The Role of Platelets in Skeletal Muscle Regeneration

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Abstract

Overview: Biomaterials such as autologous platelet-based applications have gained intense research interest. However, the mechanisms driving platelet-mediated skeletal myogenesis remain to be established. Therefore, the aim of this thesis was to optimise and determine the role of platelet releasate for skeletal myogenesis *in vitro*, *ex vivo* and *in vivo*.

Methods: Skeletal and cardiac myoblast proliferation and differentiation in response to platelet releasate was assessed by means of proliferation assays, immunohistochemistry and gene expression. We expanded these *in vitro* findings on murine single muscle fibre stem cells *ex vivo* using protein expression profiles for key myogenic regulatory factors. Most importantly, we validated these findings on an *in vivo* model of acute muscle injury *via* cardiotoxin. Finally, platelet secretomes were studied with their effect on the skeletal muscle function of a murine model of thrombospondin-1 deficiency - a potent angiostatic and pro-inflammatory factor.

Results: Preparation of platelet releasate with TRAP6 and collagen had a more pronounced effect on myoblast proliferation versus thrombin and sonication ($p<0.05$). Additionally, platelet concentration positively correlated with myoblast and cardiomyocyte proliferation. Platelet releasate increased skeletal myoblast and muscle stem cell proliferation and differentiation in a dose-dependent manner, which was mitigated by VEGFR and PDGFR inhibition. This inhibition ablated MyoD expression on proliferating muscle stem cells, compromising their commitment to differentiation ($p<0.001$). Platelet releasate was detrimental for myoblast fusion in a temporal manner. Most importantly, platelet releasate accelerates skeletal muscle regeneration *in vivo* after acute injury, and may reduce cellular senescence. Furthermore, we report for the first time that depletion of thrombospondin-1 from platelet releasate promotes more potent myoblast proliferation and skeletal muscle regeneration as compared to wild-type.

Conclusion: This study provides novel mechanistic insights on the role of platelet releasate in skeletal myogenesis and set the physiological basis for exploiting platelets as biomaterials in regenerative medicine.

Table of Contents

List of Figures

List of Tables

List of Abbreviations for tables 1.1, 1.2 & 1.3

%; percentage, **°C**; degree Celsius, ↑; an increase, ↓; a decrease, **A-PRF**; advanced-platelet-rich fibrin, **ACD**; anticoagulant citrate dextrose solution, **Akt**; protein kinase B, **ASC**; adipose derived stem cell, **BC**; bone cell, **bFGF**; basic fibroblast growth factor, **BMSCs**; bone marrow stromal cells, **C2C12**; mouse myoblast cell line, **C57bl6/J**; C57 black 6 mouse, **CAL-72**; human osteoblast cell line, **CAM model**; chicken chorioallantoic membrane model, **cbfa1**; core binding factor alpha 1, **CCl4**; chemokine (C-C motif) ligand 4, **cdk1,2;** cyclin-dependent kinase 1,2, **CGF**; concentrated growth factors, **COX-2;** prostaglandin-endoperoxide synthase 2, **CXCR4**; C-X-C chemokine receptor type 4, **DMEM**; dulbecco's modified eagle's medium, **DMEM/F-12**; dulbecco's modified eagle medium: nutrient mixture F-12, **EGF**; Epidermal growth factor, **EGM-2 SingleQuots complete medium**; endothelial cell growth medium, **EGM2-MV**; endothelial cell growth medium, **ELISA**; enzyme-linked immunosorbent assay, **EPC;** endothelial progenitor cell, **ERK1/2;** extracellular signal–regulated kinases, **FBS;** foetal bovine serum, **FCS;** foetal calf serum, **HEPES;** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, **hFOB;** transformed human osteoblasts, **HGF;** hepatocyte growth factor, **hMDPCs;** human muscle-derived progenitor cells, **HMEC-1;** human microvascular endothelial cell line, **HOS;** human osteosarcoma cells, **HSMM;** human skeletal muscle myoblast, **HSP;** heat shock protein, **HUVECs;** human umbilical vein endothelial cells, **Ibpva55;** normal human articular chondrocytes, **IGF-1Eb;** insulin-like growth factor-1 isoform-Eb, **IL-4;** interleukin 4, **IL-6;** interleukin 6, **IU;** international unit, **L-PRP;** leukocyte- and platelet-rich plasma, **MCF-7;** breast cancer cell line, **mL;** millilitres, **mM;** millimolar, **MMP-13;** matrix metalloproteinase-13, **MMP-3;** matrix metalloproteinase-3, **Mod-PRP;** modified PRP, **MPCs;** muscle progenitor cells, **MRFs;** myogenic regulatory factors, **mRNA;** messenger ribonucleic acid, **MSTN;** myostatin, **Myf5;** myogenic factor 5, **MyoD1;** myogenic differentiation 1, **myomiRNA**s; myo-micro ribonucleic acids, **NF-kB;** nuclear factor kappa-light-chain-enhancer of activated B cells, **NIH-3T3;** mouse fibroblast cell line, **NPRS;** Nirschl Phase Rating Scale, **P-PRP;** pure platelet-rich plasma, **PAR1;** thrombin protease- activated receptor 1, **PAR1-PR;** PAR1-protein releasate, **PAR4;** thrombin protease- activated receptor 4, **PAR4-PR;** PAR4-protein releasate, **Pax7;** paired box protein 7, **PBS;** phosphate-

buffered saline, **PCL;** poly(ε-caprolactone), **PCNA;** proliferating cell nuclear antigen, **PDGF;** platelet-derived growth factor, **PL**; platelet Lysate, **PMC**; platelet mediator concentrate, **PPP;** platelet-poor plasma, **PRGF**; plasma rich in growth factors, **PRGF**; Platelet Rich in Growth Factors, **PRP**; platelet-rich plasma, **PRP-Exos**; platelet-rich plasma-exosomes, **PRS**; platelet-released supernatant, **rMSCs**; rat muscle stem cells, **rpm**; rotations per minute, **RPMI-1640**; Roswell Park Memorial Institute 1640 medium, **SaOS-2**; Sarcoma osteogenic cell line, **SC**; Sodium Citrate, **SkBM-2**; skeletal muscle growth basal medium 2, **TGF-β**; transforming growth factor beta, **TGF- β1**; transforming growth factor-beta1, **TNF-α**; tumour necrosis factor-alpha, **TSP-1**; thrombospondin 1, **TXA2**; thromboxane A2, **U937 cells**; myeloid lineage cell line, **uL**; microliters, **VAS**; visual analogue scale, **VEGF**; vascular endothelial growth factor, **αMEM**; α-modification of minimum essential medium, **µg**; micrograms.

Thesis outputs

Research papers

Scully, D., Sfyri, P., Verpoorten, S., Papadopoulos, P., Muñoz-Turrillas MC., Mitchell, R., Aburima, A., Patel, K., Gutiérrez, L., Naseem, KM., Matsakas, A. Platelet releasate promotes skeletal myogenesis by increasing muscle stem cell commitment to differentiation and accelerates muscle regeneration following acute injury. ***Acta Physiologica***. 19 Oct 2018:e13207

Review articles

Scully, D., Naseem, K., Matsakas. A. Platelet Biology in Regenerative Medicine of Skeletal Muscle. ***Acta Physiologica***. 2018 Apr 06:e13071

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External Conferences

Scully, D., Sfyri, P., Verpoorten, S., Mitchell, R., Gutierrez. L., Patel, K., Matsakas, A. Skeletal Myogenesis is accelerated by key growth factors contained in the human platelet secretome. Poster presentation in **Physiology 2019**, The Physiological Society, Aberdeen, UK Jul 1, 2019

Scully, D., Sfyri, P., Verpoorten, S., Matsakas. A. Platelet releasate as a key driver for skeletal myogenesis. Poster in **Muscle Wasting Conference**, Ascona, Switzerland 24 Sep 2018

Scully, D., Sfyri, P., Verpoorten, S., Papadopoulos, P., Aburima, A., Gutierez, L., Naseem, K., Matsakas. A. Platelet-based applications for skeletal myogenesis and muscle stem cells with implications for regenerative medicine. Poster in **EUPLAN Conference** 2018, Bruges, 19 Sep 2018.

Scully, D., Sfyri, P., Verpoorten, S., Papadopoulos, P., Aburima, A., Gutierez, L., Naseem, K., Matsakas. A. Skeletal myogenesis can be driven by key growth factors contained in human platelets. Poster and Oral presented at the **Europhysiology 2018** conference, London 14 Sep 2018.

Scully, D., Sfyri, P., Verpoorten, S., Naseem, K., Matsakas, A., The cellular mechanisms driving skeletal myogenesis can be stimulated using human platelet-based applications for use in

regenerative medicine. **Poster in the North of England Cell Biology Forum**; 2018 Aug 29; Huddersfield, England

Scully, D., Sfyri, P., Verpoorten, S., Naseem, K., Matsakas, A., The Effect of Platelet Releasate on Myoblast Proliferation and Differentiation Profiles. **Poster in the North of England Cell Biology Forum**; 2017 Sep 15; Hull, England

Scully, D. Sfyri, P. Naseem, K. Matsakas, A. Understanding the Mechanisms Connecting Platelets to Skeletal Myogenesis. **Poster in the H3 symposium Muscle physiology and metabolism**, London, UK, Nov 2017.

Internal events

Scully, D., Sfyri, P., Verpoorten, S. Naseem, K. Matsakas, A. The Effect of Platelet Releasate on Myoblast Proliferation and Differentiation Profiles. **Poster in Hull York Medical School (HYMS) Postgraduate Student Conference**, Hull, UK, May 2017

Scully, D., Sfyri, P., Verpoorten, S., Papadopoulos, P., Aburima, A., Gutierez, L., Naseem, K., Matsakas. A. A novel role of human platelets for skeletal myogenesis and muscle stem cells with implications for regenerative medicine. Poster presentation in the **Allam Lecture 2018**, Hull York Medical School Apr 20, 2018. (**Awarded prized poster presentation**)

Scully, D., Sfyri, P., Verpoorten, S., Mitchell, R., Aburima, A., Gutiérrez, L., Naseem, KM., Patel, K., Matsakas, A. Exploitation of the human platelet secretome as a biomaterial for skeletal muscle regeneration. Poster presentation in the **Allam Lecture 2019**, Hull York Medical School Mar 29, 2019.

Scully, D., Sfyri, P., Verpoorten, S., Wilkinson, H., Aburima, A., Gutiérrez, L., Mitchell, R., Hardman, M., Patel, K., Matsakas, A. Novel insights into the use of platelet releasate as a therapeutic approach for tissue regeneration. Poster presentation in the **Allam Lecture 2019,** Hull York Medical School Mar 29, 2019.

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This thesis is dedicated to my grandfather Thomas Scully.

Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

Chapter 1 - General Introduction

Platelet Biology in Regenerative Medicine of Skeletal Muscle

1.1 Overview

Platelet-based applications such as platelet-rich plasma and platelet releasate have gained unprecedented attention in regenerative medicine across a variety of tissues as of late. The rationale behind utilising platelet-rich plasma originates in the delivery of key cytokines and growth factors from platelet α-granules to the targeted areas, which in turn act as cell cycle regulators and promote the healing process across a range of injuries. The aim of the present introduction is to assimilate current experimental evidence on the role of platelets as biomaterials in tissue regeneration, particularly in skeletal muscle, by integrating findings from human, animal and cellular studies. Firstly, key aspects of platelet biology preceding the preparation and use of platelet-based applications for tissue regeneration are reviewed. Secondly, relevant evidence on platelet-mediated regeneration in skeletal muscle focusing on findings is critically discussed from a) clinical trials, b) experimental animal studies and c) cell culture studies. Thirdly, the application of platelets in the regeneration of several other tissues including tendon, bone, liver, blood vessels and nerves are discussed. Finally, key technical variations in platelet preparation that may account for the large discrepancy in outcomes from different studies are reviewed. This introduction provides an up-to-date reference tool for biomedical and clinical scientists involved in platelet-mediated tissue regenerative applications.

1.2 Introduction

Platelets, also called thrombocytes, are produced from megakaryocyte projections into micro-vessels in mammalian bone marrow. Freely circulating platelets are the first cellular response following damage to vascular or tissue integrity and play a crucial role in haemostasis, innate immunity, angiogenesis and wound healing (Frojmovic and Milton, 1982). The latter aspect is receiving increased attention since the wound healing effects suggest a platelet-orientated regenerative ability for maintaining whole body integrity and homeostasis (Dimauro et al., 2014, De Pascale et al., 2015, Foster et al., 2009). Until recently, platelet-rich plasma (PRP; defined as a biologically active, autologous concentration of platelets re-suspended in plasma), was extensively used in the medical fields of connective tissue regeneration and thrombosis research, while the study of the regenerative potential of PRP in a clinical context attracted less attention. Although our understanding of the mechanisms linking platelet biology to tissue regeneration is still evolving, many aspects remain to be established due to inconsistent and conflicting scientific evidence (Stellos et al., 2010).

Data from clinical studies on the effectiveness of platelet-rich plasma applications appear to be conflicting or limited to outcomes such as improvement of quality of life, reduction of post-operative pain, improved healing or absence of any beneficial effect. This has been attributed to possible methodological differences of preparation, PRP composition, medical condition of the patients, anatomical location of the lesions as well as the type of injured tissue and has been discussed in relevant reviews of clinical interest (Navani et al., 2017, Sánchez et al., 2014, Mosca and Rodeo, 2015). Growth factors and cytokines have a crucial role in the healing process with regards to early inflammation in tissue regeneration (Borrione et al., 2014). Therefore, the rationale for utilising autologous PRP originates in the ease of availability to access and apply numerous cytokines and growth factors to the targeted area, acting as biomaterials to promote regeneration (**Figure 1.1**)(Fong et al., 2011, Masuki et al., 2016, Coppinger et al., 2004, Mumford et al., 2015, Wijten et al., 2013). These factors in turn upregulate proliferation, differentiation and migration of necessary cells in the area of regenerating tissue (Foster et al., 2009). Over the past decade, there have been amounting articles contributing to the knowledge surrounding the mechanisms of growth factors in the

regeneration of wounded or dysfunctional tissue (Dimauro et al., 2014, Martins et al., 2016, Pinheiro et al., 2016, Li et al., 2016b). Due to increasing understanding in cell signalling and growth factor biology, research and clinical attention has been drawn to the use of autologous PRP as a novel means of delivering growth factors to injured tissue such as liver, bone and skeletal muscle (See **Table 1.1:** muscle tissue regeneration**, Table 1.2:** tissue regeneration and **Table 1.3:** *in vitro* cell studies).

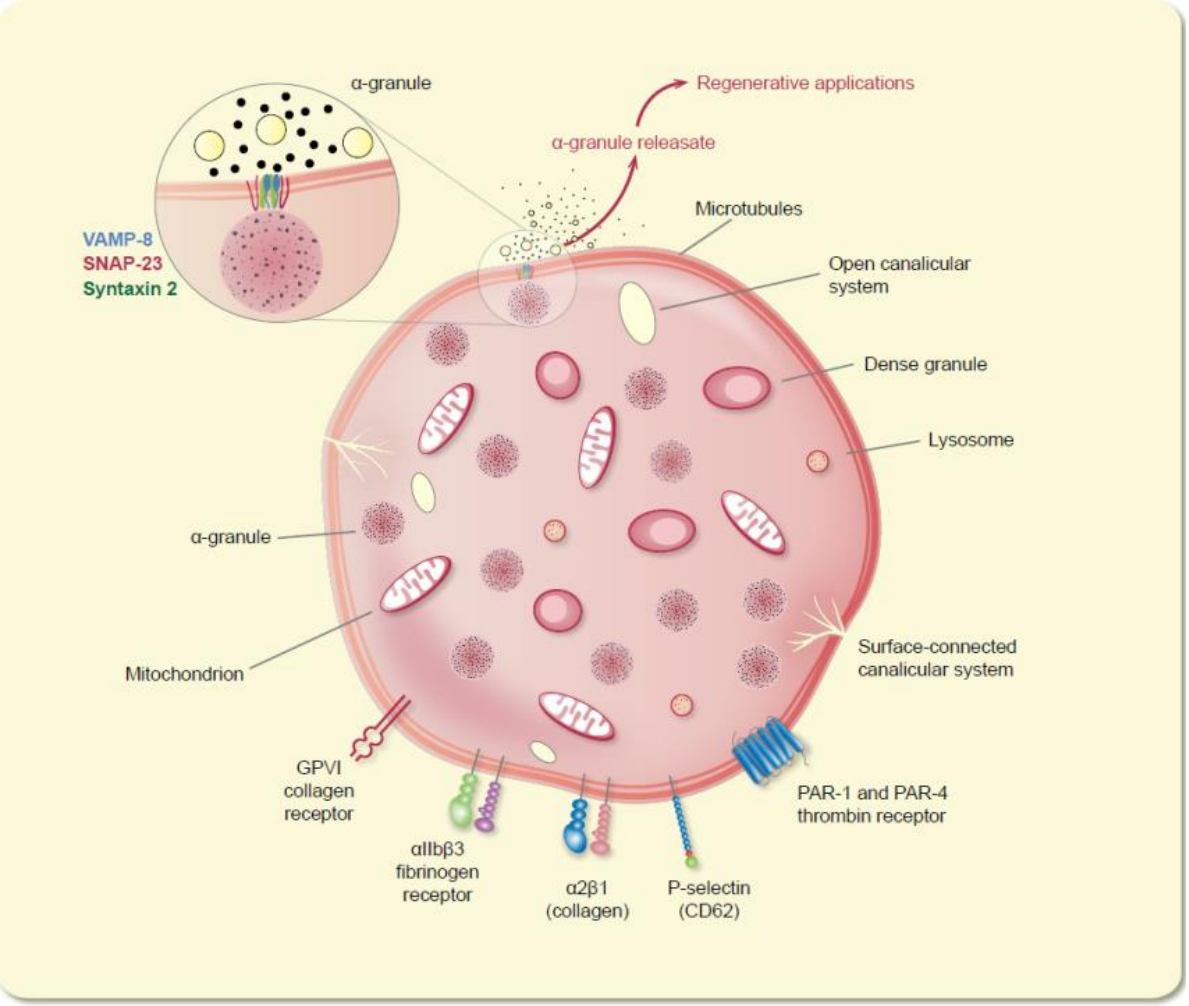


Figure 1.1 Schematic of a platelet highlighting the key surface receptors, organelles aggregation factors. These contain: adhesive proteins, clotting factors and their inhibitors, fibrinolytic factors and their inhibitors, proteases and anti-proteases, growth and mitogenic factors, chemokines, cytokines, membrane glycoproteins and anti-microbial proteins. The platelet releasate may be further used as a biomaterial in numerous applications of regenerative medicine.

Table 1.1 Platelets and skeletal muscle regeneration

Reference	Species	Intervention	Findings
(Borrione et al., 2014)	Rat	**PRP** on a flexor sublimis lesion	↑ leucocyte infiltration; ↑ early inflammatory response post muscle injury
(Dimauro et al., 2014)	Rat	**PRP** on flexor sublimis incision	↑ mRNA of pro-inflammatory cytokines, MRFs & IGF-1Eb; ↓ myo-miR-133a
(Hammond et al., 2009)	Rat	**PRP** on tibialis anterior under muscle strains	↑ myogenesis ↓ time-to-recovery after a muscle strain
(Pinheiro et al., 2016)	Rat	**PRP on** gastrocnemius muscle injury	↓ pain/claudication score
(Martins et al., 2016)	Rat	**PRP** in gastrocnemius contusion	↓ oxidative stress and ↑ enzymatic antioxidants in injured skeletal muscle
(Huang and Wang, 2012)	Rat	**PRP**-derived growth factors on rat muscle satellite cells	↑ Proliferation & osteogenic differentiation ability of satellite cells from rat masticatory muscle.
(Tsai et al., 2017)	Rat	**Rat releasate** on rat gastrocnemius muscle cells *in vitro*.	↑ proliferation; ↑ Cyclin A2, B1, cdk1, cdk2 and PCNA of protein expression (dose dependently)
(Li et al., 2016)	Rat	TGF- β1 neutralisation in PRP on a cardiotoxin-induced muscle injury model	↑ muscle regeneration; ↓ fibrosis; ↑ angiogenesis; prolonged satellite cell activation; ↑ M2 macrophages to the injury site
(Takase et al., 2017)	C2C12 myoblasts and Rat	A. **Human releasate** on C2C12 murine myoblasts. B. **Rat PRP** on **rat rotator cuff tear**	↑ Proliferation; Inhibited myogenic differentiation; ↓ expression of adipogenic genes & lipid droplet formation *in vivo*
(Denapoli et al., 2016)	Mouse	Muscle contusion injury & PRP at different time points	PRP injection 7 days after injury ↑ exercise time; ↓ fibrotic tissue; PRP at 1 and 4 days after injury ↓ exercise time; ↑ fibrotic tissue
(Notodiharjo et al., 2015)	Mouse	Gelatin hydrogel with platelet **releasate** in wound healing	↑ levels of angiogenesis ↑ wound healing rate
(Li et al., 2013)	Mouse	Human **releasate** on muscle-derived progenitor cells	↑ Proliferation of hMDPCs; PDGF further increases the proliferative effects of PRP
(Im et al., 2014)	Rabbit	Rabbit **PRP** with ASC extracts on rabbit myogenic progenitors and **Human** fibroblast culture	ASCs extracts had a stronger effect on proliferation of MPCs than PRP

(Bernuzzi et al., 2014)	Human athletes	**PRP** in grade II muscle lesions	↓ Pain in all patients and improved muscle function in 85% of patients after first injection. ↓ VAS two weeks post-treatment. 100% return to sport activities after 35 days (Non-controlled study)
(Bubnov, 2013)	Human athletes	**PRP** in acute muscle injury	93% ↓ pain after 28 days versus 80% in control; ↑ range of motion and strength
(Wetzel et al., 2013)	Human Patients	**PRP** in proximal hamstring injuries	↓ VAS and NPRS scores
(Ranzato et al., 2009)	C2C12 myoblasts	**Human PRP lysate** on C2C12 murine myoblasts	↑ C2C12 proliferation up to 20% PL but mildly cytotoxic at 100%; ↑ C2C12 scratch wound closure
(Miroshnychenko et al., 2017)	Human (*Ex Vivo)*	1) PRP 2) Releasate with depleted TGF-β1 and myostatin 3) PPP; in human skeletal muscle myoblasts	PPP and Releasate with depleted TGF-β1 and myostatin induced myoblast differentiation; ↑ myoblast proliferation with PRP
(Sassoli et al., 2018b)	C2C12s & satellite cells	PRP + BM-MSC	↑ proliferation & differentiation

- **Abbreviations for tables 1.1, 1.2 & 1.3 are in List of Abbreviations on page 14**

Skeletal muscle is a highly plastic tissue with remarkable capacity to regenerate in response to injury and trauma. The early acceleration of muscle regeneration, specifically within the first week, is a crucial time point to implement a clinical intervention due to the early inflammatory response as well as the regeneration phase taking place (Schnabel et al., 2007, Borrione et al., 2014, Dimauro et al., 2014). Therefore, understanding the molecular and physiological mechanisms that link platelet biology to tissue regeneration has the potential to identify novel opportunities in regenerative medicine in the near future.

The clinical aspects of platelet-based applications for orthopaedic regeneration in muscle, ligaments and tendons are discussed in recent relevant reviews (Mosca and Rodeo, 2015, Navani et al., 2017, Andia and Abate, 2017, Qian et al., 2017). In the present introduction, current experimental evidence on the role of platelets as biomaterials in tissue regeneration is assimilated, particularly in skeletal muscle. Firstly, key aspects of platelet biology that precede the preparation and use of platelet-related applications for tissue regeneration are reviewed. Secondly, a critique of the evidence for platelet-mediated regeneration in skeletal muscle focusing on findings

from a) clinical trials, b) experimental animal studies as well as c) cell culture studies is provided. Thirdly, the application of platelets in the regeneration of several other tissues including tendon, bone, liver, vessels and nerve is discussed.

1.3 Overview of Platelet Biology

The use of autologous PRP in clinical research has grown exponentially over recent years due to the gradually increasing understanding in the role of PRP's growth factors in tissue regeneration (Stellos et al., 2010) (**Figure 1.2**). The first publication on PRP was issued in 1954 (Kingsley, 1954). Ten years later, the first study of PRP being utilised in a therapeutic scenario was published (Levin and Freireich, 1964). This increasingly attractive therapeutic tool has made considerable advancements in many areas of regenerative medicine, particularly in the areas of wound healing and skin regeneration, dentistry, plastic and cosmetic surgery, minor wounds, fat grafting, bone regeneration, tendinopathies, ophthalmology, hepatocyte recovery, aesthetic surgery, orthopaedics, veterinary, spinal fusion, treatment of soft-tissue ulcers, heart bypass surgery and last but not least in skeletal muscle injuries (Kazakos et al., 2009, Liao et al., 2014, Cervelli et al., 2009, Frechette et al., 2005, Celotti et al., 2006, Virchenko et al., 2006, Arslan et al., 2016, Hesami et al., 2014, Kanno et al., 2005, Elgazzar et al., 2008, Masoudi et al., 2016, Dimauro et al., 2014, Li et al., 2013, Li et al., 2016a, Li et al., 2016b). Before discussing current evidence of the role of platelets in tissue regeneration, key aspects of platelet biology such as platelet formation, activation and aggregation that precede the release of growth factors and the preparation of PRP are reviewed.

1.3.1 Platelet formation and activation

Hematopoietic stem cells in the red bone marrow give rise to common myeloid progenitor cells which further differentiate to megakaryocytes (Machlus and Italiano, 2013). Platelets are anucleated products formed from long extensions into vascular sinusoids after migration of the megakaryocytes to the vascular niche (Kossev and Sokolov, 2015, Machlus and Italiano, 2013). Vascular injury leads to exposure of prothrombotic extracellular matrix proteins, which facilitate platelet adhesion and activation. In addition to minimising blood loss, a major function of platelets is to

promote healing of the damaged tissue. This is achieved through the release of cytokines, chemokines and growth factors from platelet granules. There are three major types of secretory granules in platelets including: i) α-granules, containing many growth factors and cytokines ii) dense γ-granules, which release calcium, serotonin, polyphosphates, pyrophosphates, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and iii) lysosomes, which contain a number of hydrolytic enzymes (Mumford et al., 2015).

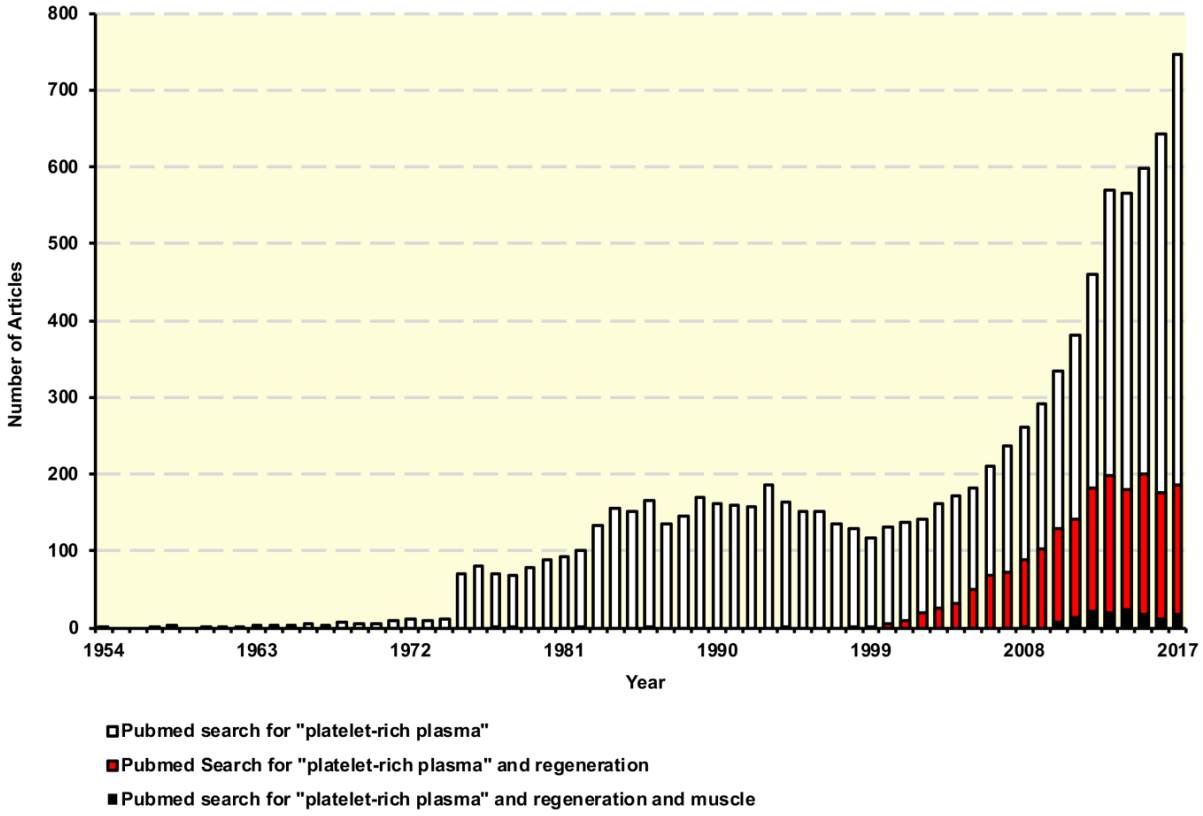


Figure 1.2 PubMed search for publications on a) "platelet-rich plasma"; b) "platelet-rich plasma" AND regeneration; and c) "platelet-rich plasma" AND regeneration AND muscle between 1954 and 2017. The diagram reveals that the publication of articles on platelet-rich plasma (PRP) have increased exponentially in the last two decades (white bars), while concomitantly scientific interest emerged for exploiting PRP in regenerative applications (red bars). Additional interest to use PRP for skeletal muscle regeneration has developed over the last decade (black bars).

In particular, there are approximately 50-80 α-granules per platelet with a typical diameter of 200–500 nanometres that can be released intracellularly or extracellularly (Frojmovic and Milton, 1982, Yeaman, 2014). Alpha-granule contents secreted by activated platelets release growth factors such as: platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), insulin-like growth factor (IGF), epithelial growth factor (EGF), endothelial cell growth factor (ECGF), and fibroblast growth factor (FGF) (Bendinelli et al., 2010). Upon platelet activation, degranulation follows and release of trophic factors occurs (Jedlitschky et al., 2012) (**Figure 1.1**). It has been suggested that interaction of these factors with the hampered tissue structures causes an ameliorated and accelerated healing response (Borrione et al., 2014).

1.3.2 Platelet aggregation

It is important to understand the mechanisms that govern platelet aggregation, as variations in aggregation strength will cause deviations in growth factor release (Cavallo et al., 2016). Differing aggregation procedures in clinical trials may be one of causes of conflicting results. Damaged cells or injured tissue cause a release of soluble platelet agonists such as ADP and thrombin, which act as platelet-activating factors. These signalling events then allow processes such as platelet spreading, consistent adhesion, granule secretion and clot retraction to occur (Li et al., 2013). In order to exocytose contents, α-granule's vesicle-associated membrane protein 8 (VAMP-8), synaptosomal-associated protein 23 (SNAP-23) and syntaxin 2 (a Q-SNARE proteins participating in exocytosis) are involved (**Figure 1.1**). After an agonist binds to its receptor, platelet shape-change occurs, followed by aggregation and granule content release.

The maximal secretion of granules, pro-coagulation, and clot-retraction responses of platelets involve binding of ligands to an integrin complex found on the platelet's glycoprotein Iib/IIIa (αIIbβ3) receptor and local platelet-platelet interaction (Shattil and Newman, 2004). Integrins are heterodimeric (i.e. αβ) type I transmembrane receptors. Platelets express these integrins as well as others such as αVβ3; α2β1; α5β1; α6β1 (Shattil and Newman, 2004). These integrins signal through a transmembrane

interaction with cognate extracellular matrix ligands orientated outside the cell and interact with cytoplasmic proteins internally. Binding at either end with ligands can stimulate cellular functions at the opposing end. Binding of adhesive ligands to the integrin αIIbβ3 can be triggered by soluble agonists such as ADP, epinephrine, thrombin, and thromboxane A2 (TXA2). These agonists then engage cognate G-protein–coupled receptors (Daniel et al., 1998). The primary receptor for von Willebrand factor (vWF); GPIb-IX-V, a platelet adhesion receptor, as well as GPVI and Iib3 can activate platelet aggregation signalling when bound and clustered by extracellular matrix ligands (Shattil and Newman, 2004). Platelets express several collagen receptors such as integrin α2β1 (see **Figure 1.1**). This receptor is vital for platelet adhesion to collagen, but collagen-induced platelet activation is prompted by the receptor glycoprotein VI (GPVI). This is important to note, as damaged cells or injured tissue releases soluble platelet agonists such as adenosine triphosphate (ADP) and thrombin in response to inflammation from platelet-activating factor, all of which contribute to platelet activation and thrombus formation. Other soluble platelet agonists are serotonin and TXA2 which are released from activated platelets, which serve to intensify platelet activation and adhere to additional circulating platelets (Shattil and Newman, 2004). The principal platelet integrin, αIIbβ3, is essential for platelet interactions with proteins in extracellular matrices (ECMs) and plasma that are important for platelet aggregation and adhesion for both haemostasis and thrombosis. Ligand-to-integrin αIIbβ3 binding both facilitate platelet adhesion and aggregation as well as initiating a series of intracellular signalling events. These signalling events then allow processes to occur such as platelet spreading, consistent adhesion, granule secretion and clot retraction (Li et al., 2013). It is important to note that VAMP-8, SNAP-23 and Syntaxin 2 are involved with the α-Granule exocytosis and release of growth factors and cytokines as visualised in **Figure 1.1**.

Platelet aggregation occurs through multiple adhesive ligands, such as vWF, fibrinogen, and fibronectin (Jackson, 2007). These three ligands have comparable binding affinities to the integrin αIIbβ3 when activated. Fibrinogen, a glycoprotein that is converted enzymatically by thrombin to fibrin, is suggested to be the primary ligand with regards to platelet aggregation in low shear environments (Jackson, 2007). With

cumulative shear pressure on the aggregation processes and platelets, the provocation of aggregation becomes gradually more reliant on vWF and fibronectin. The importance of vWF in initiating platelet-platelet adhesion occurs in a niche of rapid blood flow, where it contributes to platelet signalling over a much broader and lower blood shear range. vWF has a particularly large multimeric structure that offers a range of binding sites for the potential platelet receptors to bind (Yeaman, 2014, Jackson, 2007). Similar to vWF, fibronectin affects platelet adhesion and aggregation as cross-linking polymerized fibrin with fibronectin and added plasma fibronectin in combination enhanced platelet thrombus accumulation (Cho and Mosher, 2006). Fibronectin can bind to fibrinogen and thrombospondin, both involved in aggregation, a process that may increase the stability of platelet-platelet adherences (Jackson, 2007). Of note, the mechanism of platelet aggregation is well established (Clemetson, 2012, Smolenski, 2012, Yeaman, 2014, Jackson, 2007, Li et al., 2013).

1.3.3 The biological role of platelet releasate

Several studies have focused on platelet releasate, which is a refined, centrifuged and purified sample of growth factors released from aggregated platelets when the supernatant is collected, and cellular debris removed (Jiang et al., 2017, Chan et al., 2005, Wijten et al., 2013). Soluble bioactive molecules in the releasate secreted by the α-granules of platelets are known to enhance matrix synthesis (e.g. TGF- β), upregulate chemo-attraction and proliferation in several cell types (e.g. PDGF, VEGF, IGF-I and II, EGF and ECGF) (**Figure 1.3**) and angiogenesis (e.g. VEGF, FGF and ECGF) (Foster et al., 2009, Zammit et al., 2006a, Zammit et al., 2006b). Of note, proteins released by the α-granules, such as platelet factor 4, are inhibitors of angiogenesis, additionally; endostatins are inhibitors of endothelial cell migration. These bioactive molecules may be involved in negative feedback mechanisms to fine tune the main growth factors such as VEGF which upregulates angiogenesis, or adhesive proteins such as fibronectin that promotes cell migration and differentiation (Cavallo et al., 2016). This opposing effect is essential to create the ability for homeostasis within the injured area capable to react to the surrounding environment.

Figure 1.3

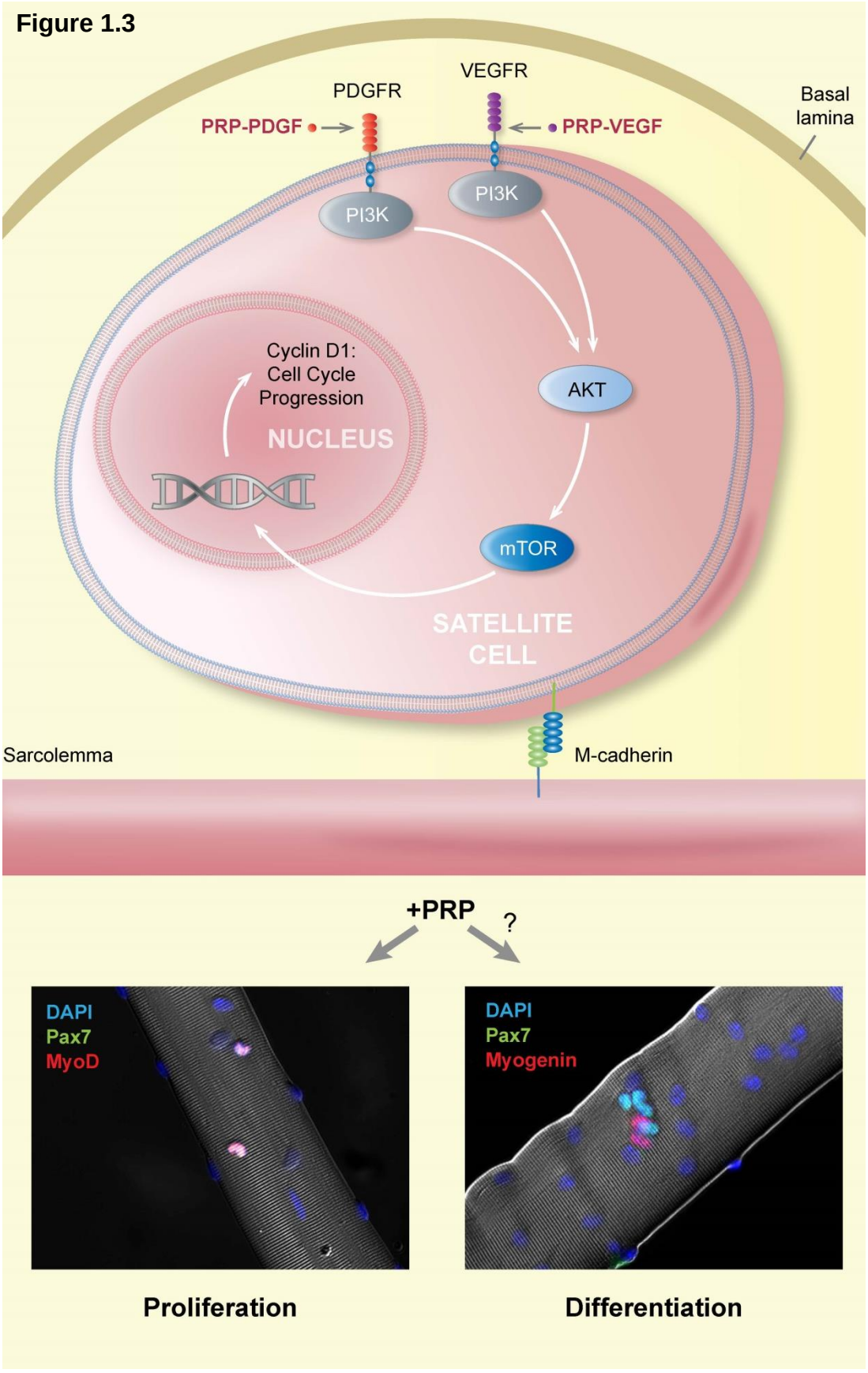

PDGFR
VEGFR
Basal lamina
PRP-PDGF
PRP-VEGF
PI3K
PI3K
Cyclin D1:
Cell Cycle
Progression
NUCLEUS
AKT
mTOR
SATELLITE CELL
Sarcolemma
M-cadherin
+PRP
?
DAPI
Pax7
MyoD
DAPI
Pax7
Myogenin
Proliferation
Differentiation

Figure 1.3 A schematic diagram showing a skeletal muscle stem cell's (i.e. satellite cell) possible response to growth factors, based on current published evidence (Tsai et al., 2017, Li et al., 2016a, Li et al., 2016b, Li et al., 2013, Miroshnychenko et al., 2017, Dimauro et al., 2014, Moench et al., 2016, Sassoli et al., 2014, Sassoli et al., 2018a). Located between the basal lamina and the sarcolemma of the muscle fibre, the satellite cell may come into contact with hundreds of growth factors and cytokines in response to platelet-rich plasma (PRP) administration. For simplicity, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which are known to be contained in PRP are presented. PDGF and VEGF interact with tyrosine kinase receptors and induce the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway to drive cell proliferation through transcription factors such as Cyclin D1 (Yuasa et al., 2004, Montarras et al., 2013, Moench et al., 2016, Hamilton et al., 2010, Yin et al., 2013, Arsic et al., 2004). The impact of PRP on muscle progenitor cell differentiation is currently debated and remains to be established.

Growth factors found in releasate are involved in promoting tissue regeneration, such as EGF which causes cell growth, recruitment and differentiation as well as cytokine exocytosis and secretion (Thornton et al., 2015). Similarly, the growth factor PDGF-BB (homodimers PDGF -AA, -BB, -CC, and –DD and the heterodimer PDGF-AB) has a physiological effect such as causing significant cell growth, cell migration, blood vessel growth, granulation, growth factor secretion and matrix formation with bone morphogenetic proteins (BMPs) (Yablonka-Reuveni et al., 1990, Yablonka-Reuveni and Rivera, 1997). The role of PDGF-BB following muscle damage is still yet to be determined (Pinol-Jurado et al., 2017). It has been shown, however, that PDGF-BB upregulates the proliferation of satellite cells (i.e. skeletal muscle stem cells) and may affect differentiation negatively (Arsic et al., 2004, Jin et al., 1991). Conversely, PRP application on satellite cells was shown to improve differentiation, indicating that the role of PRP on muscle progenitor cell differentiation has yet to be elucidated (Sassoli et al., 2018b, Miroshnychenko et al., 2017). Additionally, the VEGF and PDGF pathways both interact through the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway to induce proliferation of satellite cells (**Figure 1.3**) (Arsic et al., 2004, Montarras et al., 2013, Moench et al., 2016). Another growth factor associated with releasate is TGF-β1, this factor is known to stimulate collagen synthesis, growth inhibition, apoptosis, differentiation and activation (Celotti et al., 2006). TGF-β1 acts in both an autocrine and paracrine fashion,

inhibiting macrophage and lymphocyte proliferation, stimulating mesenchymal stem cell proliferation, while also regulating endothelial fibroblastic and osteoblastic cell mutagenesis, collagenase secretion and collagen synthesis. Additionally, releasate contains IGF-I and II, which is commonly known to cause cell growth, differentiation, recruitment and collagen synthesis when recruited with PDGF. VEGF and ECGF both target endothelial cells to cause cell growth, migration, new blood vessel growth as well as anti-necrotic properties. Finally, FGF in releasate has been shown to cause cell growth of blood vessels, smooth muscle, fibroblasts and endothelial cells as well as cell migration and blood vessel growth (Foster et al., 2009, De Pascale et al., 2015, Cavallo et al., 2016). The combination of these growth factors has been shown to be beneficial for many types of tissue regeneration (**Table 1.2**). However, there is lacking evidence surrounding the role of these growth factors from platelet-based applications in skeletal muscle regeneration (Mosca and Rodeo, 2015).

