concentration ($15x10^5$ cells/construct). 60 min polymerisation in vacuum	not attached to anchors, floating around when adding medium
3	5 mg/ml collagen, 2h vacuum	gels did not dissolve, but detached from the anchors when added medium
4	same as nr 3, 5mg/ml, more gel under anchors and pressed them towards thorough	not attached
5	same as nr 4, but 3h polymerisation	a bit loose but attached to anchors
6	Check pH. 2h vacuum stetch	no obvious detachment of the gel from the anchors when adding medium
7	Fresh NaOH solution (4g/100ml)	gels detached from the mid-part
8	testing 80% collagen solution (7.5mg/ml final concentration) 10% 10xPBS, 1M NaOH+ DMEM	detached when removing loaders->cover with silicon all over
9	Again nr 8 (w more silicon)	best so far, not so unstable when added medium
10	repeat nr 9 with cells (C2C12 1000cells/µl)	nicely attached the whole time-> stain 48h prol-> phalloidin+DAPI: checked confocal-> uneven cell distribution (more cells on the left end)
11	new hydrogel using 9.54mg/ml 80% collagen, 10xPBS and NaOH. 2h vacuum stretch +1h gradual vacuum w reduced % ->	->diff on 72h->72h diff-> 2days 6h cyclic stretch regimen 0.1Hz +10% (18h 0%). Fix and stain
12	new hydrogel: 10% 10xMEM. 80% collagen (LC: 3.57mg/ml), 1M NaOH (colour remained yellow, but pH ~7)	looking a bit loose, broken when removing loaders (problem!)
13	test 9.54mg/ml collagen: 10% 10X MEM and 80% HC collagen+ 10% 10x PBS	best so far. test elasticity to see the physiol. relevance (never happened)
14	low (3.57mg/ml) + high (9.54mg/ml) concentration collagen test again+ 0.5M cells (C2C12)-> 3000cells/µl	3h vacuum 20% 0.5Hz, both gels remained intact when removing loaders. Fix and stain: increase cell density
15	85% 3.57 mg/ml collagen + 10% DMEM+ 14µl NaOH	2h 18% stretch vacuum, no gelation!
16	2M, 4M and 6M C2C12 with 9.54mg/ml hydrogel: 85% collagen	after 2h vacuum all detached; prepare higher vol of gel to avoid bubbles, 4M did not detach.

17	9.54mg/ml hydrogel, 2M and 4M cells/scaffold	after 2h no detachment from anchors, lots of bubbles: remained intact when removed baseplate.
18	low and high concentration collagen hydrogels with Matrigel: 65% collagen, 10% MEM, 1M NaOH, 20% Matrigel	HC: lots of bubbles, RT as incubator did not work-> dried
19	new hydrogels (as nr 18) to test polymerisation	after 30min both looked gelled, did not trip when moving
20	65% 3.57mg/ml collagen, 10% MEM, 1M NaOH (~10µl), 20% Matrigel	250µl/well, few days vacuum (instead of 2h), added PM after 2h-> intact, but look dissolved in medium 2days later
21	High and low concentration collagen hydrogels (as nr 20)	vacuum for 2 days, remain attached and not dissolved when adding medium after both 2h and 2 days of polymerisation
22	65% 3.57mg/ml collagen, 10% MEM, 1M NaOH (~10µl), 20% Matrigel	total vol 300µl, loaded 2 stripes on wells. Polymerised after 2h in 37'C, when added growth medium they did not dissolve
23	Bring collagen to 3mg/ml by diluting with PBS. 65% LC collagen (3mg/ml), 10% MEM, 1M NaOH (~10µl), 20% Matrigel+cells	total vol 400µl, loaded 2 constructs with C2C12 4M/ml. No detachment/dissolvement 2h post-vacuum. Kept vacuum overnight. Stain-> nicely striated tubes!!
24	65% collagen (3mg/ml), 10% MEM, 1M NaOH (~60µl/2,4ml), 20% Matrigel+cells (4000cells/µl)	con and exercise plates old & young triplicates, 48h vacuum
25	65% collagen (3mg/ml), 10% MEM, 20% Matrigel, 1M NaOH (10µl in 0.5 ml) Final formula!	loaded w/o cells to test Flexcell (new gaskets+ connectors) vacuum 2h, loaded ~200µl/well

5.2.3 Developing re-usable inserts to generate TEM

As mentioned above, novel TEM inserts were developed to provide a cost-effective, feasible and re-usable alternative to expensive bioreactors and/or custom-made set-ups for fabricating aligned and functional TEM (see Figure 2.5). Design of the TEM insert was

partly inspired by Tissue Train Culture plates used for the Flexcell bioreactor. The development process included adjustments of insert diameter, depth of the trough, length and position of the pillars, sizing of the mesh-based anchor and sterilisation steps (see Table 5.2. for insert optimisation). To ensure *in vitro* compatibility, TEM inserts were 3D printed using biocompatible plastic, coated with resin, while anchors were made of non-oven biocompatible material. The final sterilisation process was performed as described in section 2.3.5. Images of insert-based TE constructs can be seen in Figure 5.1. Immunofluorescence staining to label F-actin and myonuclei was performed to confirm alignment of TEM myotubes cultured on inserts (Figure 5.2). To assess the functional properties of the TEM, twitch and tetanic force measurements generated by the TEM in response to electrical stimulation were performed using force transducer (Aurora Scientific) (Figure 5.3).

Table 5.2: Development of TEM insert

Version	Protocol	Results
1	Insert diameter 3.2mm.	A bit too big for Sarstedt 6-well plate.
2	Insert diameter 3mm.	Suitable for Sarstedt plates, trough not deep enough->200µl too much.
3	Maintain insert diameter, deeper trough.	Trough big enough for loaded hydrogel volume, but leaks from edges.
4	Adjust anchor size to better fit trough and pillars within the well.	Suitable anchor size achieved, but occasional leaking still occurs.
5	Same insert size, added tapes (sterile) to the edges to avoid leaking.	No leaking from edges of the insert into the well, but mould appearing in few days.
6	Using another autoclave to sterilise inserts.	No changes in contamination status- still present in culture plates.
7	Updated insert disinfection protocol after 3D printing (water, 99% Isopropyl alcohol, ultrapure water). New autoclave program (steam sterilization at 121°C for 30min) to sterilize inserts and associated tools such as tweezers, needles, tips and scissors.	After complete drying of inserts in heated cupboard, inserts were used for TEM preparation and no mould was present anymore.

8	Established insert design and cleaning procedure was used to prepare TEM for staining.	Immunofluorescence staining for phalloidin and DAPI exposed unstained/unclear areas without aligned myotubes. Occasional leaking!
9	Updated design: edges at each end of the trough to prevent leaking.	Successful culture of the hydrogel without gel running out during polymerisation and no presence of mould. Staining: problem remained.
10	Updated insert design to ensure even medium exposure together with nutrition availability for the whole hydrogel-> shorter pillars to facilitate hydrogel removal+ 2 new pillars to re-position hydrogel after polymerisation for even medium perfusion throughout hydrogel.	Hydrogel was prepared between horizontal pillars within the trough (9 to 3 direction). After 24h, hydrogel was re-positioned to vertical pillars (12 to 6 direction). Staining indicates better perfusion of the whole hydrogel.