Table 1.2 Platelets and tissue regeneration

Reference	Intervention	Tissue Type	Findings
(Arslan et al., 2016)	PMC	**Tendon** Human & murine tenocytes	↑ proliferation
(Virchenko et al., 2006)	Thrombin and platelet gel	**Tendon** Rat	↑ tendon repair (↑ in force at failure & ultimate stress)
(Schnabel et al., 2007)	PRP	**Tendon** equine flexor digitorum superficialis tenocytes	↑ TGF- β1 and PDGF-BB; ↑ expression of matrix molecules in 100% PRP; no effect on catabolic molecules (MMP-3 and MMP-13)
(Cervelli et al., 2009)	PRP suspended on collagen	**Adipose** (Fat grafting)	Chronic lower-extremity ulcers: 100% re-epithelisation, versus 40- 60% of controls
(Schmolz et al., 2011)	Human PMC	**Osteoblast** (CAL-72) & **fibroblast** (NIH-3T3)	↑ proliferation both cell line; osteoblast secretion of IL-6; ↑ differentiation of fibroblasts
(Frechette et al., 2005)	Calcium & thrombintreated PRP	**Osteoblast** and **endothelial** cells	↑ proliferation
(Celotti et al., 2006)	PRP	**Osteoblast** cell line SaOS-2	↑ chemotaxis & proliferation dose-dependently; PDGF from PRP involved in stimulating cell migration; TGF- β from PRP inhibited cell proliferation.
(Kanno et al., 2005)	PRP	**Osteoblast** HOS & SaOS-2 cell lines	↑ mRNA: Procollagen type I, osteoprotegerin, osteopontin, and cbfa1; ↑ bone regeneration

(Gruber et al., 2002) 2002)	Platelet concentrates	Human Trabecular **Bone Cells**	↑ proliferation of bone cells independent of cell-to-cell contact
(van den Dolder et al., 2006)	Goat, Rat and Human PRP-coated wells	Rat **Bone Marrow** Cells	↑ initial cell growth; Human PRP had the most growth factors per platelet; TGF- β1 was the highest growth factor in all PRPs
(Yamakawa et al., 2017)	PRP & BMSCs	**Bone marrow stromal** cells in Rat femoral defect	↑ BMSC proliferation; a concentration of platelets at 100 x 10^4 μl^{-1} with BMSCs in a collagen mixture: ↑ newly formed bone.
(Torreggiani et al., 2014)	Platelet Lysates and Platelet exosomes	**Bone marrow stromal** cells	↑ proliferation & migration dose-dependently
(Sun et al., 2010)	PRP in a polylactic-glycolic acid	**Osteochondral** articular cartilage defects in rabbits	↑ osteochondral formation
(Bendinelli et al., 2010)	PRP	**Chondrocyte** lbpva55 cells	↓ activity of NF-kB, regulating the inflammatory process; ↓ COX-2 and CXCR4 target genes; ↑ HGF, IL-4 & TNF-α
(Xu et al., 2017)	Leukocyte- and (L-PRP) and pure (P-PRP) PRP	**Chondrogenesis** of rabbit bone marrow **mesenchymal stem cells**	P-PRP ↑ both proliferation and differentiation over L-PRP group
(Meyer et al., 2015)	(Review)	**Hepatocyte** Proliferation	PRP stimulates hepatocyte proliferation by activating the Akt and ERK1/2 signalling pathways in hepatocytes.
(Hesami et al., 2014)	PRP	**Hepatocyte** (rat hepatic injury)	Hepatoprotective effects of PRP counteract the effects of CCl_4 on liver fibrosis
(Starlinger et al., 2016)	Platelet releasate	**Hepatocyte** (post-operative patients)	Patients with high TSP-1 and a low VEGF release profile have ↓ liver regenerative capacity
(Matras et al., 1973)	PPP & fibrinogen, thrombin or $CaCl_2$-	Sciatic **Nerve** Rabbit Model	Presence of functional nerve regenerates when fibrinogen, in high concentrations, plus factor XIII were used
(Elgazzar et al., 2008)	PRP	Peripheral **Nerve** (rat)	↑ number of regenerating nerve fibres
(Farrag et al., 2007)	PRP and Fibrin Sealant	Facial rat **Nerve** Regeneration	PRP with a suture established an increase in axon counts and neurotrophic effects
(Zhang et al., 2015)	Human PRP	**Brain** Reperfusion	Decreases brain injury after focal ischemia; Significantly reduces infarct volume
(Teymur et al., 2017)	PRP	**Nerve-**grafted defects (rat)	↑ nerve gap reconstruction with a 1-cm long nerve graft

(Diaz-Gomez et al., 2014)	PRP-PCL Scaffold	**Angiogenesis** in a CAM model	↑ mesenchymal stem cell attachment and proliferation on scaffold; ↓ differentiation
(Lindeboom et al., 2007)	PRP (human patients)	Oral **mucosal** wound healing	↑ capillary regeneration in mucosal wound healing
(Kazakos et al., 2009)	PRP (human)	**Cutaneous** wound healing	↑ rate of wound healing
(Notodihardjo et al., 2015)	Gelatin hydrogel & releasate	**cutaneous** murine wound healing	↑ wound area epithelialisation rate ↑ capillary formation
(Anitua et al., 2008)	Preparation PRGF	**Cutaneous** ulcers	↑ healed surface area in PRGF group
(Guo et al., 2017)	PRP-Exos (Rat)	**Cutaneous** wound healing, **Endothelial** & **Fibroblast** cell	↑ proliferation and migration of endothelial cells and fibroblasts ↑ cutaneous wound healing
(Chan et al., 2005)	1-21 day-old Human platelets	**Fibroblast**	↑ proliferation; retention of proliferative activity with old platelets
(Huang et al., 2015)	PAR1-PR & PAR4-PR	**Endothelial** progenitor cells	No effect on EPC proliferation; both ↑ cell migration; PAR1-PR ↑ vasculogenesis

- **Abbreviations for tables 1.1, 1.2 & 1.3 are in List of Abbreviations on page 14**

It has been previously argued that discrepancies in releasate content may be owing to the varying methods used for activating platelets. In this context, four main types of platelet activation used clinically (i.e. 10% of either collagen type I, $CaCl_2$, autologous thrombin, or a mixture of $CaCl_2$ + thrombin), may have an impact in the amount of growth factors and cytokines (e.g. TGFβ1, TNF-α, IL-1β, PDGF-AB, and VEGF) released by activated platelets, when collecting releasate specifically for regenerative applications (Cavallo et al., 2016). Other methods such as freeze-thaw for activation of platelets have provided insights regarding the shelf-life of platelet products. It has been reported that human platelet releasate can be stored for 21 days and retain its proliferative properties in fibroblasts as much as 2-day-old platelets (Chan et al., 2005). Standardisation of platelet activation is a crucial step in order to optimise the various growth factors and cytokines released and implemented in experimental procedures. Different protocols for different tissue or cell types have been outlined (**Table 1.3**). Other experimental variables such as platelet count and centrifugation speed have also been recognised and are schematically represented in **Figure 1.4** (Tschon et al., 2011). In terms of platelet count, the variation is donor-specific, which is why re-

suspending platelets to a standardised concentration is important. Moreover, the release of PDGF and TGF-β from platelet concentrates is pH-dependent and therefore standardisation of platelet preparation is critical, given that TGF-β1 is one of the growth factors involved in deterring stimulation of differentiation of myoblasts (Miroshnychenko et al., 2017).

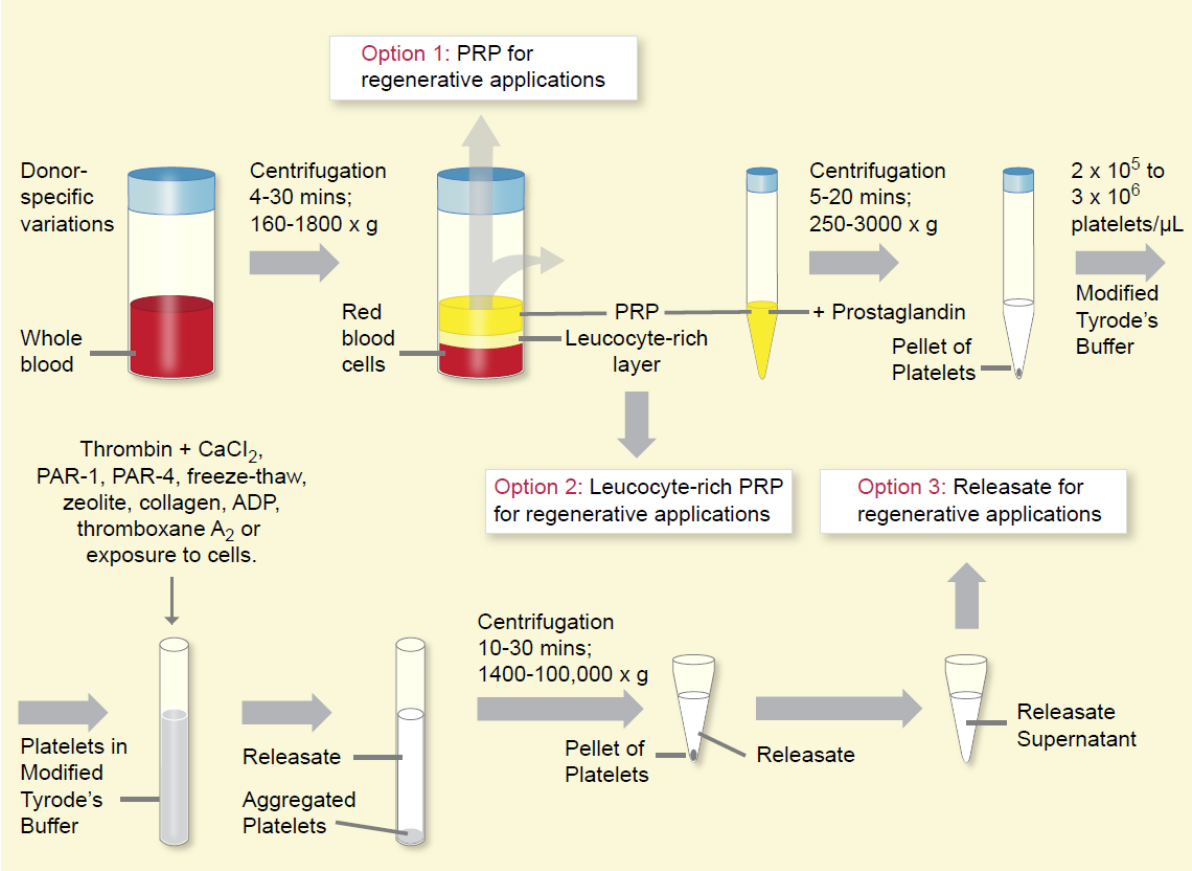


Figure 1.4 A schematic diagram showing the different stages in preparation of platelet-based applications, highlighting possible steps for experimental variations based on published evidence (see **Table 3**). Variations may include; donor-specific variability, variability in centrifugations, use of different platelet-based applications (e.g. PRP or leucocyte-rich PRP or platelet releasate), platelet concentration, buffer of resuspension, platelet agonists used for activation and the storage conditions.

Table 1.3 Platelet-based applications on cell culture studies

Reference	Cell type	Findings (Proliferation/ Differentiation)	Platelet preparation method	Sera (e.g. FBS%) in culture conditions
(van den Dolder et al., 2006)	Rat bone marrow cells	↑ proliferation between days 0 and 4; ↑ differentiation between days 8 and 12	3.8% SC @800 rpm for 15 min @25°C activated with thrombin (300 IU) & 10% $CaCl^2$.	10% FCS 10,000 cells/well (24-well) pre-coated with a PRP gel
(Yamakawa et al., 2017)	Rat bone marrow stromal cells (BMSCs)	Higher PRP concentration ↑ cell proliferation; ↑ newly formed bone with 100 x 10^4 platelets μl^{-1} & BMSCs in a collagen mixture @ 8 week	Whole blood @ 600xg for 10 mins, then 2,840xg for 15 minutes; activated with 2% $CaCl_2$ & thrombin.	α-MEM with 15% FBS; Cells were seeded on a 100-mm dish
(Masuki et al., 2016)	Human alveolar bone-derived periosteal cells	A-PRF and CGF extracts ↑ proliferation; PRP @ 2.5% showed the most proliferative properties with ↓ @ higher doses	PRP: Whole blood & ACD @ 1800g for 4 min then stored @ −80°C; PRGF: whole blood & SC @ 580g for 8 min; A-PRF & CGF clots: whole blood without anticoagulants @ 198g and 692g respectively, frozen, minced, homogenised @ 3000 rpm for 10 min	Human periodontal tissues in 10% FBS for single cells; cells were seeded at 1×10^4 in 6-well plates for 24 h in 1% FBS; PRP, PRGF, A-PRF extract or CGF extract and the cells were further incubated for 48 h
(Schmolz et al., 2011)	NIH-3T3 cells & CAL-72 cells	PMC ↑ proliferation of murine fibroblasts and human osteoblasts; ↑ NIH-3T3 angiogenesis	Platelet mediator concentrate (PMC) using an ATR system kit: human whole blood & anti-coagulant & sedimentation accelerator and aggregator	NIH-3T3 culture: DMEM with 10% FCS; CAL-72 culture: 10% FCS over-night, then 2% FCS for 24 hours followed by 10% PMC or 10% FCS
(Torreggiani et al., 2014)	Bone mesenchymal stem cells	Bone marrow stromal cells treated with platelet exosome concentrations ↑ proliferation and migration; ↑	Whole blood & heparin @ 1000 rpm for 5 mins, then 1900 rpm for 15 mins; Human PL: PRP was frozen and thawed then @ 2600xg for 30 mins; & heparin for exosome	αMEM & 10% FBS; 4 days of culture; adherent mesenchymal stem cells in αMEM & 10 % FBS.

		bFGF, VEGF, PDGF-BB & TGF-β1 in platelet exosomes than in PL.	isolation; PL @ 2000xg for 10 mins & heparin; Exosome isolation: PL @ 500xg for a series of spins then @ 30000 rpm for 1h with repeats.	
(Gruber et al., 2002)	Human trabecular bone cells	↑ mitogenic activity of BC, independent of cell-to-cell contact.	Whole blood & ACD spun to concentrate platelets ($3x10^9$); then leuco-depleted by a pall filter; washed in Tyrode's buffer, @ 1400g for 10min and resuspended in serum-free medium; activated with thrombin; @ 1400xg; supernatant @ 100,000xg	DMEM/F12 with 10% FCS
(Xu et al., 2017)	Rabbit bone marrow mesenchymal stem cells (rBMSC)	P-PRP ↓ concentrations of leukocytes and pro-inflammatory cytokines, ↑ proliferation & chondrogenesis of rBMSCs when compared to L-PRP	Whole blood & ACD-A; L-PRP: 250xg for 10 mins then @ 1000xg for 10 mins, re-suspended PPP; P-PRP: 160xg for 10 mins then 250xg for 15 mins resuspended in PPP	α–MEM 10% FBS; 96-well plates, 4,000 cells/well, then 10% of FBS, L-PRP, or P-PRP
(Kanno et al., 2005)	Human osteosarcoma cells & SaOS-2	↑ viability of HOS and SaOS-2 cells dose-dependently; ↑ levels of procollagen type I, osteopontin, osteoprotegerin and core binding factor alpha 1 (cbfa1) mRNA.	Human whole blood & SC. Centrifuged at 2,000 rpm for 5 minutes. PRP Centrifuged again at 2,000 rpm for 20 minutes.	SaOS- 2 were cultured in RPMI-1640 medium 10% FCS
(Frechette et al., 2005)	HUVECs hFOB	Growth factor concentration variations between individuals. ↑ osteoblast and endothelial cell divisions.	PRP & Thrombin and $CaCl2{\cdot}_2H_2O$ x 1, x 5 and x 25; @ 4000xg for 10 min	10% FBS with endothelial cell growth supplement; hFOB cells in DMEM/F12 with 10% FBS, glutamine, and G-418
(Celotti et al., 2006)	SaOS-2 (Sarcoma	PRP ↑ chemotaxis & cell proliferation; PDGF ↑ cell	Whole blood & Citrate phosphate dextrose @ 180xg for 20 mins, then 580xg for 15 mins; resuspended in PPP;	DMEM with 10% FCS

	osteogenic)	migration & TGF-β ↓ proliferation	Jellified with calcium gluconate/batroxobine; then @ 1400 x g for 10 mins	
(Bendinelli et al., 2010)	Ibpva55 & U937 cells	PRP in chondrocytes ↓ activity of NF-kB & ↓ expression of COX-2 & CXCR4 target genes; ↑ HGF, IL4 & TNF-a; PRP in U937-monocytic cells ↓ chemokine transactivation & CXCR4-receptor expression	Whole-blood prepared using the platelet concentrate collector system GPS II; activated with thrombin and CaCl2·2H2O; then centrifuged for 10 min at 4,000xg.	DMEM & 20% FBS for Ibpva55 cells; RPMI-1640 medium & 10% FBS for U937 cells
(Huang et al., 2015)	EPCs	No benefit on proliferation; ↑ EPC migration; ↑ vasculogenesis	Whole blood @ 190xg for 20 min; then @ 900xg for 10 min & PGE_1; resuspended in Tyrode's HEPES buffer @ 2 x 10^9 mL^{-1}; PAR1-AP or PAR4-AP for 10 min; @ 15000xg for 10 min	EGM-2 SingleQuots complete medium 10% FBS
(Battinelli et al., 2011)	MCF-7 breast cancer cells & HUVECs	Platelet releasates activated with 1) ADP: ↑ migration & formation of capillary structures by HUVECs; 2) TXA_2: inhibited migration & formation of capillary structures	Human platelets @ 2x10^8 mL^{-1} activated by ADP, Thromboxane A_2, PAR4, or exposure to MCF-7 cells @ 3 x 10^6 mL^{-1}	HUVECs (1 x 10^4 mL^{-1}) in serum free media on 0.5% gelatin pre-coated/ transwell plate with 2 x 10^8 mL^{-1} platelets in the bottom chamber
(Diaz-Gomez et al., 2014)	Human adipose-derived MSCs	MSC seeded on the PRP–PCL nanofibers showed an increased adhesion and proliferation compared to pristine PCL fibres.	The buffy coat was centrifuged @ 400xg for 15 min; 3x10^6 platelets μL^{-1} activated by freeze–thaw cycles; @ 12000xg for 10min	Human MSCs (2x10^4 cells) were seeded in 24-well plates with 1 mL of MSC medium or 1 mL of PRP-rich medium.

(Cervelli et al., 2009)	Adipose tissue-derived stem cells	PRP accelerates chronic skin ulcer reepithelization; ↑ proliferation	SC as an anticoagulant with a 1100xg for 10 min spin; Ca^{2+} for activation	DMEM with 10% FBS @ 2500–5000 cell/cm^2.
(Im et al., 2014)	Rabbit Myogenic progenitors & ASCs & Human fibroblasts	ASCs had an anabolic paracrine effect on proliferation of MPCs; PRP ↑ proliferation of MPCs; ASC-extracts ↑ proliferation more than PRP	Rabbit whole blood & SC; @ 400×g for 10 mins; then PRP @ 800×g for 10 mins.	Rabbit MPCs & ASCs cultured in EGM-2MV containing 5% FBS; Human fibroblasts in DMEM with 10% FBS
(Tsai et al., 2017)	Rat gastrocnemius muscle cells	Releasate ↑ proliferation of skeletal muscle cells by transitioning cells from G1 phase to S phase and G2/M phases	Whole blood & ACD @ 800xg for 30 mins then @ 3000xg for 20 mins; 10% thrombin with $CaCl_2$; Then @ 5500xg for 15 mins and filtered	DMEM with 10% FBS & 5% chick embryo extract
(Takase et al., 2017)	Murine C2C12 myoblasts	PRP ↑ proliferation & inhibited both myogenic and adipogenic differentiation	Whole blood & SC @ 2400 rpm for 10 mins; then @ 3600 rpm for 15 mins; activated by freeze-thawing then @ 10000 rpm for 10 mins	DMEM with 10% FBS; for myogenesis, DMEM without FBS was used @ 5.0×10^4 cells/well
(McClure et al., 2016)	Murine C2C12 myoblasts	PRP ↑ both myogenic proliferation & differentiation	PRGF: human whole blood in SmartPRePW 2 centrifugation system; Then a freeze–thaw–freeze process to lyse platelets and release their granule contents	High-glucose DMEM with 10% FBS for Proliferation; High-glucose DMEM with 2% horse serum for Differentiation
(Ranzato et al., 2009)	Murine C2C12 myoblasts	PL ↑ C2C12 proliferation and motility	Platelet lysates; centrifuged, washed, repeatedly frozen and thawed & centrifuged to eliminate debris	DMEM with 10% FBS
(Miroshnychenko et al., 2017)	Human myoblasts	↑ in proliferation; both PPP & MSTN and TGF-β1 depletion in PRP ↑ myoblast differentiation	Pure PRP kit; 1. PPP 2. PRP 3. Mod-PRP with TGF- β1 and MSTN depletion 4. PRP second spin 550g 5 mins 5.	10,000 cells/cm^2; 2% horse serum for differentiation; 10% FBS for proliferation

(Huang and Wang, 2012)	Rat MSCs (Muscle satellite cells)	PRP-derived growth factors ↑ proliferation on rMSCs & ↑ osteogenic differentiation potential with scaffolds subcutaneously in nude mice	Rat whole blood platelet pellet; re-suspended in plasma snap frozen & thawed then repeated; Plasma was separately spun @ 3000xg for 15 mins after clotting	rMSCs cultured in 1) DMEM with 10% FBS, 2) serum or 3) PRP-derived growth factors with NHA/PLGA scaffolds in 10% FBS; Osteogenic differentiation: DMEM with 10% FBS, b-glyceraldehyde-3-phosphate, L-ascorbic acid & dexamethasone
(Li et al., 2013)	hMDPC ;Myo-endothelial cells; Pericytes	PRP ↑ proliferation; antibody neutralisation of PDGF ↓ proliferative effects of PRP and maintained differentiation of hMDPCs	PRP @ 3000xg for 10 mins re-suspended in PPP; activated with thrombin then @ 3000xg spin for 30 mins and filtered	hMDPCs, myo-endothelial cells & pericytes: 20% FBS
(Guo et al., 2017)	HMEC-1 & Primary dermal fibroblasts	↑ proliferation and migration of endothelial cells and fibroblasts	Whole blood in ACD-A; A series of centrifuges from 160×g for 10 min; to 100,000×g for 70 min to pellet the exosomes in PBS	HMEC-1 cells and primary dermal fibroblasts were cultured with 10% FBS
(Chan et al., 2005)	Human dermal fibroblasts	21-day-old platelets were as stimulatory as 2-day-old platelets on fibroblast proliferation; Total protein, PDGF & TGF- concentrations	Platelet activation using zeolite (1 g 10 mL^{-1}) and 1 mL of a 10% calcium chloride; Spun at 48000xg	DMEM with 10% FBS; $1x10^4$ Cells in a 24 well plate; adhere for 6h; incubating in 10% platelet extract in media without FBS.
(Arslan et al., 2016)	Human & Murine Achilles tenocytes	PMC ↑ human tendon cell growth and viability in a dose-dependent manner	An ATR kit was used to prepare PMC in which collected the platelets and activated them; then frozen at –80°C.	Human tenocytes - DMEM + 10 % FCS; Murine tenocytes - 50% FCS; reduced to 10 % after 1 week

Abbreviations for tables 1.1, 1.2 & 1.3 are in List of Abbreviations on page 14

Current preparation methods of platelet-based applications are outlined in **Table 1.3**.

There seems to be significant variations between preparation methods from different

laboratories, even when producing the same product such as PRP. Typically, either the whole blood is inhibited with an anticoagulant such as citrate dextrose solution (ACD) or sodium citrate and then either centrifuged or processed using a kit. The first centrifugation step can be processed at a speed between 160 to 1800 x g and between 4 to 30 minutes (Masuki et al., 2016, Guo et al., 2017, Tsai et al., 2018a, Tsai et al., 2017). The resultant platelet-rich plasma is then either isolated and used as it is, or inhibited with further anticoagulants such as prostaglandin or prostacyclin and centrifuged between 5 to 20 minutes at 250 to 3000 x g in order to pellet the platelets (Miroshnychenko et al., 2017, Tsai et al., 2017, Xu et al., 2017). Pelleted platelets are then resuspended in either a buffer or platelet-poor plasma (PPP) to a standardised concentration, varying between physiological levels (2×10^5 platelets μL^{-1}) to 3×10^6 platelets μL^{-1}. This resuspension is then aggregated using many methods including thrombin and calcium chloride, thrombin protease- activated receptor (PAR)-1, PAR-4, freeze-thaw cycles, zeolite, calcium, calcium gluconate/ batroxobine, ADP, thromboxane A_2 or exposure to cells (Frechette et al., 2005, Huang et al., 2015, Diaz-Gomez et al., 2014, Chan et al., 2005, Cervelli et al., 2009, Celotti et al., 2006). Thrombin is very frequently used in PRP preparations; however, one characteristic of thrombin is that it is a protease and may cleave or damage proteins in the releasate sample. For this reason, using a thrombin protease- activated receptor -1, or -4 (PAR-1) or (PAR-4) peptide may be an appropriate method for releasate preparation, activating the thrombin receptors without the potential of damaging the sample's contents enzymatically (Huang et al., 2015). Finally, the releasate can be spun down to remove cellular debris. Expectedly, there is a large variation between publications for this final centrifugation step. In fact, the final centrifugation, excluding platelet exosome isolation, varies from 1400 to 100,000 x g between 10 and 30 minutes. Such discrepancies in the preparation methods are illustrated in **Figure 1.4**. At present, it cannot be ruled out that technical inconsistencies such the ones mentioned above may possibly account for the variable outcomes reported in several studies.

1.3.4 PRP Alternatives

It is essential to characterise the current forms of platelet-based applications that are derived from PRP methods in order to cope with the current methodological limitations

(**Table 3**). One alternative to PRP that is noteworthy to mention is platelet mediator concentrate (PMC). PMC contains similar factors as PRP such as PDGF-BB, TGF-β1, VEGF, BMP-2, BMP-4, TNF-α BMP-7, and IL-6 (Arslan et al., 2016). Additionally, it has been reported that low levels of TNF-α and IL-6 in PMC are suitable for tendon healing and reduced scar formation (Arslan et al., 2016). Platelet-rich fibrin (PRF; a fibrin clot in which contains the platelet cellular debris with their cytokines) also contains many of the above-mentioned growth factors such as PDGF, TGF-β, VEGF, EGF and IGF-1 (Dohan et al., 2006, Arunachalam et al., 2016). These alternatives may be particularly useful in patient-dependent cases where local injection may not be applicable. Several alternatives to PRP could also be implemented such as advanced platelet-rich fibrin (A-PRF) as well as a concentrated growth factor (CGF) which have higher levels of TGF-β1, PDGF-BB and VEGF as well as higher platelet counts (Masuki et al., 2016). Leukocyte-rich PRP and pure PRP (i.e. leukocyte-poor PRP) is an emerging new delineation where hampering effects of having white blood cell contamination in PRP preparations can be studied (Xu et al., 2017). Other novel platelet therapy methods could be implemented if PRP continues to show promising results in terms of skeletal muscle regeneration. One may speculate that delivering higher concentrations of these factors to the localised injury site may cause further recovery, however an undesirable ratio of growth factors and cytokines may cause an imbalance in homeostasis which may be detrimental. Attempting to get this ratio correct results in many variations and alternatives in the platelet preparation methods. Novel platelet-based applications such as CGF or A-PRF would have to be a localised implantation (Dohan et al., 2006). These physical manipulations, as well as leaving the injury site open for minor surgery, may cause dissimilar factors released from the platelets, resulting in non-comparable results to PRP (Cavallo et al., 2016, Fioravanti et al., 2015). It is important to outline the role of platelet aggregation when denoting the ratio of the release of growth factors that are crucial in relation to tissue regeneration, and choosing the correct method of platelet-based application.

1.4 Platelet-mediated skeletal muscle regeneration

Current evidence on the role of platelet-based applications in skeletal muscle regeneration derives mainly from: a) human clinical trials, b) experimental animal studies, as well as c) *in vitro* cell studies.

1.4.1 Clinical trials with platelet-based applications targeting skeletal muscle

Given the recent development of platelet-based applications, the number of studies in this area has increased exponentially, with more than 1000 articles being published in the last two years (**Figure 1.2**). The studies reviewed in this thesis introduction were identified through a PubMed search using combinations of the following key words: platelet-rich plasma, regeneration, skeletal muscle or any other tissue as discussed. Relevant references were reviewed to identify further original research on platelet-based applications and regeneration, focusing on cellular, animal and human studies. There have been several clinical trials examining the use of PRP for muscle regeneration (see **Table 1.1**). One study describing human athletes with muscle lesions (partially torn) of whom were injected locally with autologous PRP every 7 days for 21 days. Despite not having a control group, this study determined that the autologous PRP injections are a safe and effective treatment for varying muscle lesions (Bernuzzi et al., 2014). Ultrasound-guided injections of PRP in professional athletes had an augmented pain relief score as well as decreased pain on resisted flexion, strength and range of motion after only 7 days versus conventional treatment (Bubnov, 2013). Similarly, pre- and post-treatment of proximal hamstring injuries with PRP was carried and evaluated using the Nirschl Phase Rating Scale and Visual Analogue Scale for pain rating (Wetzel et al., 2013). The results of this clinical study showed that pain reduction was augmented in the PRP group over the conventional treatments. A recent study analysed hamstring injuries in professional football players over a 31-month period (Zanon et al., 2016). This study conversely reported that lesions showed a non-significant healing rate over patients treated with Actovegin; however, it reported that the use of PRP in human patients was a safe practice.

It has been previously argued that the conclusion of several trials opposing the use of PRP in sport injuries may be attributed to technical inconsistencies and methodological limitations of the studies (Mosca and Rodeo, 2015). Such limitations are poor sample

sizes, non-blinded studies, lack of control studies, inconsistency in PRP preparation methods, platelet concentration and growth factor levels are inconsistent, selection bias in clinical studies as well as a specific demographic used, such as healthy and fit male athletes as opposed to the general population or patients (Rettig et al., 2013, Bernuzzi et al., 2014, Wetzel et al., 2013, Mosca and Rodeo, 2015). One of the major flaws in PRP studies for muscle healing in human subjects is the lack of physiological data and mechanistic insights as an outcome measure, rather, pain scores and return to respective sporting fields have been used.

There have been many speculations made in recent scientific reviews upon the beneficial use of platelet-based applications in musculoskeletal injuries (Lee et al., 2011, Nguyen et al., 2011, Sánchez et al., 2014, Mosca and Rodeo, 2015, Brossi et al., 2015, Navani et al., 2017, Andia and Abate, 2017, Qian et al., 2017). These previous reviews of clinical trials using PRP in orthopaedic injuries seem to consistently indicate insufficient results while they recognise that platelet-based applications may hold promise in future applications. A systematic review covering the effects of PRP on muscle lesions in both humans and horses showed that PRP has positive results in 46.7% of the clinical studies (Brossi et al., 2015). Further to this, Sanchez et al. delineate a common protocol used for PRP in muscle injuries in clinical settings as well as the post-infiltration protocol for follow up potential complications (Sánchez et al., 2014). The protocol here for human patients seems thought out, thorough and effective in follow up treatment. In fact, this may be a promising approach to go forward for optimisation in clinical trials. With the gathered evidence over recent years, one may surmise that there is an effect between platelet-based applications in the early stages of inflammation and with increased skeletal muscle healing rate (Dimauro et al., 2014, Borrione et al., 2014).

Experimental evidence from animal and cellular studies remains to be applied to clinical practice. Optimisation of platelet preparation is essential to be standardised or tailored for individual patients, such as depletion of deleterious cytokines (Li et al., 2016b). Additionally, the timing of PRP application is pertinent. For example, addition of PRP on days 1 and 4 post-injury hinder skeletal muscle regeneration, but on day 7 has been shown to be beneficial in a rat model (Denapoli et al., 2016). Of note,

inconsistencies between clinical and experimental data need to be narrowed, given the current diverse methodologies in platelet preparation among different laboratories as listed in **Table 1.3**.

1.4.2 Animal Studies with platelet-based applications targeting Skeletal Muscle

Despite the limitations in human trials with respect to PRP, there have been numerous progressions in the field of skeletal muscle regeneration in rodent models (Hammond et al., 2009, Pinheiro et al., 2016, Borrione et al., 2014, Dimauro et al., 2014). Accumulating data from animal studies on the role of PRP on skeletal muscle recovery after varying types of injury has emerged (**Table 1.1**). Dimauro et al. showed promising data regarding the delivery of PRP on cell proliferation and differentiation as well as satellite cell recruitment that resulted in improved skeletal muscle regeneration (Dimauro et al., 2014). They report that PRP downregulated myo-miR-133 and increased Paired box protein-7 (Pax7) and MRFs involved in both myoblast proliferation and differentiation *in vivo*. Myo-miR-133 upregulates myoblast proliferation through the suppression of serum response factor (SRF) and impedes myotube differentiation (Chen et al., 2007, Chen et al., 2010). Conversely, myotube differentiation was increased with the application of 20nM of double-stranded micro-ribonucleic acid (miRNA) for myo-miR-1, 133 and 206 (Nakasa et al., 2010). The conflicting data suggests that the role of miRNAs on muscle regeneration remains to be fully elucidated. It has been reported that the concentrations of the specific growth factors TGF- β1, PDGF-AA, PDGF-AB, and PDGF-BB in human PRP are greater over goat and rat growth factors per platelet (van den Dolder et al., 2006). Interestingly, TGF- β1 was found to be the highest growth factor concentration across all 3 species' PRP. Both b-FGF and IGF-1 have been found in the α-granules of platelets; these factors are known to independently promote regeneration *in vivo* in a murine model. For example, gastrocnemius muscles of mice injected with (100ng ml^{-1}) IGF-1 or with (100ng) b-FGF on days 1, 3 and 5 after injury showed faster muscle healing and tetanic strength recovery (Menetrey et al., 2000). An influential study addressing the effect of TGF- β1 neutralisation in PRP on a rat muscle injury, reported that modified PRP with depleted TGF- β1 boosted myofibre regeneration and decreased fibrosis (Li et al., 2016b). The study also reported an increase in angiogenesis and greater M2

macrophage localisation in the injury site; which are known to have an anti-inflammatory function and regulate wound healing. Additionally, satellite cell number was increased in response to TGF- β1-depleted PRP two weeks post-injury. This finding indicates that PRP composition may be modified in order to optimise benefits towards skeletal muscle regeneration. In a previous mouse study from the same group, it was shown that human muscle-derived progenitor cells cultured in either PRP or foetal bovine serum (FBS) had the same capacity to regenerate myofibres *in vivo* upon transplantation into injured gastrocnemius muscle (Li et al., 2013).

Interestingly, local delivery of PRP can shorten recovery time after a muscle strain or multiple muscle strain injuries in rat models leading to faster functional recovery of the tibialis anterior muscle (Hammond et al., 2009). Additional evidence from a mouse injury model suggests that the optimal time point for a platelet-pure PRP (i.e. a leukocyte-poor PRP) injection was 7 days post-injury, leading to reduced fibrosis and better exercise tolerance. However, addition of PRP on 1- or 4-days post-injury that coincide with the period of myoblast fusion and commitment to differentiation causes fibrosis and shortens exercise tolerance (Denapoli et al., 2016). The mechanistic insights of this finding remain to be determined. One possibility is that platelet releasate has a more potent effect on myoblast proliferation, while its use during the early phases of regeneration or cell differentiation may be compromised by inflammatory pathways (Borrione et al., 2014, Li et al., 2016b). This notion is supported by *in vitro* data, where PRP releasate upregulated myoblast proliferation but inhibited myoblast fusion (Miroshnychenko et al., 2017).

Two recent articles relate the role of reactive oxygen species (ROS) and muscle regenerative capacity (Martins et al., 2016, Kozakowska et al., 2015) et al., 2015). The role of ROS in myogenic differentiation is multifaceted as cellular responses alter acutely to minute changes in ROS stress levels. Martins et al. showed that PRP is capable of modulating the oxidative impairment determined by muscle contusion, defined as a section of damaged tissue where capillaries have been ruptured (Martins et al., 2016). The prevalence of contusions is very common both in the general population and in sporting athletes affecting the function of the musculoskeletal system (Mosca and Rodeo, 2015). The contusion, by dropping a 200g mass directly onto the

gastrocnemius muscle, was shown to increase the levels of oxidative stress markers (i.e. thiobarbituric acid and oxidized dichlorofluorescein) in both muscle tissue and in erythrocyte preparations (Martins et al., 2016). Application of PRP was able to attenuate oxidative stress and increase enzymatic antioxidant defence in injured skeletal muscle. These data suggest that the beneficial effects of PRP on muscle regeneration may, at least in part, be brought about by lower levels of oxidative stress. It is evident that both animal and human studies have revealed largely dissimilar results when analysing the impact of PRP in various tissues and treatments. The supportive evidence of platelet-based applications from experimental animal studies remains to be validated and extrapolated into human studies with a more thorough experimental design and biological or functional end-point measures. Identification and in-depth assimilation of the mechanisms behind the effect of platelet releasate on these tissues is crucial in order to design and conduct better human trials.

1.4.3 Cell Studies with platelet-based applications

Newly emerging studies are finding beneficial results with platelet releasate in muscle regeneration similar to PRP treatment outcomes (Tsai et al., 2017, Muto et al., 2016, Li et al., 2013, Takase et al., 2017). One crucial and recent study added platelet releasate to a primary culture of rat gastrocnemius muscle cells with the aim of investigating the impact of releasate on cell proliferation (Tsai et al., 2017). It was revealed that releasate increases the proliferative potential of the cells in a dose-dependent manner. This finding was attributed to a continuation of the cell cycle from the G1 phase to the S phase, driving progression through expressions of cyclin and cyclin-dependent kinase (cdk) protein (Tsai et al., 2017).

One recent study examined the role of releasate on both myogenesis and adipogenesis in rats as well as in an immortalised mouse skeletal myoblast C2C12 *in vitro* cell line (Takase et al., 2017). C2C12 cells are an immortalised mouse myoblast cell line capable of differentiation. The localised sub-acromial injection of PRP proved significantly effective in reducing the instance of adipogenic gene expression as well as supressing adipogenic differentiation. In the C2C12 cells, there was substantially increased proliferation when PRP was administered as well as inhibiting muscle and adipocyte differentiation. This finding mirrors the effect of PRP on myoblasts, namely

an upregulation in proliferation but presumably an inhibitory role in differentiation (Miroshnychenko et al., 2017). Thrombin-activated PRP has been reported to be detrimental in both Saos-2 cells (Sarcoma osteogenic cell line) and marrow stromal in terms of cell viability *via* a 48-hour MTT assay (Han et al., 2009). However, a recent study has shown that co-cultures of adipose-derived stem cells (ASCs) or PRP with myogenic progenitor cells had an augmented effect on myogenic proliferation (Im et al., 2014). Notably, this study reported that the ASCs promoted both myogenic progenitor and C2C12 cell proliferation with PRP. An interesting tissue engineering study looked at C2C12 cells in a PRP treatment embedded in fibres of polydioxanone and polycaprolactone which were electro-spun (McClure et al., 2016). This study showed proliferative benefits using the electro-spun scaffold in myoblasts. Co-culturing myoblasts with a micro-environmental niche such as with ASCs or with a fibrous scaffold may be a more accurate representation of myogenesis *in vivo* than single cell culture. Conclusively, myoblast cell lines proliferate in response to platelet releasate; however the role of releasate in cell differentiation is still being discussed.

In order to determine if there is any merit to using platelet-based applications as an effective form of regulating cell proliferation and differentiation to support regeneration; key cell culture studies were analysed as outlined in **Table 3**. With the exception of endothelial progenitor cells in one study (Huang et al., 2015), platelet preparations seemed to produce a positive proliferative effect on various cell types across species. Some of the cell types in **Table 3** include myogenic progenitor cells, bone-derived periosteal cells, osteosarcoma, endothelial, trabecular bone cells, human adipose-derived mesenchymal stem cells, fibroblasts, tenocytes, myo-endothelial cells, pericytes, C2C12 cells, adipose-derived stem cells and muscle satellite cells. Variable levels of differentiation have been reported for different cell types; e.g. increased differentiation for rat bone marrow cells, human skeletal muscle myoblasts, rat muscle satellite cells, rabbit bone marrow mesenchymal stem cells, and C2C12 myoblasts or maintained effect with hMDPCs, myo-endothelial cells and pericytes compared to control conditions (Miroshnychenko et al., 2017, Huang and Wang, 2012, Xu et al., 2017, van den Dolder et al., 2006, McClure et al., 2016, Sassoli et al., 2014, Kelc et al., 2015, Li et al., 2013, Sassoli et al., 2018a). Conversely, some studies reported that

platelets inhibited differentiation of C2C12 myoblasts (Miroshnychenko et al., 2017, Takase et al., 2017). This discrepancy in the current literature may provide the basis for a thorough re-consideration of the technical aspects in platelet preparations that may affect the final outcome in a study. For example, despite the majority of studies applying 10% PRP, a recent study used higher concentrations of platelets in smaller volumes (i.e. 1-2%) to induce differentiation, assuming that the concentration of growth factors was not altered (Sassoli et al., 2018a). Finally, an interesting study carried out by Miroshnychenko et al. has led to a new insight in PRP and PPP effects *in vitro* on human skeletal muscle myoblast cells (Miroshnychenko et al., 2017). This study looked at PRP, PRP with depleted TGF-β1 and myostatin and PPP in culture with the myoblasts. TGF-β1 and myostatin were depleted due to their detrimental effects on muscle regeneration (Dimauro et al., 2014). The study has reported that PPP and leukocyte-poor PRP with a second centrifugation to remove whole platelets induced myoblast differentiation, however unmodified leukocyte-poor PRP increased myoblast proliferation (Miroshnychenko et al., 2017). An interesting aspect of this study is that PRP did not seem to induce muscle differentiation; rather it was more inclined to induce a proliferative property. This study is pertinent due to the method of removing unwanted growth factors from the PRP which resulted in altered biological properties on the myoblast cell line used. Further studies aiming to eliminate additional releasate factors could be implemented to optimise skeletal muscle regeneration and possibly expand clinical application in the near future.

1.5 Current evidence on platelet-mediated regeneration in other tissues

There has been an intense interest in determining the effect of platelets and platelet-related application on the regeneration of several tissues other than skeletal muscle such as tendon, adipose tissue, bone, liver, nerve, vascular tissue, wound healing etc.

1.5.1 Tendon regeneration

One recent study analysed PMC, a centrifugation-free method of preparing human platelet releasate, co-cultured with Achilles tenocytes *in vitro* from both human and murine tendons (Arslan et al., 2016). This study reported that PMC concentrations

caused an elevation of important growth factors and markers for cell viability in tenocytes, suggesting that autologous PMC may be a useful future therapy in tendon recovery. However, a cross-comparison with PRP or releasate would be useful in determining which treatment at what dose would be optimal for tendon recovery *in vivo*. An *in vivo* study looked at platelet gel; a re-suspended pellet of platelets activated with thrombin and calcium, in relation to the effect on the transected Achilles tendon of female rats after 14 days (Virchenko et al., 2006). The results, when compared to a saline injection, showed a 42% increase in the force at tendon failure when subject to being pulled at a consistent speed of 1mm second^{-1}. There was also a 61% increase in ultimate stress (MPa) observed in the tendons when compared to the saline injections, suggesting an increased tendon recovery time. Notably, the platelet gel seemed to have a significantly lower force at failure score than the platelet gel, 24% and 42% respectively. This suggests that the activated PRP with thrombin and calcium is required for optimised tendon recovery. Currently, leukocyte-poor PRP progresses tendon healing and is considered a more viable option for the clinical treatment of tendinopathy after a comparative use in a rabbit model (Yan et al., 2017). In general, the current consensus of platelet-based applications on tendon regeneration is a positive one with tenocyte proliferation reported *in vivo*, as well as structural optimisation of the tissue being upregulated.

1.5.2 Adipose and Endothelial tissue regeneration

A clinical and *in vitro* study analysing the application of PRP for tissue engineering, specifically fat grafting, reports an accelerated chronic skin ulcer re-epithelisation (Cervelli et al., 2009). Co-culture of adipose tissue-derived stem cells (ADSCs) with PRP shows a proliferative effect. The clinical application of fat grafting with PRP showed consistently higher re-epithelisation from 3 weeks until 18 months over a control group. Fat grafts were maintained with PRP up to a 69% restoration rate when compared to 31% for the control. In an earlier study, activated PRP co-cultured with human umbilical vein endothelial cells or with transformed human osteoblasts showed increased endothelial proliferation (Frechette et al., 2005). Interestingly, the non-activated PRP group in osteoblast cells were more proliferative than in the activated group, suggesting that PRP secretions gave no supplementary benefit on osteoblast

proliferation over the 3 groups. However, when compared to the minimal medium group there was a substantial increase in all PRP conditions showing that platelet extracellular growth factors were expressed in adequate quantities to induce substantial proliferation. These results indicate towards an increased proliferative rate of ADSCs with platelet-based applications, with a possible benefit for cosmetic utilisation.

1.5.3 Osteoblast and Chondrocyte tissue regeneration

Many studies now see the potential mechanisms of PRP acting on osteoblast proliferation and migration (Kanno et al., 2005, Schmolz et al., 2011, Celotti et al., 2006, Frechette et al., 2005). Using the SaOS-2 osteoblast line, both TGF-β and PDGF were analysed for both cellular proliferation and migration. Notably, TGF-β appeared to have an inhibitory effect on proliferation, while PDGF was reported to upregulate migration. Similarly, Kanno et al., have shown the link between a PRP treatment and osteogenesis *in vitro* in a dose-dependent method (Kanno et al., 2005). This study suggests that growth factors in PRP, such as TGF-β, prompt pre-osteoblasts to undergo division increasing their quantities through chemotaxis, stimulating differentiation into mature osteoblasts. In connection to osteogenesis, one study makes the connection to bone and cartilage regeneration (Bendinelli et al., 2010). In this study, the mechanisms connecting PRP to chondrocyte differentiation and regeneration were assessed by the means of regulating local inflammation in cartilage through decreasing chemotaxis of anti-inflammatory agents such as HGF. Releasate from the PRP was found to be accountable for the inhibition of NF-kB-trans-activating activity due to the upregulation of HGF (Bendinelli et al., 2010). It is still unclear whether the stimulatory effects of PRP, in osteoblast proliferation, for example, are connected to the growth factors present or to other factors present in the cytoplasm or cell membranous structures from activated platelets (Gruber et al., 2002). This study also stated that cell-to-cell contact was not reportedly required for upregulated osteoblastic proliferative effects of platelets. In summary, recent studies indicate that there is a positive proliferative and differentiative effect of platelet-based applications in osteogenesis. PRP also promoted growth and proliferation in chondrogenesis and may be beneficially applicable in cartilage repair.

1.5.4 Hepatocyte tissue regeneration

A study evaluated the *in vivo* effect of platelet-rich plasma on carbon tetrachloride-induced hepatotoxicity in male rats. Animals received PRP treatment twice a week for 8 weeks (Hesami et al., 2014). After the 8 weeks, the rats were bled and their livers were analysed histologically, showing a hepatocytic protection of the PRP as well as showing that PRP itself is not toxic for at least a 3-week period. Further to this, a recent review highlighted the factors released by PRP such as VEGF, HGF and IGF-1 to promote hepatocyte proliferation (Meyer et al., 2015). It has been hypothesised that an unidentified receptor on the liver sinusoidal endothelial cells interacts directly with the platelets in PRP in which stimulate proliferation in the hepatocytes. Similarly, a recent study followed up from this, analysing patients who underwent hepatic resection (Starlinger et al., 2016). It was shown that a rapid accumulation of platelets to the resection was correlated to regeneration of the liver. Interestingly, an unfavourable ratio of growth factors such as an increased TSP-1 level as well as a lower VEGF level displayed hampered regenerative properties. These studies reveal a proliferative effect in hepatocytes when using platelet-based applications.