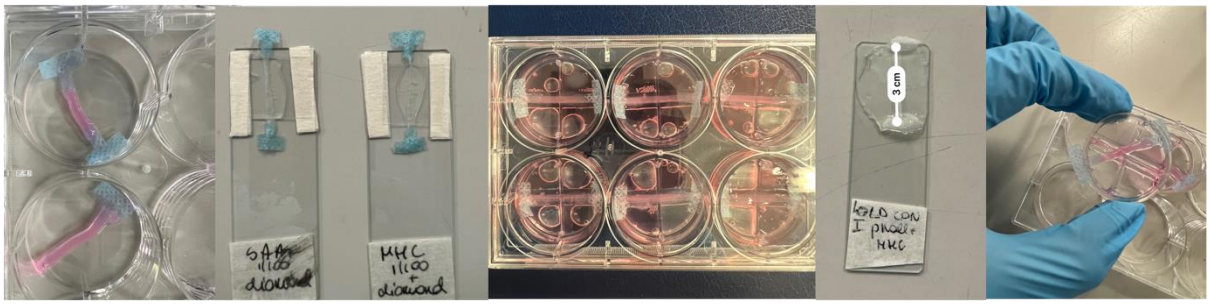

Figure 5.1: Representative images of TEMs prepared using novel re-usable inserts. *Re-usable inserts were developed by our lab and is currently under patent filing process. Printing of the TEM inserts was performed by Dr Yang Wei (Department of Engineering, Nottingham Trent University).*

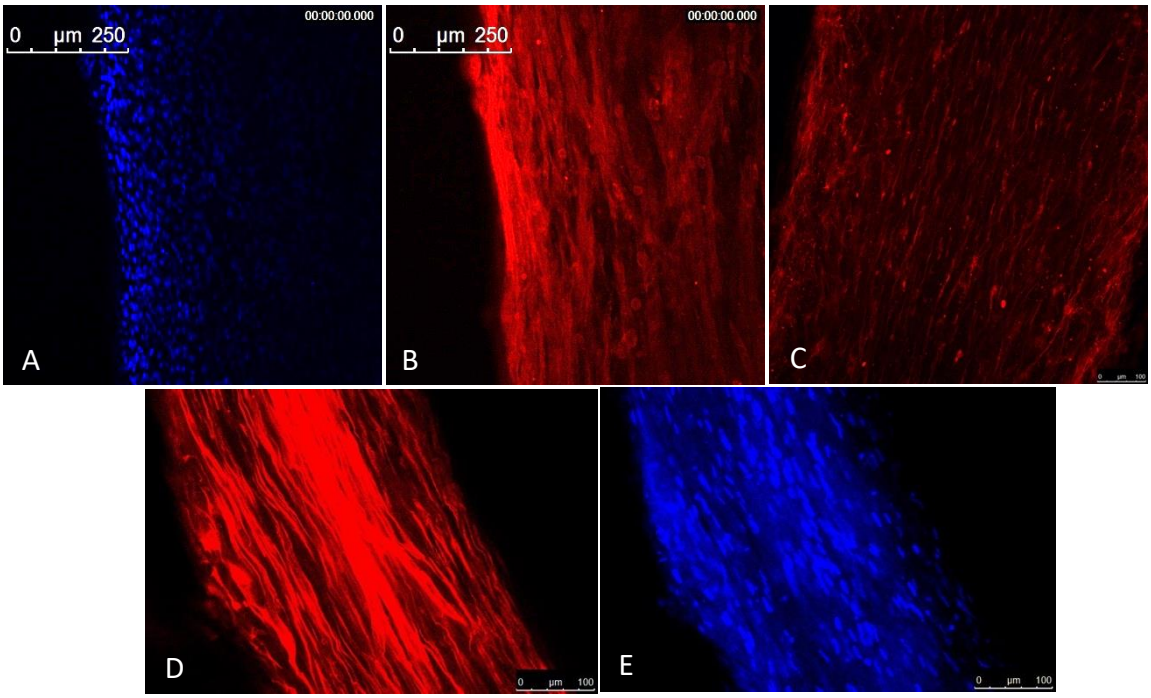

Figure 5.2: Immunofluorescence images of TEM.
Staining for F-actin (red, phalloidin) and nuclei (blue, DAPI) show aligned myotubes in bioreactor- (A, B, C) and insert-based (D. E) TEM constructs of C2C12 mouse (A, B, D, E) and human (C) muscle cells.

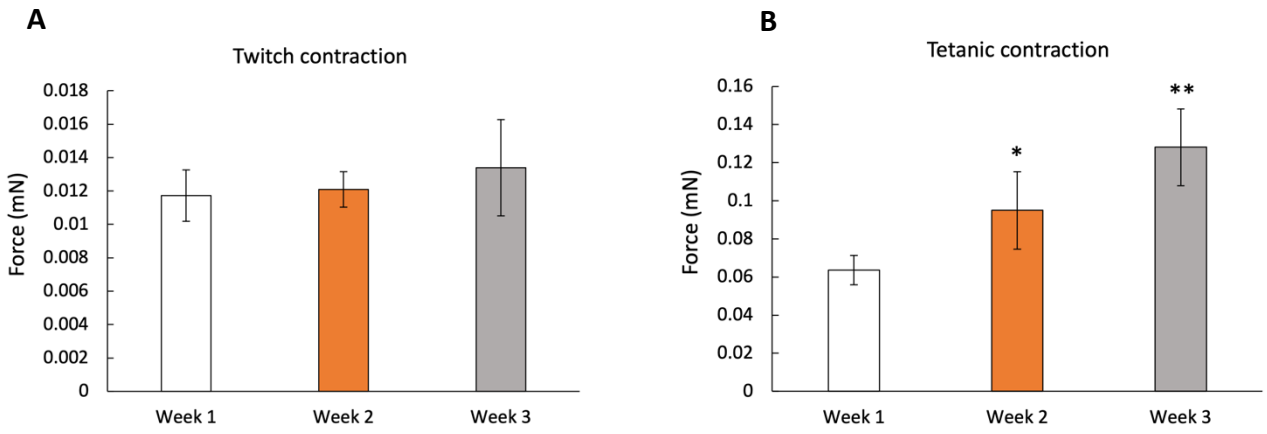

Figure 5.3: Contractility measurements of C2C12 TEM constructs.
*Graphs depicting twitch force (100Hz for 10ms) of C2C12 TEM at week 1, 2 and 3 (A) and tetanic force (100Hz for 1s in trainmode) at week 1, 2 and 3 (B). The contractility measurements at every time-point were obtained from 4 samples (n=4), each undergoing three repetitions. (*P<0.05 (week 2 vs week 1), **P<0.01 (week 3 vs week 1)). Error bars represent standard deviation (SD). Experiments on force transducer were performed by our lab member Dr Chris Gabbott.*

5.3 Discussion

Different tissue engineered culture systems have shown to be useful for drug screening, disease modelling and for tissue/organ level co-culturing (Bhatia and Ingber 2014, Allen et al. 2005, Lee and Vandenburgh 2013, Wagner et al. 2013). There is a necessity for culture systems that can recapitulate the biological tissue environment to ensure that traditional monolayer formats are exposed to a similar complex *in vivo* muscle milieu. With the growing recognition of the importance of the extracellular niche in regulating cell dynamics, biomaterials have been implemented in TEM fabricating to better control the structure, composition and mechanics of culture model (Caliari and Burdick 2016, Baker and Chen 2012). Hydrogels as scaffolds are proven to be the most promising in TEM culture applications as their mechanics is like that of soft tissues and they possess fundamental details of *in vivo* ECMs that facilitate cell adhesion and signalling (Lutolf and Hubbell 2005, Tibbitt and Anseth 2009). Collagen is the main intercellular organic component in many tissues, which is the reason for collagen being widely used to encapsulate cells in 3D (Shoulders and Raines 2009, Burkel et al. 2016). As such, type I collagen-based hydrogels have been utilized to engineer skeletal muscle constructs (Capel et al. 2019, Clause et al. 2010, Juhas and Bursac 2014, Prüller et al. 2019) hence it was the choice of the biomaterial to prepare TEM in this study. The composition of the hydrogel was based on the literature (Somers et al. 2019, Fleming et al. 2019, Madden et al. 2015) with modifications to make it suitable for available culture set-ups (see Table 5.2.1). Once the best formulation was achieved, muscle cells from mouse (C2C12) and human (young and aged) were embedded for up to a month culturing period. As established markers to confirm structural maturity (Madden et al. 2015, Wragg et al. 2019, Capel et al. 2019, Khodabukus et al. 2019), immunofluorescence staining was performed to visualize aligned myotubes, sarcomeric α-actinin and nuclei. The computer controlled Flexcell bioreactor uses a vacuum that creates a uniaxial strain by deforming the flexible culture surface, thus creating a (physiological)