1.5.5 Nerve tissue regeneration

One of the first experimental uses of platelet-based applications was the use of PPP in a rabbit model in 1973 (Matras et al., 1973). This study analysed a plasma clot welding of nerves with regained myo-neural function and no sign of substance rejection. A more recent study performed a bilateral sciatic neurotomy in rats, followed by being promptly re-anastomosed with a cyanoacrylate glue used in order to study the regenerative properties of PRP in relation to nerve regeneration (Elgazzar et al., 2008). The biopsies were harvested 12-weeks post-operation with the aim to see if the PRP treatment promoted peripheral nerve healing. The article suggests that through distal axon counts, neurotisation indexing and density analysis, a PRP-treated group has potential in enhancing peripheral nerve regeneration. In terms of facial nerve regeneration, a study analysing the effect of PRP and fibrin sealant in a rat model was conducted (Farrag et al., 2007). Male rats were subject to transection in survival and non-survival surgery groups of the left facial nerve and treated with either PPP, PRP or fibrin sealant using the right facial side as the control. Axon counts and facial nerve

motor action potentials were analysed resulting in a faster recovery in the PRP group, the study reported overall that PRP was notably the better option when sutured compared to the other two interventions. A more recent study explored the benefit of PRP lysate on an ischemic stroke in rats (Zhang et al., 2015). The outcomes were measured by means of analysing neurological deficit score and infarct volume. One of the more interesting points that this article tackles is the use of human PRP lysate in a rat model and how it shows a significant benefit in recovery after an induced stroke. Overall, platelet-based applications show a beneficial effect on nerve regeneration in animal models.

1.5.6 Angiogenesis

In a recent study to analyse angiogenesis on a PRP-seeded poly (ε-caprolactone) scaffold, it was reported that this PRP application method may be beneficial for tissue engineering due to the consistent delivery of growth factors without loss of activity (Diaz-Gomez et al., 2014). Not only was there an increase in angiogenesis, the chicken chorioallantoic membrane (CAM) model also increased the hydrophilicity, attachment of mesenchymal stem cells and cell proliferation on the scaffold. The therapeutic value of PRP in angiogenesis can be seen in a study aiming to evaluate the application of platelet-enriched plasma in oral mucosal healing in terms of capillary count and density in a randomised split-mouth design in patients (Lindeboom et al., 2007). The results showed that for the initial two weeks, capillary density and capillary count was higher in the PRP treatment over the placebo treatment administered to the contralateral side. A gelatin hydrogel was used with releasate in a recent study to analyse the aspect of angiogenesis in wound healing (Notodihardjo et al., 2015). This study used male mice to demonstrate that capillary formation was enhanced after 2 weeks in the gelatin hydrogel with PRP group, supporting angiogenesis when compared to a control saline group and a single PRP injection group. The article also reported that augmented wound healing through wound area analysis and angiogenesis using anti-vWF immunohistochemical staining was significantly higher in the treatment group. This study suggests a more specified application of PRP through a hydrogel with releasate can steadily release the growth factors over a period of time being more beneficial than a single PRP injection. It would be interesting to see if the beneficial effects of platelet-

based applications on angiogenesis were directly due to the concentration of VEGF released from the α-granules in platelets, or due to the ratio of growth factors released. Taking these studies into consideration, platelet-based applications are seen to increase angiogenesis.

1.5.7 Cutaneous wound healing

PRP has been shown to be increasingly used in wounds that are difficult to heal such as tissue injuries. In order to address if PRP was beneficial for acute cutaneous trauma wounds such as open and closed fractures as well as epithelial necrosis and friction injuries, a study looked at patients receiving a local injection of PRP (Kazakos et al., 2009). With conventional treatments given to patients as a control group, the PRP group showed a faster rate of recovery in comparison; measured by the time taken for the wound to heal to such a degree that plastic surgery is applicable. This trend of a faster regenerative rate can also be seen in chronic cutaneous ulcers. A study had shown that a localised 100-200 µl injection of autologous platelet-enriched plasma in patients significantly increased the percentage area of healed cutaneous ulcer (Anitua et al., 2008). This study suggests that topical application of platelet-enriched plasma is cheap and effective treatment at tackling chronic ulcers in modern healthcare.

1.6 Stress on the Muscle Microenvironment

Minor amplifications of ROS modulate signal transduction pathways, however while after a certain threshold of ROS, cellular necrosis can be triggered. For example, activated muscle stem cells and primary myoblasts, when isolated, express high levels of dual oxidase 1 (DUOX1), a ROS producing enzyme. DUOXA1 overexpression in these stem cells induces apoptosis and inhibits myogenic differentiation (Sandiford et al., 2014). Interestingly however, ROS can intensify Nuclear Factor κB (NF-κB) activity in muscle satellite cells and myoblasts which is an upstream cause in the reduction of Myogenic differentiation-1 protein (MyoD) levels as well as increasing cyclin D1 transcription and cell proliferation (Kozakowska et al., 2015). These findings indicate that an inhibition of myoblasts' differentiation is taking place, however myogenesis can be controlled by opposing NF-κB pathways (Bakkar et al., 2008). Interestingly, NF-κB signalling was shown to be required for myogenesis (Kozakowska et al., 2015). Similar

to the NF-κB signalling, ROS can also regulate, in a dual manner, the Insulin-like Growth Factor-1 (IGF-1; a skeletal muscle anabolic factor) signalling pathway, which can increase myoblast differentiation and hypertrophy. For example, ROS can increase phosphorylation of the IGF-1 receptor while decreasing IGF-1 transcription (Kozakowska et al., 2015).

A recent study (Martins et al., 2016) has shown that PRP is capable of modulating the oxidative impairment induced *via* skeletal muscle injury. This study explored the effects of PRP on a gastrocnemius contusion in rats in response to oxidative damage (Martins et al., 2016). A localised injection of PRP was applied after injury with subsequent injections every 48 hours. Analysis of the gastrocnemius muscles was carried out 1, 3, 5, or 7 days after injury. Lipid peroxidation levels were analysed as oxidative markers. The contusion was shown to increase the levels of oxidative stress markers in both muscle tissue and in erythrocyte preparations. Interestingly, PRP was able to modulate the damage from ROS in the skeletal muscle post-contusion.

1.7 Signalling Pathways in Satellite Cell Differentiation and Proliferation

Complementary signalling pathways affect both proliferating and differentiating satellite cells during skeletal muscle regeneration (Brack et al., 2008). Two key signalling pathways reported in satellite cell asymmetric division are wingless/integrated (Wnt) signalling (activated during muscle regeneration *in vivo*) and Notch signalling (Wen et al., 2012). It has been suggested that antagonism between Barx2 homeobox protein (Barx2) and Pax7 in direction of Wnt signalling may support in facilitating the switch from myoblast proliferation to differentiation (Zhuang et al., 2014). Barx2 is downstream of canonical Wnt signalling and can control mechanisms of both the Wnt and Notch signalling cascades. Furthermore, Wnt signalling has been shown to induce differentiation, typically asymmetric differentiation potentially due to the gradient change in the newly formed cells during proliferation, in murine myoblast cells (Tanaka et al., 2011, Brack et al., 2008). In differentiation media, Wnt3a amplified the amount of elongated myocytes and fused cells within 18 hours (Zhuang et al., 2014).

In contrast, activation of Notch upregulates the *Pax7* gene and stimulates the symmetric replication of skeletal muscle satellite cells. A myogenic cell line, $NICD^{OE}$,

(overexpression of constitutively activated Notch 1 intracellular domain) was used to show an inhibition of MyoD as well as myogenic differentiation while stimulating Pax7 and encouraging self-renewal (Wen et al., 2012). In mammals, Notch signalling involves four Notch receptors and five ligands (Delta-like 1, 3, 4 and Jagged 1-2). The de-differentiation of myocytes into Pax7 positive quiescent muscle stem cells is induced by MLC-Cre mediated constitutive Notch1 activation (Shan et al., 2017). Additionally, Notch1 signalling also controls the expression of myogenic transcription factors and cell cycle factors (Qin et al., 2013). As this data indicates, Notch and Wnt signalling affect skeletal muscle satellite cell proliferation and differentiation temporally, these signalling cascades are useful tools to analyse when mapping out the mechanisms underlying the effects of platelet releasate on muscle regeneration.

1.8 Current insights into the potential misuse of platelet-based applications for doping in sports

There has been much debate in recent scientific literature both for and against the notion that there is an advantageous use of platelet-based applications in musculoskeletal adaptations (Lee et al., 2011, Nguyen et al., 2011, Sánchez et al., 2014, Mosca and Rodeo, 2015, Brossi et al., 2015, Navani et al., 2017, McClure et al., 2016, Andia and Abate, 2018). The reason for this particular application to attract such consideration relates to the experimental evidence both *in vitro* and *in vivo* showing promising benefit for skeletal muscle recovery after injury (Li et al., 2016b, Li et al., 2013, Saury et al., 2018, Dimauro et al., 2014). However, the use of platelet-based applications fails to consistently translate to positive results in clinical trials, especially for skeletal muscle injuries (Rettig et al., 2013, Bernuzzi et al., 2014, Wetzel et al., 2013, Mosca and Rodeo, 2015). Studies now suggest that the discrepancy in the literature may, at least in part, be due to the differing preparation methods between laboratories and the clinical setting (Andia and Abate, 2018). Beyond the potential benefits of platelets as biomaterials for therapeutic purposes, it has previously been speculated that at least 86,000 athletes in the United States have used platelet-rich plasma injections annually (Wasterlain et al., 2013).

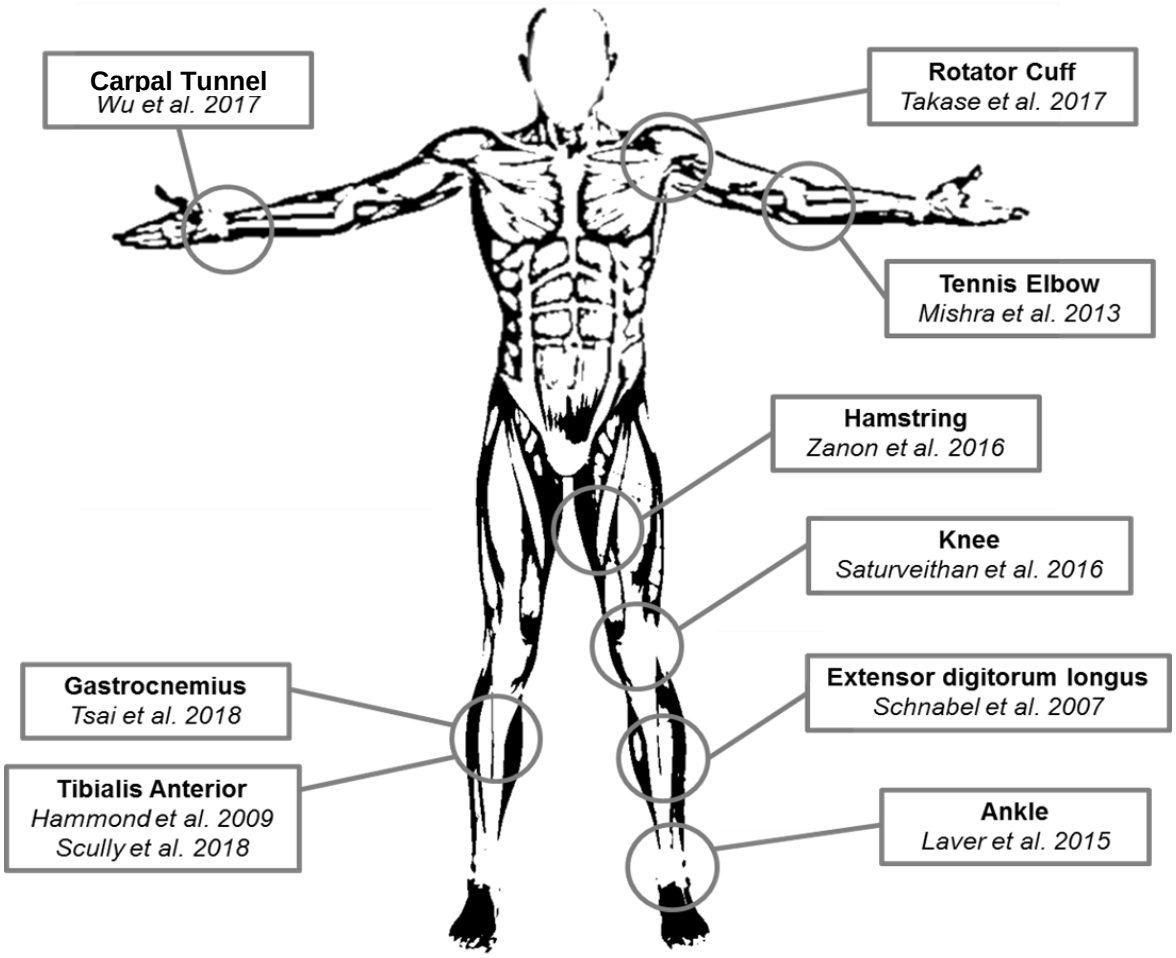


Figure 1.5 A schematic depicting clinical and pre-clinical evidence for the beneficial effects of platelet-based applications on different areas of the body for tendon, ligament and skeletal muscle regeneration. Based on data from the following sources (Takase et al., 2017, Hammond et al., 2009, Wu et al., 2017, Mishra et al., 2012, Zanon et al., 2016, Saturveithan et al., 2016, Schnabel et al., 2007, Laver et al., 2015, Scully et al., 2018).

In 2011, the World Anti-Doping Agency (WADA) removed autologous platelet-rich plasma (PRP) for skeletal muscle, tendon and ligament injections from the prohibition list due to the lack of convincing evidence regarding its performance enhancing and anabolic effects. The WADA's current stance on the status of platelet-derived preparations is that PRP does not exhibit any potential for performance augmentation other than a possible therapeutic effect. Despite the presence of variable cytokines

and growth factors in platelet-derived preparations, they were removed from the prohibition list (WADA, World Anti-Doping Agency. 2011, accessed on 23/11/2018).

In the WADA prohibition lists from 2010-2018 (i.e. section 2 regarding peptide hormones, growth factors and related substances), the agency declared a prohibition against the individual growth factors; "*Growth Hormone, Insulin-like Growth Factor-1, Fibroblast Growth Factors, Hepatocyte Growth Factor, Mechano-Growth Factors, Platelet-Derived Growth Factor, Vascular-Endothelial Growth Factor*". Interestingly, they continued stating that "*any other growth factor affecting muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilization, regenerative capacity or fibre type switching; and other substances with similar chemical structure or similar biological effect(s)*" are also prohibited. This was followed in the same section from 2010 by a prohibition against "*Platelet-derived preparations (e.g. Platelet Rich Plasma, "blood spinning") administered by intramuscular route*" for competing athletes (WADA, World Anti-Doping Agency. 2011, accessed on 23/11/2018, WADA, World Anti-Doping Agency. 2010, accessed on 23/11/2018). However, several of these individual growth factors are found in high concentrations in platelet releasates and are still on the WADA's prohibition list when administered independently as refined constituents (Wijten et al., 2013, WADA, World Anti-Doping Agency. 2010, accessed on 23/11/2018).

Due to the fact that the majority of the above-mentioned growth factors are contained in platelet releasate, the aim of this introductory section is to provide current insights in the potential abuse of platelet-based applications in competitive sports. Another aim is to debate whether there is any merit to consider re-establishing PRP and analogues as a performance enhancing applications (Burkhart et al., 2012). The use of PRP and various other platelet-based applications such as platelet releasate (defined as an acellular preparation of platelet granule secretions after aggregation) have been shown to be beneficial in skeletal muscle injury models (Bernuzzi et al., 2014, Borrione et al., 2010, Dimauro et al., 2014, Denapoli et al., 2016, Hammond et al., 2009, Huang and Wang, 2012, Im et al., 2014, Li et al., 2016b, Li et al., 2013, Martins et al., 2016, Notodihardjo et al., 2015, Pinheiro et al., 2016, Takase et al., 2017, Tsai et al., 2018b) (**Figure 1.5**). For this reason, this introductory segment will focus on the 2011 WADA

statement that "*any growth factors affecting muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilization, regenerative capacity and other substances with similar chemical structure or similar biological effect(s)"* are prohibited by athletes, and that platelet-based applications may be re-established in this category.

1.8.1 Can platelet-based applications be exploited to improve sports performance?

A study determined the effect of a single intramuscular injection of autologous conditioned plasma on the levels of circulating cytokines and growth factors banned by the WADA, in the blood of the recipients (Schippinger et al., 2012). This study reported that amongst the levels of Insulin Like Growth Factor-1 (IGF-1), Endothelial Growth Factor-1 (EGF-1), Platelet-Derived Growth Factor-AB, -BB (PDGF-AB, -BB), Fibroblast Growth Factor (FGF), Vascular-Endothelial Growth Factor-1 (VEGF-1) and Transforming Growth Factor-β1, or -β2 (TGF-β1, -β2), only TGF-β2 showed a significant increase in circulating blood 3 hours and 24 hours post-injection. This may increase the potential for fibrosis according to the authors. If intramuscular injections of PRP do not increase banned substances in the circulatory system, this impedes methods of Anti-doping detection. Unlike blood doping, however, platelet-based applications are autologous, and are supposed unlikely to be ergogenic (Mishra et al., 2012). One may argue that concentrating a substance (e.g. PRP) by removal, refinement and re-introduction to a specific location, such as intramuscular injections; may augment tissue function without being detected in the circulatory system.

Opinions regarding the removal of PRP from the WADA prohibition list have been expressed in detail previously (Creaney and Hamilton, 2008, Engebretsen et al., 2010). It has been argued that the unbound half-life of IGF-1 (10 minutes) would not be able to exert systemic effects, and therefore, any potential benefits are limited to the area of injection. The same study also states that the concentration of IGF-1 has to be 500 times higher than that found in PRP in order to exert systemic anabolic effects (Creaney and Hamilton, 2008). However direct evidence that platelet-based applications are ergogenic in uninjured skeletal muscle is still lacking, providing further

grounds for the removal of PRP from the WADA prohibition list in 2011 (WADA, World Anti-Doping Agency. 2010, accessed on 23/11/2018, Engebretsen et al., 2010). A later publication looked at the systemic effects of PRP in circulation after intra-tendinous injections, using similar PRP preparation methods as Creaney and Hamilton in 2008. This study reported that increased levels of the constituents banned by WADA were detected systemically after injection of PRP (Wasterlain et al., 2013, WADA, World Anti-Doping Agency. 2010, accessed on 23/11/2018). In fact, serum IGF-1, VEGF, and bFGF levels were significantly elevated after PRP injection, associated with an ergogenic effect of PRP, exclaiming that VEGF could be used as a potential marker for PRP doping.

It is important to emphasise the fact that taking PRP off the prohibition list does not restrict the athletes from using higher concentrations of platelet-released growth factors and apply them more frequently. As of yet, there has been no standardised clinical preparation method for PRP, indicating that the system is susceptible to being abused in the context of sports performance. Moreover, additional platelet-based applications can be utilised, such as platelet releasate, platelet lysate, platelet-rich fibrin, leukocyte-rich PRP to further customise and optimise the preparation of PRP for use in athletic competition. Previous studies have attempted to customise PRP for further benefits in skeletal muscle recovery, such that by removing TGF-β it may avoid fibrosis and inflammation (Miroshnychenko et al., 2017). However, the impact of platelet releasate in muscle stem cell function and myogenesis in highly trained healthy individuals such as athletes remains to be determined.

1.8.2 Recent evidence for platelet-based applications to remodel skeletal muscle and potentially increase sports performance

There has been recent robust evidence that platelet-based applications positively affect myoblast proliferation, early inflammatory response, myogenic regulatory factors, regeneration time and muscle fibre hypertrophy with a decrease in pain, claudication score, oxidative stress and time-to recovery (Borrione et al., 2010, Borrione et al., 2014, Dimauro et al., 2014, Hammond et al., 2009, Pinheiro et al., 2016, Martins et al., 2016, Huang and Wang, 2012, Tsai et al., 2018a, Tsai et al., 2017, Denapoli et al., 2016, Im et al., 2014, Li et al., 2016b, Li et al., 2013, Takase et al.,

2017, Bernuzzi et al., 2014, Bubnov, 2013, Wetzel et al., 2013, Miroshnychenko et al., 2017, Sassoli et al., 2014, Sassoli et al., 2012, Sassoli et al., 2018a, Borrione et al., 2018). In particular, platelet releasate has been shown to increase skeletal muscle regeneration *in vivo* (Tsai et al., 2018a, Tsai et al., 2017). This supports the notion that the positive effect on skeletal muscle is derived from platelet granule secretions as opposed to the plasma or cell-to-cell contact. With this being the case, the potential to concentrate the growth factors and increase the dose in an autologous or allogeneic manner is a matter of concern that could be abused by athletes. Growth factors directly derived from platelet-based applications are known to affect other tissues and this is in conflict with the WADA statement that prohibits the use of "any growth factor(s) affecting muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilization, regenerative capacity". As outlined in previous papers, platelet-based applications affect tendon and skeletal muscle tissue and cellular vascularisation, regenerative capacity, protein synthesis and skeletal muscle energy utilization (Arslan et al., 2016, Virchenko et al., 2006, Schnabel et al., 2007, Tsai et al., 2017, Dimauro et al., 2014, Borrione et al., 2014) (See **Figure 1.5**).

The main argument against using platelet based applications is typically due to the lack of detectable growth factors in the circulatory system after intramuscular injection (Creaney and Hamilton, 2008). Although this is a prominent argument whereby platelet-based applications may lack systemic effects; the experimental evidence suggests a localised injection through ultrasound guidance is quite effective at regeneration (Sengodan et al., 2017, Davidson and Jayaraman, 2011). It is indeed important to speed up recovery and injury in sports and orthopaedics; however, the major lacking evidence is the effect on uninjured skeletal muscle. Moreover, *in vivo* studies looking at non-regenerating myofibres remain to be conducted to obtain a better understanding whether platelet-based applications are effective in increasing performance. Additionally, platelet concentration typically takes up to seven days to return to baseline in the blood after donation with prolonged storage of platelet releasate, supra-physiological levels of growth factors can also be achieved (Thokala et al., 2016).

Interestingly, one study looking at uninjured skeletal muscles from racing horses showed an increase in embryonic and type I myosin heavy chain mRNA expression two and seven days after PRP injection, respectively (Fukuda et al., 2017). Whether this can be translated to muscle fibre growth or hyperplasia, was not examined in that particular study and the physiological significance of this finding remains to be established. This is currently the only report on PubMed that identifies using PRP into intact muscle, where a potentially beneficial effect was observed.

A recent article has shown how application of platelet releasate can alter skeletal muscle stem cell fate and drive myogenesis (Dimauro et al., 2014). Myogenic regulatory factors such as MyoD expression have been shown to be altered on myofibres from mice *ex vivo* after platelet releasate application in a dose-dependent manner (Li et al., 2016b). Both Pax7 and MyoD are important markers for skeletal muscle stem cells. Pax7 is expressed in quiescent and activated cells; however MyoD is expressed in activated and proliferating cells (Zammit et al., 2004). Loss of Pax7 in MyoD-positive cells is seen after application of platelet releasate, causing an enhanced commitment to differentiation, without sacrificing the pool of Pax7-positive stem cells returning to quiescence (Li et al., 2016b). Since regular exercise training drives skeletal muscle functional adaptations by regulating muscle stem cell function, the above recent findings raise concerns about the WADA 2018 prohibition list on peptide hormones, growth factors, related substances, and mimetics and/towards potential abuse in sports (Kadi et al., 2005, WADA, World Anti-Doping Agency. 2018, accessed on 23/11/2018). However, it becomes apparent that further studies into intact muscle *in vivo* need to be carried out before any assumption can be made about whether platelet-based applications are beneficial for athletes.

1.8.3 The Food and Drug Administration's stance on platelet-based applications

Although platelet-based applications are not on the United States' Food and Drug Administration's (FDA) banned substance list, there are rules and regulations for commercially using them therapeutically and cosmetically. In terms of using PRP for sports-related or therapeutic augmentations, there are many legalities and such as that clinicians are made to maintain records of use and effects, have a biologics licence and to keep up-to-date with the current evidence in the field (Beitzel et al., 2015). These

restrictions, in turn, help regulate safety and consistency and keep record of PRP procedures. PRP is considered a “biologic”, or biological product by the FDA. Biologics are regulated by the FDA’s centre for biologics evaluation and research. However, the use of autologous growth factors had not been specifically considered by WADA, prior to 2010 (Mishra et al., 2012, Bachl et al., 2009). The ability, however difficult and expensive, to currently treat the public, patients and athletes with PRP and platelet-rich products is perfectly acceptable under FDA and WADA regulations. This leaves the possibility for the application of concentrated supra-physiological platelet-based applications, or frequently used intramuscular injections to be potentially abused. Supra-physiological levels of platelets used to make platelet releasate have been shown to dose-dependently upregulate myoblast proliferation *in vitro*, which may contribute to a faster recovery after training or exercise, similar to anabolics (Li et al., 2010).

1.8.4 Safety of platelet-based applications for use by athletes

Despite the plausible potential for athletes to abuse platelet-based applications to manipulate sports performance, another crucial factor to consider is the safety of application. There is currently increasing evidence in the literature as to whether platelet-based applications affect cellular senescence, apoptosis, reactive oxygen species and mutagenesis. Some promising articles addressing these aspects show a reduction in apoptosis of *in vivo* injured skeletal muscle (Tsai et al., 2018b). Similarly, cellular survival has been shown to be increased with platelet-based applications *in vitro* (Sassoli et al., 2018b). Platelet-based applications are known to be pro-proliferative in many tissues and to induce angiogenesis, which has sparked interest in their effects on cancers. It has recently been shown that platelet releasate causes *in vitro* breast cancer growth and angiogenesis *via* VEGF signalling (Jiang et al., 2017). In fact, platelets are known to drive cellular proliferative signals, cell survival, metastasis and angiogenesis (Franco et al., 2015). This microenvironment may be potentially harmful in already existing cancers, however platelet-based applications are argued to be autologous and non-harmful as they do not induce mutagenesis (Salamanna et al., 2015). On the other hand, clinical trials report an effective and safe

outcome to using platelet-based applications for multiple treatments with no observed side effects (Bernuzzi et al., 2014, Bubnov, 2013, Wetzel et al., 2013, Zanon et al., 2016, Le et al., 2018). Additionally, allogeneic platelet-rich plasma has recently been deemed safe for osteoarthritis patients according to a human pilot study (Bottegoni et al., 2016). This suggests that PRP may be a safe treatment even if non-autologous; although this remains to be established in large scale studies. At present, the WADA does not distinguish between the use of autologous and allogeneic PRP (Anitua et al., 2017a, Anitua et al., 2017b). Subsequently, a potential route towards performance manipulation cannot be ruled out, such that master-class athletes are currently not restricted from using allogenic platelet-rich plasma from young athletes (Anitua et al., 2017c). A recent study has established a differential profile of platelet-rich plasma-contained growth factors amongst individuals in terms of age and sex, showing higher levels of growth factors (e.g. IGF-1) and lower levels of inflammatory and fibrosis-inducing cytokines (e.g. TGF-β) in young males (Xiong et al., 2018). Consequently, with allogeneic PRP becoming a safe and viable option for patient treatment, and no ban on its use in athletes, the risk of exploitation is not prohibited.

On a final note, there is a noticeable difficulty of testing for autologous platelet-based applications. PRP's involvement in altering the biological passport of an athlete could be a way of detecting potential abuse for sporting applications. Perhaps the World Anti-Doping Agency might consider revising the use allogeneic platelet-based applications in their prohibition list. The option for athletes to use their own platelet's innate ability to induce regeneration is an important tool in use in clinics worldwide. Additionally, allogeneic platelet-based applications should not be seen as unlike to utilising a cocktail of recombinant growth factors for a competitive edge in sports.

1.8.5 Future perspectives

There is limited evidence on whether platelet-based applications can improve sports performance and/or skeletal muscle transcriptional signature in healthy, non-injured individuals. Therefore, more studies are needed to assess experimentally whether such platelet-based approaches are able to improve muscle strength, and/or increase muscle mass, endurance, athletic performance, and/or affect recovery time, protein

synthesis and/or alter redox homeostasis. To this aim, it is essential to establish and further develop techniques in order to detect abuse of platelet-based applications in competitive sports. For example, VEGF has been previously suggested as a marker for PRP-doping in the blood (Wasterlain et al., 2013).

Knowledge in the field of platelet-based applications affecting skeletal muscle, ligament and tendon is continuously accumulating. Despite the absence of direct evidence linking platelet applications with increased sports performance, current evidence suggests that platelet-rich plasma drives skeletal myogenesis *in vitro*, *ex vivo* and accelerates regeneration *in vivo*. This falls within the current doping definition regarding manipulation of regenerative capacity and begs the question whether platelet-based applications have to be reconsidered as prohibited substances or methods for competitive athletes.

1.9 Conclusions

In conclusion, there is mounting evidence on the use of platelet-based applications in tissue regeneration. There is currently a large discrepancy in the effectiveness of platelet-based applications in the scientific literature, especially between human and experimental animal studies. This may be attributed to methodological differences in platelet preparation and platelet releasate composition among different research groups. At present, there is an intense interest in the field worldwide with tremendous possibilities for exploitation in regenerative medicine. The current consensus with the use of PRP and especially modified PRP (where individual factors are depleted) in skeletal muscle regeneration remains promising, despite an incomplete understanding of mechanistic insights in both knowledge of platelet-satellite cell interactions, as well as PRP preparation optimisation. Most importantly, the molecular mechanisms linking platelet biology to skeletal muscle, or other tissue regeneration, have just begun to unravel and are expected to transform our understanding in using platelets as a biomaterial for tissue healing.

Current insights and biological evidence that set the ground for exploitation and misuse in competitive sports and provide reasoning that may help to develop strategies to combat this are discussed. With studies reporting no increase in systemic levels of

growth factors in the blood after intra-muscular PRP injections, there is reasonable merit to not consider PRP a banned substance. However, with more studies emerging, additional consideration needs to be deliberated, such as manipulation, customisation and concentration of PRP preparation methods that can potentially be abused by athletes. Allogeneic applications from healthier and younger individuals that were overlooked previously in terms of performance manipulation may require re-evaluation. The 2011-2018 WADA prohibition lists declare that any substance that increases muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilization or regenerative capacity is banned. As current evidence has shown that such methods strongly impact skeletal muscle, tendon, ligament and orthopaedics in terms of regeneration and potential for sports performance, the risk to abuse platelet-based applications remains valid.

Chapter 2

General Materials and Methods

2.1 Practical Methods

2.1.1 Animals

Mice were accessed from the University of Hull. The study was approved by the local Ethics Committee of the University. Mice homozygous for deletion of Thrombospondin-1 (TSP-1), Cluster of Differentiation 36 (CD36), and Apolipoprotein E (ApoE) on a C57Bl/6J background (The Jackson Laboratory, Bar Harbor, ME) and wild type (C57Bl/6J) mice were used in accordance with UK Animals (Scientific Procedures) Act 1986 and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). Only male mice were used in order to eliminate confounding variables from sex hormones. Mice were housed in a temperature-controlled room with a 12 hour light/dark cycle and had free access to water and food. The mice were euthanized by Schedule 1 according to the 2013 Edition of AVMA Guidelines for the Euthanasia of Animals.

2.1.2 *In Vivo* cardiotoxin-induced muscle injury.

In order to examine the rate of regeneration for platelet releasate treatment on murine muscle, a cardiotoxin injury was implemented. For the experimentation in Chapter 3, the aim was to see the effect of platelet releasate on skeletal muscle regeneration, which was kindly carried by Dr. Robert Mitechell out at the laboratory of Professor Ketan Patel of the University of Reading. To achieve this, on day 1; mice (12 week old) were tail vein injected either with 100 µL platelet releasate or 100 µL phosphate-buffered saline (PBS; Oxoid, UK, cat. BR0014) (n=5 per group). 30 minutes later, mice were injected with a total of 30µL, 10µM *Naja pallida* cardiotoxin (CTX; Latoxan, Valence France) into the tibialis anterior muscle. 24 hours later (day 2), the mice received a second identical intravenous injection of either the platelet releasate or PBS. At 5 days, mice were sacrificed, the TA muscles were collected, immediately frozen and 12µm cryo-sections were processed for immunohistochemistry.

For the *in vivo* regeneration experimentation in Chapter 4, two time-points were considered (Day 5 and day 10) as there was a platelet releasate treatment and a

genotype difference (WT and TSP-1 KO). In our laboratory, at time-point 0 hours, C57Bl/6J mice (12 week old) and TSP-1 KO mice (12 weeks old) were anesthetised with ketamine/xylazine (100mg/kg and 10mg/kg respectively). Mice were then injected with a total of 30µL, 10µM CTX into the tibialis anterior muscle. Mice were then injected intraperitoneally (IP) either with or without 200 µL platelet releasate (n=3/group/time-point). 24 hours later, the mice in the releasate groups received a second identical IP injection of platelet releasate, and a third injection at 72 hours. After 5 or 10 days, mice were sacrificed and the TA muscles were collected, immediately frozen and 12µm cryo-sections were processed for immunohistochemistry.

2.1.3 Tissue harvesting and freezing

Murine tissue was dissected in order to analyse skeletal muscle differences between genotypes, muscle weights, cross sectional area, culture of single muscle fibres and analysis of the effects of platelet releasate on injured skeletal muscle. Extensor Digitorum Longus (EDL), Tibialis Anterior (TA), Gastrocnemius (Gas), Quadriceps (QDs), Biceps Brachii (BB) and soleus muscles were excised from both limbs. The connective tissue surrounding the EDL, TA and soleus muscles was removed and the muscles were weighed and subsequently frozen in isopentane (2-methylbutane) cooled in liquid nitrogen in order to avoid freezing artefacts, autolysis or putrefaction. Quadriceps and gastrocnemius were snap frozen in liquid nitrogen for molecular analysis, stored on dry ice before transporting to the -80°C.

2.1.4 Tissue embedding and cryo-sectioning

Skeletal muscle morphology and fibre-typing of the EDL, TA and soleus muscles were evaluated by histological and immunohistochemical procedures. Specimens were embedded in optimal compound temperature tissue mounting medium (OCT; VWR, UK cat. 00411243) and 100% ethanol on dry ice. A cryo-mould was filled with OCT and was subsequently submerged in cooled ethanol. Once the OCT began freezing, the tissues were transferred, oriented for cryo-sectioning and fully submerged in the OCT. The frozen blocks (tissue in OCT) were stored at -80°C.

Prior to cryo-sectioning, blocks were equilibrated to -21°C for 20 minutes in the cryostat chamber. Transverse sections (12µm thickness) from the mid-belly of the muscles were obtained and mounted on microscopy glass slides coated with Poly-L-lysine (Thermo Scientific, UK, cat. J2800AMNT). The microscopy glass slides were air dried for 30 minutes at room temperature and stored at -80°C until further analysis.

2.1.5 Haematoxylin and eosin

For *in vivo* cardiotoxin-induced murine tibialis anterior injury, haematoxylin and eosin staining (H&E) was conducted in order to detect if the cryo-sections are indeed in the area of injury. When haematoxylin becomes oxidised, one of the products are haematin, which colours the nuclei of cells blue. The nuclear staining is counterstaining with an aqueous solution of Eosin (Sigma, UK, cat. E.4382), which colours eosinophilic structures in various shades of red, pink, and orange (Chan, 2014). Cryo-sectioned slides were retrieved from the -80°C freezer and let to air-dry for 15-20 minutes. Borders were next drawn around the tissue sections on the optical slides with a hydrophobic PAP pen (Sigma Aldrich, cat. Z377821, UK) and let dry for a further 10 minutes. Tissue sections were re-hydrated with 1000µL of PBS for 2 minutes. Slides were then fixed with 4% paraformaldehyde (PFA; 200µL, Sigma Aldrich, UKP6148-500G) for 15 minutes. Sections were then washed twice with PBS for 2 minutes. Haematoxylin staining (1:10 in dH_20; Haematoxylin Solution, Harris Modified, Sigma Aldrich, UK, cat. HHS32) was added at 200µL per slide for 5 minutes. In a Coplin jar, the slides were rinsed in distilled (d) H_20 for 3 minutes on a low flow pressure. Eosin staining was conducted for 200µL per slide for 30 minutes. Next, the staining was washed off for 3 minutes in a Coplin jar with dH_20 on a low flow pressure. Slides were then mounted with PermaFluor™ Aqueous Mounting Medium (Thermo Scientific, UK, cat. TA-030-FM) and a coverslip was added before imaging.

2.2 Preparation of Platelet Releasate

In order to test the effects of platelets on skeletal muscle satellite cells and skeletal myoblasts, platelets from human blood were collected and isolated. Blood sampling

from healthy human volunteers was performed with written informed consent and was approved by the University's Ethics Committee.

2.2.1 Human Platelet Releasate

Human platelet releasate was prepared in acid citrate dextrose (ACD) to whole blood at a ratio of 1:5, centrifuged in a swing-out rotor at 190g for 15 minutes on 0 breaks at 22°C. This was followed by PRP collection by a Pasteur pipette and inactivation of platelets using prostaglandin I_2 (534nM; Cayman Chemical). The PRP was then centrifuged in a swing-out rotor at 800g for 12 minutes at 22°C on 3 breaks and the platelet-poor plasma supernatant was then removed. Modified Tyrode's buffer at a pH of 7.4 (NaCl; 150mM, HEPES (Na^+ Salt); 5mM, NaH_2PO_4 (anhydrous); 0.55mM, $NaHCO_3$ (Anhydrous); 7mM, KCl; 2.7mM, $MgCl_2$ (Hexa (6) hydrated); 0.5mM and D-Glucose (anhydrous); 5.6mM) was used to re-suspend the platelet pellet at a concentration of $2.5x10^8$ platelets mL^{-1} to $10x10^8$ platelets mL^{-1} (unless otherwise stated) using a cell counter (Beckman Coulter; Z1-Series Coulter® Particle Counter). The platelet preparation was activated using a PAR1 agonist (TRAP6; 20μM; AnaSpec; cat. AS-60679), thrombin (0.05-0.1 NIH Units mL^{-1}; Sigma Aldrich; cat.9002-04-4), or collagen (10μg mL^{-1}; BioData; cat. 101562), in an aggregometer (CHRONO-LOG® Model 490 4+4 Optical Aggregation System, USA). Alternatively, after counting and re-suspending in modified Tyrode's buffer, platelets were sonicated for 2 minutes using a 0.5 cycle at a gradually increasing amplitude of 60–100% (Hielscher Ultrasonic Processor - UP200S). Platelet aggregates were stored on wet ice until centrifuged. Platelets were centrifuged at 9500g for 10 minutes, unless otherwise stated, and the releasate supernatant was aliquoted and stored at -80°C (or used freshly where indicated).

2.2.2 Murine Platelet Releasate

Mouse platelets from wild-type and knock-out TSP-1, CD36 and ApoE mice were extracted locally. Mouse platelet releasate from GPR55, FPR1 and FPR2 KO mice were extracted and donated kindly from the University of Reading; using the same method as described below. Murine blood prepared with 200μL ACD in a 1mL syringe

& 25-gauge needle, gently mixed before transferring to 500µL modified Tyrode's buffer with 500µL ACD, per mouse, and centrifuge in a swing-out rotor at 100g for 5 minutes with no breaks. The PRP supernatant was then transferred using a Pasteur pipette to a clean Eppendorf tube and centrifuged at 800g for 5 minutes with no breaks. The platelet-poor plasma was discarded and platelets were resuspended in modified Tyrode's buffer to a concentration of $2.5x10^8$-$10x10^8$ platelets mL^{-1} using a Beckman Coulter particle counter. Collagen (10µg mL^{-1}) was used to activate platelets in an aggregometer for 5 minutes at 37ºC (reaching > 80% aggregation) before centrifuging at 9500g and storing releasates at -80°C.

2.2.3 Platelet releasate growth factor multiplex immunoassay.

Growth factor analysis was conducted with collaborators as denoted in the Declaration of this Thesis. Releasate was sent *via* shipment on dry ice. Growth factors contained in the platelet releasate were measured by two methods, i) using high-performance multiplex immunoassays with the Bio-Plex Pro™ Human Cancer Biomarker Panel 1 and 2, (BioRad, UK, Cat. 171AC500M and 171AC600M respectively) and ii) ProcartaPlex Human kits (Invitrogen) which test a panel of 37 molecules, including growth factors, cytokines, chemokines and immune stress markers. The Bio-plex array system includes a blend of magnetic bead-based assays for a number of biomarkers involved in cell division such as Angiopoietin-2, sCD40L, EGF, Endoglin, sFASL, HB-EGF, IGFBP-1, IL-6, IL-8, IL-18, PAI-1, PLGF, TGF-α, TNF-α, uPA, VEGF-A, VEGF-C, VEGF-D, sEGFR, FGF-basic, Follistatin, HGF, sHER-2/neu, sIL-6Rα, PECAM-1, PDGF-AB/BB, SCF, sTIE-2, sVEGFR-1, sVEGFR-2 as defined in **Table 3.1**. Bio-plex assays were performed using the Bio-Plex 200 system according to the manufacturers' instructions. The heat-map of hierarchical clustering and principal component analysis of Bio-Plex data was performed on the Perseus software (version 1.5.5.3).

For the ProcartaPlex Human kits, all releasate samples were analysed by multiplex immunoassay based on Luminex 200 technology (Luminex Corporation, USA). The multiplex assay was performed following the manufacturer's instructions and the plates were read using the xPONENT software (Luminex Corporation, USA). The specific factors analysed were: Caspase-3, CD40L, EGF, FGF-2, FGF-23, G-CSF (CSF-

3),GM-CSF, GITRL, Granzyme B, GRO alpha (KC/CXCL1), HGF, ICAM-1, IFN gamma, IL- 1a, IL-1b, IL-2, IL-6, IL-7, IL-8 (CXCL8), IL-10, MIP-1a (CCL3), MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MIP-1b (CCL4), Osteopontin, PDGF-BB, PECAM-1, P-Selectin, RANTES (CCL5), SDF-1α, Thrombopoietin (TPO), TGFβ, TNFα, VCAM, VEGF-A and VEGF-D.

2.3 Cell cultures and treatments

Dr. Montrose Thomas Burrows was recognised with coining the phrase "tissue culture", and is the first to use plasma instead of lymph in tissue culture of cells from warm blooded animals (Carrel and Burrows, 1911). 108 years later, our lab aimed to assess the effect of platelet releasate on skeletal and cardiac muscle, using both a standardised immortalised myoblast cell line; murine C2C12 skeletal myoblasts (American Type Culture Collection, VA) and a rat H9C2 cardiomyocyte cell line (LGC-PromoChem, Teddington, UK)(Yaffe and Saxel 1977). C2C12 cells were cultured in growth medium (GM); Dulbecco's Modified Eagle's Medium (DMEM; HyClone (High glucose, no sodium pyruvate, FischerScientific, UK SH30022.01)) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, UK, cat. F0804), 1% penicillin/streptomycin (PS; Sigma-Aldrich) and 0.1% amphotericin B (AB; Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2. Rat H9C2 cardiomyocytes were cultured in the same conditions; however, the DMEM contained glutamine and sodium pyruvate (Thermo Fischer, UK, cat. 10569010). cardiac (H9C2) and skeletal muscle (C2C12) cells have been studied in laboratories through the implementation of the anti-cancer drug, Doxorubicin; a DNA and RNA synthesis inhibitor developed in the 1960s (Bielak-Zmijewska et al., 2014, Chen et al., 2018, Richardson and Johnson, 1997, Sin et al., 2016). In Chapter 5, H9C2 cells, C2C12 cells and single EDL muscle fibres were treated with Doxorubicin (0.0625μM, 0.125μM, 0.25μM or 0.5μM Cayman chemical cat. 15007) in order to simulate a dose-response of cellular senescence.

2.3.1 Cell Thawing

Importantly, frozen cells are very fragile during handling (Bakhach, 2009). Cryo-vials were taken carefully from liquid nitrogen and put onto dry ice for transport. Next, they

were put in a pre-warmed water bath set to 37°C for 35 seconds to begin thawing. Cryo-vials were immediately diluted in a sterile cell culture hood in 15mL of pre-heated growth medium to 37°C to dilute the dimethyl sulfoxide (DMSO) contained in the freezing solution (90% FBS and 10% DMSO) from 10% to 0.62%. The cell suspension was then centrifuged at 210g for 5 minutes and the pellet was gently homogenised in 1 mL of growth medium and counted to a set concentration for cell seeding (see section 2.3.2. and section 2.3.3.).

2.3.2. Cell Freezing

In order to keep passage numbers low and maintain a high-quality cell line, sub-passages of cells were cultured and frozen down to be used when the working cell line becomes too old. All medium was removed from cell culture flask by tipping into a virkon waste bath under sterile cell culture hood conditions. The T75cm flask containing cells was washed gently with 5mL of pre-heated (37°C for 30 minutes) PBS twice per flask. PBS was removed and 3mL of 0.25% Trypsin solution (preheated at 37°C for 30 minutes) per flask added and incubated for 7 minutes at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the flask was tapped gently to detach cells from the flask. Cells were checked by microscopy that they had indeed detached. 5mL of growth medium was added per T75cm flask in order to stop the digestion reaction of the trypsin on the cells. Cells were then collected into a 50mL falcon tube before centrifuging at 210g for 5 minutes at room temperature. The supernatant was removed gently before re-suspending in 1mL of freezing solution (90% FBS and 10% DMSO). Cells were mixed 20 times gently by pipette before counting (see section 2.3.3). Cells were placed in labelled cryo-vials at -20°C for 1 hour, and then transferred to -80°C for 48 hours before transporting on dry ice to freeze in liquid nitrogen.

2.3.3 Cell Counting

As in the previous section (2.3.2), and in cell passaging, standardising the number of cells by counting them is an important step in maintaining a cell culture. In a suspension of cells of 1mL (i.e. freezing solution), 50µL re-suspended cells were pipetted 6 times

and made homogenous. This 50µL was added to 50µl of PBS and mixed. 50µL of this suspension was further diluted in 50µL of trypan blue to have a dilution factor of 1:4. This diluted cell suspension in trypan blue is then pipetted onto a haemocytometer to count using the following formula:

- **(**Total number of cells counted x 10,000 x 4(dilution factor))/4(squares measured on the haemocytometer**)** = Total cells/mL
- Cell culture flasks of T75 were seeded with 75,000 cells per passage. Therefore, the following calculation was used to determine the volume of cells per flask in millilitres;
- **(**75,000 cells/ (total cell count) x 1000µL**)** = volume of cell suspension that equates to 75,000 cells.

2.3.4 Differentiation of C2C12 and H9C2 cells

C2C12 cells are capable of undergoing differentiation, which consists of cell cycle inhibition and expression of myogenic factor-4 (Myogenin) in order to fuse with neighbouring cells with the objective of forming myotubes (Flamini et al., 2018). To induce differentiation, C2C12 cells were cultured in GM until reaching 80% confluence before switching to differentiation media (DM), containing DMEM plus 2% horse serum (HS; Gibco) 1% PS and 0.1% AB for either 6, 9 or 12 days. Depending on the experimental condition, platelet releasate was added either once during proliferation at time-point 0, or at every media change (either every 12 hours or 24 hours depending on the experiment) or during/after differentiation, unless otherwise stated. For the use of inhibitors, they were added during DM media changes when indicated using 5µM PDGFR Inhibitor (Tyrphostin AG 1295, Santa Cruz, UK) or a 130nM VEGFR Inhibitor (AAL-993, Merck, UK). The myofusion index for C2C12 cells was calculated as Myogenin^{+ve} cells per myotube (with a minimum threshold of 3 nuclei per myotube) divided by DAPI (4',6-diamidino-2-phenylindol-stained (Dako) -stained cells as a percentage. For proliferation in all experimental groups, unless otherwise stated, C2C12 cells were cultured in serum free (SF) conditions (DMEM, 1% PS and 0.1% AB). Inhibitors used for proliferation and differentiation experiments were VEGFR

Inhibitor (AAL-993; 23nM, 130nM or 1.30μM) and PDGFR Inhibitor (AG-1295; 250nM, 500nM or 5μM).

2.3.5 Single fibre isolation and culture

Single-fibre skeletal muscle cell culture has been conducted previously as a useful tool to analyse the proliferation and differentiation profiles of the satellite cells in their micro-environmental niche (Zammit et al., 2006b). For single-fibre culture, murine C57Bl/6J single fibres were isolated from the extensor digitorum longus muscle (EDL). Limb muscles were dissected and subjected to pre-warmed collagenase (Sigma Aldrich; cat. C2674) digestion medium (2mL of DMEM with 1% Glutamine, 1% PS and 0.2% collagenase stock (20mg/mL) in a Bijoux) for 3-4 hours at 37°C and 5% CO_2 stirring gently every 30 minutes with the lid of the Bijoux left partially unscrewed. The digested muscle was then placed in a 35mm diameter pre-coated (0.7mL horse serum, removed and air dried for 30 minutes) tissue culture petri-dish. The collagenase was carefully removed using a wide-bore fire-polished glass pipette (UV glass pipettes before using for cell culture). Preheated (37°C) wash medium consisting of DMEM with 1% Glutamine and 1% PS was added (2mL) to the petri-dish and gently triturated with a glass pipette. Fibres that came off the muscle were immediately transferred to a second pre-coated (horse serum) petri-dish (35mm diameter) during trituration. Approximately 50 fibres were added to each replicate well of a 6-well plate. Fibres were then cultured for 48 and 72 hours in single fibre media (FM; DMEM with Glutamine and sodium pyruvate, 1% PS, 10% horse serum, 0.5% chick embryo extract), or serum free media (SF) with the addition of 10% platelet releasate (R) 3 times during culture every 16 hours (unless otherwise stated). Inhibitors used for proliferation and differentiation experiments were VEGFR Inhibitor (AAL-993; 23nM, 130nM or 1.30μM) and PDGFR Inhibitor (AG-1295; 250nM, 500nM or 5μM).

2.3.6 Myofibre fixation and staining

Proliferation and differentiation profiles of the single-fibre skeletal muscle satellite cells were previously defined as the percentage of muscle stem cells per fibre expressing the myogenic regulatory factors (MRFs). Specific MRFs define the

proliferative/differentiative state of a satellite cell such as; $Pax7^{+ve}/MyoD^{+ve}$ (cell activation), $Pax7^{-ve}/MyoD^{+ve}$ (commitment to differentiation) and $Pax7^{+ve}/MyoD^{-ve}$ (quiescence) at 48 hours after seeding, or for 72 hours; cells per fibre expressing $Pax7^{-ve}/Myogenin^{+ve}$ (differentiating cells) and $Pax7^{+ve}/Myogenin^{-ve}$ (returning to quiescence)(Zammit et al., 2006a). 6-well plates were taken from the incubator and the media was removed carefully. 1.5mL of 4% PFA was added per well for 15 minutes at room temperature. Using the wide-bore fire-polished glass pipette, the PFA was then removed and 2.5mL of PBS was used to wash each well. During this and the second wash of PBS, dead fibres and debris were removed. Next, myofibres were carefully transferred to a labelled 1.5mL Eppendorf tube. Of note, the time-point 0 fibres were fixed and stored at 4°C in order to conduct all (T48 and T72 hour) staining simultaneously.

From the 1.5mL Eppendorf tubes, as much PBS was removed as possible without disturbing the fibres. 500mL of Permeabilisation Buffer (0.5% Triton X-100, 3mM $MgCl_2$, 20mM HEPES, 300mM sucrose, 50mM NaCl) was used In order to detect intracellular antigens; as cells must first be permeabilised to allow access for the antibodies to bind (Jamur and Oliver, 2010). After removal of the permeabilisation buffer, wash buffer (1 litre consists of 948.5mL PBS, 50mL FBS, 1mL Sodium Azide and 500µL Triton-X 100) was applied for 30 minutes at room temperature. During these 30 minutes, primary antibodies were prepared. Fibres were stained for mouse monoclonal anti-Pax7 (1:200, Santa Cruz; cat. sc-81648), rabbit polyclonal anti-MyoD (1:200; Santa Cruz; cat. sc-760) and rabbit polyclonal anti-Myogenin (Santa Cruz; cat. sc-576), or with anti-Cyclin D1 (1:200 Santa Cruz; cat. sc-450) or Scrib (Santa Cruz; cat. sc-374139). The 1:200 dilutions were made up in wash buffer, to avoid unspecific binding. T0 comprised of Pax7, T48 had Pax7 plus MyoD and the T72 samples had Pax7 plus Myogenin co-staining of primaries. The primary antibody solutions were left overnight at 4°C in the dark. Next, the first secondary antibody (Alexa fluor 488 Goat-anti-mouse; Life Technologies; cat. A11029) was prepared in wash buffer while the primary antibody was removed. Wash buffer was added to the fibres for 30 minutes to remove the primary antibody solutions. 200µL of secondary antibody was added for 1 hour at room temperature in the dark per sample of fibres. This was followed by 30

minutes in wash buffer and application of the second secondary (1:200 Alexa fluor 594 Goat-anti-rabbit; Life Technologies; cat. A11037) in wash buffer for 1 hour at room temperature in the dark. The samples were then washed in wash buffer (500µL three times). The fibres were next transferred to an optical slide and mounting medium with DAPI before adding a coverslip for imaging. The commitment index was calculated as MyoD-stained muscle stem cells divided by DAPI-stained cells as a percentage. The list of antibodies used is detailed in the **Appendix I**.

2.3.7 Satellite cell isolation from single fibres

Next, the effect of platelet releasate on primary satellite cells stripped from the single myofibres with the aim of deciphering the effect of the satellite cells off of their micro-environmental niche was analysed (Charville et al., 2015, Montarras et al., 2005). For primary myoblasts, satellite cells (i.e. primary muscle stem cells) were derived from both the EDL and biceps brachii muscles of wild-type C57Bl/6J mice as above. Limb muscles were dissected and subjected to 0.2% collagenase digestion for 4 hours at 37°C. The digested muscle was then gently transferred to 35mm petri-dishes where the collagenase was removed. Single muscle fibres were isolated in serum-free culture medium, by means of a gentle mechanical trituration with a glass pipette and then cultured for 72 h in Matrigel (1mg mL-1; Corning Matrigel; cat. 354234) -treated 6-well plates, at 37°C in a humidified atmosphere of 5% CO_2, in satellite proliferation media containing DMEM, 30% FBS, 1.5% chick embryo extract (C.E.E.; APS, UK, MD-004D-UK) plus 1% P.S. Next, the myofibres were removed and the satellite cells were cultured as per experimental conditions. Differentiation of primary satellite cells (stem cells) was achieved through culturing for 3-4 days in appropriate proliferation medium as per experimental condition before switching to differentiation medium (5% Horse Serum, 0.5% C.E.E., 1% P.S. and 0.1% A.B.) for a further 4-5 days.

2.4 Technical Methods

Primary and secondary antibodies for immunofluorescence and immunohistochemistry are given in **Appendix I**.

2.4.1 Immunofluorescence

An antibody (immunoglobulin) is a Y-shaped protein produced mainly by plasma cells that bind to antigens (Noll et al., 1982). Immunofluorescence is the procedure by which antigens are detected with the use of antibodies that are conjugated with fluorescent dyes. Immunofluorescence was conducted in order to examine whether a specific antigen is expressed in tissues and cells. Immunofluorescence is usually comprised of two steps. Firstly, the primary antibody binds to the target antigen in the cells or tissues and in the second step; a secondary antibody conjugated with a fluorescent dye is used to bind to the primary antibody.

2.4.2 Immunohistochemistry

C2C12 cells and H9C2 cells or primary muscle stem (satellite) cells, as appropriate, were seeded on coverslips in 1mL of media in 24-well plates (Corning® Costar® TC-Treated 24-Well Plates). Staining of single myofibres' satellite cells *ex vivo* was conducted as per section 2.3.6. Media was removed at the end of experiments with 4% PFA added for 15 minutes, followed by two washes in PBS. Permeabilisation buffer was then added for 20 minutes followed by two washes in wash buffer before transferring onto optical slides. Primary antibodies for anti-Pax7 (Santa Cruz, cat. sc-81648), anti-MyoD (Santa Cruz; cat. sc-760), anti-Myogenin (Santa Cruz; cat. sc-52903), mouse monoclonal anti-IGF-1Rα (Santa Cruz; cat. sc-271606), anti-PDGF B (Santa Cruz; cat. sc-365805), anti-VEGF (Santa Cruz; cat. sc-7269), anti-Ki-67 (ThermoFisher. Cat. 14-5698-80), anti-IL-10 (Santa Cruz; cat. sc-8438), anti- VEGF-C (Santa Cruz; cat. sc-374628), anti-PCNA (Santa Cruz; cat. sc-56), anti-TLR4 (Santa Cruz; cat. sc-293072), ICAM-1 (Santa Cruz; cat. sc-8439), anti-myosin heavy chain 3 (Santa Cruz; cat. sc-53091), anti-Cyclin D1 (Santa Cruz; cat. sc-450) or anti-Scrib (Santa Cruz; cat. sc-374139) were added (1:200 in wash buffer) overnight. Primary antibodies were removed with 3 washes in wash buffer, followed by addition of secondary antibodies (Alexa fluor 488 Goat-anti-mouse; Life Technologies; cat. A11029 or Alexa fluor 594 Goat-anti-rabbit; Life Technologies; cat. A11037) in wash buffer (1:200). Cells were measured by the intensity of fluorescence per cell divided by

DAPI-stained nuclei as a percentage. All antibodies are detailed further in **Appendix I**.

2.4.3 Immunofluorescence for myofibre composition

In order to examine if there is a skeletal muscle genotype switch between mouse models, fibre-typing was conducted. Fibre-typing was used for examining the distribution of the two myofibre groups (i.e. types IIB - IIA or types IIA - I). Identification of type IIX myofibres in muscle sections was conducted simultaneously (i.e. serial sections stained with types IIB - IIA and types IIA - I)(Larsson et al., 1991). MHC type I, IIA and IIB were identified by using A4.84 IgM, A4.74 IgG and BF-F3 IgM monoclonal primary antibodies respectively, as summarised in Appendix I (Matsakas et al., 2009). Primary antibodies were visualised using Alexa Fluor secondary antibodies as summarised in Appendix I (Matsakas et al., 2009). Firstly, cryo-sections were equilibrated to room temperature and rehydrated with phosphate buffered saline (PBS) very carefully for 2 minutes. Cells were permeabilised with permeabilisation buffer and incubated with blocking buffer (5% FBS, 0.05% Triton X-100, 0.1% Sodium Azide) to enhance cell membrane permeability and reduce non-specific background staining, respectively. Specimens were incubated with the primary antibody for type IIA myofibres overnight at 4°C. On the following day sections were washed with wash buffer and incubated with the secondary antibody Alexa Fluor 488 (**Appendix I**). Sections were rinsed with wash buffer and were incubated with the second primary antibody for either type IIB or type I overnight at 4°C. On the following day, specimens were washed in wash buffer and the Alexa Fluor 633 secondary antibody was applied for 1 hour at room temperature and kept away from light sources (**Appendix I**). Sections were rinsed with wash buffer, mounted with fluorescent mounting medium containing DAPI and imaged (see section 2.4.5 for imaging). Quantification of myofibre number, type and cross-sectional area (CSA) was performed manually with the use of ZEN lite imaging software (Zen Software Zen 2.3 Blue Edition ©Carl Zeiss microscopy GmbH, 2011). Myofibres negative for Mhc IIA and IIB on double stained sections were considered as type IIX, after subtracting the number of type I stained myofibres from serial muscle sections.

2.4.4 Cell proliferation and viability analysis

Cellular proliferation was evaluated *via* the pyrimidine analogue EdU incorporation assay using the fluorescent Click-iT® EdU Cell Proliferation Assay (BaseClick, Germany, cat. BCK-EdU488) according to the manufacturer's instructions. The advantage of this assay was that EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analogue to thymidine which can be incorporated into Deoxyribonucleic acid (DNA) during cellular proliferation, a live staining of cellular replication. In brief, a 10mM stock solution of EdU was prepared in DMSO so that a 1µL of EdU stock solution in a 999µL well of medium in a 24-well plate equates to a final concentration of 10µM EdU. The incubation time for the majority of cellular proliferation assays was 3 hours with EdU before fixation and staining as detailed in the manufacturer's instructions. Proliferating cells were measured as EdU divided by DAPI-stained nuclei as a percentage. In addition, cellular viability was assessed by seeding 50,000 C2C12 cells in a 35mm petri dish for 24 hours in either serum-free, 10% FBS growth medium, 10% releasate or 10% FBS growth medium plus 10% releasate, and counting the ratio of live to dead cells using trypan blue.

2.4.5 Digital imaging and analysis

A Zeiss Axio Observer Z1 microscope was used (Zeiss, Germany) with x5, x10, x20 and x40 objective lenses and an Axiom 105 microscope camera (Zeiss, Germany) with Zen Software Zen 2.3 Blue Edition ©Carl Zeiss microscopy GmbH, 2011. Fluorescent imaging and SDH bright-field imaging was performed on a Zeiss Axioimager A.1 microscope (Zeiss, Germany) with x5, x10, x20 and x40 objective lenses and an AxioCam MRm monochrome digital camera (Zeiss, Germany) coupled with ZEN imaging software. The four filter sets used for taking epifluorescence images were Alexa 488, Alexa 594, Alexa 633 and Alexa 355 for visualisation of the nuclei of the myofibres and depended on the excitation and emission of the Alexa fluor dyes conjugated to the secondary antibodies (**Appendix I**). Fluorescence intensity was determined with the Zen 2 blue light edition automatic quantification software that measured pixel number in relation to image area in μm^2.

2.4.6 Two-step quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

The polymerase chain reaction (PCR) was first credited to Kary Mullis in which he was awarded the Nobel Prize in chemistry in 1993 (Mullis et al., 1986). PCR is used to make copies of a specific DNA segment. A DNA sequence is exponentially amplified to generate thousands to millions of copies of that particular DNA segment (Higuchi et al., 1993). This is achieved through a series of denaturing, annealing and elongation steps as described in more detail in section 2.4.9. Real time, or quantitative, (RT-qPCR) was used for the quantification of genes of interest, monitoring the amplification of a gene during the PCR, i.e. in “real-time”. Total RNA was isolated from cells and muscle samples and was used for the two-step qRT-PCR. The two-step qRT-PCR included the reverse transcription of total RNA into cDNA using a reverse transcriptase and this first-strand cDNA synthesis reaction was primed with the use of Oligo deoxythymine (dT; single-stranded sequence used for priming reactions catalysed by reverse transcriptase). Next, with the use of sequence-specific primers, the number of copies of the gene of interest was measured with the fluorescent dye SYBR Green (Thermo Fisher Scientific; cat. 4364344) in RT-qPCR.

2.4.6.1 Total RNA extraction

In order to test the gene expression of cellular and tissue samples, total RNA from these samples needs to be extracted. This process is problematical due to the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA (Peirson and Butler, 2007). RNA extraction was performed with the E.Z.N.A. Total RNA kit I (Omega Bio-Tek, USA, R6834-01) according to the manufacturer’s instructions. Briefly, cell cultures were washed once with PBS and 500µL of pre-chilled TriZol (AMRESCO RiboZol™ RNA Extraction Reagent) was added for 5 minutes, aspirated up and down using a pipette (p1000). The homogenised cell sample was transferred on ice in an Eppendorf tube and 100µL of 100% chloroform was added for separation of molecules, after shaking vigorously for 15 seconds. This mixture was left at room temperature for 2 minutes and subsequently centrifuged at

4°C for 15 minutes at 12,000xg. The upper aqueous phase (containing RNA) was carefully transferred in a new tube with 250µL of 100% Isopropanol and incubated for 5 minutes at room temperature for precipitation of nucleic acids. The mixture was loaded onto a HiBind RNA column and centrifuged at 4°C for 1 minute at 10,000xg and the flow-through was discarded. 500µL of RNA Wash Buffer I was added in the HiBind RNA column and centrifuged at 4°C for 1 minute at 10,000xg and the flow-through was discarded. Collection tubes were changed and 500µL of RNA Wash Buffer II was subsequently added in the HiBind RNA Column and centrifuged at 4°C for 1 minute at 10,000xg and the flow-through was discarded. The procedure was repeated once more and the HiBind RNA Column was centrifuged at 4°C for 2 minutes at 14,000xg in order to remove the remaining ethanol. 20µL of ultrapure MilliQ dH_20 was added in the HiBind RNA column in order to elute the RNA. The column was left for 2 minutes at room temperature before being centrifuged at 4°C for 2 minutes at 12,000xg. The concentration ($ng \cdot \mu L^{-1}$) and purity (A260/A280) of RNA isolated was determined on a NanoDrop spectrometer (ThermoFisher Scientific, UK) prior to sample storage at -80°C.

2.4.6.2 *cDNA* synthesis

Reverse transcription was used to synthesise complementary DNA (cDNA). The reverse transcription was performed with the -RevertAid H Minus First Strand cDNA Synthesis Kit- (ThermoFisher Scientific, UK). As per the manufacturer's instructions, a reverse transcription master mix per RNA sample was prepared; 5x Reaction Buffer; 4µL, RiboLock RNase Inhibitor; 1µL, 10mM dNTP mix; 2µL, Ultrapure Water; to 20µL, Revertaid Reverse Transcriptase; 1µL (200 U/µL). With 8µL of this mastermix, a further mix per RNA sample was prepared that included 1µL of 100µM Oligo(dT)18 Primer, 3µL of ultrapure water and a volume of 8µL and concentration of at least $2\mu g \cdot \mu L^{-1}$ of RNA sample; giving a total volume of 20µL. Reverse transcription was performed using a Veriti thermal cycler (Applied Biosystems, UK) at 42°C for 60 minutes, followed by 70°C for 10 minutes. cDNA was diluted in MilliQ dH20 by a factor 1:18 to correct for loading on qPCR. Prior to the dilution, 3µL of cDNA were kept for evaluation of the primers. The concentration ($\mu g \cdot \mu L^{-1}$) and purity (A260/A280) of cDNA was determined

on the NanoDrop spectrometer prior to dilution and sample storage at -20°C. Primers used to quantify gene expression are listed in **Appendix I.**

2.4.6.3 Quantitative real time PCR assay

In order to measure gene expression differences for genes of interest for C2C12 proliferation (*Vegfa165, Vegfr1, Igf-1, Igf-1r, Pdgfα, Pdgfrα, Pdgfβ, Pdgfrβ, Pax7, Myf5, Myod, Prmt1, Ogg1, Gadd45g, Parp1, Ngf, Bdnf, Ntf3, TrkA, TrkB and TrkC*) and differentiation (*Pax7, Myod, Pdgfβ, Vegfa165, Myogenin, Mhc1, Mhc2a, Mhc2b, Acta1, Tmem8c, Bex1 and Igf-1*), qPCR was employed. Primer sequences are given in **Appendix I**. The Primer master mix was prepared on ice as follows; ultrapure water; 3.2µL, forward primer; 0.15µL, reverse primer; 0.15µL, SYBR Green PCR Master Mix; 7.5µL, which equates to 11µL per well, followed by the subsequent addition of cDNA template; 4µL. Two technical replicates were performed per sample in PCR grade 96-well plates (ThermoFisher Scientific, UK) and enclosed with corresponding adhesive covers (ThermoFisher Scientific, UK). No template controls (NTCs) for each gene were included on each experimental plate to control for unspecific binding. Plates were pulse centrifuged for 5 seconds and subsequently loaded onto a Step-onePlus Real-Time PCR machine (Applied Biosystems, UK). The following cycling programme used was;

Holding stage; 50°C for 2 minutes, 95°C for 10 minutes, cycling stage; 95°C for 15 seconds, denaturation; 61°C for 1 minute annealing/extension, melt curve stage; 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds.

The initial holding stage and the melt curves were achieved in a single cycle while the cycling stage was set to run for 40 cycles. Following from this, the annealing and extension steps were combined in a single step. Melt curves were performed to determine the specificity of amplification. The melting point represents the temperature at which 50% of the DNA has dissociated and is relative to the DNA size (Gundry et al., 2003).

2.4.6.4 qRT-PCR analysis *via* the comparative CT method ($2^{-\Delta\Delta CT}$ method)

SYBRgreen is a cyanine dye that interacts in different ways with double stranded DNA (dsDNA) through intercalation between base pairs and electrostatic interaction (Dragan et al., 2012). SYBRgreen binding to dsDNA leads to an increase of the yield about 1,000-fold. Additionally, the excitation and emission of the SYBRgreen/dsDNA complex is 497nm and 520nm respectively (Dragan et al., 2012). The increase in the amount of dsDNA is due to the qPCR thermal cycles and is represented by an increase in the fluorescence signal (Kubista et al., 2006).

Reference gene normalisation *Cyp* and *Hprt* (**Appendix I**) was utilised in order to detect differences in gene expression among biological samples. To attain this, first, the baseline threshold cycle values were determined automatically from the relative amplification rate using StepOne software V2.0 (Kubista et al., 2006). Gene expression data was determined using the comparative cycle threshold (CT) method according to the following published recommendation (Livak and Schmittgen, 2001). In brief, the CT of the sample for the gene of interest is normalised to the mean CT of the reference genes both for the sample. Next, the change (Δ) in CT of the sample is normalised.

2.4.7 Seahorse XFp extracellular flux measurements

In order to analyse the bioenergetics of myoblasts the Seahorse XFp Analyzer (Seahorse Biosciences, UK) was conducted kindly by Dr. Sandrine Verpoorten. To achieve this, C2C12 myoblasts and isolated satellite cells were seeded at a density of 10,000 cells per well in 8-well XF plates. Cells were pre-incubated under serum-free conditions or treated with 10% releasate, 10% FBS or 10% releasate plus 10% FBS for 24 hours. Prior to the experiment, sensor cartridges were hydrated with XF calibrate solution (pH 7.4), as instructed by the manufacturer's guidelines and incubated at 37°C in a non-CO_2 environment for 24 hours. Seahorse analysis was kindly conducted by Sandrine Verpoorten. The cell culture medium was replaced with assay medium containing 1mM sodium pyruvate and incubated for one hour in a non-CO_2 incubator. Carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP; 5 µM working concentration, Sigma) and antimycin (2.5 µM working concentration) were diluted in the assay medium. The Seahorse XFp Analyzer was used to measure the oxygen

consumption rate (OCR) in real time. Baseline measurements of OCR were taken before sequential injection of FCCP and antimycin. After three basal assay cycles, FCCP was injected to measure maximal mitochondrial respiration by uncoupling ATP synthesis from electron transport followed by injections of antimycin to measure the non-mitochondrial respiratory rate. Data were normalised to protein levels (BCA; Pierce Biotechnology).

2.5 Statistical Analysis

Statistical analysis was performed on the SPSS software (IBM SPSS Statistics version 24). All values are expressed as mean ± standard deviation (SD). Data for satellite cell expression profiles on *ex vivo* myofibres were expressed as mean ± standard error of the mean (SEM). Data normal distribution was tested with the Kolmogorov-Smirnov test. Main effects of the two independent variables and their interaction were statistically tested by either one-way or two-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis (e.g. **Figure 3.1C**). The one-way ANOVA is used to determine whether there are any statistically significant differences between the means of two or more independent groups. A two-way ANOVA was used to establish if there was an interaction between two independent variables on the dependent variable (e.g. **Figure 4.2B** – total numbers of satellite cells). Where appropriate, statistical analysis between two experimental groups was performed by the Student's *t*-test (e.g. **Figure 5.5B**). The Student's *t*-test is a robust test that can be used to analyse a single variable or compare two variables that may be either correlated or independent. Chi-Square was implemented for satellite cell frequency. A statistically significant result was indicated by a probability level of $p \leq 0.05$.

Chapter 3

Platelet releasate promotes skeletal myogenesis by increasing muscle stem cell commitment to differentiation and accelerates muscle regeneration following acute injury

3.1 Abstract

Aim: The use of platelets as biomaterials has gained intense research interest. However, the mechanisms regarding platelet-mediated skeletal myogenesis remain to be established. The aim of this study was to determine the role of platelet releasate in skeletal myogenesis and muscle stem cell fate *in vitro* and *ex vivo* respectively.

Methods: The effect of platelet releasate on proliferation and differentiation of C2C12 myoblasts was analysed by means of cell proliferation assays, immunohistochemistry, gene expression and cell bioenergetics. *In vitro* findings were expanded on single muscle fibres by determining the effect of platelet releasate on murine skeletal muscle stem cells using protein expression profiles for key myogenic regulatory factors.

Results: TRAP6 and collagen used for releasate preparation had a more pronounced effect on myoblast proliferation versus thrombin and sonicated platelets ($p<0.05$). In addition, platelet concentration positively correlated with myoblast proliferation. Platelet releasate increased myoblast and muscle stem cell proliferation in a dose-dependent manner, which was mitigated by VEGFR and PDGFR inhibition. Inhibition of VEGFR and PDGFR ablated MyoD expression on proliferating muscle stem cells; compromising their commitment to differentiation in muscle fibres ($p<0.001$). Platelet releasate was detrimental for myoblast fusion and affected differentiation of myoblasts in a temporal manner. Most importantly, platelet releasate promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis and accelerates skeletal muscle regeneration after acute injury.

Conclusion: This study provides novel mechanistic insights on the role of platelet releasate in skeletal myogenesis and set the physiological basis for exploiting platelets as biomaterials in regenerative medicine.

3.2 Overview

Skeletal muscle is a remarkably plastic tissue with a strong capacity to regenerate after injury due to a small population of skeletal muscle stem cells, termed satellite cells. These muscle stem cells are typically quiescent, but become activated upon myofibre injury, proliferate and either return to quiescence or differentiate to support regeneration (Zammit et al., 2004). However, this regenerative ability typically comes at a cost, such as incomplete functional recovery, stiffness, excessive inflammation or fibrosis (Burks and Cohn, 2011, Miroshnychenko et al., 2017, Li et al., 2016b). There is currently an increasing interest in the field of skeletal muscle regenerative research. This is driven by the increasing prevalence of injuries in both office and sporting vocations, a lack of effective treatments for myopathies, neuromuscular disease and sarcopenia, and finally, an intense interest in preventing skeletal muscle atrophy in microgravity (von Haehling et al., 2010, Jarvinen et al., 2005, Dunbar, 2018, Hides et al., 2016). Taking all these factors into consideration, skeletal muscle injuries, myopathies and research into these areas are of great economical interest to both governmental bodies and private-sector industries alike. At present, conventional treatments to musculoskeletal injuries include rest, ice, compression and elevation (RICE), nonsteroidal anti-inflammatory drugs (NSAIDs) and physical therapy (Baoge et al., 2012).

Lacking evidence for the effectiveness of RICE and NSAIDs leave the field of skeletal muscle regeneration somewhat redundant (van den Bekerom et al., 2012, McAnulty et al., 2007). Autologous platelet rich plasma (PRP; an autologous biomaterial where platelets are re-suspended in plasma) treatment has emarged as an alternative to the above-mentioned methods. This is due to the cost, ease of availability to numerous cytokines and growth factors (such as IGF-1, VEGF and PDGF contained in the alpha-granules of the platelets) to the targeted area, acting as biomaterials to regulate cell cycle, promote wound healing and stimulate regeneration (Sassoli et al., 2018a, Masuki et al., 2016, Wijten et al., 2013). Growth factors and cytokines are essential components of the early inflammatory response and subsequent successful tissue regeneration (Borrione et al., 2014). However, there are inconsistencies in the literature as to whether PRP proves beneficial in skeletal muscle regeneration (Mosca

and Rodeo, 2015). It has been speculated that methodological variability may account for the observed discrepancies among studies on the effectiveness of PRP in wound healing and tissue regeneration (Navani et al., 2017, Andia and Abate, 2017). Another leading proposition for the debatable effects of PRP on skeletal muscle regeneration may be the abundance of cytokines that can induce fibrosis, such as transforming growth factor beta-1 (TGF-β1) (Miroshnychenko et al., 2017, Li et al., 2016b, Li et al., 2013, Denapoli et al., 2016, Kelc et al., 2015).

Data from clinical studies on the effectiveness of PRP-based applications for skeletal muscle regeneration is limited. Therefore, the need for a better understanding of the effects of platelets on skeletal muscle regeneration is necessary. Recent data suggests that platelet releasate (a refined suspension of growth factors released from aggregated platelets, where the platelet cellular-debris is removed) can be used as an alternative method to PRP administration for improving musculoskeletal regeneration (Mosca and Rodeo, 2015, Tsai et al., 2018a, Wijten et al., 2013, Tsai et al., 2017, Notodihardjo et al., 2015).

3.3 Aims

Therefore, the aim of this study was: i) to determine the impact of methodological variability in platelet preparations on skeletal myogenesis, ii) dissect the effect of platelet releasate on myoblast and muscle stem cell's myogenic potential *in vitro* and *ex vivo* respectively and iii) to define the molecular mechanisms of platelet releasate in regulating muscle stem cell fate and recruitment. Briefly, it is shown here that platelet releasate is capable to drive myoblast and muscle stem cell proliferation and differentiation both *in vitro* and *ex vivo*. This effect is mediated by the PDGF/VEGF pathway that promotes muscle stem cell commitment to differentiation and ultimately enhances myogenesis. The findings of this study set the basis for exploiting platelets as biomaterials to promote tissue regeneration in future studies.

3.4 Methods

3.4.1 Ethics statement

The study was approved by the local Ethics Committee of the University. The animal experiments were performed under a project license from the United Kingdom Home Office in agreement with the revised Animals (Scientific Procedures) Act 1986 and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). Blood sampling from healthy human volunteers was performed with their written informed consent and was approved by the University's Ethics Committee.

3.4.2 Preparation of platelet releasate

Human platelet releasate was prepared as described in section 2.2.1. Human platelet releasate was prepared in acid citrate dextrose (ACD) to whole blood at a ratio of 1:5, centrifuged at 190g for 15 minutes followed by PRP collection and inactivation using prostaglandin I_2 (534nM). The PRP was then centrifuged in a swing-out rotor at 800g for 12 minutes and the PPP was then removed. Modified Tyrode's buffer was used to re-suspend the platelet pellet to a concentration of $2.5x10^8$ platelets mL^{-1} (unless otherwise stated) using a cell counter. The platelet preparation was activated using a PAR1 agonist; TRAP6; 20μM, thrombin (0.05-0.1 NIH Units mL^{-1}) or collagen (10μg mL^{-1}) in an aggregometer. Alternatively, after counting and re-suspending in modified Tyrode's buffer, platelets were sonicated for 2 minutes. Platelets were centrifuged at 9500g for 10 minutes, unless otherwise stated (see **Figure 3.2**), and the releasate supernatant was aliquoted until further use.

Murine platelets were prepared as previously described in section 2.2.2. Murine platelets were prepared with 200μL ACD in a 1mL and gently mixed before transferring to 500μL modified Tyrode's buffer with 500μL ACD, per mouse, and centrifuged in a swing-out rotor at 100g for 5 minutes with no breaks. The PRP supernatant was then centrifuged at 800g for 5 minutes with no breaks. The platelet-poor plasma was discarded and platelets were resuspended in modified Tyrode's buffer to a concentration of $2.5x10^8$ platelets mL^{-1} using a Beckman Coulter particle counter.

Collagen (10µg mL^{-1}) was used to activate the platelets in an aggregometer for 5 minutes at 37ºC before centrifuging at 9500g.

3.4.3 Cell cultures and treatments

Murine C2C12 skeletal myoblasts were cultured in GM at 37°C in a humidified atmosphere of 5% CO_2. To induce differentiation, C2C12 cells were cultured in GM until reaching 80% confluence before switching to differentiation media (DM) for either 6, 9 or 12 days. Releasate was added either once during proliferation at time-point 0, or at every media change (either every 12 hours or 24 hours depending on the experiment) or during differentiation, unless otherwise stated. Inhibitors were added during DM media changes when indicated using 5µM PDGFR Inhibitor or a 130nM VEGFR Inhibitor. The myofusion index was calculated as $Myogenin^{+ve}$ cells per myotube (with a minimum threshold of 3 nuclei per myotube) divided by DAPI-stained cells as a percentage. For proliferation in all experimental groups, unless otherwise stated, C2C12 cells were cultured in serum free (SF) conditions. Inhibitors used for proliferation and differentiation experiments were VEGFR Inhibitor (AAL-993; 23nM, 130nM or 1.30µM) and PDGFR Inhibitor (AG-1295; 250nM, 500nM or 5µM).

3.4.4 Cell proliferation and viability analysis

C2C12 myoblast cell proliferation was also evaluated by the pyrimidine analogue EdU incorporation assay using the fluorescent Click-iT® EdU Cell Proliferation Assay according to the manufacturer's instructions. Proliferating cells were measured as EdU divided by DAPI-stained nuclei as a percentage. Cellular viability was assessed by seeding 50,000 C2C12 cells in a 35mm petri dish for 24 hours in either SF, GM, 10% releasate or GM+10% releasate, and counting the ratio of live to dead cells using trypan blue.

3.4.5 Single fibre isolation and culture

For single-fibre culture, murine single fibres were isolated from the extensor digitorum longus muscle (EDL). Limb muscles were dissected and subjected to collagenase (0.2%) digestion for 3-4 hours. The digested muscle was then gently triturated in

DMEM with 1% PS. Fibres were then cultured for 48 and 72 hours in single fibre media (FM), or SF with the addition of 10% releasate 3 times during culture every 16 hours (unless otherwise stated). Fibres were stained for mouse monoclonal anti-Pax7, rabbit polyclonal anti-MyoD, and rabbit polyclonal anti-Myogenin, or with anti-Cyclin D1 or Scrib and with DAPI. The commitment index was calculated as MyoD-stained muscle stem cells divided by DAPI-stained cells as a percentage. PDGF and VEGF were inhibited in the myofibres using either a 5μM PDGFR Inhibitor or a 130nM VEGFR Inhibitor.

3.4.6 Satellite cell isolation from single fibres

For primary myoblasts, satellite cells (i.e. primary muscle stem cells) were derived from the EDL of wild-type mice. Briefly, limb muscles were dissected and subjected to 0.2% collagenase digestion for 4 hours at 37°C. The digested muscle was then gently transferred to 35mm petri dishes where the collagenase was removed. Single muscle fibres were isolated in SF culture medium, by means of a gentle mechanical trituration and then cultured for 72 hours in Matrigel (1mg mL^{-1}) -treated 6-well plates, at 37°C, in satellite proliferation media containing DMEM, 30% FBS, 1.5% C.E.E. plus 1% PS. Next, the myofibres were removed and the satellite cells were cultured as per experimental conditions. Differentiation of primary satellite cells (stem cells) was achieved through culturing for 3 days in appropriate proliferation medium as per experimental condition before switching to DM for a further 5 days.

3.4.7 Immunohistochemistry

C2C12 cells or primary muscle stem cells as appropriate were seeded on coverslips in 1mL of media in 24-well plates. Primary antibodies for anti-Pax7, anti-MyoD, anti-Myogenin, mouse monoclonal anti-IGF-1Rα, anti-PDGF B, anti-VEGF, anti-Ki-67, anti-myosin heavy chain 3, anti-Cyclin D1 or anti-Scrib were added to fixed and permeabilised cells at 1:200 in wash buffer overnight. Primary antibodies were removed with 3 washes in wash buffer, followed by the addition of the appropriate secondary antibodies (**Appendix I**). Cells were measured by the intensity of fluorescence per cell divided by DAPI-stained nuclei as a percentage.

3.4.8 Platelet releasate growth factor multiplex immunoassay

Growth factors contained in the platelet releasate were measured by using high-performance multiplex immunoassays with the Bio-Plex Pro™ Human Cancer Biomarker Panel 1 and 2. This array system includes a blend of magnetic bead-based assays for a number of biomarkers involved in cell division such as Angiopoietin-2, sCD40L, EGF, Endoglin, sFASL, HB-EGF, IGFBP-1, IL-6, IL-8, IL-18, PAI-1, PLGF, TGF-α, TNF-α, uPA, VEGF-A, VEGF-C, VEGF-D, sEGFR, FGF-basic, Follistatin, HGF, sHER-2/neu, sIL-6Rα, PECAM-1, PDGF-AB/BB, SCF, sTIE-2, sVEGFR-1, sVEGFR-2 as defined in **Table 3.1**. All the assays were performed using the Bio-Plex 200 system according to the manufacturers' instructions. The Heatmap of hierarchical clustering and principal component analysis of Bio-Plex data was performed on the Perseus software.

3.4.9 RNA extraction and real-time PCR analysis

Quantitative PCR was performed as described previously (Matsakas et al., 2012a). In brief, $2.4x10^5$ C2C12 myoblasts were seeded per well of a 6-well plate in either SF or GM with or without 10% platelet releasate. After 24 hours, cells were harvested in for RNA isolation and qPCR. Total RNA (2 μg) was reverse-transcribed to cDNA with RevertAid H MinusFirst Strand cDNA synthesis kit and analysed by quantitative real-time RT-PCR on a StepOne Plus cycler, using the Applied Biosystems SYBRGreen PCR Master Mix. Primers were designed using the software Primer Express 3.0. Details of primers are given in **Appendix I**. Relative expression was calculated using the ΔΔCt method with normalisation to the reference genes encoding *Cyp* and *Hprt* (Matsakas et al., 2006). mRNA levels of *Vegfa165, Vegfr1, Igf-1, Igf-1r, Pdgfα, Pdgfrα, Pdgfβ, Pdgfrβ, Pax7, Myf5, Myod, Prmt1, Ogg1, Gadd45g, Parp1, Ngf, Bdnf, Ntf3, TrkA, TrkB and TrkC* were measured in proliferation cell cultures. Similarly, mRNA levels of *Pax7, Myod, Pdgfβ, Vegfa165, Myogenin, Mhc1, Mhc2a, Mhc2b, Acta1, Tmem8c, Bex1* and *Igf-1* were measured in differentiation cell cultures.

3.4.10 Seahorse XFp extracellular flux measurements

C2C12 myoblasts and isolated satellite cells were seeded at a density of 10,000 cells per well in 8-well XF plates. Cells were pre-incubated under SF or GM conditions or treated with/without 10% releasate for 24 hours. The cell culture medium was replaced with assay medium containing 1mM sodium pyruvate and incubated for one hour in a non-CO_2 incubator. FCCP (5 µM working concentration) and antimycin (2.5 µM working concentration) were diluted in the assay medium. The Seahorse XFp Analyzer was used to measure the OCR in real time. Baseline measurements of OCR were taken before sequential injection of FCCP and antimycin. After three basal assay cycles, FCCP was injected to measure maximal mitochondrial respiration by uncoupling ATP synthesis from electron transport followed by injections of antimycin to measure the non-mitochondrial respiratory rate. Data were normalised to protein levels.

3.4.11 *In Vivo* cardiotoxin-induced muscle injury

On day 1; mice (12 week old) were tail vein injected (IV) either with 100 µL platelet releasate or 100 µL PBS (n=5 per group). 30 minutes later, mice were injected with a total of 30µL, 10µM CTX into the tibialis anterior muscle. 24 hours later (day 2), the mice received a second identical intravenous injection of either the platelet releasate or PBS. At 5 days, mice were sacrificed, the TA muscles were collected, immediately frozen and 12µm cryo-sections were processed for immunohistochemistry.

3.4.12 Statistical analysis

Data are reported as mean±SD. Cell culture experiments were conducted with n=6-27 replicates as indicated in figure legends. Statistical differences among experimental groups were determined by one-way ANOVA followed by the Tukey post-hoc test. Differences between two groups were detected by using Student's *t* test. Statistical differences were considered as significant for $p<0.05$.

3.5 Results

3.5.1 The effect of platelet agonists and centrifugation speed during releasate preparation and dosage of releasate on C2C12 myoblast proliferation

There is currently large controversial evidence on the role of PRP in tissue regeneration (Mosca and Rodeo, 2015, Sánchez et al., 2014, Navani et al., 2017, Nguyen et al., 2011, Andia and Abate, 2018). Therefore, it was next aimed to determine the effect of methodological variations during platelet releasate preparation on C2C12 myoblast proliferation. To this aim, C2C12 myoblasts were cultured for 24 hours in serum-free media (SF) supplemented with releasate (R) derived from either collagen, TRAP6 (a PAR1 agonist), or thrombin-activated platelets, which were centrifuged at different speeds after stimulation (i.e. 1400, 5500 and 9500g) (**Figure 3.1A-C and Figure 3.2**). These were compared to cells incubated with lysates from sonicated platelets (i.e. S.L.). Treatment of cells with 10% releasate resulted in significantly increased cell proliferation (i.e. 32-55%) independent of the agonist and centrifugation speed as compared to SF-treated cells (i.e. 5%; negative control). C2C12 myoblast proliferation in response to treatment with TRAP6- and collagen-activated releasate was comparable to cells treated with 20% growth medium (GM, containing 20% FBS; positive control). However, the highest percentage for myoblast proliferation amongst groups was found for PAR1-activated platelet releasate independent of centrifugation speed. Obtaining platelet lysates by sonication did not show any additional advantage on myoblast proliferation as compared to releasate activated with either TRAP6 or collagen. Of note, collagen-, TRAP6- and thrombin-activated platelets had similar levels of aggregation and total protein (mg mL^{-1}) (**Figure 3.3, Figure 3.1D** respectively). Taken together, these findings suggest that growth factors from thrombin-activated or sonicated platelets improve C2C12 myoblast proliferation to a lesser extent than collagen- or PAR1-activated platelet releasate, while the final centrifugation speed during platelet preparation does not appear to play an important role. Therefore, TRAP6 at 9500g to obtain platelet releasate for all subsequent experiments was used. The proliferative effect using this method of platelet preparation was supported with Ki-67 and cell viability experimentation (**Figure 3.4**).

Next, the dose-response of platelet releasate on C2C12 cell proliferation was determined. Cells were treated with 10-, 20- or 30% releasate for 24 hours. Administration of 10-30% releasate resulted in significant myoblast proliferation compared to serum free (SF) conditions (50-62% vs. 5% respectively; **Figure 3.1E**)**.** In addition, 20- and 30% releasate exhibited a higher proliferative effect on murine myoblasts as compared to the 10% releasate group (**Figure 3.1E**). These data are indicative of a dose-dependent effect of releasate on myoblast proliferation.