strain. Although the Flexcell bioreactor has been used to tissue engineer myobundles (Juffer et al. 2013, Chen et al. 2013, Hua et al. 2016) and has unique and adjustable technique, it is expensive and not easily accessible. Therefore, we sought to develop re-usable inserts to culture morphologically and functionally mature TEMs that are less expensive and more environment friendly, while enabling high-throughput analysis. We anticipate that a high accessibility of this insert culture method will facilitate *in vitro* disease modelling, drug screening and potential treatment for muscle wasting related disorders. Different stages of the optimisation included adjustments of the insert diameter to fit within conventional 6-well culture plates; depth of the trough to make sure enough hydrogel/cell solution was prepared; design/length of the pillars to facilitate removal of the analysable TEM; size of the anchors to ensure gel attachment during the compactment period; position of the additional pillars (total 4) to enable culture medium (i.e., nutrients) perfusion throughout the culture period. The final version of the inserts resulted in myobundle formation with aligned/mature myotubes as indicated by cytoskeleton stainings of C2C12 TEMs. Most importantly, initial contractility measurements (twitch and tetanic force) of the C2C12 mouse muscle indicate the presence of functional properties of the inserts which more closely resemble *in vivo* skeletal muscle. Due to the novelty of the force transducer utilized in this study, the measurements displayed considerable variability. Consequently, not all collected data has been included, as certain force graphs exhibited atypical characteristics that raised doubts about their reliability. Furthermore, these results were disregarded because, in addition to data collection, the samples were employed for learning purposes, involving the implementation of various methodologies that did not consistently elicit the expected responses, such as altering or reducing muscle stretching. Consequently, the resulting graphs deviated from the patterns reported in previous literature. The measurements were reviewed by an academic from another institution, and both video evidence and data were shared with the manufacturer of the TEM. Based on their feedback, we have confidence in the functionality of the TEM and its response to stimulation. Observations indicate that the

TEM's contractile ability increased as it matured from day 7 to day 21 for all periods. This suggests that the TEM is capable of proliferation and differentiation, enabling a substantial augmentation in the forces it generates. In conclusion, the efforts dedicated to developing a TEM using a hydrogel formation and the finalized methodology have successfully resulted in an available TEM that can be assessed using a force transducer.

5.4 Conclusion

As an alternative to existing tissue engineered culture systems, we developed high throughput inserts to prepare TEM that could significantly benefit research on muscle ageing. Re-usable inserts provide an easily accessible tool to generate TEMs with structural and functional resemblance to native muscle, and with a size that enables both functional and downstream analysis (protein and gene expression).

Chapter 6: Main Discussion

6.1 General discussion

The ageing population is an economic burden on healthcare across the globe (Seals et al. 2016, Harper 2014). The increased proportion of elderly is accompanied by a higher prevalence of sarcopenia, metabolic diseases, falls, mortality and reduced independency (Cristea et al. 2020, Cesari et al. 2014, Dennison et al. 2017). Composing approximately 40% of body mass, maintaining skeletal muscle tissue throughout lifetime prevents many of these morbidities. Despite the conducted studies on animal and humans investigating muscle atrophy (Clemens et al. 2021, Brett et al. 2020, Brook et al. 2021, Gharahdaghi et al. 2019), underpinning mechanisms mediating sarcopenia are not completely established.