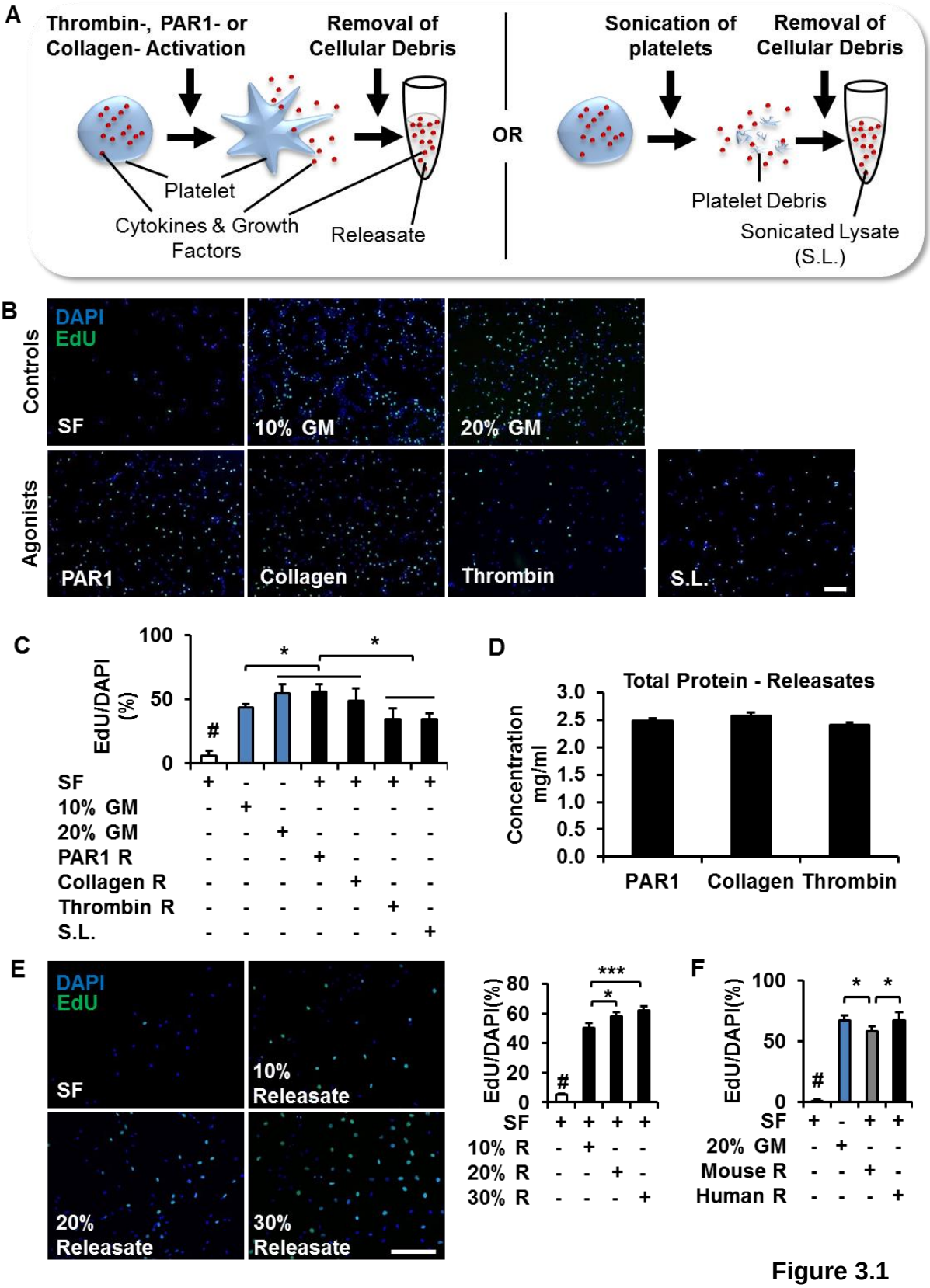

A
Thrombin-, PAR1- or Collagen- Activation
Removal of Cellular Debris
Platelet
Cytokines & Growth Factors
Releasate
OR
Sonication of platelets
Removal of Cellular Debris
Platelet Debris
Sonicated Lysate (S.L.)
B
Controls
Agonists
DAPI
EdU
SF
10% GM
20% GM
PAR1
Collagen
Thrombin
S.L.
C
EdU/DAPI (%)
100
50
0
SF
10% GM
20% GM
PAR1 R
Collagen R
Thrombin R
S.L.
D
Total Protein - Releasates
Concentration mg/ml
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PAR1
Collagen
Thrombin
E
DAPI
EdU
SF
10% Releasate
20% Releasate
30% Releasate
EdU/DAPI(%)
80
60
40
20
0
SF
10% R
20% R
30% R
F
EdU/DAPI(%)
100
50
0
SF
20% GM
Mouse R
Human R

Figure 3.1

Figure 3.1. The effect of platelet agonists and species-of-origin during releasate preparation on C2C12 myoblast proliferation. (**A**) A schematic depicting the methods of obtaining platelet releasate or platelet lysate. (**B-C**) Representative images and quantitative data for C2C12 myoblast proliferation (EdU) and nuclear staining (DAPI) by releasate from platelets activated with collagen, TRAP6 (a PAR1 agonist) or thrombin (x5 magnification, scale bar 200μm). Lysates from mechanically-activated platelets was achieved by sonication (S.L.). Control conditions included serum-free (SF) DMEM ± 10-20% FBS (GM). (**D**) Total protein concentrations (mg mL^{-1}) for the 3 platelet releasates activated with different platelet agonists. (**E**) Dose-dependent effect of PAR1-activated platelet releasate (i.e. 10, 20, 30% v/v) on C2C12 myoblast proliferation after 24h. Representative images for proliferating C2C12 myoblasts (EdU) and total nuclear staining (DAPI); (x10 magnification, scale bar 200μm). (**F**) Quantitative data on the effect of human (Human R; stimulated with PAR1) and mouse platelet releasate (Mouse R; stimulated with collagen) on C2C12 myoblast proliferation (EdU). Data are mean±SD (n=3/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *$p<0.05$, ***$p<0.001$ and #$p<0.001$ vs. every other group.

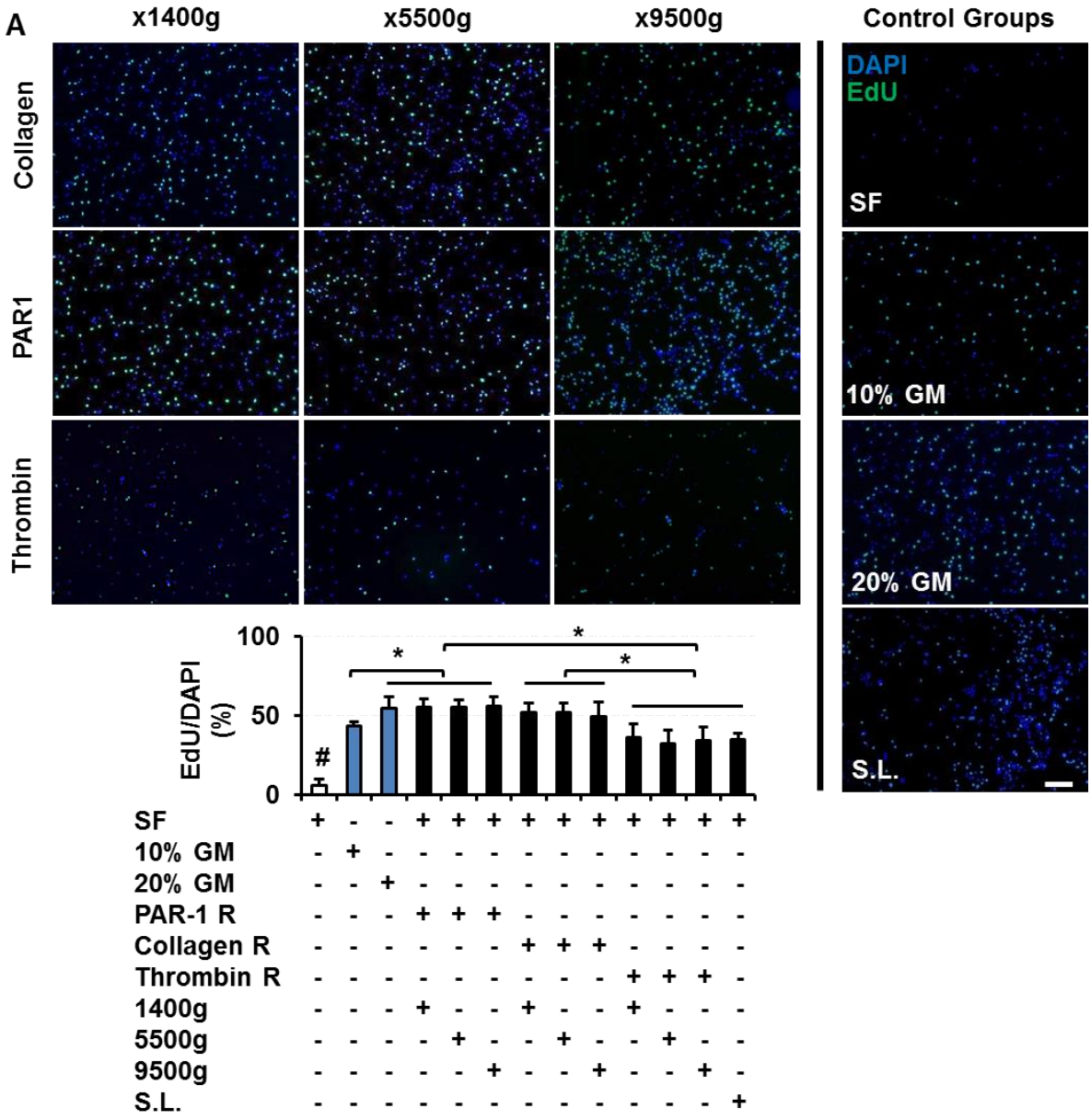


Figure 3.2 The effect of platelet agonists and centrifugation speed during releasate preparation on C2C12 myoblast proliferation. Representative images and quantitative data for C2C12 myoblast proliferation (EdU) and nuclear staining (DAPI) by releasate (R) from platelets activated with collagen, TRAP6 (a PAR1 agonist) or thrombin at three different centrifugation speeds (x5 magnification, scale bar 200 μm). Platelet lysates from mechanically-activated platelets was achieved by sonication (S.L.). Control conditions included serum-free (SF) DMEM ± 10-20% FBS (GM). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *p<0.05 and #p<0.001 vs. every other group.

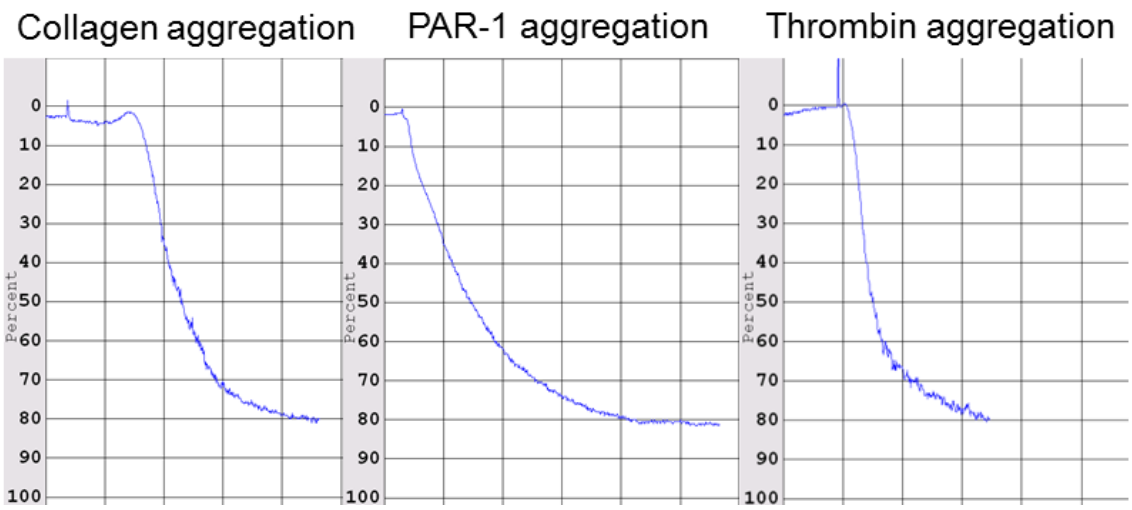


Figure 3.3 Representative platelet aggregation plots. A threshold of at least 80% aggregation was used for the preparation of platelet releasate using light transmission aggregometry.

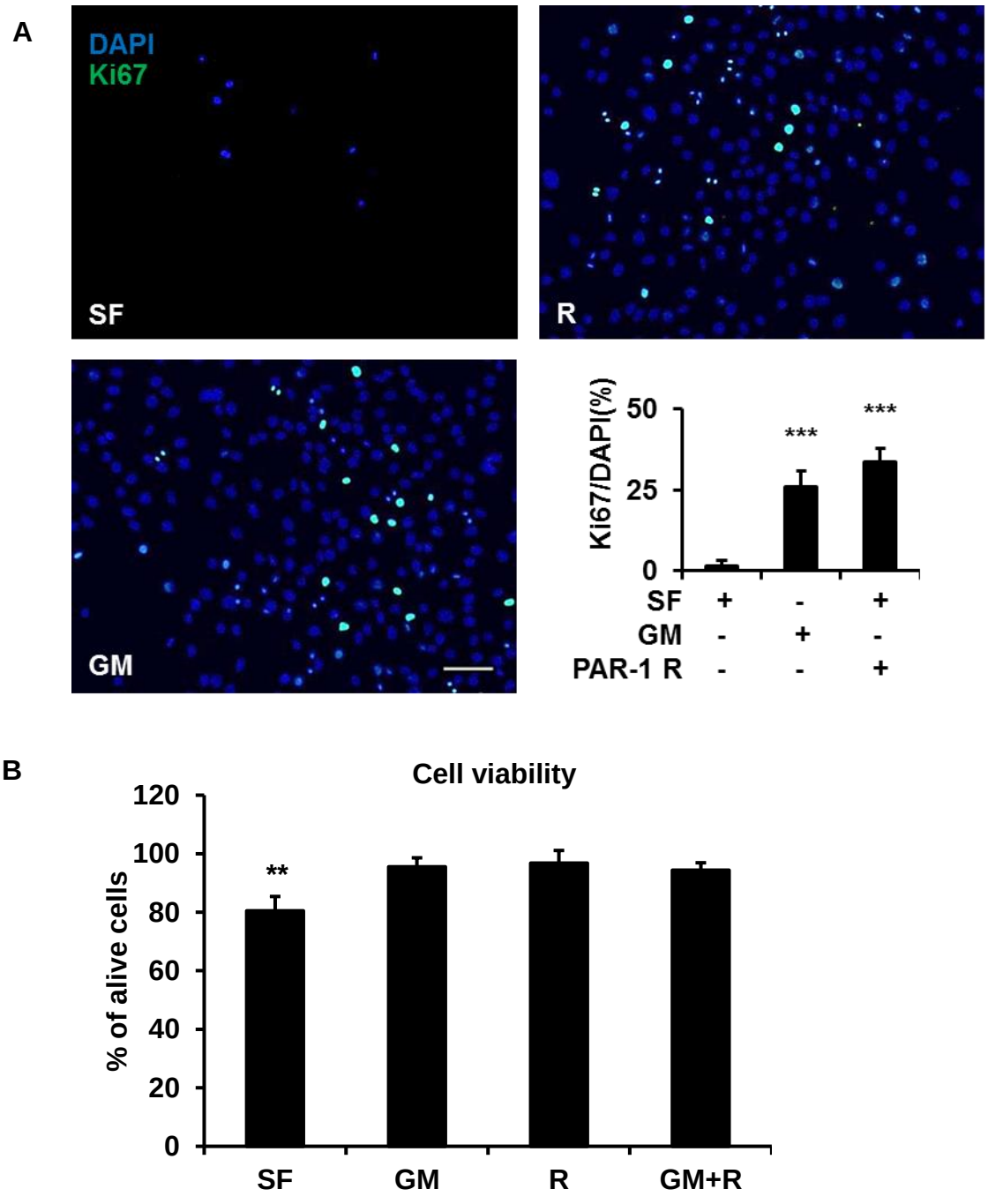


Figure 3.4 Supportive evidence on the effect of releasate on myoblast proliferation and viability. (**A**) C2C12 cell proliferation in response to PAR-1-activated platelet releasate treatment for 24 hours was visualised by Ki-67 immunofluorescence staining. (**B**) Cell viability of C2C12 cells cultured for 24 hours in serum free, growth medium, releasate or growth medium plus releasate (assessed by Trypan blue staining). Differences are **p<0.01, and ***p<0.001 vs. every other group.

3.5.2 Human releasate has a stronger proliferative effect on C2C12 cells than murine releasate

The effect of human and mouse platelet releasate on myoblast proliferation was next established. From an ethical point of view, human platelet releasate is more readily available without the need for murine sacrifice (Kilkenny et al., 2010). Therefore, the species-specific variability of murine versus human platelet releasate treatment on murine C2C12 myoblast proliferation was investigated. Next, it was sought to establish whether using the same concentration of platelets ($2.5x10^8$ mL^{-1}; physiological levels of platelets in human blood) of 10% murine platelet releasate (Mouse R) on murine cells would yield better proliferation than 10% human platelet releasate (Human R) due to differing species-dependent platelet concentrations. Serum-free conditions and 20% FBS (20% GM) were used as negative and positive controls respectively. Mouse releasate was found to be significantly less affective by 10% in promoting cell division than human releasate (**Figure 3.1F**). This may be accountable to the fact that nominal levels of platelets in murine blood are 3.5-7 times higher than human blood (i.e. $12.5x10^8$ mL^{-1}; physiological levels of platelets in mouse versus $2.5x10^8$ mL^{-1} in human blood). In addition, the platelets of mice are considerably smaller in size (2-4 times) than human platelets (Schmitt et al., 2001). Since murine platelets do not express the PAR-1 receptor, collagen was used as a platelet agonist (Arachiche et al., 2014). Correcting for these variables may optimise the proliferative capacity of murine releasate.

3.5.3 Releasate from physiological platelet concentrations is beneficial for myoblast proliferation

The effect releasate from varying platelet concentrations on myoblast proliferation was next explored. To address this, platelet concentrations of $0.1x10^8$ – $10x10^8$ platelets mL^{-1} from human blood were used (**Figure 3.5A**). Releasate prepared from sub-physiological levels of platelets (i.e. $0.1\text{-}1x10^8$ platelets mL^{-1}) resulted in diminished myoblast proliferation compared to cultures supplemented with growth medium (GM; 10% FBS, positive control). However, releasate from physiological platelet concentrations (i.e. $2.5x10^8$ platelets mL^{-1}) resulted in significantly higher myoblast

proliferation compared to releasate from sub-physiological platelet concentrations and serum free conditions, which was similar to the growth medium (GM) group. There was a positive correlation between the platelet concentration used for releasate preparation and myoblast proliferation (**Figure 3.6**). Interestingly, releasate from supra-physiological levels of platelets exhibited additional effects on myoblast proliferation by approximately 30% compared to the physiological concentration. These findings indicate that the supra-physiological levels of platelets have a stronger effect on the proliferative capacity of the C2C12 myoblasts.

Figure 3.5

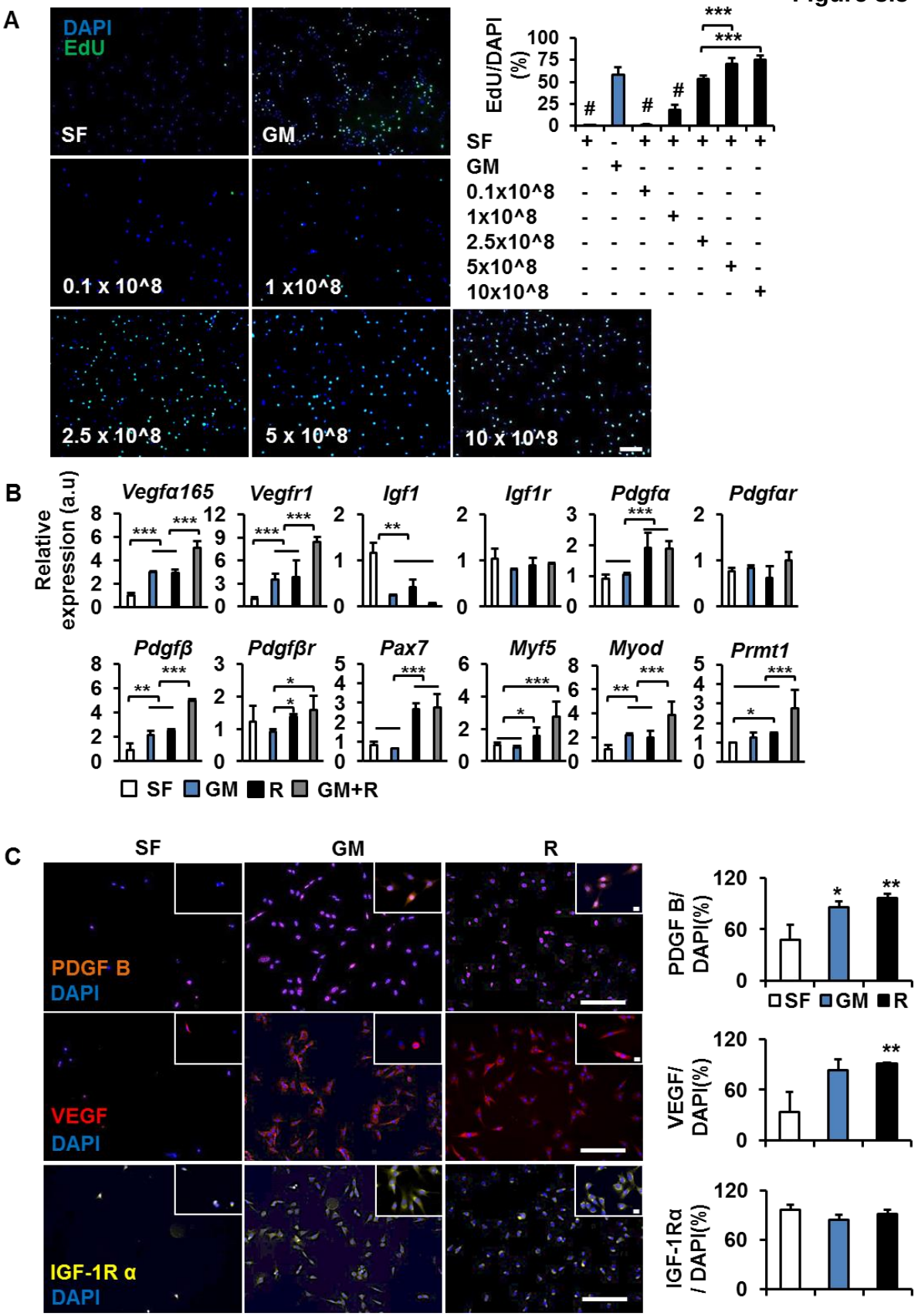

A
DAPI
EdU
SF
GM
0.1 x 10^8
1 x10^8
2.5 x 10^8
5 x 10^8
10 x 10^8
EdU/DAPI (%)
SF
GM
0.1x10^8
1x10^8
2.5x10^8
5x10^8
10x10^8
B
Relative expression (a.u)
Vegfa165
Vegfr1
Igf1
Igf1r
Pdgfa
Pdgfar
Pdgfβ
Pdgfβr
Pax7
Myf5
Myod
Prmt1
SF GM R GM+R
C
SF
GM
R
PDGF B
DAPI
VEGF
DAPI
IGF-1R α
DAPI
PDGF B/ DAPI(%)
VEGF/ DAPI(%)
IGF-1Rα / DAPI(%)

Figure 3.5 The effect of releasate from different platelet concentrations on C2C12 myoblast proliferation and myoblast gene and protein expression patterns (**A**) Representative images for C2C12 myoblast proliferation (EdU) and nuclear staining (DAPI) (x5 magnification, scale bar 200μm) in response to physiological (i.e. $2.5x10^8$) and non-physiological platelet concentrations. Quantitative data showed a significant effect of physiological platelet concentration on C2C12 myoblast proliferation that was comparable to 10% FBS (GM) and supra-physiological platelet concentrations (i.e. 5-$10x10^8$). Control conditions included serum-free (SF) DMEM ± 10% FBS (GM). Data are mean±SD (n=3/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Statistically significant differences are *p<0.05, **p<0.01 and ***p<0.001 and # for p<0.001 compared to GM and 2.5-$10x10^8$ respectively. (**B**) Myoblast gene expression patterns using releasate from physiological platelet concentration (i.e. $2.5x10^8$) by qPCR for *Vegfa165, Vegfr1, Igf1*, *Igf1r*, *Pdgfα*, *Pdgfrα*, *Pdgfβ*, *Pdgfrβ*, *Pax7*, *Myf5*, *Myod* and *Prmt1* on 10% releasate vs. control (DMEM). Data are mean±SD (n=6/group). Statistical analysis was performed by unpaired Student's *t*-tests. Differences are ***p<0.001, **p<0.01. (**C**) Immunohistochemical staining of PDGF B, VEGF or IGF-1R*α* in C2C12 myoblasts cultured with SF, 10% FBS (GM) or 10% R for 24 hours; (x10 magnification, scale bar 200μm, inlay images are x40 magnification with 20μm scale bars). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, and **p<0.01 vs. the SF control group.

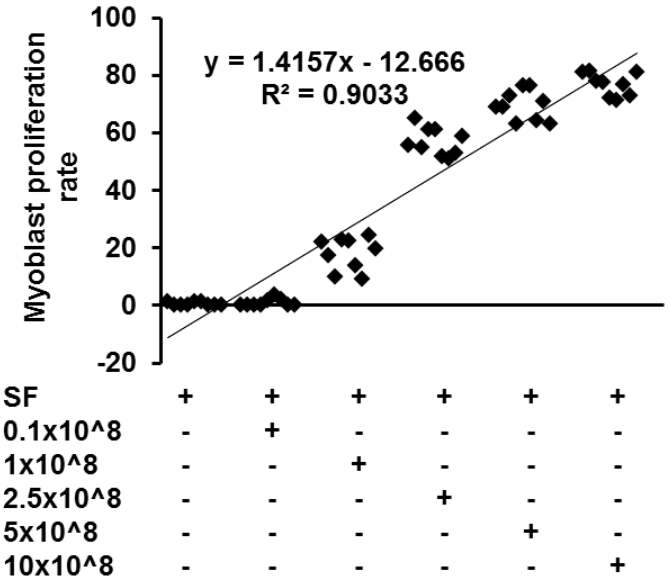


Figure 3.6. Correlation between platelet concentration (i.e. 0.1-10x10^8 platelets) used in the preparation of platelet releasate versus myoblast proliferation rate. Data are taken from **Figure 3.5A**. A positive correlation was detected at p<0.01 by using a bivariate Pearson Correlation test.

3.5.4 The effect of releasate on gene and protein expression patterns of C2C12 myoblasts

Releasate was next applied from physiological levels of human platelets ($2.5x10^8$ platelets mL^{-1}) on proliferating C2C12 myoblasts for 48 hours and gene expression by quantitative real-time RT-PCR was measured. There were significantly higher mRNA

levels ($p<0.001$) for *Vegfα165, Vegfr1, Pdgfα*, *Pdgfβ* (growth factors and their receptors involved in myogenesis), *Pax7, Myf5* and *Myod* (myogenic regulatory factors) and *Prmt1* (a regulator of muscle stem cell fate) in response to releasate treatment in serum-free or serum-rich conditions compared to non-releasate controls (i.e. SF and GM respectively). Significantly lower ($p<0.01$) mRNA levels for *Igf1* (growth factor involved in myogenic differentiation) in all groups as compared to the SF group was observed. Transcript levels of the *Igf1r* and *Pdgfαr* receptors were unchanged between groups. *Pdgfβr* mRNA levels were upregulated by releasate in the GM group only (**Figure 3.5B)**. mRNA levels for the DNA repair genes *Ogg1* (8-Oxoguanine glycosylase), *Gadd45g* (Growth arrest and DNA-damage-inducible protein 45 gamma) and *Parp1* (Poly [ADP-ribose] polymerase 1) were increased for all groups versus the SF group (**Figure 3.7**). Gene expression of neurotrophins and their receptors (i.e. Nerve growth factor; *Ngf*, Brain-derived neurotrophic factor; *Bdnf*, Neurotrophin-3; Ntf-3 and their receptors Tropomyosin receptor kinase A, B and C; *TrkA*, *TrkB* and *TrkC* respectively) was not affected by the administration of releasate on proliferating myoblasts (**Figure 3.7**). Platelet releasate also induced higher PDGFB and VEGF protein levels in proliferating C2C12 myoblasts (**Figure 3.5C and Figure 3.8**). These

findings suggest that platelet releasate regulates PDGF and VEGF mRNA and protein expression.

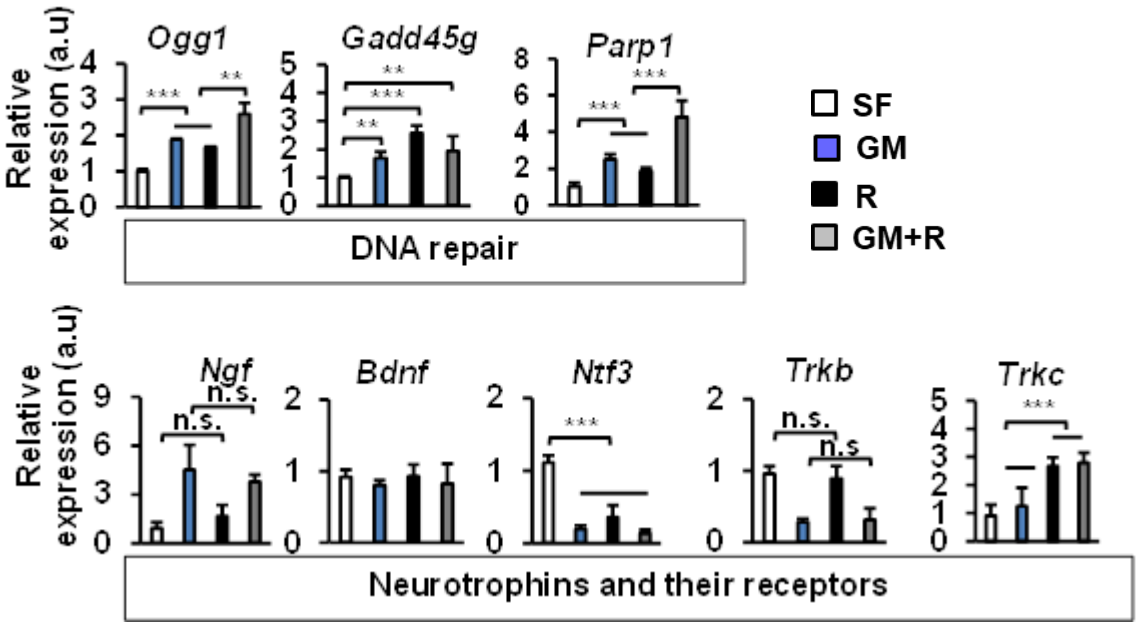


Figure 3.7 mRNA levels of DNA repair genes (*Ogg1*, *Gadd45g* and *Parp1*), neurotrophins (*Ngf*, *Bdnf* and *Ntf3*) and their receptors (*Trkb* and *Trkc*) in response to releasate in serum free and serum rich conditions during myoblast proliferation. mRNA levels from C2C12 myoblasts were measured after proliferating for 24 hours in serum-free (SF), growth medium (GM; 10% FBS), releasate (R) and GM+R respectively. Of note, *TrkA* was undetectable by qPCR in proliferating cells in any group. Data are mean±SD (n=5/group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Statistically significant differences are **p<0.01, and ***p<0.001, n.s.: not significant.

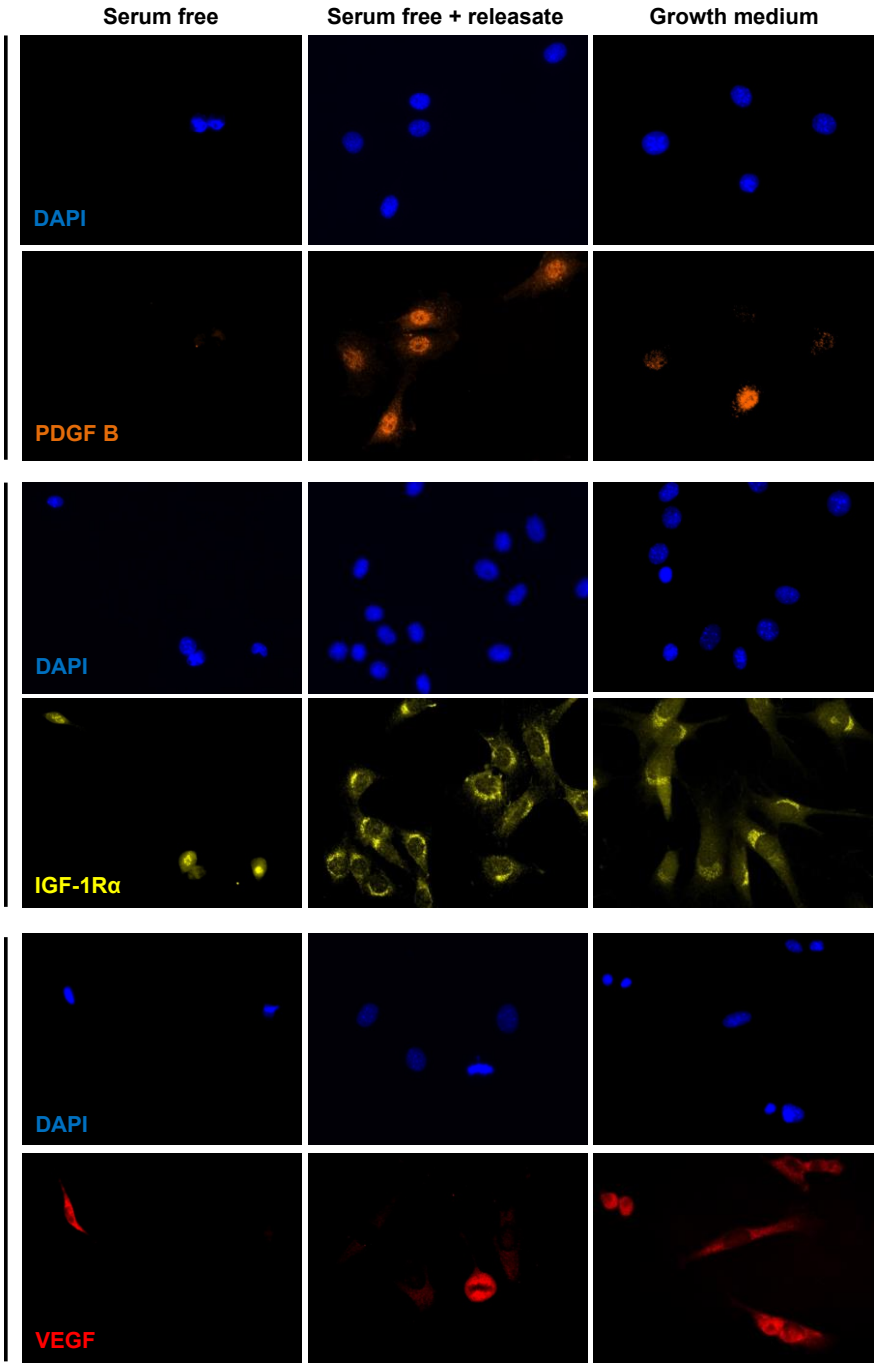


Figure 3.8 Unmerged immunohistochemical staining for PDGFB (orange), IGF-1Rα (yellow), VEGF (red) and DAPI (blue). Data are taken from main **Figure 3.5C** (Magnification is taken at x40).

3.5.5 Growth factors in platelet releasate

The next aim was to get insights into the levels of relevant growth factors contained in platelet releasate. For this purpose, stimulated washed platelets from human blood with agonists that induce platelet aggregation and degranulation [i.e. TRAP6 (a PAR1 agonist) or collagen] or left the platelet suspension untreated were used. Samples generated for this thesis were sent to Laura Gutierrez for processing as mentioned in the Acknowledgements. The levels of 32 biomarkers involved in cell proliferation and differentiation were measured. All biomarkers were increased several-fold in TRAP6 and collagen induced releasate compared to unstimulated platelets (**Figure 3.9**). In particular, it was found that between unstimulated and stimulated (Collagen and TRAP6) groups, two clusters based on relative heatmap intensity have emerged. One cluster with a moderate increase including uPA, IL-18, Angiopoietin-2, Follistatin, IGFBP-1, Endoglin, IL-6, sCD40L, PLGF, TGF-α and TNF-α and a second cluster with >10-fold increase including VEGF-A, -C and -D, SCF, PDGF, EGF, sFASL, HGF, sVEGFR-1, -2, sTIE2, FGF and sEGFR. Lower levels of some biomarkers were detectable in unstimulated platelets. Published evidence regarding the role of these biomarkers in myoblast proliferation and differentiation is summarised in supplementary **Table 3.1.**

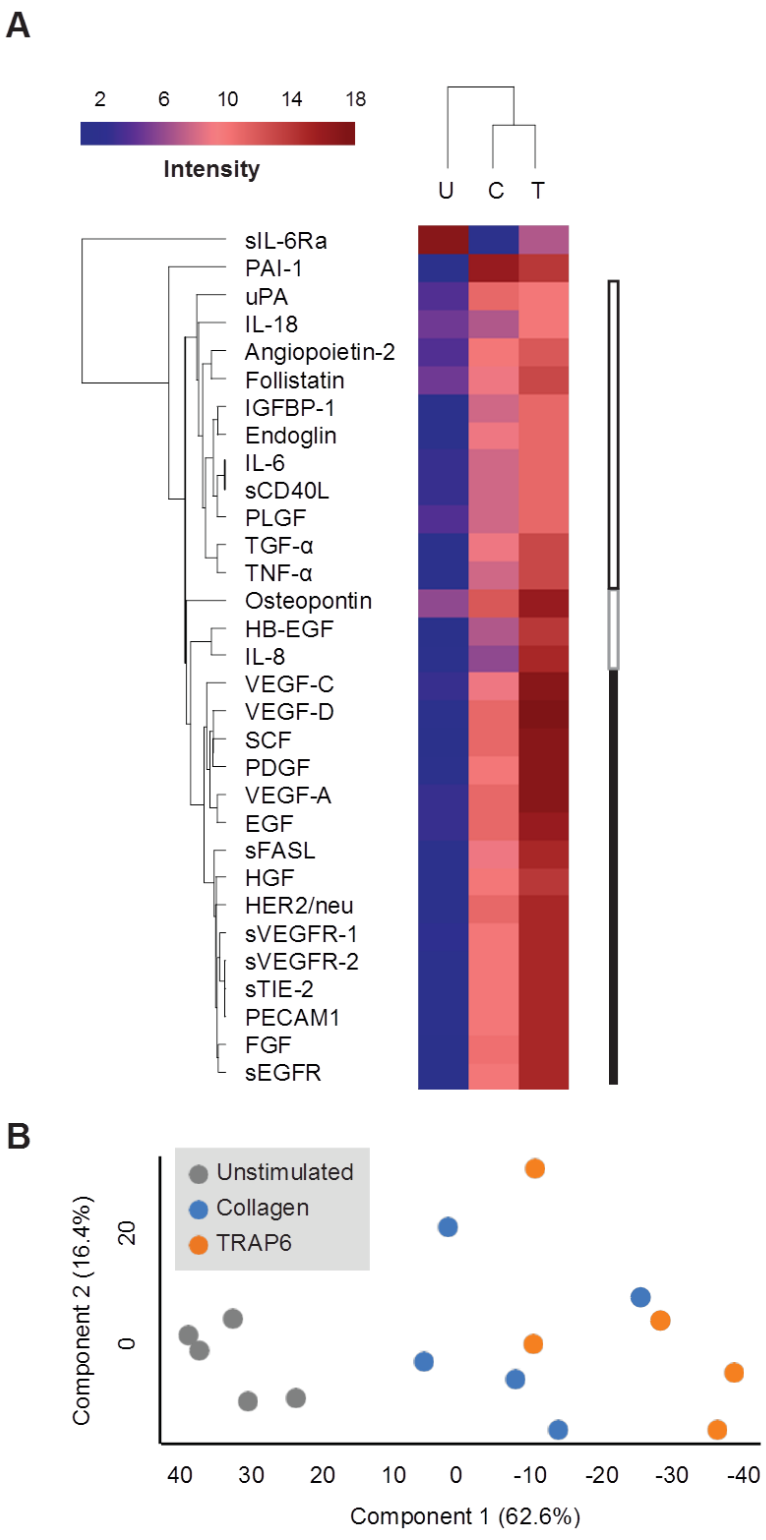


Figure 3.9 Growth factors measured in platelet releasate Human platelet suspensions were aggregated using either TRAP6 (a PAR1 agonist), or collagen, or were left unstimulated. Concentrations of specific analytes contained in BIOPLEX ONCO I and II panels were measured in platelet releasate. Platelets from n=6 independent donors were used in this assay. (A) Hierarchical clustering representing the average expression intensity of each analyte per condition (U, unstimulated; C, collagen; T, TRAP6). (**B**) Principal Component Analysis (PCA) representing the distribution and grouping of samples considering the analytes measured as a whole.

Table 3.1 Overview of effects of releasate components on myoblast proliferation and differentiation based on published evidence

Reference	Factor	Experimental evidence	Proliferation	Differentiation
(Thornton et al., 2015) (Leroy et al., 2013)	Epidermal growth factor receptor (sEGFR)	Blocking EGFR causes a loss of proliferation of satellite cells. sEGFR down-regulation triggers human myoblast differentiation	↑	↓
(Milasincic et al., 1996)	Fibroblast growth factor (FGF)	A strong MAP kinase agonist, is both a potent mitogen and inhibitor of myogenic differentiation	↑	↓
(Zhu et al., 2011) (Kocamis et al., 2004)	Follistatin	Myostatin antagonist, Follistatin, improves skeletal muscle healing. Follistatin alters myostatin gene expression in c2c12 muscle cells	↑	
a) (Chen et al., 2014) b) (Leroy et al., 2013)	human epidermal growth factor receptor 2 (ErbB2) (sHER2/neu)	a) (ErbB2) is not upregulated in proliferation or differentiation, b) however epidermal growth factor receptor (EGFR) down-regulation triggers human myoblast differentiation	-	↓
(Walker et al., 2015)	Hepatocyte growth factor (HGF)	2 ng/ ml promoted cell division but reduced myogenic commitment and fusion, 10 ng/ml HGF reduced proliferative capability, but increased differentiation	↑↓	↓↑
(Serrano et al., 2008)	Soluble Interleukin 6 receptor alpha (sIL-6Ra)	IL-6 expression ↑ hypertrophic muscle growth regulating myoblast proliferation and migration	↑	↑
(Barbosa-Souza et al., 2011)	Osteopontin	A pro-fibrotic factor in skeletal muscle and myoblasts	-	(↑ Myogenin)
a) (Jin et al., 1991) b)(Yablonka-Reuveni et al., 1990)	PDGF-AB/BB	a) Platelet-derived Growth Factor-BB stimulates growth and inhibits differentiation b) PDGF-AA and PDGF-AB have little or no effect on proliferation and differentiation	PDGF-BB ↑ PDGF AB -	PDGF-BB ↓ PDGF AB -
(Deasy et al., 2001)	Stem cell factor (SCF)	SCF increases skeletal muscle stem cell number	↑	
(McClung et al., 2015)	Soluble Angiopoietin-1 receptor (sTIE-2)	Angiopoietin1 (Ang-1), Tie1, and Tie2 mRNA increased during differentiation.	-	↑
(Bryan et al., 2008)	Vascular Endothelial Growth Factor Receptor-1 (sVEGFR-1)	VEGF acting on VEGFR1 was increased during proliferation and differentiation.	↑	↓

(Bryan et al., 2008)	(sVEGFR-2)	VEGFR2 was expressed minimally during proliferation and increased during differentiation.	↓	↑
(Mofarrahi and Hussain, 2011)	Angiopoietin-2	Enhanced differentiation and survival, no influence on proliferation or migration	-	↑
(Leroy et al., 2013)	EGF	EGF stimulates myoblast proliferation in growth but not in differentiation medium	↑	↓
(Scherner et al., 2007)	Endoglin(CD105)	Increased proliferation and migration and counteracts TGF-1	↑	↓
(Horikawa et al., 1999)et al., 1999) (Chen et al., 1995)	Heparin-binding epidermal growth factor (HB-EGF)	Inhibiting HB-EGF rendered myotubes sensitive to apoptotic cell death and is upregulated in differentiation	-	↑
a)(Haywood et al., 2017) B)(Milasincic et al., 1996)	Insulin-like growth factor-binding protein 1 (IGFBP-1)	a) IGFBP-1 enhances insulin signalling b) A weak activator of the MAP kinase cascade, is mitogenic but ultimately enhances the differentiated phenotype	↑	↑
(Serrano et al., 2008)	IL-6	IL-6 expression ↑ Hypertrophic Muscle growth IL-6 Regulates Myoblast Proliferation and Migration	↑	↑
(Pedersen and Febbraio, 2008)	IL-8	Interleukin 8 (IL-8) acts as an angiogenic factor	-	-
(Su et al., 2017)	IL-18	IL-18 ↑ angiogenesis	-	-
(Tamura et al., 2017) (Mann et al., 2011)	PAI-1	Paracrine PAI-1 is involved in glucocorticoid-induced muscle wasting, negative role of PAI-1 in muscle regeneration with increase in fibrosis	↓	↓
(De Falco, 2012)	PLGF	Placenta growth factor (PLGF) to enhance vascularization	-	-
(Luetteke et al., 1993)	Transforming Growth Factor-α (TGF-α)	TGF-α did not C2C12 differentiation. Overexpressing in mice causes a smaller fibre cross-sectional area.	?	-
a) (Chen et al., 2007) b) (Zhao et al., 2014) c) (De Larichaudy et al., 2012)	Tumour necrosis factor (TNF-α)	a) Recombinant TNF-α in differentiation medium stimulated myogenesis at 0.05 ng/ml, but inhibited it at 0.5 and 5 ng/ml b) TNF-α inhibits myogenic differentiation of C2C12 cells through NF-kB. c) Myotube atrophy was induced by TNF- α	?	↓
(Suelves et al., 2002)	uPA	Urokinase-type plasminogen activator induces myoblast fusion and differentiation	-	↑

(Sassoli et al., 2012)	VEGF-A	b) VEGF causes myoblast proliferation	↑	↑
(Kuwahara et al., 2013)	VEGF-C	VEGF-C plays a role in angiogenesis and lymphangiogenesis in a murine model of hind limb ischemia	?	?
(Rissanen et al., 2003) (Nakamura et al., 2012)	VEGF-D	VEGF-D is the most potent (angiogenesis and lymphangiogenesis) member of the VEGFs when delivered via an adenoviral vector into skeletal muscle	-	?

"↑/↓": indicate increase/decrease respectively, "-": indicates no effect and "?": indicates that no relevant reference was detected

3.5.6 Platelet releasate drives myoblast proliferation through PDGF and VEGF

Given the above observations that mRNA and protein levels of PDGF and VEGF are increased in proliferating myoblasts (**Figure 3.5**), as well as their abundance in the releasate (**Figure 3.9**), it was reasoned that these growth factors may be governing cell proliferation, as shown when individually targeted (Sassoli et al., 2014, Yablonka-Reuveni and Rivera, 1997). To further investigate this notion, low, medium and high doses of VEGFR or PDGFR Inhibitors with or without releasate were used to assess C2C12 cell proliferation (**Figure 3.11A, B**). Treatment with VEGFR and PDGFR inhibitors resulted in a dose-dependent attenuation of myoblast proliferation in groups treated with platelet releasate (**Figure 3.11B**). A similar trend was observed under serum free (SF) conditions with the VEGFR and PDGFR inhibitors. Additionally, VEGFR and PDGFR inhibition compromised C2C12 proliferation under serum-rich (i.e. growth medium) conditions as indicated by decreased total cellular number (**Figure 3.10**). Taken together, these data suggest that myoblast proliferation is, at least in part, driven by the VEGF and PDGF growth factors contained in the platelet releasate.

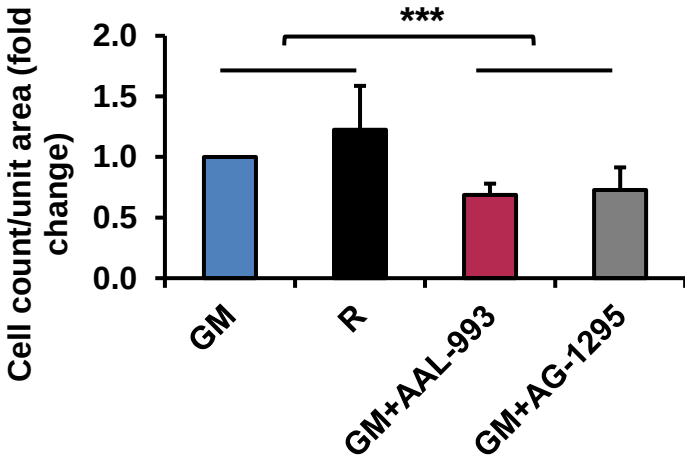


Figure 3.10 The effect of VEGFR and PDGFR inhibition on C2C12 cell proliferation under serum rich conditions. Quantitative data representing the effects of 5μM AG-1295 and 1.3μM AAL-993 on total cell count per unit area. Data are mean±SD (n=8/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are ***p<0.001.

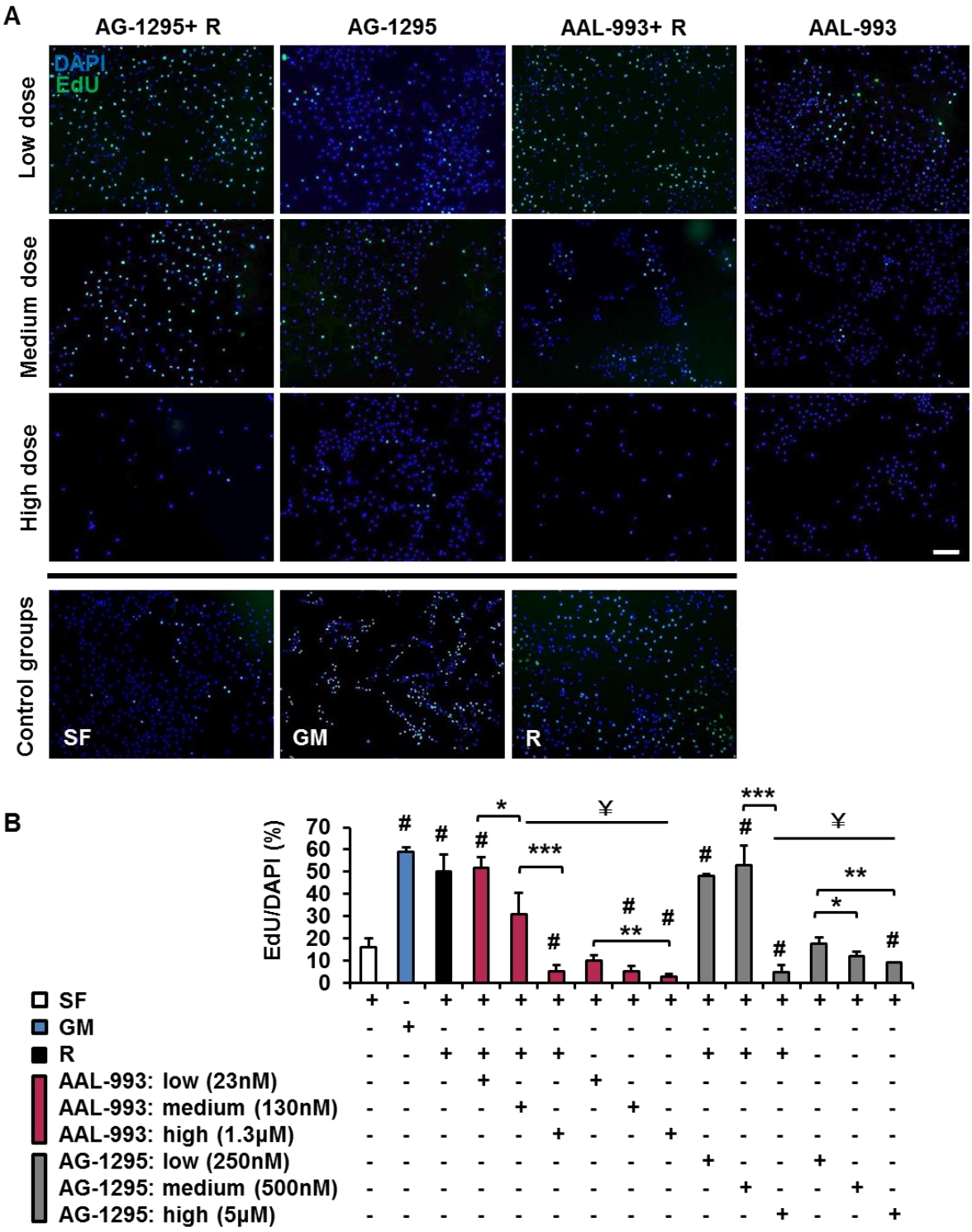


Figure 3.11 VEGFR and PDGFR inhibitors dose-dependently attenuate the effect of releasate on C2C12 myoblast proliferation. C2C12 cells were treated with either 10% FBS (GM) or 10% Releasate ± VEGFR Inhibitor (AAL-993; 23nM, 130nM or 1.30μM) or 10% releasate ± PDGFR Inhibitor (Tyrphostin AG 1295; [AG-1295] 250nM, 500nM or 5μM). Control conditions included serum-free (SF) DMEM ± 10% FBS (GM)

or 10% Releasate (R). (**A**) Representative images for C2C12 myoblast proliferation (EdU) and nuclear staining (x5 magnification, scale bar 200μm) (DAPI). (**B**) Quantitative data on the effect of VEGFR and PDGFR inhibition show a dose-dependent attenuation of myoblast proliferation with or without releasate treatment. Data are mean±SD (n=8/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #p<0.05 vs. the SF (control) group, ¥p<0.05 vs. the R group.

3.5.7 Temporal effects of platelet releasate on myoblast differentiation

It is known that growth factors contained in platelet granules such as Hepatocyte growth factor (HGF), TGF-β1, Insulin-like growth factor 1 (IGF-1), Soluble Angiopoietin-1 receptor (sTIE-2), Angiopoietin-2 and Vascular Endothelial Growth Factor Receptor-2 (sVEGFR-2) are beneficial for myotube fusion (McClure et al., 2016, Kelc et al., 2015, Creaney and Hamilton, 2008, Miroshnychenko et al., 2017, Wijten et al., 2013). Therefore, it was next hypothesised that releasate would be beneficial in C2C12 myoblast differentiation into myotubes. To test this hypothesis, myotube differentiation in response to releasate administration either i) during the proliferative phase (day 0-2, GM+R;DM) or ii) throughout the proliferation and differentiation (day 0-9, GM+R:DM+R) was assessed. A significant decrease in myogenic fusion and differentiation mRNA (*Myogenin, Mhc1, Mhc2a, Mhc2b, Acta1, Tmem8c, Bex1* and *Igf1*) for the continuously added releasate group (GM+R;DM+R) versus the other two groups (GM:DM and GM+R;DM; which were non-significantly different for all expression) was found. *Myod, Pgdfβ* and *Vegfα165* mRNA expression was increased in all groups that had received releasate treatment. *Pax7* mRNA expression was decreased for GM+R;DM and increased for GM+R;DM+R versus the GM;DM positive control group (**Figure 3.12A**). Strikingly, continuous administration of releasate during day 0-9 resulted in a robust inhibition of myoblast differentiation as shown by impaired myofusion, myotube number, Myogenin expression, myotube length and myotube thickness with increased nuclei number. Subsequently, it was asked whether platelet releasate improves myotube terminal differentiation when administered after the initiation of myoblast fusion (days 5-12, GM;DM+R.D5-12). The addition of releasate during terminal differentiation resulted in significantly increased total nuclear number,

myotube number and Myogenin expression without affecting the myofusion index or the myotube length and thickness (**Figure 3.12B**). Taken together, these findings suggest that continuous administration of releasate causes myoblasts to fail exiting the proliferative phase and has detrimental effects on myotube formation. Conversely, administration of platelet releasate after the initiation of myotube formation (i.e. terminal differentiation) is beneficial for myoblast differentiation.

3.5.8 PDGFR and VEGFR inhibition reduces myoblast differentiation

To gain mechanistic insights into the role of releasate on myoblast differentiation, differentiating myoblasts were treated with PDGFR and VEGFR inhibitors with or without platelet releasate (**Figure 3.13A**). It was found that PDGFR inhibition reduced the total nuclei number, Myogenin expression and total myotube number in cultures with or without platelet releasate. Similarly, VEGFR inhibition during myoblast differentiation resulted in decreased total nuclei number, Myogenin expression and myotube number in cultures with or without platelet releasate (**Figure 3.13B-C**). These results indicate that PDGF and VEGF contained in releasate both are important for terminal myoblast differentiation.

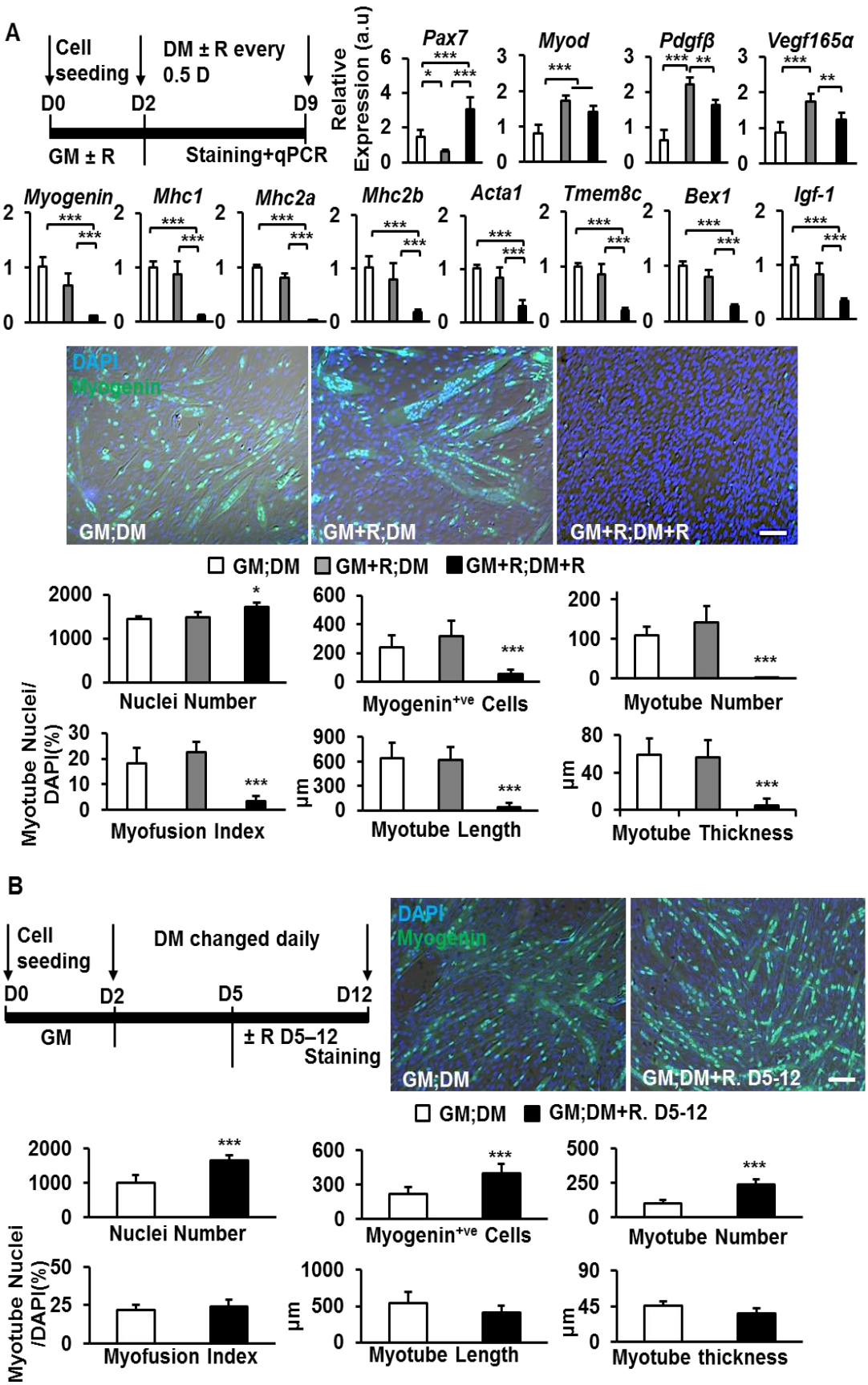

A
Cell seeding
DM ± R every 0.5 D
D0
D2
D9
GM ± R
Staining+qPCR
Relative Expression (a.u)
Pax7
Myod
Pdgfβ
Vegf165α
Myogenin
Mhc1
Mhc2a
Mhc2b
Acta1
Tmem8c
Bex1
Igf-1
DAPI
Myogenin
GM;DM
GM+R;DM
GM+R;DM+R
GM;DM
GM+R;DM
GM+R;DM+R
Nuclei Number
Myogenin+ve Cells
Myotube Number
Myotube Nuclei/ DAPI(%)
Myofusion Index
Myotube Length
Myotube Thickness
B
Cell seeding
DM changed daily
D0
D2
D5
D12
GM
± R D5–12
Staining
DAPI
Myogenin
GM;DM
GM;DM+R. D5-12
GM;DM
GM;DM+R. D5-12
Nuclei Number
Myogenin+ve Cells
Myotube Number
Myotube Nuclei /DAPI(%)
Myofusion Index
Myotube Length
Myotube thickness

Figure 3.12

Figure 3.12 Releasate causes C2C12 myoblasts to fail exiting the proliferative phase dependent on the timing of application. (**A**) Schematic depicting the experimental setup; C2C12 myoblasts were seeded with either 10% releasate (R) and/or 10% FBS (GM) for 2 days followed by adding differentiation medium (DM; 2% horse serum) every 0.5 days ±R for a total of 9 days culture. Representative images for Myogenin and DAPI (x5 magnification, scale bar 200µm). The myotube fusion index was calculated by Myogenin^{+ve} nuclei in myotubes/ DAPI as a percentage. Outcome measures include the nuclei number, number of Myogenin^{+ve} nuclei, myotubes (n=3 nuclei/ myotube), myotube thickness and length (µm). Gene expression for *Pax7, Myod, Pdgfβ, Vegf165α, Myogenin, Mhc1, Mhc2a, Mhc2b, Acta1, Tmem8c, Bex1 and Igf-1* was measured by qPCR for these 3 groups (i.e. GM;DM, GM+R;DM and GM+R;DM+R). (**B**) Schematic depicting experimental setup, as well as representative images (x5 magnification, scale bar 200µm). C2C12 cells were seeded with 10% FBS (GM) (GM;DM, GM;DM+R.Day 5-12). For the next 8 days, DM was added daily for every group and releasate added daily with each media change on day 5-12. Outcome measures include the number of myotubes, nuclei, myonuclei, myotube thickness and length (µm). Data are mean±SD (n=9/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001, vs. the positive control (GM:DM).

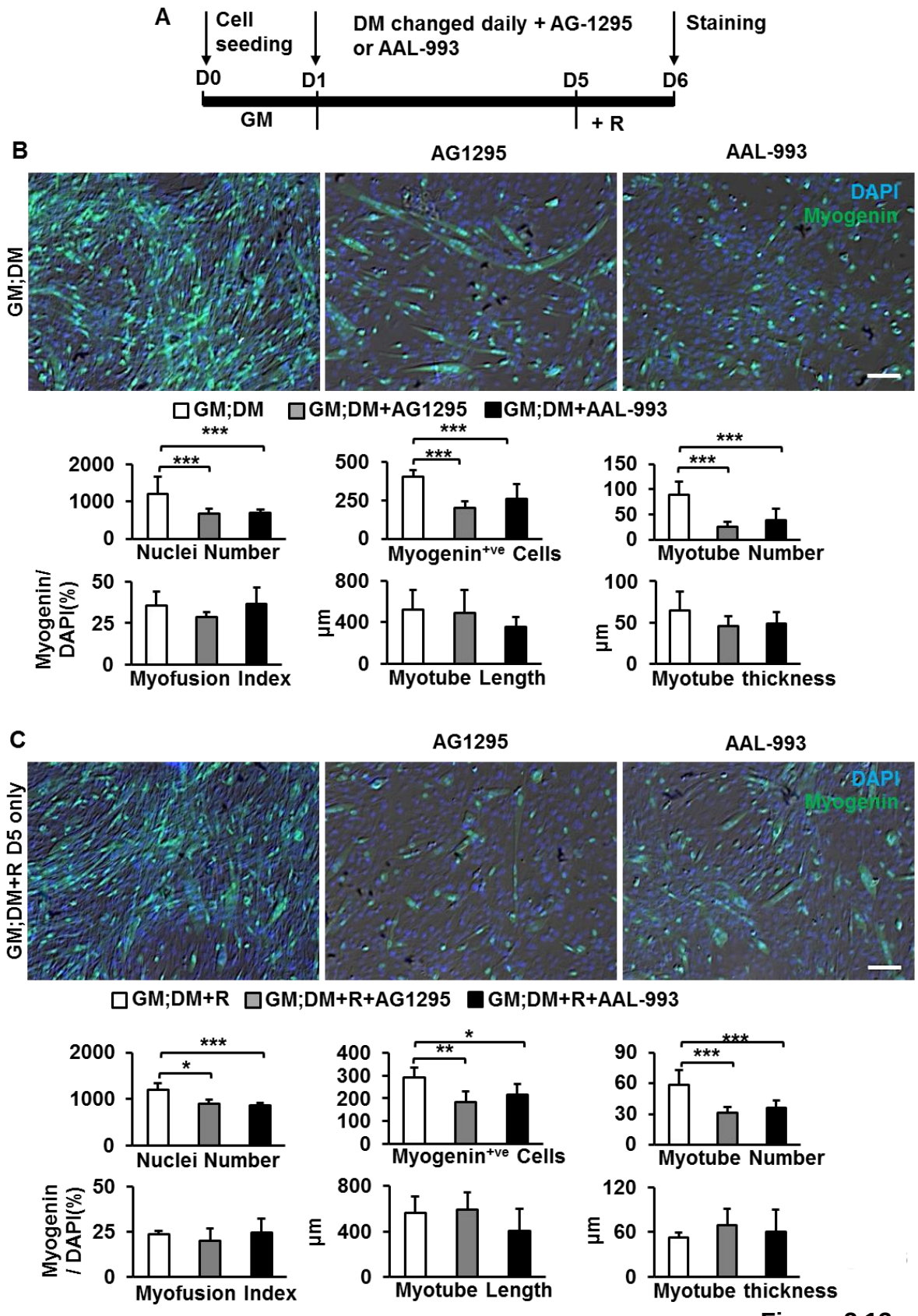

A
Cell seeding
DM changed daily + AG-1295 or AAL-993
Staining
D0
D1
D5
D6
GM
+ R
B
AG1295
AAL-993
DAPI
Myogenin
GM;DM
GM;DM
GM;DM+AG1295
GM;DM+AAL-993
Nuclei Number
Myogenin+ve Cells
Myotube Number
Myogenin/ DAPI(%)
Myofusion Index
Myotube Length
Myotube thickness
µm
C
AG1295
AAL-993
DAPI
Myogenin
GM;DM+R D5 only
GM;DM+R
GM;DM+R+AG1295
GM;DM+R+AAL-993
Nuclei Number
Myogenin+ve Cells
Myotube Number
Myogenin / DAPI(%)
Myofusion Index
Myotube Length
Myotube thickness
µm

Figure 3.13

Figure 3.13 PDGFR and VEGFR Inhibitors have a detrimental effect on nuclei number, Myogenin expression and myotube number during differentiation. (**A**) Schematic depicting the experimental setup; C2C12 cells were seeded with either 10% FBS (GM), 10% Releasate (R). After 1 Day of proliferation, media was changed to 2% horse serum (DM) with or without a 5μM PDGFR Inhibitor (AG-1295) or a 130nM VEGFR Inhibitor (AAL-993). Media was changed daily with inhibitors added on each change. Releasate was added on day 5 only, 1 day before experimental termination (GM;DM+R D.5). (**B**) PDGFR and VEGFR inhibition in control cultures (GM:DM). (**C**) PDGFR and VEGFR inhibition in cultures supplemented with releasate on day 5 (GM:DM+R D5 only). (**B-C**) Representative images for Myogenin and DAPI. The myofusion index was calculated by myotube nuceli/ total DAPI as a percentage. Outcome measures include the number of nuclei, Myogenin^{+ve} nuclei, myotubes, myotube thickness and length (μm). Data are mean±SD (n=3/group, 2 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 vs. the positive control (GM;DM).

3.5.9 Platelet releasate increases myofibre stem cell commitment to differentiation through VEGF and PDGF in a dose-dependent manner

Next, whether platelet releasate affected muscle stem cell expression profiles by using the single fibre *ex vivo* model was assessed. Immunohistochemical detection of Pax7^{+ve}/MyoD^{-ve}, Pax7^{+ve}/MyoD^{+ve} and Pax7^{-ve}/MyoD^{+ve} cells identified quiescent, activated/proliferating and committed-to-differentiation muscle stem cells, respectively (**Figure 3.14A**). Platelet releasate added 1-3 times (x) with media change within 48 hours of culture resulted in a dose-dependent increase of Pax7^{-ve}/MyoD^{+ve} cells compared to control (i.e. either serum free or fibre media conditions; **Figure 3.14B**). For example, treatment of fibres with platelet releasate x3 resulted in a 300-400% increase of Pax7^{-ve}/MyoD^{+ve} cells in either serum free or fibre media (i.e. serum rich) conditions, indicating that platelet releasate increased muscle stem cell commitment to differentiation. Furthermore, administration of releasate three times (x3) during a 48-hour culture induced a 50-100% increase in committed stem cells (i.e. Pax7^{-ve}/MyoD^{+ve}) as compared to a single addition of platelet releasate (x1). The commitment index, defined as the number of Pax7^{-ve}/MyoD^{+ve} cells per single fibre, was increased with releasate addition compared to serum free conditions and releasate supplemented with fibre medium had an additional cumulative effect (**Figure 3.14C**). Interestingly, inhibition of the VEGF and PDGF receptors independently

reversed the effect of releasate on both the number of committed stem cells (i.e. Pax7^{-ve}/MyoD^{+ve}) to control levels (i.e. fibre media) as well as the total stem cell number to serum free conditions (**Figure 3.14D**). In an attempt to identify key downstream factors involved in muscle stem cell progression, Cyclin D1 and Scrib were targeted, which have been acknowledged as two important regulators of cell cycle and muscle stem cell fate respectively (Tsai et al., 2017, Ono et al., 2015). It was found that platelet releasate resulted in a significant co-localisation of Cyclin D1 and Scrib with MyoD on muscle stem cells respectively, as well as a robust expression in proliferating C2C12 cells (**Figure 3.15A-C and Figure 3.16**). Ultimately, administration of platelet releasate on single fibres resulted in significantly higher muscle stem cell progeny independent of the use of serum in culture (i.e. SF and FM groups; **Figure 3.14D**). Collectively, these data suggest that use of platelet releasate has a powerful effect on driving muscle stem cell commitment to differentiation and similar to C2C12 cells, the effects of releasate are mediated by the VEGF and PDGF pathways through cell cycle and cell fate regulation.

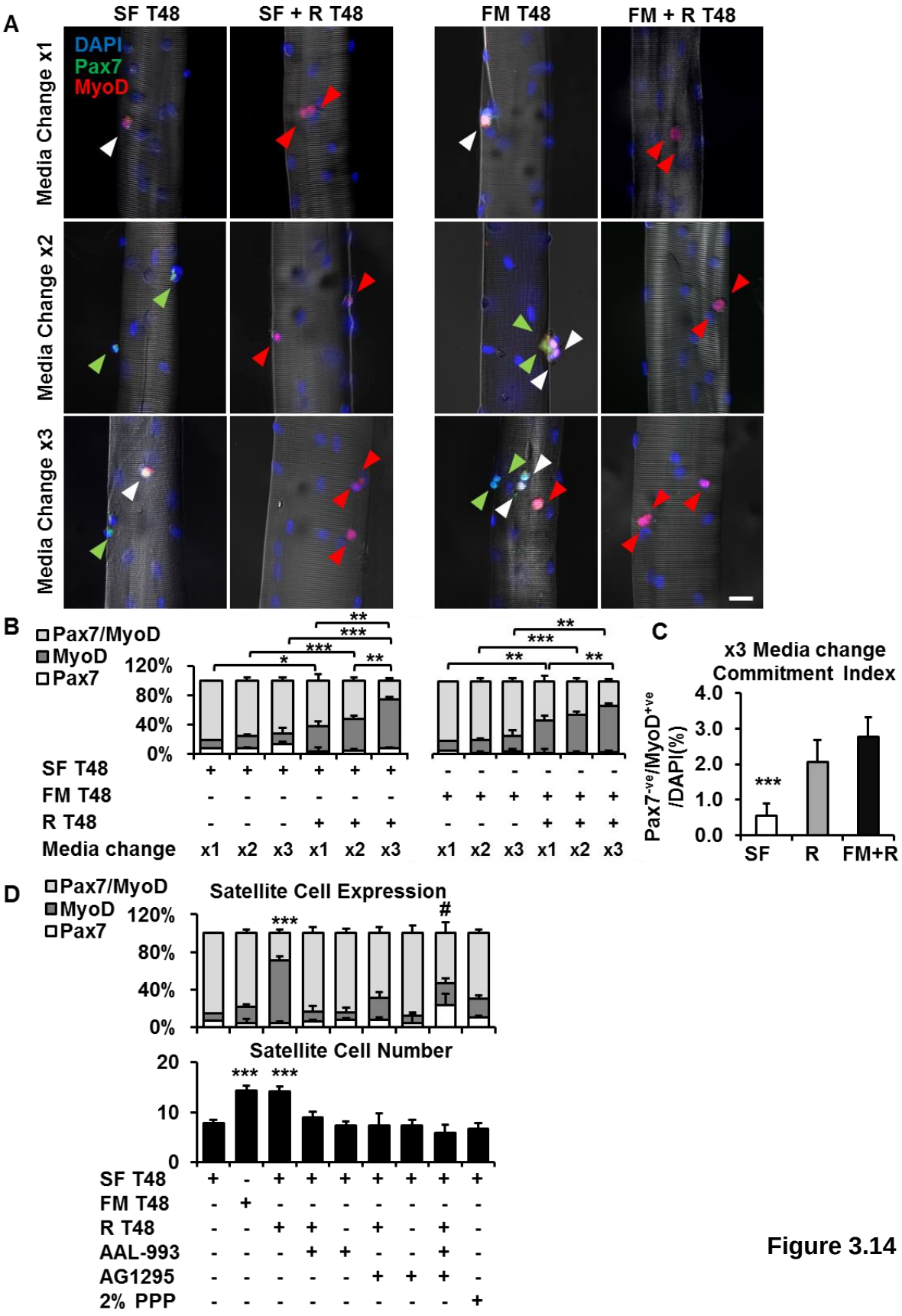

A
SF T48
SF + R T48
FM T48
FM + R T48
DAPI
Pax7
MyoD
Media Change x1
Media Change x2
Media Change x3
B
Pax7/MyoD
MyoD
Pax7
120%
80%
40%
0%
SF T48
FM T48
R T48
Media change
x1 x2 x3 x1 x2 x3 x1 x2 x3 x1 x2 x3
C
x3 Media change
Commitment Index
Pax7-ve/MyoD+ve
/DAPI(%)
4.0
3.0
2.0
1.0
0.0
SF
R
FM+R
D
Pax7/MyoD
MyoD
Pax7
Satellite Cell Expression
120%
80%
40%
0%
Satellite Cell Number
20
10
0
SF T48
FM T48
R T48
AAL-993
AG1295
2% PPP

Figure 3.14

Figure 3.14 Platelet releasate increases myofibre stem cell commitment to differentiation through VEGF and PDGF in a dose-dependent manner. Murine C57Bl/6J single fibres were isolated from the extensor digitorum longus muscle (EDL) for 48 hours culture in single fibre media (FM), or serum free media (SF) with the addition of releasate (R) either x1 or x2 (every 24 hours) or x3 (every 16 hours) media changes. (**A-B**) The percentage of muscle stem cells/ fibre expressing myogenic regulatory factors (MRFs) $Pax7^{+ve}/MyoD^{+ve}$ (White arrows) for cell activation, $Pax7^{-ve}/MyoD^{+ve}$ (Red arrows) for commitment to differentiation and $Pax7^{+ve}/MyoD^{-ve}$ (Green arrows) for quiescence (n=10 mice, 17 EDL muscles; 50-130 fibres quantified per condition (x40 magnification, scale bar 20μm). (**C**) Percent of $Pax7^{-ve}/MyoD^{+ve}$ cells per total nuclei (DAPI) per fibre. (**D**) Murine C57BL6 single fibres were isolated from the EDL for 48 hours culture in FM or serum free media (SF) with the addition of 10% releasate (R) (every 16 hours) ± PDGFR (AG-1295) or VEGFR (AAL-993) Inhibitors. Quantitative data representing the percentage of muscle stem cell expression per fibre expressing MRFs (upper graph) and total muscle stem cell number (lower graph). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. every other group for $Pax7^{-ve}/MyoD^{+ve}$ cells, and #$p<0.05$ for $Pax7^{+ve}/MyoD^{-ve}$ cells vs. every other group.

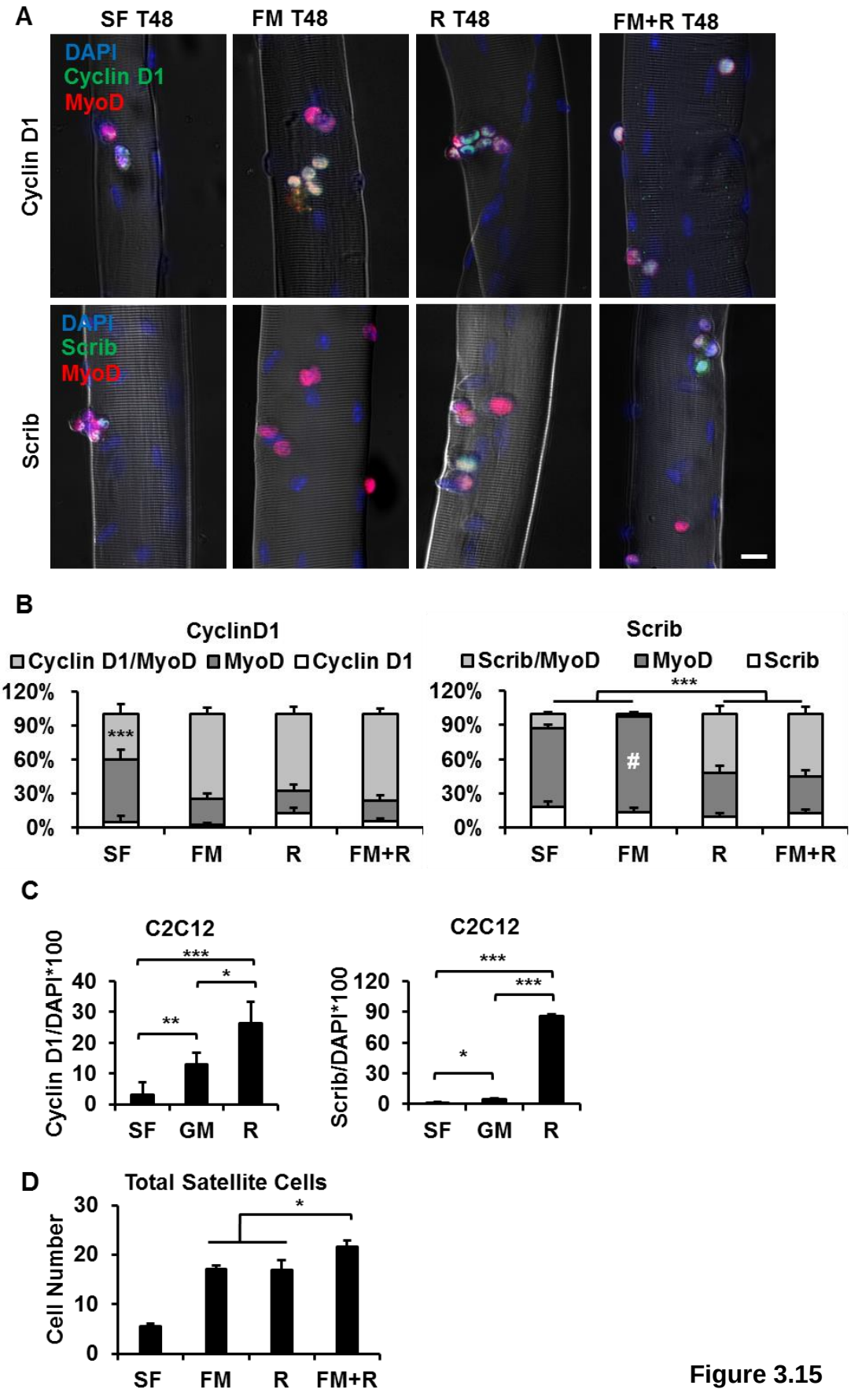

A
SF T48
FM T48
R T48
FM+R T48
DAPI
Cyclin D1
MyoD
Cyclin D1
DAPI
Scrib
MyoD
Scrib
B
CyclinD1
Cyclin D1/MyoD
MyoD
Cyclin D1
120%
90%
60%
30%
0%
***
SF
FM
R
FM+R
Scrib
Scrib/MyoD
MyoD
Scrib
***
#
C
C2C12
Cyclin D1/DAPI*100
***
*
**
40
30
20
10
0
SF
GM
R
C2C12
Scrib/DAPI*100
***
***
*
120
90
60
30
0
D
Total Satellite Cells
Cell Number
*
30
20
10
0
SF
FM
R
FM+R

Figure 3.15

Figure 3.15 Platelet releasate causes an increase in muscle stem cell proliferation and regulates cell fate through upregulation of Cyclin D1 and Scrib respectively. (**A**) Representative images from single fibres stained for Cyclin D1, Scrib, MyoD and DAPI (x40 magnification, scale bar 20μm). (**B**) The relative expression (%) of MyoD, Cyclin D1 and Scrib and DAPI for nuclei staining on muscle stem cells. Results are shown for serum-free (SF), Fibre Medium (10% HS; FM), 10% Releasate (R) and FM+R. (**C**) Cyclin D1 and Scrib immunohistochemical expression in C2C12 as a percentage per DAPI for serum-free, 10% FBS growth medium, and 10% releasate conditions. (**D**) Total muscle stem cell number per condition at time-point 48 hours of single fibre isolation *ex vivo*. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #p<0.01 vs. every other group. Differences for (B) refer to Cyclin D1/MyoD (double staining) ***p<0.001.

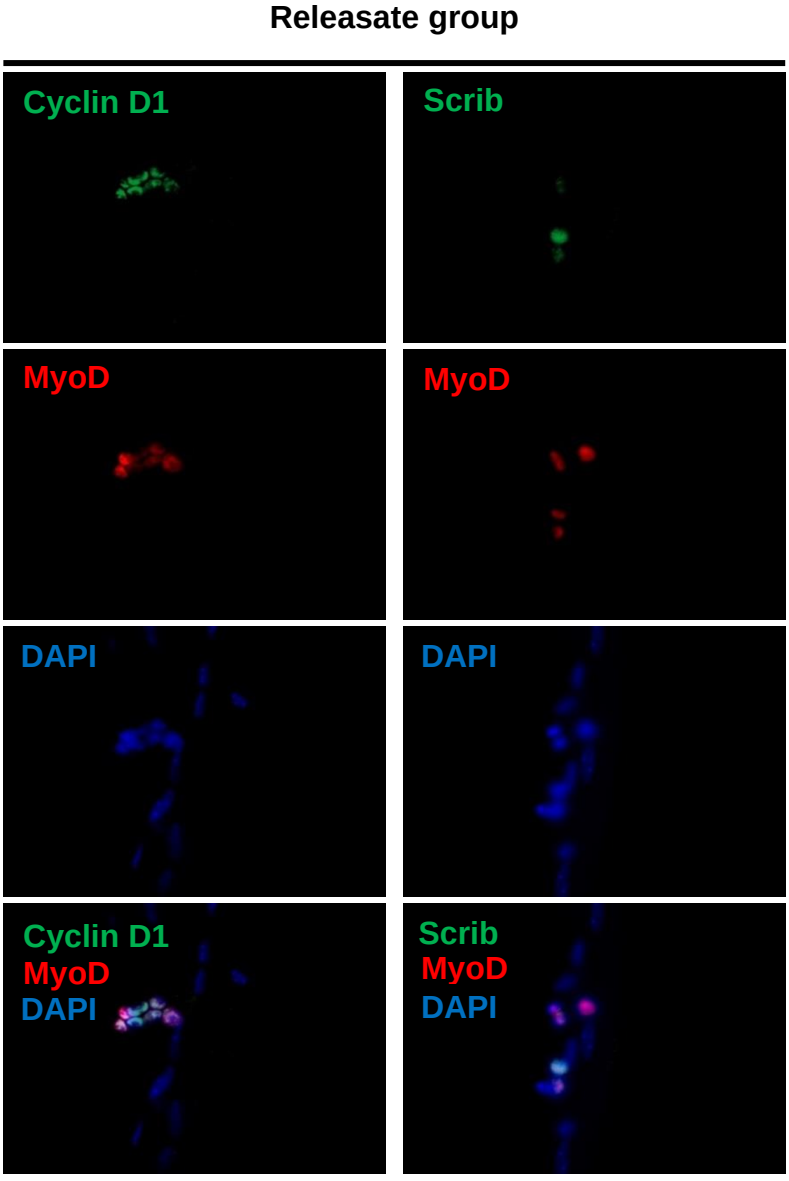


Figure 3.16 Unmerged immunohistochemical staining for Cyclin D1 (left panel, green), or Scrib (right panel, green), co-stained with MyoD (Red) and DAPI (Blue). Data are taken from main **Figure 3.15A** (Magnification is taken at x40).

3.5.10 Platelet releasate increases the total number of Myogenin^{+ve} muscle stem cells and clusters per fibre

Single myofibre cultures were conducted next, at 72 hours, where muscle stem cell progeny is expected to have differentiated to give rise to new myonuclei driven by high expression of Myogenin (Zammit et al., 2004). It was found that significantly increased Pax7^{-ve}/Myogenin^{+ve} cells in the releasate group as compared to serum free conditions, without any change in the relative expression patterns among groups (**Figure 3.17A-B**). The relative expression of Pax7^{+ve}/Myogenin^{-ve} cells (i.e. quiescent muscle stem cell pool) was not affected by the use of releasate, however was significantly increased between the FM and SF group. In addition, the total progeny stem cell number and the number of cell clusters per fibre were significantly higher in the fibre media and platelet releasate groups as compared to serum free media (**Figure 3.17B**). These data provide convincing evidence that the use of platelet releasate is beneficial for muscle stem cell differentiation *ex vivo*. Crucially, the normalised standardised rank curves illustrating the distribution of total muscle stem cell progeny number per myofibre were similar between the fibre medium (FM) and releasate (R) groups as compared to serum free (SF) conditions after 48- and particularly 72- hours in culture. Furthermore, regression analysis correlating 48-hour and 72-hour muscle stem cell progeny numbers revealed different gradient values (i.e. slopes) indicating the muscle stem cell proliferation rates were different between SF cultures versus FM and releasate conditions (**Figure 3.17C**). These results suggest that the muscle stem cells that developed under the influence of releasate treatment retained their normal capacity to proliferate. Taken together, these findings suggest that platelet releasate promotes myogenesis to a similar extent as found for standard serum-rich cultures.

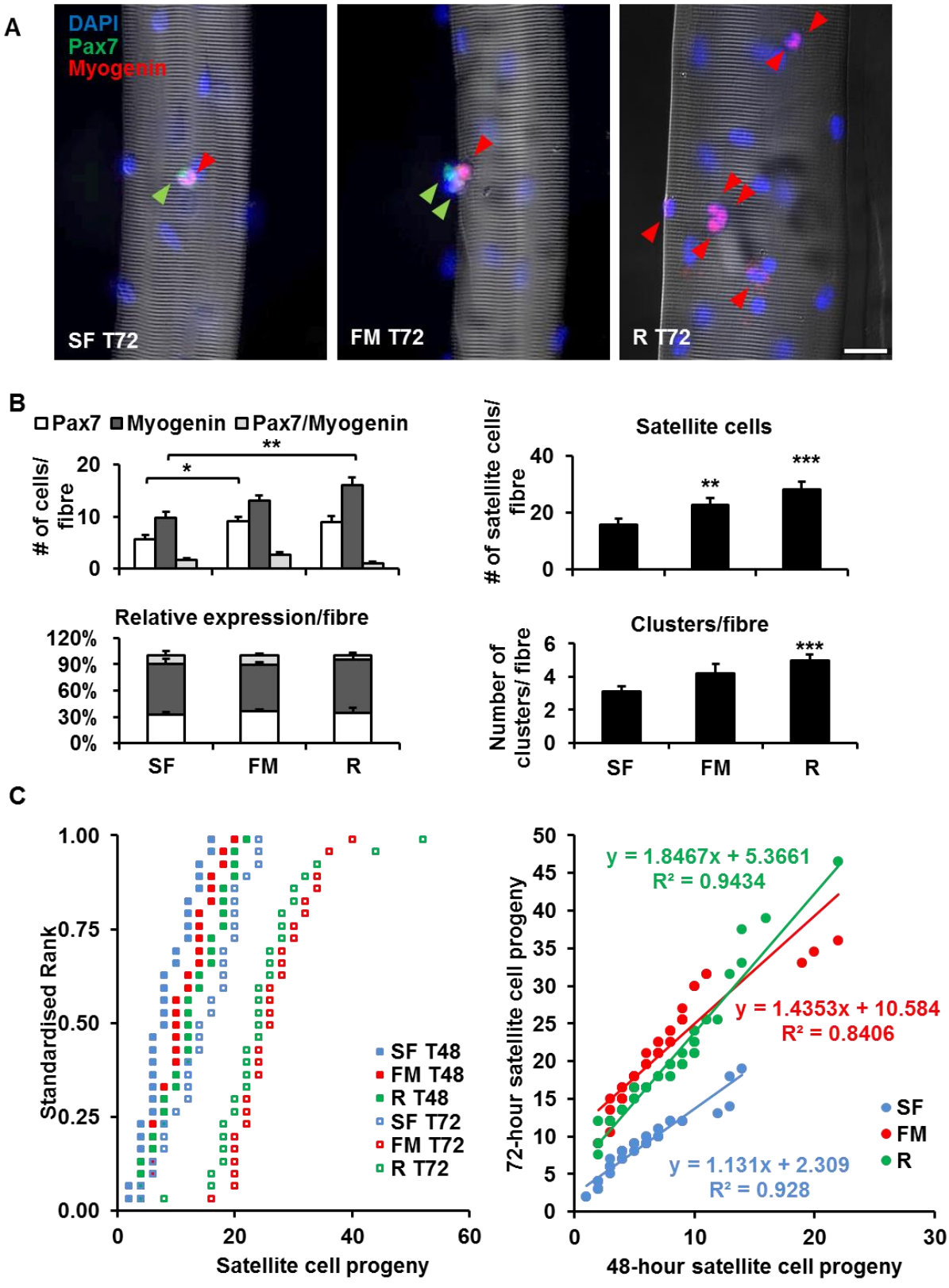

A
DAPI
Pax7
Myogenin
SF T72
FM T72
R T72
B
Pax7
Myogenin
Pax7/Myogenin
# of cells/ fibre
Satellite cells
# of satellite cells/ fibre
Relative expression/fibre
Clusters/fibre
Number of clusters/ fibre
SF
FM
R
C
Standardised Rank
Satellite cell progeny
SF T48
FM T48
R T48
SF T72
FM T72
R T72
y = 1.8467x + 5.3661
R² = 0.9434
y = 1.4353x + 10.584
R² = 0.8406
y = 1.131x + 2.309
R² = 0.928
72-hour satellite cell progeny
48-hour satellite cell progeny

Figure 3.17 Releasate increases muscle stem cell progeny without affecting the relative expression pattern (%) after 72 hours in serum-free conditions Male murine C57BL6 single fibres were isolated from the extensor digitorum longus muscle for 72 hours culture in single fibre media (FM), or serum free media (SF) with or without the addition of releasate (R) every 24 hours. (**A**) Representative images for Pax7 and Myogenin staining (x40 magnification, scale bar 20μm). The percentage of muscle stem (satellite) cells per fibre expressing $Pax7^{+ve}$/$Myogenin^{+ve}$ (white arrows), $Pax7^{-ve}$/$Myogenin^{+ve}$ (red arrows) for differentiating cells and $Pax7^{+ve}$/$Myogenin^{-ve}$ (green arrows) for return to quiescence. (**B**) Outcome measures include satellite cell expression pattern (number and percentage per fibre), total number of satellite cells and number of clusters per fibre. (**C**) Standardised rank of the satellite cell progeny for SF, FM and R conditions for T48 and T72 hours. Regression analysis correlating T48 and T72 hour satellite cell progeny numbers revealed different gradient values (i.e. slopes) indicative of the satellite cell proliferation rates. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs. the SF control group.