To better understand sarcopenia, the present Thesis i) examine the transcriptome of the ageing muscle using AI (chapter 3); ii) develop an *in vitro* model that enables cellular and molecular high-throughput investigations of muscle regeneration across ageing (chapter 4); iii) and develop two methods to generate TEM, one involving a bioreactor and the other using a cell culture insert recently invented and patented by our lab (chapter 5). Importantly, these methods enable identification of genes, interactions and potential biomarkers associated to ageing and exercise; to investigate muscle regeneration across ageing; and fabricate TEM using a well-established bioreactor system and novel cell culture inserts. These findings and models expand our current understanding of sarcopenia and provide new and high-throughput tools to further elucidate underlying molecular and cellular pathways regulating skeletal muscle mass during ageing, and to test potential therapies.

Advanced techniques are required to further shed light on muscle mass regulation. As such, artificial neural network inference (ANNI) analysis is a subset of AI machine-learning previously used to predict cancer and neurodegenerative disease related genes (Tran et al. 2021, Tong et al. 2014, Bhagwat et al. 2019). Although ANNI has contributed to a better

understanding of other diseases, it has not been used in the field of skeletal muscle mass regulation which motivated us to apply ANNI to discover new genes to better understand and predict sarcopenia or be used as targets for therapies that could benefit research on sarcopenia.

Tissue engineering is an advanced cell culture method to generate myobundles that recapitulates the structure and function of native muscle closer than conventional monolayer cultures (Rao et al. 2018, Rimington et al. 2021, Gholobova et al. 2020, Madden et al. 2015). However, established TE methods are either expensive, custom-made, low-fidelity or generate relatively small myobundles (Prüller et al. 2018, Somers et al. 2019, Heher et al. 2015, Khodabukus et al. 2019). Given the limitations, developing a TE method to produce high throughput, cost-effective and easily reproducible muscle constructs would reduce the ethical matter of animal models, improve drug screening and aid identification of potential interventions to combat sarcopenia.

6.2 Key findings

The expression of genes involved in mitochondrial protein synthesis, energy metabolism, cytokine signalling, cellular senescence, apoptosis and myogenesis are different in young and aged muscle (Robinson et al. 2017, Tumasian et al. 2021, Welle et al. 2003). In chapter 3, using the ANNI model to predict for the first time the most influential (drivers) and influenced (target) genes associated with muscular ageing and adaptions to exercise interventions identified genes previously not associated with age-related and/or exercise-induced changes in skeletal muscle. *USP54, JAK2, CHAD* and *ZDBF2* were the top genes associated with skeletal muscle ageing that also showed significant different expression (except *CHAD*) in aged vs young muscle when performing RT-PCR analysis on human biopsies (Figure 3.13). Similarly, distinct from previously reported genes related to skeletal

muscle adaptation during ageing, ANNI identified *EIF4A2, NIPAL3, SCFD1* and *KDM5D* as hitherto unidentified genes with the strongest interaction in aged muscle exposed to long-term resistance training. However, no differential expression of these genes was confirmed by RT-PCR (Figure 3.14) which could probably be due to differences in training regimens between the studies from which these genes were derived by ANNI and our samples that were of a shorter training duration (3 vs 6 months). Nevertheless, this does not exclude the non-activation of these genes in response to exercise as online MetaMEx database showed presence of these genes in previous transcriptome analyses of chronic strength training (Figure 3.15). The novel findings in Chapter 3 suggest that AI has a potential to benefit the field of muscle ageing and sarcopenia.