3.5.11 Platelet releasate increases mitochondrial respiration of C2C12 myoblasts and skeletal muscle stem cells

Since mitochondria play a central role in the regulation of cell proliferation, the use of platelet releasate affects the bioenergetic capacity of C2C12 myoblasts and muscle stem cells were determined, by measuring the OCR (Antico Arciuch et al., 2012, Hargrave et al., 2016). Significantly increased OCR in C2C12 myoblasts under serum free conditions but no change in serum rich cultures was found (**Figure 3.18A**). Conversely, there was significantly higher spare respiratory capacity in muscle stem cells supplemented with platelet releasate in either serum-free or -rich conditions (**Figure 3.18B**). These data indicate that platelet releasate robustly affects the bioenergetics of muscle stem cells.

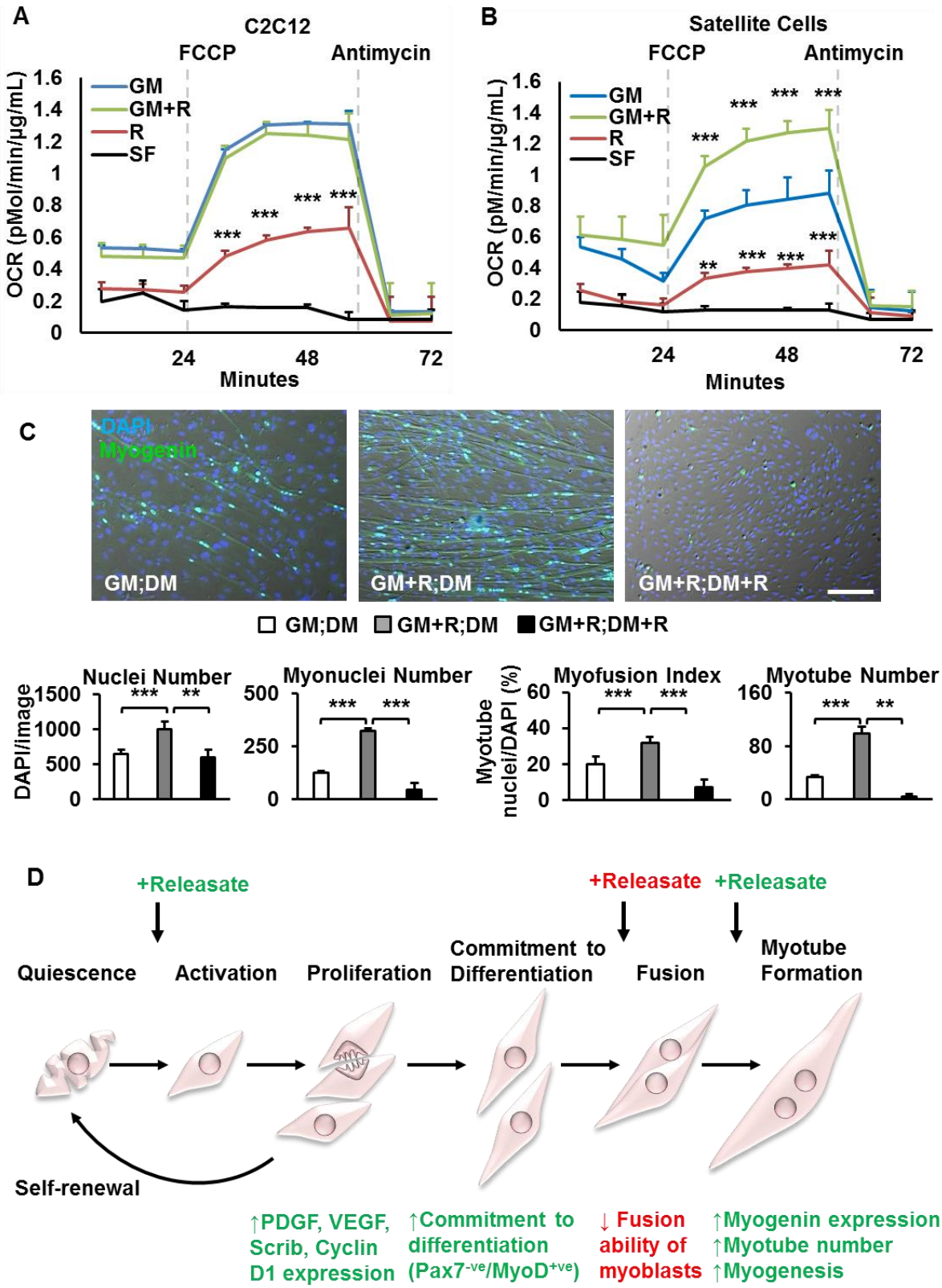

A
C2C12
FCCP
Antimycin
OCR (pMol/min/µg/mL)
GM
GM+R
R
SF
Minutes
B
Satellite Cells
FCCP
Antimycin
OCR (pM/min/µg/mL)
GM
GM+R
R
SF
Minutes
C
DAPI
Myogenin
GM;DM
GM+R;DM
GM+R;DM+R
GM;DM
GM+R;DM
GM+R;DM+R
Nuclei Number
DAPI/image
Myonuclei Number
Myofusion Index
Myotube nuclei/DAPI (%)
Myotube Number
D
+Releasate
+Releasate
+Releasate
Quiescence
Activation
Proliferation
Commitment to Differentiation
Fusion
Myotube Formation
Self-renewal
↑PDGF, VEGF, Scrib, Cyclin D1 expression
↑Commitment to differentiation (Pax7-ve/MyoD+ve)
↓ Fusion ability of myoblasts
↑Myogenin expression
↑Myotube number
↑Myogenesis

Figure 3.18 Platelet releasate affects myoblast or muscle stem cell metabolism (A) C2C12 myoblasts were seeded in Seahorse plates for 24h in either serum free (SF) ±10% releasate (R), or 10% FBS growth medium (GM) ±10% R. (**B**) Muscle stem cells were isolated from EDL (n=8) single fibres from male C57BL6 mice and cultured for 72 hours in growth media containing 30% FBS and 1.5% chick embryo extract. Cells were then transferred to a Seahorse plate in either growth media with GM, R or GM+R. The OCR was measured in response to FCCP and antimycin injections. Statistical analysis was performed by two-way ANOVA followed by Tukey's post-hoc test. Differences are **p<0.01, ***p<0.001 for either GM vs. GM+R or SF vs. R. (**C**) Isolated primary skeletal muscle stem cells from biceps brachii (BB) of C57Bl/6J mice (WT) cultured for 3 days in growth media (GM) or growth media plus releasate (GM+R), before changing to differentiation media for an additional 5 days with or without daily addition of 10% releasate. Representative data outcomes include cell number, myonuclei number, myofusion index and myotube number. (**D**) Schematic highlighting the beneficial effect of platelet releasate on muscle stem cell proliferation and differentiation. These findings suggest that platelet releasate increases muscle stem cell commitment to differentiation and promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis. Data are mean±SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are **p<0.01, ***p<0.001.

3.5.12 Platelet releasate accelerates regeneration of skeletal muscle following acute damage with cardiotoxin

Having shown that platelet releasate had a profound effect on myoblast proliferation and differentiation; its impact on the regeneration of adult mouse skeletal muscle, a process that is dependent on the generation of muscle precursors and their differentiation from the resident muscle stem cells, was next examined. To this end, the impact of platelet releasate was determined on muscle that has been acutely damaged using cardiotoxin (CTX) which causes muscle fibre necrosis, followed by macrophage-mediated debris clearance. Subsequent formation of new muscle fibres originates from the progeny of satellite cells, which undergo proliferation, differentiation and fusion. Newly formed regenerating muscle fibres were identified by their expression of embryonic myosin heavy chain (eMHC). It was found that generation of newly formed fibres was significantly enhanced (p<0.001) in mice that had been treated with releasate compared to those that received PBS (**Figure 3.19A**). Furthermore, a profound impact of platelet releasate on the clearance of dead and dying fibres was found. The number and size of dying fibres (identified through the infiltration of IgG into fibres) present at 5 days after CTX damage in PBS-treated mice

was significantly higher than those treated with platelet releasate ($p<0.001$; **Figure 3.19B**). These results show that platelet releasate promotes muscle regeneration by acting not only on muscle cells but possibly *via* other components of the regeneration process.

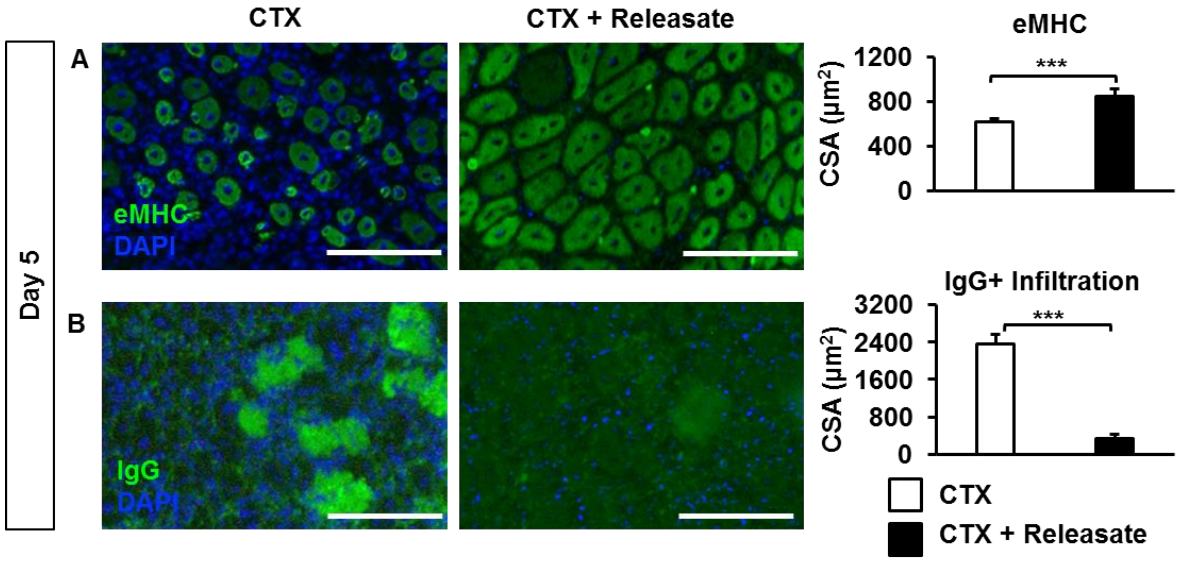


Figure 3.19 Platelet releasate accelerates skeletal muscle regeneration of tibialis anterior *in vivo* (A-B) Representative images for the identification of regenerating muscle fibres (through the expression of embryonic myosin heavy chain (eMHC), and damaged and dying fibres (identified through the presence of IgG inside muscle fibres on day 5 after cardiotoxin injury and platelet releasate treatment. (Scale bar 100µm). Data are mean ±SD. Statistical analysis was performed by Student's *t*-test. Differences are ***$p<0.001$ between the cardiotoxin (CTX) and the releasate (CTX + Releasate) group.

3.6 Discussion

Platelet-based applications have gained much attention recently as an effective way to promote muscle regeneration (Navani et al., 2017, Andia and Abate, 2018). However, data are inconsistent and there is currently a limited understanding of the molecular events that govern skeletal myogenesis using platelet-based products as biomaterials (Li et al., 2016b, Mosca and Rodeo, 2015, Tsai et al., 2018a). Due to largely inconsistent protocols in platelet preparation, it was hypothesised here that methodological variables such as platelet agonists, final centrifugation speed and platelet count may be crucial in determining experimental outcomes among studies (Miroshnychenko et al., 2017, Takase et al., 2017, Tsai et al., 2017, Im et al., 2014). Therefore, the effect of platelet agonists and final centrifugation speed and species-of-origin during releasate preparation on C2C12 myoblast proliferation was determined. Increased myoblast proliferation in response to releasate administration was observed, which is in line with previously published data on the use of platelet releasate was found (Tsai et al., 2017, Takase et al., 2017, Li et al., 2013, Tsai et al., 2018a, Saury et al., 2018).

3.6.1 Optimisation of platelet releasate for myoblast proliferation

However, the presented data also suggest that platelet releasate from collagen and TRAP6; two widely used platelet agonists, have a stronger effect on myoblast proliferation as compared to thrombin or mechanically lysed platelets by sonication, independent of the final centrifugation speed used. This finding may be explained by possible detrimental effects of proteases on releasate growth factors, since thrombin itself is a serine protease, as well as digestive enzymes released in the ablation of sonicated platelets (Duvernay et al., 2013, Zhao et al., 2014). In addition, the use of 10-30% of releasate resulted in a dose-dependent increase of myoblast proliferation, possibly due to higher amounts of releasate components. Since human platelet releasate was more powerful at promoting myoblast proliferation than mouse releasate, human releasate for all of the subsequent experiments was used. Platelet concentration was previously recognised as a potentially important variable for the diverse and conflicting data of PRP or releasate on tissue regeneration (Tschon et al.,

2011). The presented data in this thesis indicates that there is a linear relationship between platelet concentrations used for the production of releasate and myoblast proliferation and have to be taken into consideration in future studies. Collectively, despite a positive role of platelet releasate on myoblast proliferation, the data in this thesis suggests that methodological variations such as: i) platelet agonists, ii) amount of releasate administered, iii) species of platelet origin and iv) platelet concentration may account for some of the discrepancies among studies. In order to be consistent with the amount of releasate used in several previous studies, physiological levels of platelets for the production of releasate in all the subsequent experiments was used (Dimauro et al., 2014, Li et al., 2013, Miroshnychenko et al., 2017, Sassoli et al., 2018a, Tsai et al., 2018b).

3.6.2 How platelet releasate affects myoblasts on a genetic and protein level

It was next sought to determine the effect of releasate on myoblast gene and protein expression for factors known to regulate myogenesis. Gene and protein expression data from proliferating C2C12 myoblasts revealed a consistent and significant increase in PDGF and VEGF in response to human releasate as compared to serum free (control) conditions. The physiological effect of PDGF on cell growth and C2C12 myoblast proliferation has been recognised previously (Smith et al., 1999, Yablonka-Reuveni and Rivera, 1997). In fact, PDGF is found as homodimers of AA, BB, CC and DD or a heterodimer of AB isoforms. Although PDGF-AA and PDGF-AB have little or no effect on myogenic proliferation and differentiation, it has been shown that PDGF-BB promotes muscle stem cell proliferation but inhibits differentiation (Yablonka-Reuveni et al., 1990, Jin et al., 1991, Arsic et al., 2004). In turn, VEGF165α has been shown to enhance C2C12 myoblasts migration and exhibit anti-apoptotic effects (Germani et al., 2003). Higher mRNA levels of *Pax7*, *Myf5* and *Myod* in proliferating C2C12 myoblasts in response to releasate treatment may be part of a coordinated response of myogenic regulatory factors that are known to promote myogenic population expansion (Collins et al., 2009). This is in line with previous evidence showing failure to withdraw from the cell cycle and commit to differentiation in response to releasate use (Tsai et al., 2017). Impaired *Igf1* mRNA levels followed by unchanged

mRNA and protein levels of IGF1Rα indicate that the effect of releasate on proliferating myoblasts is not mediated by IGF1. IGF1 is known to increase embryonic myoblast proliferation, but *Igf1* mRNA levels tend to be higher during myogenic differentiation (Yoshiko et al., 2002, Smith et al., 1999). Increased mRNA levels of *Prmt1* in response to releasate administration are in line with previous data reporting that arginine methylation by *Prmt1* regulates muscle stem cell fate and it is important for myoblast fusion (Blanc et al., 2017). Taken together, these data suggest that the observed effects on myoblast proliferation by platelet releasate may be at least in part mediated by the PDGF and VEGF growth factors.

Given that *Ngf* has been shown previously to affect myogenic cell proliferation and differentiation and its expression is induced by platelet-rich plasma, it was questioned whether platelet releasate affects neurotrophic growth factor (e.g. *Ngf, Bdnf, Ntf-3, TrkA, TrkB* and *TrkC*) gene expression in proliferating myoblasts (Rende et al., 2000, Zheng et al., 2016). The data in this thesis suggests that administration of releasate on proliferating myoblasts does not upregulate the gene expression of neurotrophic growth factors. However, one has to bear in mind that neurotrophic factors and their receptors are more important for myogenic differentiation and their expression patterns in myotubes in response to platelet releasate remains to be established.

3.6.3 Growth factor composition of physiological platelet releasate

By using a multiplex immunoassay several growth factors in human platelet releasate in response to known platelet agonists were quantified (i.e. TRAP6 and collagen). The heatmap of those factors suggests that their amount increases several-fold in platelet releasate resulting in two clusters of moderate and higher intensity respectively. Of note, the factors SCF, FGF-β, HGF, HER2, Follistatin, VEGFR-1, EGFR, PDGF-AB and PDGF-BB (known to regulate proliferation (Deasy et al., 2001, Milasincic et al., 1996, Walker et al., 2015, Kocamis et al., 2004, Wright et al., 2014, Bryan et al., 2008, Thornton et al., 2015, Leroy et al., 2013, Jin et al., 1991, Yablonka-Reuveni et al., 1990) and VEGFR-2, IL-6, TIE-2 and HB-EGF (known to induce differentiation (Bryan et al., 2008, Serrano et al., 2008, McClung et al., 2015, Horikawa et al., 1999, Chen et al., 1995) showed a 4-20-fold increase in the TRAP6-activated platelet releasate as

compared to unstimulated platelets. EGF, FGF, VEGF and PDGF-BB have been shown to promote myoblast proliferation and consistently inhibit myogenic differentiation, while VEGFR2 expression is increased during differentiation (Wallaschofski et al., 2004, Bryan et al., 2008, Sassoli et al., 2012, Leroy et al., 2013, Milasincic et al., 1996, Jin et al., 1991). Moreover, SCF increases skeletal muscle stem cell number, IL-6 regulates myoblast proliferation and migration, while TNF-α inhibits myogenic differentiation in C2C12 cells through NF-κB and promotes myotube atrophy (Deasy et al., 2001, Serrano et al., 2008). Conversely, IGFBP-1, Angiopoietin-2and uPA have been shown to promote myogenic differentiation (Milasincic et al., 1996, Mofarrahi and Hussain, 2011, Suelves et al., 2002). Of note, it cannot be ruled out that other growth factors (not included in the Bioplex kits used in this study) contained in the platelet releasate may potentially affect myogenesis. Taken together these data suggest the numerous growth factors, cell cycle regulators and transcription factors present in platelet releasate can be used to promote skeletal myogenesis.

3.6.4 Inhibiting key growth factors in releasate-treated myoblast proliferation

The role of PDGF and VEGF contained in the human platelet releasate in skeletal myogenesis was next sought to be determined. For this reason, C2C12 myoblasts were cultured with releasate and administered either a PDGFR or VEGFR known inhibitor (Hutton et al., 2012, Ban et al., 2010). It is shown here that a dose-dependent attenuation of myoblast proliferation for both inhibitors used separately, independent of the use of platelet releasate. These findings suggest that the powerful proliferative effect of platelet releasate on C2C12 myoblasts is, at least in part, mediated through PDGF and VEGF signalling. The proliferative effect of releasate is abolished by the highest dosage of PDGF or VEGF inhibitors. This may possibly be due to a crosstalk with other growth factor receptors present in the releasate such as EGFR when used in high concentrations (Manley et al., 2002). This notion is strengthened by previous studies showing that PDGF/VEGF inhibition resulted in diminished myoblast proliferation and recombinant PDGFs improved skeletal muscle recovery after injury (Sugg et al., 2017, Jin et al., 1991, Yablonka-Reuveni et al., 1990, Sassoli et al., 2014, Arsic et al., 2004, Bryan et al., 2008, Germani et al., 2003, Li et al., 2013).

3.6.5 How platelet releasate affects myoblast differentiation

In contrast to myoblast proliferation, the use of platelet releasate during myogenic differentiation seems to be more complex. It is shown here that, on one hand; treatment of myotubes with releasate during terminal differentiation promotes myogenic differentiation (i.e. Myogenin^{+ve} cells and myotube number). On the other hand, administration of platelet releasate during both proliferation and differentiation of C2C12 myoblasts results in attenuated differentiation based on largely impaired *Myogenin, Mhc1, Mhc2a, Mhc2b, Acta1, Tmem8c, Bex1, Igf-1* mRNA, myogenin protein expression, myotube number, myofusion index, myotube length and thickness, which is in line with recent studies using platelet-rich plasma (Miroshnychenko et al., 2017, Saury et al., 2018, Kelc et al., 2015). This is indicative of a failure to withdraw from the cell cycle, supported by the increased nuclei number (**Figure 3.12A**). Importantly, evidence is provided that platelet releasate is beneficial for myogenic differentiation leading to higher Myogenin expression and larger myotubes, when it is administered after the early differentiation phase, where myoblast fusion into myotubes has started. Taken together, these data suggest that the timing of application of releasate to C2C12 myoblasts or myotubes is crucial and may account for discrepancies in the literature over whether releasate hampers or improves differentiation (Denapoli et al., 2016, Dimauro et al., 2014, Mosca and Rodeo, 2015).

3.6.6 Inhibiting key growth factors during releasate-treated myoblast differentiation

Similar to myoblast proliferation, inhibition of PDGFR and VEGFR resulted in attenuated C2C12 myotube differentiation as shown by impaired Myogenin expression and myotube number independent of releasate administration. This finding highlights the importance of PDGF and VEGF during myogenic differentiation. Previous research data suggest that VEGF is important for Myogenin expression as well as myoblast and myotube number (Sassoli et al., 2012, Sassoli et al., 2014). Moreover, addition of PDGF-BB to myoblasts inhibits differentiation (Pinol-Jurado et al., 2017, Yablonka-Reuveni et al., 1990, Yablonka-Reuveni and Rivera, 1997, Jin et al., 1991). Of note,

the myotube fusion index did not significantly differ amongst groups, indicating proliferation factors may be necessary for proper differentiation capacity and that lower total nuclei number may be accountable for the attenuated differentiation. Therefore, it appears that PDGF plays an important role during myoblast differentiation, which is in line with previous evidence that PDGFR inhibition decreases muscle mass (Sugg et al., 2017).

3.6.7 Platelet releasate alters the expression pattern of *ex vivo* single myofibres

Collectively, it is shown so far that platelet releasate is capable of promoting myoblast proliferation and exhibits opposing effects on myogenic differentiation dependent on the time of application. The importance of PDGF and VEGF is also highlighted for both myogenic proliferation and differentiation. PDGF and VEGF pathways interact through the Akt/mTOR pathway to induce skeletal muscle stem cell proliferation (Arsic et al., 2004, Yuasa et al., 2004, Moench et al., 2016). In order to determine the effect of platelet releasate on muscle stem cell proliferation and differentiation profiles murine single muscle fibres in culture were used. It is shown here for first time that platelet releasate administration on single myofibres results in a significant increase of $Pax7^{-ve}/MyoD^{+ve}$ muscle stem cells in a dose-dependent manner. These findings depict an increased commitment of proliferating muscle stem cells to differentiation as shown by $MyoD^{+ve}$ cells per total myonuclear number (i.e $MyoD^{+ve}$/DAPI/fibre). The increase in committed muscle stem cells to differentiation in response to platelet releasate is evident for both serum free and standard fibre growth conditions, indicating that releasate may act as an appropriate substitute for the regular fibre growth medium. To this end, several studies have aimed to replace bovine serum by autologous platelet-based applications (Saury et al., 2018, Li et al., 2013).

Although upregulation of MyoD mRNA and protein in rat skeletal muscle stem cells by PRP has been shown previously on muscle sections, evidence is provided here that this is taking place with the concomitant downregulation of Pax7 which results in higher number of stem cells following the differentiation pathway to support myofibre regeneration (Zammit et al., 2006b, Dimauro et al., 2014). Importantly, the effect of platelet releasate on MyoD is reversed by PDGFR and VEGFR inhibition, indicating a

possible crosstalk between these factors. Indeed, addition of PDGF-BB decreases myoblast fusion, however it upregulates MyoD during myogenesis (Yablonka-Reuveni and Rivera, 1997). Interestingly, application of platelet releasate resulted in a robust increase of Cyclin D1 and Scrib expression on muscle stem cells. Cyclin D1 driven by platelet releasate is known to regulate the cell cycle and is important for cell proliferation (Tsai et al., 2017). Most importantly, Scrib is a significant regulator of myogenic progression after commitment to differentiation that dictates muscle stem cell fate and is indispensable for muscle repair (Ono et al., 2015).

It has been claimed that the beneficial effects of platelet-rich plasma on myogenesis can be brought about by the plasma per se (Miroshnychenko et al., 2017). However, this possibility can be ruled out, since the data in this thesis indicates the use of platelet poor plasma does not mimic the findings on muscle stem cells observed with platelet releasate. Beyond that, platelet preparations were conducted with washed platelets in modified tyroid's buffer and not plasma in this study.

3.6.8 How platelet releasate affects primary skeletal muscle stem cells

Despite previous evidence on compromised myogenic differentiation in response to PDGF administration, it is found here that platelet releasate was beneficial for muscle stem cell differentiation (Yablonka-Reuveni and Rivera, 1997). In particular, it is reported in this thesis that platelet releasate on single muscle fibres leads to significantly higher expression levels of Myogenin in differentiated muscle stem cells, significantly higher stem cell progeny and total stem cell numbers as well as number of stem cell clusters per fibre. Further to this, isolated primary skeletal muscle stem cells have an increased number, fusion index and myotube number when co-cultured with platelet releasate and growth medium simultaneously; mitigating the negative effects that releasate has on differentiation alone. Altogether, these findings provide novel evidence on a powerful effect of platelet releasate on muscle stem cell proliferation and commitment to differentiation through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis (**Figure 3.18D**).

3.6.9 Bioenergetics of myoblasts treated with platelet releasate

In order to obtain insights in the metabolic homeostasis of proliferating cells exposed to platelet releasate, the oxygen consumption rate was measured at the cellular level. We found that administration of releasate resulted in significantly higher spare oxidative capacity in isolated muscle stem cells under either serum free or serum rich conditions. This finding indicates that platelet releasate may alter metabolic homeostasis of muscle stem cells, which is important for myogenesis and most importantly for muscle regeneration. This notion is supported by previous studies showing that cardiac myoblasts (i.e. H9C2 cells) had an increased respiratory reserve capacity in response to platelet-rich plasma (Hargrave et al., 2016). In the current study, platelet releasate did not upregulate the OCR of C2C12 myoblasts under serum-rich conditions, as opposed to primary murine stem cells. A possible explanation of this finding may originate in the recently described role of MyoD in regulating skeletal muscle oxidative metabolism with implications for energy availability and muscle contraction (Shintaku et al., 2016). Evidence also suggests that reactive oxygen species (ROS) are essential for activating several growth factors including PDGFs (Handayaningsih et al., 2011). Increased ROS production may overwhelm the antioxidant capacity of the cell resulting in oxidative stress. It can be speculated that the increased mRNA levels of DNA repair enzymes (i.e. *Ogg1*, *Gadd45g* and *Parp1*) in response to releasate treatment reported in this study may be part of a cellular response to the enhanced energetic state and potentially increased stress during accelerated myoblast proliferation.

3.6.10 Platelet releasate during skeletal muscle regeneration

Skeletal muscle regeneration following acute damage is a process that is reliant on a number of cell types that coordinate their activity to reconstitute working tissue (Harris, 2003). This includes not only the resident stem cell population (i.e. satellite cells), which regenerate most of, if not all of, the muscle cells that eventually fuse to form muscle fibres, but also macrophages and angioblasts (Collins et al., 2005, Latroche et al., 2017, Munoz-Canoves and Serrano, 2015). Here it is shown that platelet releasate promotes muscle regeneration following cardiotoxin injection, evidenced by the

presence of larger newly formed muscle fibres. This outcome is in keeping with *in-vitro* data in this thesis showing that platelet releasate impacts on the proliferation and differentiation of myoblasts. Herein, the accelerated regeneration is postulated to be a consequence of the direct impact of releasate on satellite cells. An additional possibility is that the angiogenic factors in the releasate could act on satellite cells indirectly so that they firstly promote angiogenesis in the regenerating tissue which then impacts on the activity of satellite cells. Indeed, angiogenesis is a major factor in muscle regeneration and can even compensate for a large decrease in muscle stem cells and still deliver robust regeneration (Omairi et al., 2016). Finally, it is shown here that the number of dying fibres and their size (an indicator of the clearance process) was greatly reduced by platelet releasate, a process controlled by macrophages. This may imply a role for the platelet releasate in controlling the pro-inflammatory/ anti-inflammatory properties of macrophages. This could be of particular importance to setting of chronic muscle damage in a clinical context (such as Duchenne Muscular Dystrophy) in which the inflammatory process induces fibrosis and attenuates muscle regeneration (Gosselin and McCormick, 2004).

3.7 Conclusion

The present study provided evidence that methodological variability may account for discrepancies among researchers on the role of platelet releasate in skeletal muscle regeneration. Platelet releasate promotes myoblast proliferation and terminal differentiation both *in vitro* and *ex vivo*. However, the timing of releasate application appears to be critical due to the inhibitory role of releasate on myoblast fusion as shown here and elsewhere (Saury et al., 2018). Most importantly, it is shown for the first time that platelet releasate increases muscle stem cell commitment to differentiation and promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis and accelerates skeletal muscle regeneration after acute injury. Collectively, the present findings can be exploited in future studies to pinpoint the role of platelets as biomaterials in skeletal muscle regeneration.

Chapter 4

The role of platelet releasate in skeletal myogenesis of the Thrombospondin-1 null mouse

4.1 Abstract

Aim: Thrombospondin-1 (TSP-1); a negative regulator of excessive capillarisation, is produced by platelets and tissues such as skeletal muscle. There is currently accumulating knowledge regarding the impact of platelet releasate on skeletal muscle cell proliferation and differentiation. The aim of this study was to determine properties of platelet releasate from the TSP-1 null mouse with regards to myogenesis and to profile its skeletal muscle.

Methods: The effect of platelet releasate made from TSP-1 null platelets as well as several knock-out murine models that effect platelet function (CD36, GPR55, FPR1, FPR2 and ApoE) were analysed for C2C12 myoblast proliferation. The proliferation and differentiation of skeletal muscle satellite cells were further characterised both on intact myofibres and in primary satellite cell culture between wild-type and TSP-1 KO. The weight and fibre-type of several skeletal muscles were then assessed on TSP-1 KO mice as compared to wild-type. Cardiotoxin-induced tibialis anterior injury was treated with or without platelet releasate for 5-10 days.

Results: TSP-1 KO murine platelet releasate promoted significantly greater myoblast proliferation compared to wild-type releasate. Platelet releasate significantly increased the total number of satellite cells during proliferation on single fibres but not on stripped primary satellite cells. Importantly, TSP-1 KO stripped satellite cells have an overall reduced differentiation capacity which can be rescued by human platelet releasate. TSP-1 KO had a comparable skeletal muscle weight, cross-sectional area and fibre-type to wild-type. TSP-1 KO mice had a faster regenerative genotype with platelet releasate having an additive effect.

Conclusion: This study provides novel insights into the role of platelet releasate in skeletal myogenesis of the TSP-1 null mouse. TSP-1 null myoblasts have a more proliferative phenotype than wild-type mice, which may account for the enhanced regeneration observed. Further to this, depleting TSP-1 from platelet releasate may be an active way of further optimising the platelet-based application for clinical use, providing enhanced skeletal muscle recovery.

4.2 Introduction

TRAP-6-activated platelet releasate has enhanced skeletal muscle regeneration *in vivo*, upregulated proliferation of skeletal muscle satellite cells *ex vivo* and increased commitment to differentiation on cultured single fibres. Interestingly, during the preparation of platelet releasate, TRAP-6-induced platelet activation has been shown to result in a 5.6-fold increase in Thrombospondin-1 (TSP-1) release over other agonists such as collagen and ATP (Coppinger et al., 2007). TSP-1 is one of the most abundant proteins in TRAP-6-activated platelet releasate (O'Connor et al., 2010).

TSP-1 binds to activated platelets upon platelet stimulation and interacts with fibrinogen which is an important step in platelet aggregation. TSP-1 has previously been speculated to be an important risk factor for spontaneous thrombosis in ageing (Cini et al., 2015). TSP-1 is an important endogenous negative regulator of angiogenesis that prevents excessive capillarisation in both heart and skeletal muscles, partially acting through the CD36 scavenger receptor (Audet et al., 2013, Isenberg et al., 2007a, Isenberg et al., 2007b). Knock-out models of TSP-1 show an enhanced exercise capacity, altered blood flow and vessel diameter (Malek and Olfert, 2009, Isenberg et al., 2007a). Myoblast cell lines can synthesise and secrete TSP-1 where it acts as a growth stimulatory component of the extracellular matrix during tissue remodelling (Adams and Lawler, 1994). TSP-1 mRNA levels are increased after acute exercise but protein levels are unaffected after 4 weeks of exercise training in human vastus lateralis (Hoier et al., 2012). Moreover, studies in CD47 (a TSP-1 receptor) null and TSP-1 null mice show enhanced physical performance, lower reactive oxygen species (ROS) production and a more efficient metabolism (Frazier et al., 2011). Additionally, TSP-1 has been shown to suppress insulin signalling and activates stress signalling pathways in myotubes (Matsugi et al., 2016).

As TSP-1 plays a role in skeletal muscle function and angiogenesis, and is abundantly present in TRAP-6-activated platelet releasate, the aim was to determine: i) whether platelet releasate from TSP-1 Knock-out (KO) mice differentially affect myoblast proliferation as compared to wild-type and other null mouse lines, ii) to study the effect of both human and TSP-1 KO platelet releasate on TSP-1 KO and wild-type satellite

cell differentiation and iii) to identify potential differences in fibre-type composition and size from wild-type. To this end, human and TSP-1 KO platelet releasate on C2C12 myoblasts, satellite cells on single fibres (wild-type and TSP-1 KO) in *ex vivo* culture and primary satellite cells for both proliferation and differentiation were compared. Additionally, the satellite cell expression profiles, skeletal muscle fibre-typing and skeletal muscle weights were characterised for TSP-1 KO versus wild-type mice.

In this Chapter, it is reported that TSP-1 KO platelet releasate is more beneficial for myoblast proliferation *in vitro* as compared to releasate from any other mouse line studied here. TSP-1 KO releasate enhances satellite cells progeny at T48 and T72 in both WT and TSP-1 KO fibres. Finally, we show that TSP-1 KO murine muscle has normal fibre-type composition and size; however in response to injury, they have accelerated regeneration, further assisted by platelet releasate.

4.3 Methods

4.3.1 Ethical standards

This study was approved by the Ethics Committee of the University. The animal experiments were performed under a project license from the United Kingdom Home Office in agreement with the revised Animals (Scientific Procedures) Act 1986 and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). TSP-$1^{-/-}$ and wild-type (WT) mice on the C57BL/6 background were housed under standard environmental conditions provided standard chow and water ad libitum. Blood sampling from healthy human volunteers was performed with their written informed consent and was approved by the Ethics Committee of the University.

4.3.2 Preparation of platelet releasate

Human platelet releasate was prepared as described in section 2.2.1. In brief, whole blood in acid citrate dextrose was centrifuged followed by inactivation of platelets using prostaglandin I_2. The inhibited platelet-rich plasma was then centrifuged and the platelet pellet was resuspended in Modified tyrode's buffer at a concentration of 2.5 - $10x10^8$ platelets mL^{-1}. The platelet preparation was activated using a PAR-1 (TRAP-6; 20μM). Platelet debris was then removed and the secretome was collected.

Mouse platelets were prepared as previously described in section 2.2.2. In brief, whole blood was collected in acid citrate dextrose and centrifuged. The platelet-rich plasma layer was then further centrifuged and resuspended in Modified tyrode's buffer to a concentration of $2.5x10^8$ platelets mL^{-1}. Thrombin (0.05-0.1 NIH Units mL^{-1}) was used to activate the platelets in an aggregometer for 5 minutes at 37ºC (reaching >70% aggregation) before centrifuging at 9500g and storing releasates at -80°C. Thrombin was used to activate the PAR receptors (i.e. PAR-4) in all murine whole blood.

4.3.3 Cell cultures and treatments

Murine C2C12 skeletal muscle cells were cultured as described previously in section 2.3. In brief, cells were cultured in GM. All releasate treatments were cultured in serum-

free conditions when not indicated to be in a GM group. To induce differentiation, isolated primary satellite cells were cultured in GM until reaching 80% confluence (4 days) before switching to DM for a further 4 days. Releasate was added once during proliferation at T0 hours. The myofusion index was calculated as Myogenin-stained cells per myotube (n=3 nuclei/myotube) divided by DAPI-stained cells as a percentage.

4.3.4 Cell proliferation analysis

C2C12 myoblast and primary skeletal muscle satellite cell proliferation was also evaluated by the pyrimidine analogue EdU incorporation assay using the fluorescent EdU cell proliferation assay according to the manufacturer's instructions. Proliferating cells were measured as EdU divided by DAPI-stained nuclei as a percentage.

4.3.5 Single fibre isolation and culture

For single-fibre culture, murine C57Bl/6J and TSP-1 KO single fibres were isolated from the EDL muscle. Limb muscles were dissected and subjected to collagenase (0.2%) as described previously (Matsakas et al., 2009). Fibres were then cultured for 48 and 72 hours in FM, or SF media with the addition of 10% releasate 3 times during culture every 16 hours, unless otherwise stated. Fibres were stained for mouse monoclonal anti-Pax7, rabbit polyclonal anti-MyoD and rabbit polyclonal anti-Myogenin, and with DAPI. The commitment index was calculated as MyoD-stained satellite cells divided by DAPI-stained cells as a percentage. VEGF was inhibited in the myofibres using 1.3μM VEGFR Inhibitor (AAL-993). For **Figure 4.12B**, time-point 0 fibres were stained for Pax7 and DAPI only.

4.3.6 Satellite cell isolation from single fibres

Primary satellite cells were isolated from the EDL and BB of wild-type and TSP-1 KO mice. Briefly, limb muscles were dissected and subjected to 0.2% collagenase digestion for 4 hours at 37°C. The digested muscle was then gently transferred to 35mm petri dishes where the collagenase was removed. Single muscle fibres were isolated in SF culture medium, by means of a gentle mechanical trituration with a Pasteur pipette and then cultured for 72 h in Matrigel (1mg mL^{-1}) –treated 6-well plates,

at 37°C in a humidified atmosphere of 5% CO_2, in satellite proliferation media containing DMEM, 30% FBS, 1.5% C.E.E. plus 1% PS. Next, the myofibres were removed and the satellite cells were cultured as per experimental conditions.

4.3.7 Immunofluorescence

Cells were seeded on coverslips in 1mL of media in 24-well plates. Cell fixation and permeabilisation was conducted as in section 2.3.6. Primary antibodies for anti-Pax7, anti-Myogenin and anti-MyoD, or anti-eMHC or CD31 or CD68 or anti-VEGF were added overnight. Primary antibodies were removed with 3 washes in wash buffer, followed by addition of the secondary antibody (Alexa fluor 488 Goat-anti-mouse and Alexa fluor 633 Goat-anti-mouse) of 1:200 in wash buffer. Cells were measured by the intensity of fluorescence per cell divided by DAPI-stained nuclei as a percentage.

4.3.8 Immunofluorescence for myofibre composition

Fibre typing was used for examining the distribution of two myofibre types (i.e. types IIB and IIA; extensor digitorum longus and tibialis anterior) or types IIA and I (Soleus) simultaneously and identification of type IIX myofibres (remaining non-stained fibres) in muscle sections. Myosin heavy chain (Mhc) type I, IIA and IIB were identified by using A4.84 IgM, A4.74 IgG and BF-F3 IgM monoclonal primary antibodies respectively. Alexa fluor 488 Goat-anti-mouse and Alexa fluor 633 Goat-anti-mouse were used to visualise the primary antibodies diluted 1:200 in wash buffer. The cross-sectional area (CSA) was measured per fibre type (I, IIA and IIB) on Zen Software Zen 2.3 Blue Edition ©Carl Zeiss microscopy GmbH, 2011.

4.3.9 RNA extraction and real-time PCR analysis

Quantitative PCR was performed as described previously (Matsakas et al., 2012b). In brief, gastrocnemius muscle of uninjured mice injected with 3 doses of human platelet releasate on time-point 0h, 24h and 72h was minced in Trizol for RNA isolation and qPCR. Total RNA (2 μg) was reverse-transcribed to cDNA with RevertAid H MinusFirst Strand cDNA synthesis kit and analysed by quantitative real-time RT-PCR on a StepOne Plus cycler, using the Applied Biosystems SYBRGreen PCR Master Mix.

Primers were designed using the software Primer Express 3.0. Details of primers are given in **Appendix I**. Relative expression was calculated using the ΔΔCt method with normalisation to the reference genes encoding Cyp and Hprt. mRNA levels of *Vegfa165, Vegfr1, Fgf1, Hgf, Iix, Iib, Pgc1α, Mstn, Sirt1, Igf1, Pdgfα, Pdgfβ* and *Bdnf* were measured.

4.3.10 *In Vivo* cardiotoxin-induced muscle injury

C57Bl/6J and TSP-1 KO mice were injected with 30µL, 10µM *Naja pallida* cardiotoxin (CTX) into the tibialis anterior (TA) muscle under anaesthesia with ketamine/xylazine (100mg/kg and 10mg/kg respectively). Mice (n=5/group) were then injected with 200µL platelet releasate intraperitoneally (IP) 0, 24 and 72 hours after injury. TA muscles were surgically removed on day 5 and 10 post-injury and were processed for immunohistochemistry.

4.3.11 Statistical analysis

Data are reported as mean±SD. Statistical differences among experimental groups were determined by either one-way or two-way ANOVA followed by the Tukey post-hoc test as indicated in the figure legends. Differences between two groups were detected by using Student's *t*-test. Chi-Square was implemented for satellite cell frequency. Statistical differences were considered as significant for $p<0.05$. Statistical analysis was performed on the SPSS software.

4.4 Results

4.4.1 Platelet releasate derived from human blood and several KO mouse lines differently affect skeletal myoblast proliferation

It is shown here that a physiological concentration of platelets used to make platelet releasate was a powerful inducer of skeletal muscle satellite cell proliferation. Therefore, it was next aimed to establish the proliferative effect of platelet releasate from several knock-out models known to either effect skeletal muscle or platelet function on C2C12 cells (**Figure 4.1A-B**). Platelet releasate from FPR2; Formyl peptide receptor 2 and ApoE; Apolipoprotein E null mice were found to induce a lower level of C2C12 myoblast proliferation compared to wild-type (WT) platelet releasate. In contrast, platelet releasate from FPR1; Formyl peptide receptor 1 (FPR1) and Cluster of differentiation 36 (CD36) knock-out mice induced the same level of C2C12 proliferation as WT platelet releasate. Strikingly, TSP-1 KO releasate induced greater levels of proliferation compared to that from wild-type mice and matched the levels induced by human platelet releasate. These data indicate a potential benefit to removing Thrombospondin-1 from the platelet releasate to support myoblast proliferation.