While altered transcriptome profile is one of the potential underlying causes of declined musculoskeletal health (Tumasian et al. 2021, Zeng et al. 2020, Han et al. 2021), impaired regeneration is considered to be another hallmark of sarcopenia (Brzeszczynska et al. 2018, Farup et al. 2015). To assess the regenerative response of aged muscle cells, we developed *in vitro* injury model using the chemical toxin $BaCl_2$ (Jung et al. 2019, Morton et al. 2019, Sicherer et al. 2020) to destroy the cytoskeleton of differentiated muscle cells to then evaluate the recovery from damage of young and aged human muscle cells. Experiments performed in Chapter 4 indicate that although the proliferation capacity was similar to that of young cells, aged muscle cells do not recover to pre-injury level (Figure 4.5). Interestingly, morphological findings were supported by RNAseq analysis where downregulation of GO processes related to muscle adaptation, contraction and hypertrophy was detected (Figure 4.13). The feasibility and reproducibility of the injury model opens further possibilities to study muscle ageing, responses to different stimuli and to test potential therapies mediating regeneration in skeletal muscle.

Although monolayer systems have been widely used to study regulation of skeletal muscle mass during ageing and disease (Tarum et al. 2017, Fleming et al. 2019, Dugan et al. 2014), they do not consider the 3D structure of a muscle nor the impact of the niche of the muscle

fibres and supporting cells, such a fibroblast, satellite cells and endothelial cells. To mimic the *in vivo* situation better, advanced tissue engineered models have been developed (Sharples et al. 2012, Madden et al. 2015, Khodabukus et al. 2019, Capel et al. 2019), such as those based on bioreactors, molds or custom-made set-ups. Thus, making TEMs is either expensive, low-throughput and/or complex. The novel high throughput inserts described in Chapter 5 were developed to fabricate TEMs cost-effectively and ecologically. Furthermore, morphological analysis on TEMs show aligned myotubes indicating the physiological structure of the myobundles. In addition, unlike available TE models, novel re-usable inserts enable to produce numerous TEMs simultaneously making it a high-throughput and time-efficient method to facilitate research on muscle ageing and adaptations to different stimuli such as injury, electrical stimulation and knock-down of selected age-related genes.

Chapter 7: Conclusion and Future Work

The work presented in this thesis describes developed methods for investigating skeletal muscle ageing at the transcriptome, cellular and structural level. These different models provide insight in the age-related changes in gene expression and regenerative response to *in vitro* injury while also introducing newly developed inserts to generate tissue engineered muscle.

Artificial neural network analysis identified genes that were differently expressed in young and old muscles and during adaptations to long-term strength training in older people. By applying AI-derived network analysis for the first time in the field of the musculoskeletal system, genes and interactions were discovered that had previously not been reported as potential biomarkers of muscle ageing and exercise. These findings encourage the use of advanced AI bioinformatic analysis to discover genes and associated signalling pathways underpinning muscle loss.

The developed muscle model to examine regeneration generated additional data on muscle adaptations during ageing. More specifically, RNAseq, cell cycle and morphological analyses confirmed impaired repair capacity of older vs younger cells in response to injury that was not attributable to impaired proliferation.

To study skeletal muscle and associated conditions more closely, advanced tissue engineered models have been developed that more closely mimic *in vivo* muscle than traditional mono-culture monolayers. Given the costly and low-throughput methods currently available for TE, in this study an insert suitable for conventional 6-well plates to fabricate TEM was developed. The 3D-printed biocompatible inserts are re-usable and eco-friendly to generate TEM in high quantities thereby facilitating efficiency of research conducted on muscle ageing.

The findings in this thesis provide a robust framework for future studies that could elucidate underlying mechanisms of muscle ageing. The main driver and target genes identified by ANNi analysis, with differential expression of these genes in human muscle biopsies

confirmed by RT-PCR, suggest additional *in vitro* experiments to either check the expression or perform knockdown of these genes in insert-based TEM of aged human muscle cells. In addition, the *in vitro* injury monolayer model could be replicated on TE inserts to assess human skeletal muscle regeneration using an advanced method. Furthermore, pre-liminary experiments have shown the force generating potential of the developed inserts which could be a basis for future experiments to assess the repair process of the stimulated myobundles following injury. Thus, evaluating the potential role of exercise in regeneration capacity of (aged) muscle. In addition, assessing ANNi-identified gene expressions in contracting insert-TEMs would add to the knowledge of underpinning molecular pathways in exercised aged muscle.

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