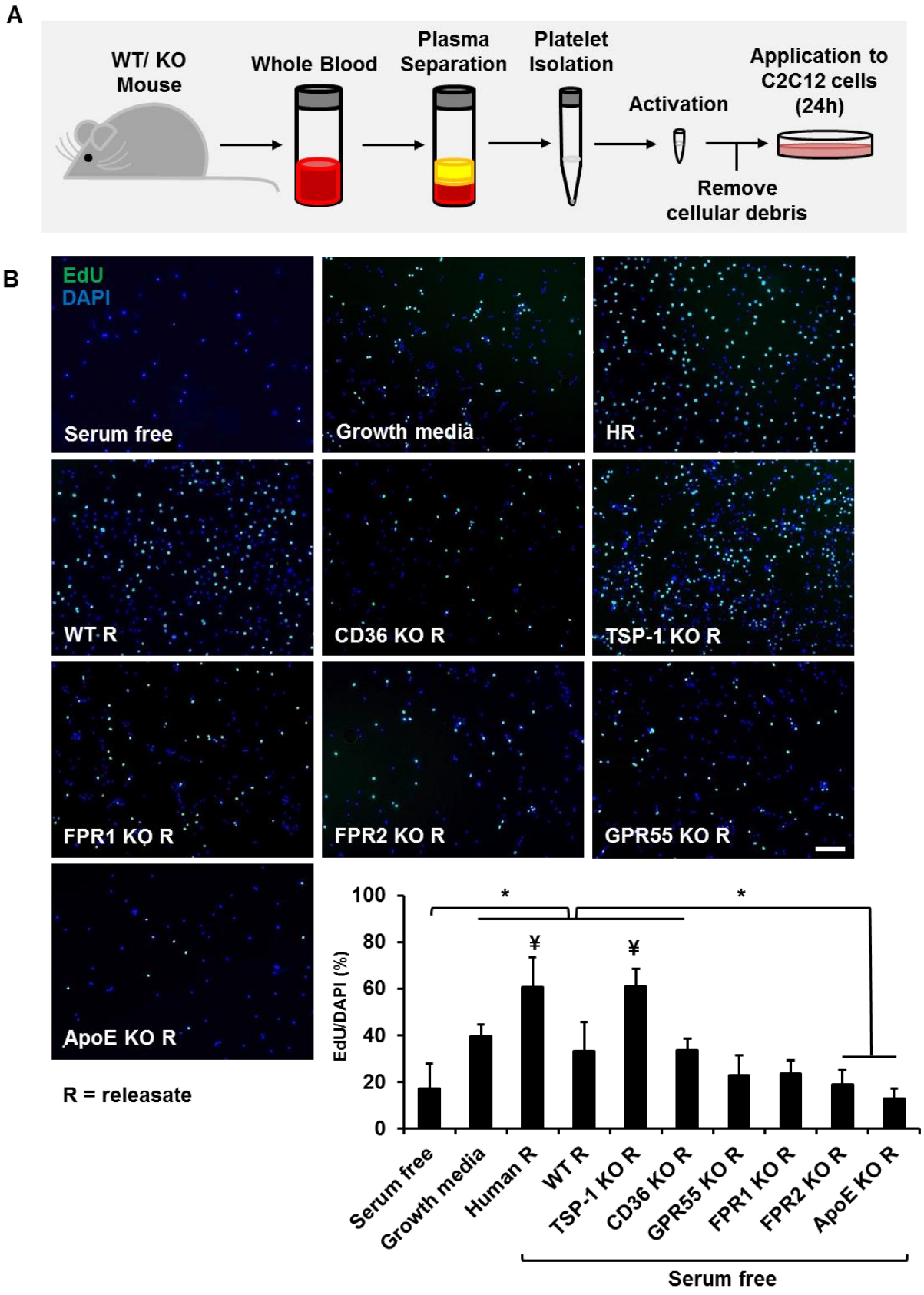

A
WT/ KO Mouse
Whole Blood
Plasma Separation
Platelet Isolation
Activation
Application to C2C12 cells (24h)
Remove cellular debris
B
EdU
DAPI
Serum free
Growth media
HR
WT R
CD36 KO R
TSP-1 KO R
FPR1 KO R
FPR2 KO R
GPR55 KO R
ApoE KO R
R = releasate
EdU/DAPI (%)
100
80
60
40
20
0
*
*
¥
¥
Serum free
Growth media
Human R
WT R
TSP-1 KO R
CD36 KO R
GPR55 KO R
FPR1 KO R
FPR2 KO R
ApoE KO R
Serum free

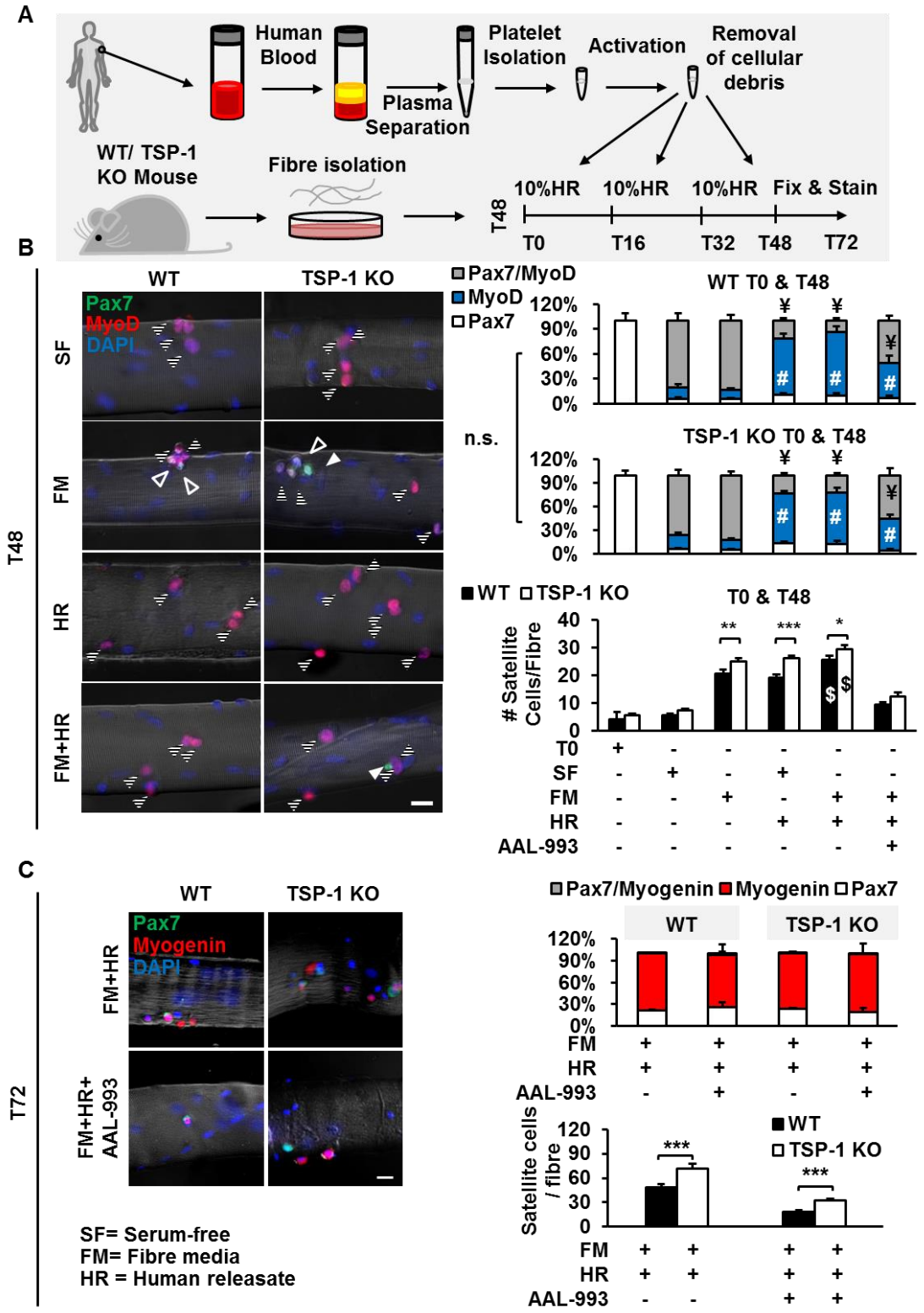
Figure 4.1

Figure 4.1 Platelet releasate derived from human blood and several KO mouse lines differently affect skeletal myoblast proliferation (**A**) A schematic depicting the methodology behind the experimental setup. EdU proliferative live-staining was conducted for 3-hours after 21 hour incubation totalling 24-hours of proliferation in serum-free, growth media (10% FBS), 10% human releasate (HR) in serum-free conditions, or murine releasate (WT; wild-type R, TSP-1 knockout (KO) R, CD36 KO R, GPR55 KO R, FPR1 KO R, FPR2 KO R or ApoE KO R). (**B**) Representative images and quantitative data for EdU staining with DAPI co-staining (x5 magnification, scale bar 200 μm). All releasate was made with $2.5x10^8$ platelets/mL. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. n=3 independent experiments with 9 technical replicates per experiment. Differences are *p<0.05, ¥p<0.01 vs. GM, WT R, CD36 KO R, GPR55 KO R and FPR1 KO R, FPR2 KO R and ApoE R.

4.4.2 TSP-1 KO myofibres have a larger satellite cell progeny compared to WT independent of treatment with human platelet releasate

The effect of human platelet releasate on skeletal muscle satellite cell functionality of the TSP-1 KO versus WT murine myofibres was established. A normal expression pattern for quiescence ($Pax7^{+ve}/MyoD^{-ve}$), proliferation ($Pax7^{+ve}/MyoD^{+ve}$) and commitment to differentiation ($Pax7^{-ve}/MyoD^{+ve}$), was found; which was assessed for T48 hours on isolated myofibres between wild-type (WT) and TSP-1 KO mice. A VEGF receptor- specific inhibitor (1.3μM VEGFR Inhibitor; AAL-993) was used to further differentiate the potential effects of TSP-1 KO on skeletal muscle satellite cell proliferation, however no significant difference was detected versus WT. There were no significant differences between WT and TSP-1 KO for expression patterns, indicating a normal phenotype. Total satellite cell number on the fibres was counted to indicate proliferation at 48 hours. TSP-1 KO showed a benefit for normal culture conditions (10% FBS), for 10% releasate and FM+R groups as compared to the WT conditions (**Figure 4.2A-B**). A normal expression pattern for quiescence ($Pax7^{+ve}/Myogenin^{-ve}$) and satellite cells committed to differentiation ($Pax7^{-ve}/Myogenin^{+ve}$) assessed for T72 hours on isolated myofibres from wild-type and TSP-1 KO mice (**Figure 4.2C; upper graph**) was found. A VEGF receptor- specific inhibitor (1.3μM VEGFR Inhibitor; AAL-993) was used to further differentiate the potential effects of TSP-1 KO on skeletal muscle satellite cell differentiation, however no significant difference was detected versus WT for expression patterns but there was a

significant increase in total cell count with and without the inhibitor (**Figure 4.2C; lower graph**). These results indicate that there is a healthy satellite cell expression pattern



throughout proliferation and differentiation; however a TSP-1 KO model produces overall more total satellite cells versus WT, independent of a VEGF inhibitor.

Figure 4.2 TSP-1 KO myofibres have a larger satellite cell progeny compared to WT independent of treatment with human platelet releasate (**A**) A schematic depicting the methodology behind the experimental setup. (**B**) Representative images and quantitative data for the expression profiles of satellite cells cultured on myofibres for 48 hours (proliferation). Expression for quiescence ($Pax7^{+ve}/MyoD^{-ve}$; white arrow heads), proliferation ($Pax7^{+ve}/MyoD^{+ve}$; striped arrow heads) and commitment to differentiation ($Pax7^{-ve}/MyoD^{+ve}$; open arrow heads) was assessed for T48 hours (x40 magnification, scale bar 20µm). A VEGF receptor- specific inhibitor (1.3µM VEGFR Inhibitor; AAL-993) was used to further differentiate the potential effects of TSP-1 KO on skeletal muscle satellite cell proliferation, however no significant difference was detected versus WT. Human platelet releasate caused an increase in the percentage expression of $Pax7^{-ve}/MyoD^{+ve}$ cells. (**C**) T72 single fibre culture for differentiation was analysed on TSP-1 KO and wild-type satellite cells when cultured with or without 10% human releasate as depicted in the schematic. Satellite cell expression profiles on single fibres for quiescence ($Pax7^{+ve}/Myogenin^{-ve}$) and commitment to differentiation ($Pax7^{-ve}/Myogenin^{+ve}$) was assessed (top graph) with total satellite cell number per fibre (bottom graph) for 10% FBS fibre medium (FM), 10% human releasate and AAL-993 for both TSP-1 KO and WT. Representative images (x40 magnification, scale bar 20µm). Statistical analysis was performed by two-way ANOVA followed by Tukey's post-hoc test. n=3 independent experiments. Differences are *p<0.05, **p<0.01, ***p<0.001, $p<0.05 versus total satellite cells number per fibre for FM and releasate groups, #p<0.05 versus the FM group $MyoD^{+ve}$ percentage and ¥p<0.05 versus FM group for $Pax7+^{ve/}MyoD^{+ve}$ percentage co-expression.

4.4.3 Isolated TSP-1 KO satellite cells proliferate normally compared to WT cells, but have impaired differentiation that can be rescued by the addition of human platelet releasate

Next, primary satellite cell proliferation potential in culture was investigated, eliminating the muscle-fibre to satellite cell interaction (i.e. their micro-environmental niche) as a potential source of variability in satellite cell proliferation. Interestingly, satellite cells derived from the EDL muscle shows no additional proliferative response in serum-free, or growth medium conditions with or without platelet releasate. The surprising effect was confirmed by repeating the experiment on the biceps brachii with the same non-significance (**Figure 4.3A-B**). Additionally, there was no significant difference on these cells cultured under different proliferative conditions, i.e. releasate made using physiological levels of releasate ($2.5x10^8$ platelets/mL) or supra-physiological levels of

platelet releasate ($10x10^8$ platelets/mL) (**Figure 4.3C**). Primary satellite cell differentiation potential in culture was next investigated after no effects were observed on proliferation. Interestingly, satellite cells derived from the EDL muscle of TSP-1 KO muscle shows a diminished differentiative phenotype additional proliferative response in growth medium conditions and is rescued with the addition of platelet releasate. This effect was confirmed by repeating the experiment on the biceps brachii with the same significance (**Figure 4.3D**).

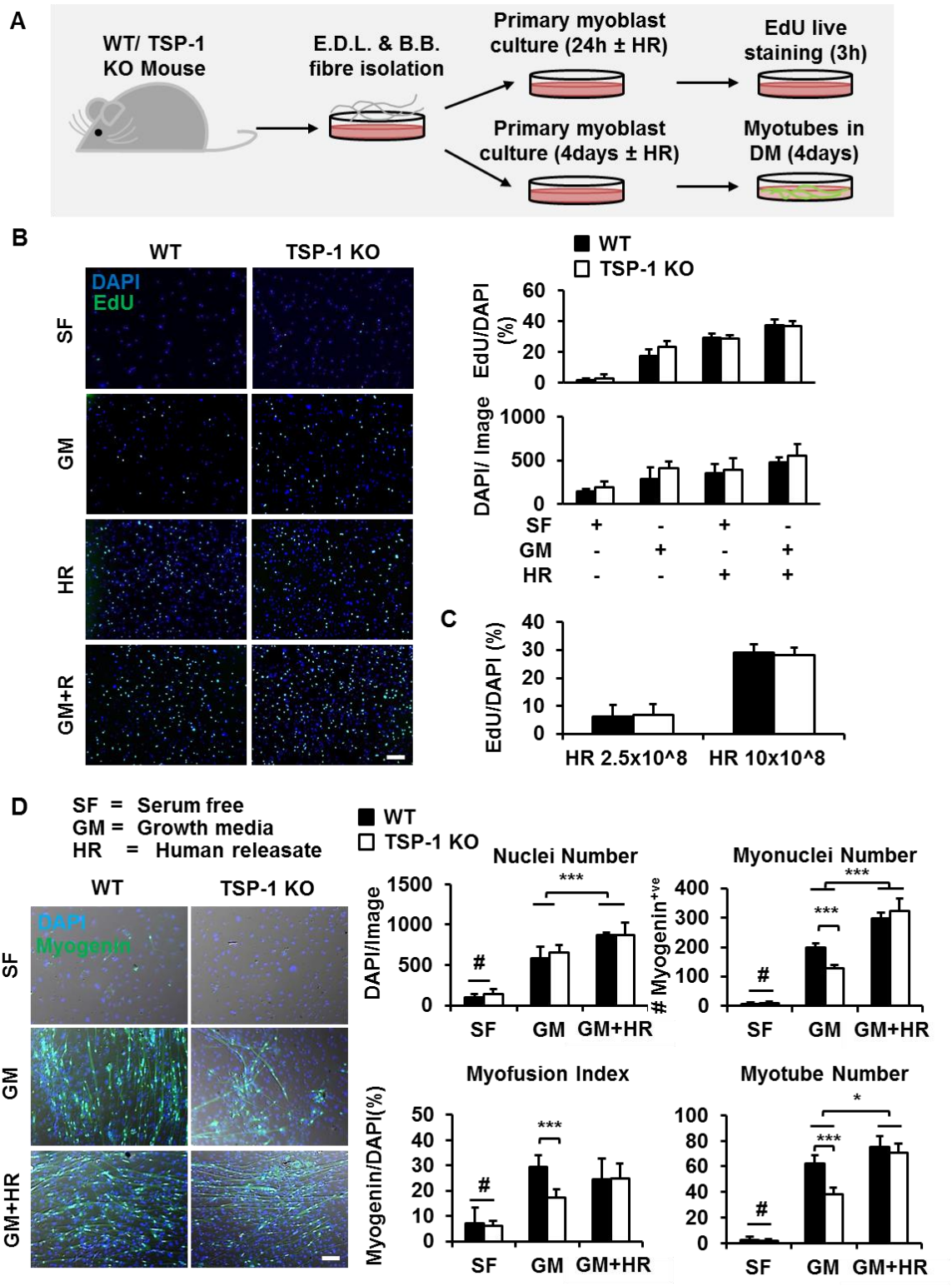

A
WT/ TSP-1 KO Mouse
E.D.L. & B.B. fibre isolation
Primary myoblast culture (24h ± HR)
EdU live staining (3h)
Primary myoblast culture (4days ± HR)
Myotubes in DM (4days)
B
WT
TSP-1 KO
DAPI
EdU
SF
GM
HR
GM+R
EdU/DAPI (%)
DAPI/ Image
SF + - + -
GM - + - +
HR - - + +
C
EdU/DAPI (%)
HR 2.5x10^8
HR 10x10^8
D
SF = Serum free
GM = Growth media
HR = Human releasate
DAPI
Myogenin
Nuclei Number
Myonuclei Number
DAPI/Image
Myogenin+ve
Myofusion Index
Myotube Number
Myogenin/DAPI(%)
SF
GM
GM+HR

Figure 4.3

Figure 4.3 Primary TSP-1 KO satellite cells show normal proliferation, but impaired differentiation that can be rescued by the addition of human platelet releasate (**A**) A schematic depicting the methodology behind the experimental setup. EdU proliferative live-staining was conducted for 3-hours after 21 hour incubation totalling 24-hours of proliferation in serum-free (SF), growth medium (GM; 10% FBS), 10% human releasate (HR) in serum-free conditions, or GM+HR. (**B**) Representative images and quantitative data for EdU staining with DAPI co-staining (x5 magnification, scale bar 200 μm). Human releasate was made with $2.5x10^8$ platelets/mL. (**C**) Normal proliferation was observed for both physiological levels of platelets ($2.5x10^8$ platelets/mL) and supra-physiological levels ($10x10^8$ platelets/mL) for making platelet releasate when comparing WT to TSP1 KO primary satellite cells. (**D**) SF, GM and GM+HR conditions were next used for differentiation of stripped primary satellite cells during the proliferation phase (4 days). Myogenin levels were measured after 7 days in differentiation medium (5% horse serum). (x10 magnification, scale bar 100μm) for extensor digitorum longus (EDL) muscles. Outcome measures include the nuclei number, number of Myogenin^{+ve} nuclei, myotubes (n=2 nuclei/ myotube) and myotube fusion index; calculated by Myogenin^{+ve} nuclei in myotubes/ DAPI as a percentage. Data are mean±SD (n=4 independent experiments with 9 technical replicates per experiment). Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #P<0.05 vs. every other group.

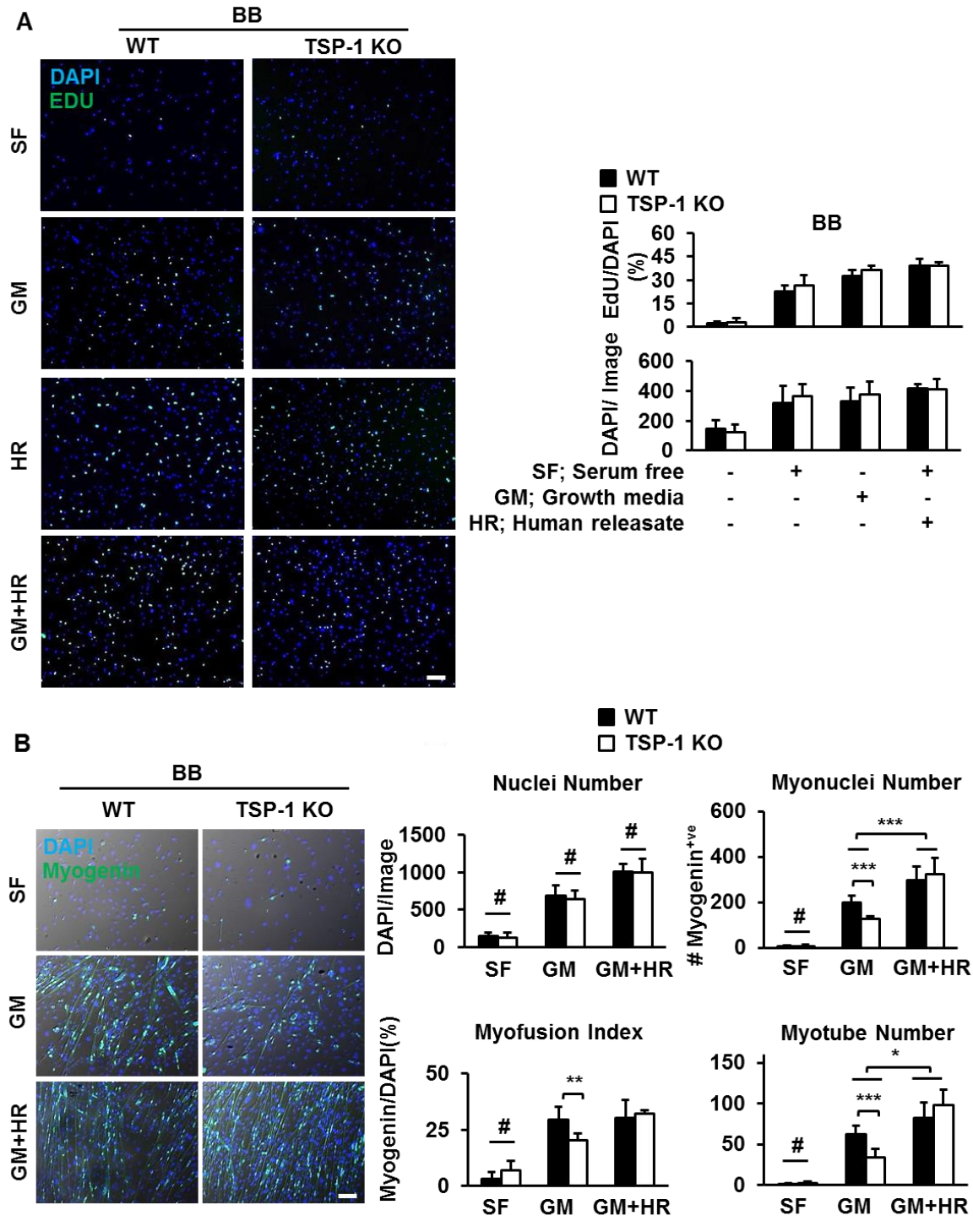


Figure 4.4

Figure 4.4 Primary TSP-1 KO satellite cells show normal proliferation, but impaired differentiation that can be rescued by the addition of human platelet releasate (**A**) As in **Figure 4.3**; stripped satellite cells were assessed for proliferation with EdU live-staining was conducted for 3-hours after 21 hour incubation totalling 24-

hours of proliferation in serum-free (SF), growth medium (GM; 10% FBS), 10% human releasate (HR) in serum-free conditions, or GM+HR. Representative images and quantitative data for EdU staining with DAPI co-staining (x5 magnification, scale bar 200 µm). (**B**) SF, GM and GM+HR conditions were next used for differentiation of stripped primary satellite cells during the proliferation phase (4 days). Myogenin levels were measured after 7 days in differentiation medium (5% horse serum). (x10 magnification, scale bar 100µm) for biceps brachii (BB) muscles. Outcome measures include the nuclei number, number of Myogenin^{+ve} nuclei, myotubes (n=2 nuclei/ myotube) and myotube fusion index; Myogenin^{+ve} nuclei in myotubes/ DAPI as a percentage. Data are mean±SD (n=4 independent experiments with 9 technical replicates per experiment). Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #P<0.05 vs. every other group.

4.4.4 TSP-1 KO platelet releasate causes TSP-1 KO satellite cells to proliferate faster than wild-type satellite cells

After establishing that TSP-1 KO murine platelet releasate had a strong proliferative effect on C2C12 cells, and that TSP-1 KO satellite cells have a better proliferative phenotype versus WT cells, the next aim was to apply TSP-1 KO platelet releasate (TR) and WT platelet releasate (WR) on both TSP-1 KO and WT satellite cells. TR and WR were applied to TSP-1 KO and WT myofibres for T48 and T72 to gauge their impact on proliferation (**Figure 4.5A**) and differentiation (**Figure 4.5B**) phases, respectively. There was no significant difference at T48 between WT and TSP-1 KO for expression patterns, however as expected; MyoD was increased dramatically in all WR and TR groups (**Figure 4.5A**). Additionally, there was a significant genotype difference between WT and TSP-1 KO for total satellite cell numbers at T48 with the application of TR on TSP-1 satellite cells yielding the highest mean cell count. Furthermore, it was found that a normal expression pattern for quiescence (Pax7^{+ve}/Myogenin^{-ve}) and differentiating satellite cells (Pax7^{-ve}/Myogenin^{+ve}) were present in all groups. The trend was the same for T72 however, TSP-1 KO treated with TR had the highest cells per fibre (**Figure 4.5B**). Interestingly, this suggests that when in contact with the fibre, satellite cells can proliferate to greater numbers than wild-type satellite cells. Additionally, TSP-1 KO platelet releasate has a more potent effect on the cells giving further proliferation and differentiation overall, with a normal expression pattern.

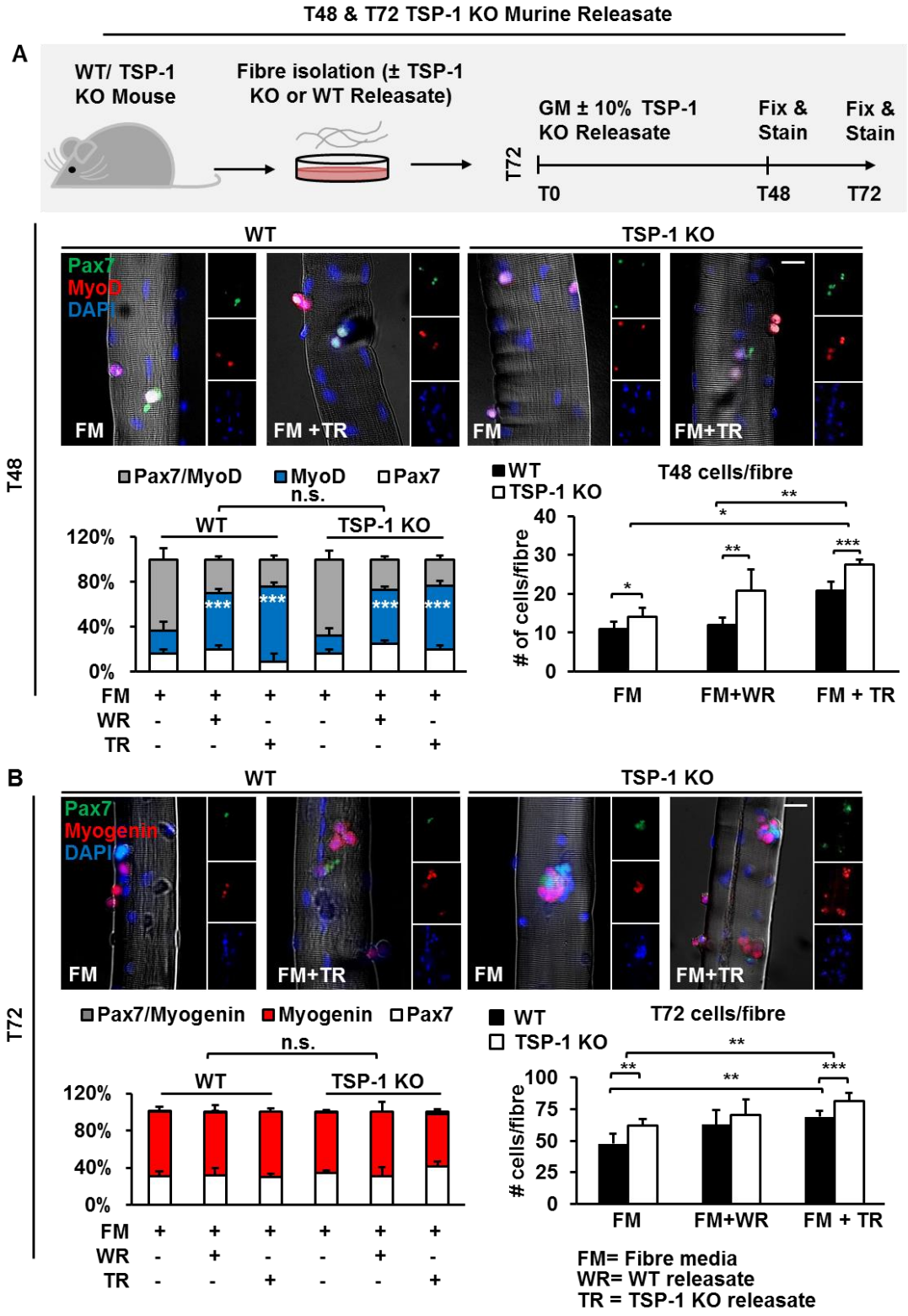


Figure 4.5 TSP-1 KO platelet releasate causes TSP-1 KO satellite cells to proliferate faster than wild-type satellite cells (**A-B**) A schematic depicting the experimental set up. T48 and T72 hours in culture for proliferation and differentiation, respectively, was assessed on TSP-1 KO and WT single fibres when cultured in fibre

medium (FM) with or without 10% platelet releasate from the whole blood of WT (WR) or TSP-1 KO mice (TR). Expression for quiescence ($Pax7^{+ve}/MyoD^{-ve}$), proliferation ($Pax7^{+ve}/MyoD^{+ve}$) and commitment to differentiation ($Pax7^{-ve}/MyoD^{+ve}$) was assessed for T48 hours (**A**). Satellite cell expression profiles on single fibres for quiescence ($Pax7^{+ve}/Myogenin^{-ve}$) and commitment to differentiation ($Pax7^{-ve}/Myogenin^{+ve}$) was assessed for T72 (**B**). Graphs on the right-hand side represent the average total number of satellite cells per fibre for each condition at T48 and T72. Representative images (x40 magnification, scale bar 20μm). Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test. n=3-5 animals per group. Differences are *p<0.05, **p<0.01, ***p<0.001 versus the same phenotype without TR and #p<0.05 versus every other group.

4.4.5 Human platelet releasate promotes faster regeneration and shows less necrosis in a TSP-1 KO muscle-injury model.

Having shown that TSP-1 KO satellite cells have a higher progeny after 48 and 72 hours in culture and that platelet releasate was able to rescue the differentiation deficit; these results were expanded for an *in vivo* cardiotoxin-induced murine tibialis anterior injury. Human platelet releasate was applied to WT and TSP-1 KO injuries and the effect was analysed after day 5 and day 10 of injury. On day 5, cardiotoxin injury induced a significant muscle mass loss in WT +/- releasate and TSP-1 KO without releasate. However, the TSP-1 KO with releasate was the only group that had regained its weight comparted to non-injury contralateral TA muscle (**Figure 4.6A-B**). On day 10, TA muscle weights were normalised for both TSP-1 KO with and without releasate, as opposed to WT groups. It was found that there was no genotypic effect on the size of regenerating fibres as categorised *via* embryonic myosin heavy chain expression on day 5; however there was a profound increase in fibre size for both TSP-1 KO and WT when treated with platelet releasate. On day 10, the cross-sectional area was greatly increased in all groups versus the WT group without releasate (**Figure 4.6C**). Following from this, the number of IgG-infiltrated fibres was analysed, which indicate necrotic fibres, on day 5 and 10. On day 5, there was a significant reduction of cardiotoxin-induced necrosis with both TSP-1 KO groups compared to WT and with treatment of platelet releasate. However, on day 10, there was very little necrosis, with only a reduction in the TSP-1 KO treated with releasate versus the WT without releasate (**Figure 4.6D**). Representative images were taken *via* haematoxylin and eosin staining

(H&E) to determine equal injured areas were analysed among groups (**Figure 4.7**). Interestingly, both TSP-1 KO and platelet releasate contribute to a larger $Pax7^{+ve}$ pool of satellite cells after cardiotoxin injury in the uninjured contralateral EDL muscle. This novel data suggests that both TSP-1 KO and platelet releasate may have additive effects when undergoing skeletal muscle adaptations after injury.

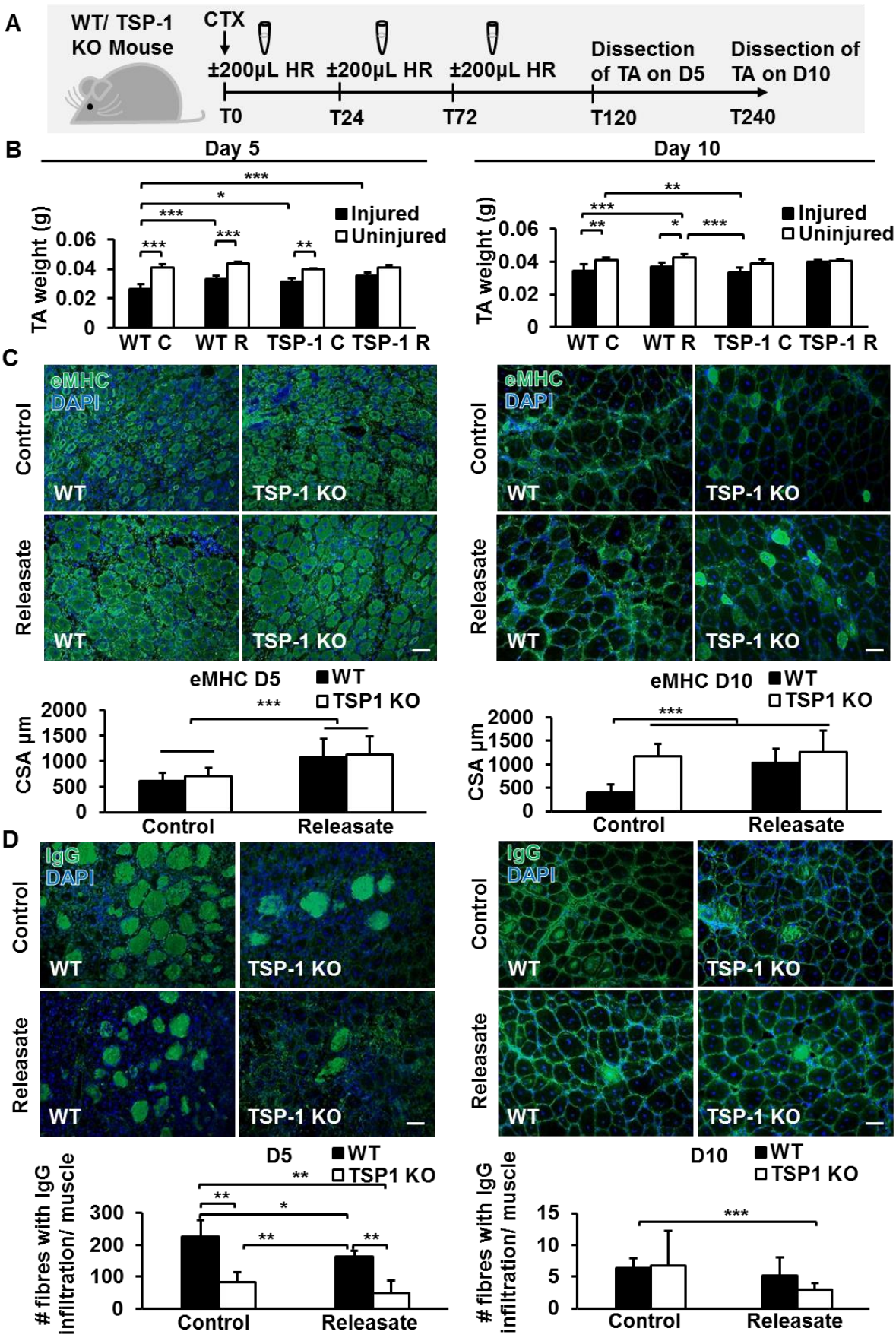

A
WT/ TSP-1 KO Mouse
CTX
±200µL HR
±200µL HR
±200µL HR
Dissection of TA on D5
Dissection of TA on D10
T0
T24
T72
T120
T240
B
Day 5
Day 10
TA weight (g)
Injured
Uninjured
WT C
WT R
TSP-1 C
TSP-1 R
C
Control
Releasate
eMHC
DAPI
WT
TSP-1 KO
eMHC D5
eMHC D10
CSA µm
TSP1 KO
D
IgG
DAPI
D5
D10
fibres with IgG infiltration/ muscle

Figure 4.6 Human platelet releasate promotes faster regeneration and shows less necrosis in a TSP-1 KO muscle-injury model. (**A**) A schematic depicting the experimental set up. Cardiotoxin (CTX) was injected with human platelet releasate (R) or without R (control) at time-point 0 (T0), followed by two follow up injections of R at T24 and T72. (**B**) Injured and uninjured tibialis anterior muscles were then collected and weighed before snap freezing. (**C**) eMHC staining of cryo-sections from cardiotoxin-induced tibialis anterior injury that was treated with or without 3 doses of human platelet releasate (R) and collected on D5 or D10. (**D**) IgG infiltration into necrotic fibres on tibialis anterior sections for D5 and D10. Representative images (x20 magnification, scale bar 50μm). Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test. n=3-5 animals per group. Differences are *p<0.05, **p<0.01, ***p<0.001.

4.4.6 TSP-1 deficiency and systemic administration of platelet releasate independently induce skeletal muscle angiogenesis.

TSP-1 is a negative regulator of excessive capillarisation, and platelet-rich plasma has been shown to increase capillarisation in an *in vivo* injury model (Isenberg et al., 2007a, Li et al., 2016b). For both these reasons, CTX-injured TA muscle sections were stained with CD31 (i.e. PECAM1; Platelet endothelial cell adhesion molecule) in order to determine the rate of angiogenesis in TSP-1 KO with or without platelet releasate as compared to WT. It was found that there were significantly more CD31-positive capillaries in the injured TA on day 5 for both the TSP-1 KO with and without platelet releasate treatment. Additionally, wild-type mice were shown to have an increased angiogenic response to releasate treatment 5-days after injury (**Figure 4.8A**). When the extent of angiogenesis 10 days after injury was assessed, the capillary density per unit area increased between the genotypes and WT without HR having significantly less capillarisation than both TSP-1 KO groups. Furthermore, CD68 staining for macrophage infiltration into the muscle showed a clear significant loss of macrophages on day 5 for both genotype and treatment with releasate. On day 10 after injury the loss of macrophages had increased dramatically for the TSP-1 KO groups as compared to the WT (**Figure 4.8B**).

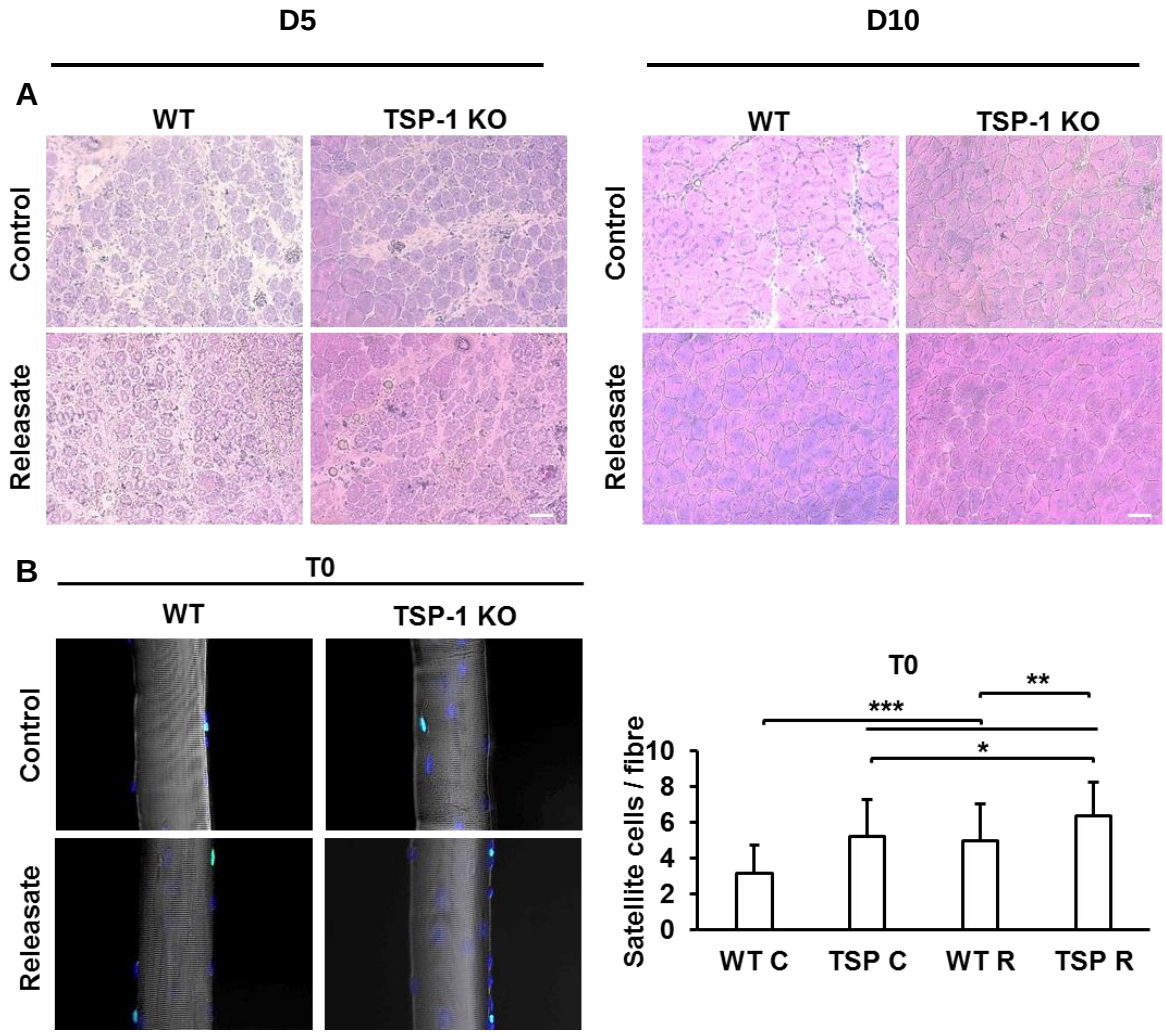


Figure 4.7 Both TSP-1 KO and platelet releasate contribute to a larger Pax7^{+ve} pool of satellite cells after injury. Cardiotoxin (CTX) was injected with human platelet releasate (R) or without R (control) at time-point 0, followed by two follow up injections of R at T24 and T72. (**A**) Haematoxylin and eosin staining was conducted on injured D5 and D10 TA muscle to determine correct area of injury is being examined. This was determined through observational centrally located nuclei in the muscle fibres. (**B**) Representative images and quantitative data for the number of satellite cells on single muscle fibres that are stained with Pax7 and DAPI of the uninjured EDL muscle. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. n=3-5 animals per group. Differences are *p<0.05, **p<0.01 and ***p<0.001.

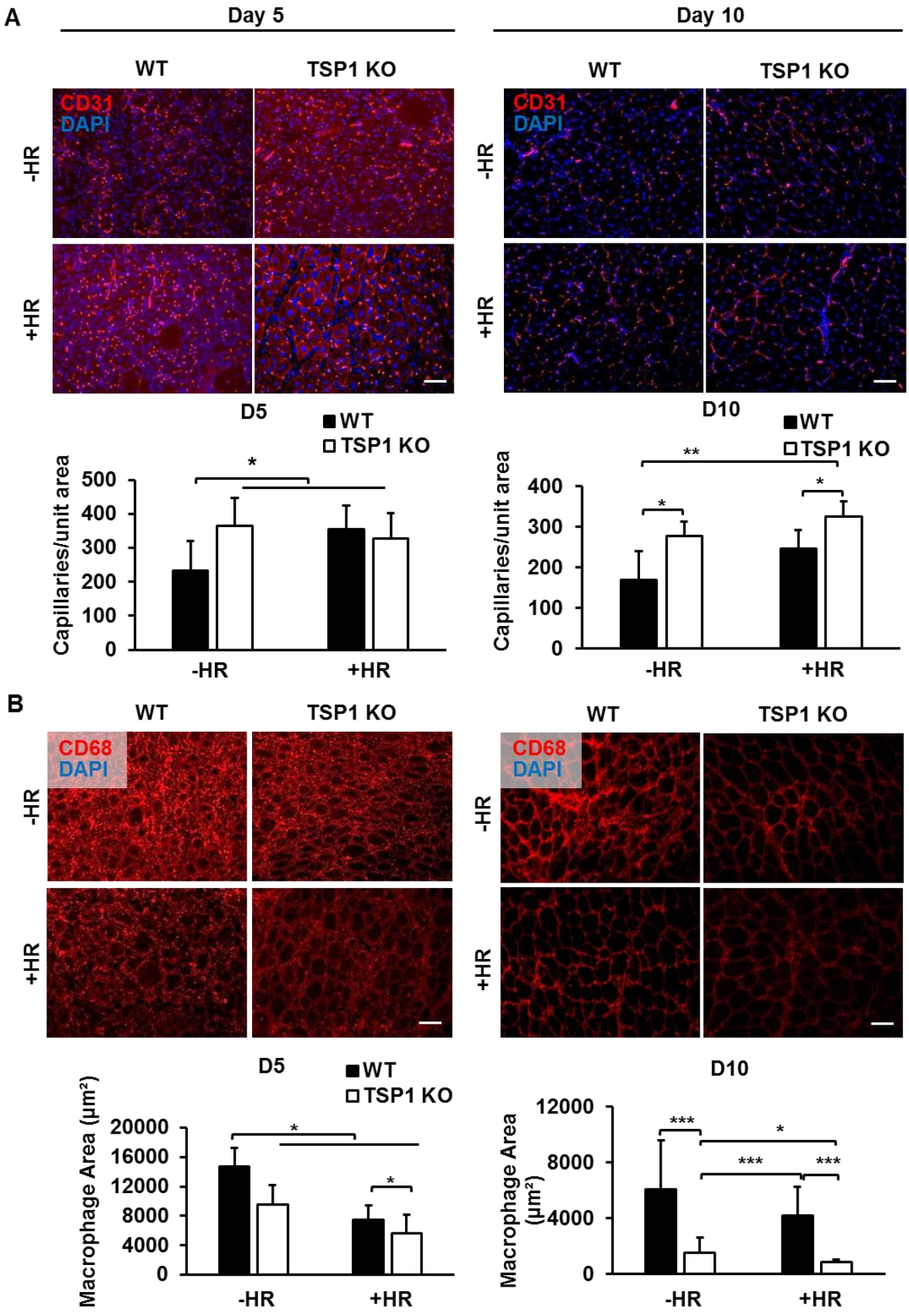

A
Day 5
Day 10
WT
TSP1 KO
CD31
DAPI
-HR
+HR
D5
D10
WT
TSP1 KO
Capillaries/unit area
500
400
300
200
100
0
*
**
-HR
+HR
B
CD68
DAPI
Macrophage Area (µm²)
20000
16000
12000
8000
4000
0
12000
8000
4000
0
***

Figure 4.8

Figure 4.8 Platelet releasate and TSP-1 deficiency independently affect capillarisation and macrophage infiltration in injured muscle. Cardiotoxin (CTX) was injected into tibialis anterior muscles of WT and TSP-1 KO mice, with human platelet releasate (HR) or without HR (control) at time-point 0 (T0), followed by two follow up injections of HR at T24 and T72. (**A**) Representative images and quantitative data for CD31 and (**B**) CD68, immunohistochemical staining, conducted on the injured tibialis anterior muscles, co-stained with DAPI to visualise nuclei on day 5 and day 10 after injury. Representative images (x20 magnification, scale bar 50μm). Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test. n=3-5 animals per group. Differences are *p<0.05, are **p<0.01, ***p<0.001

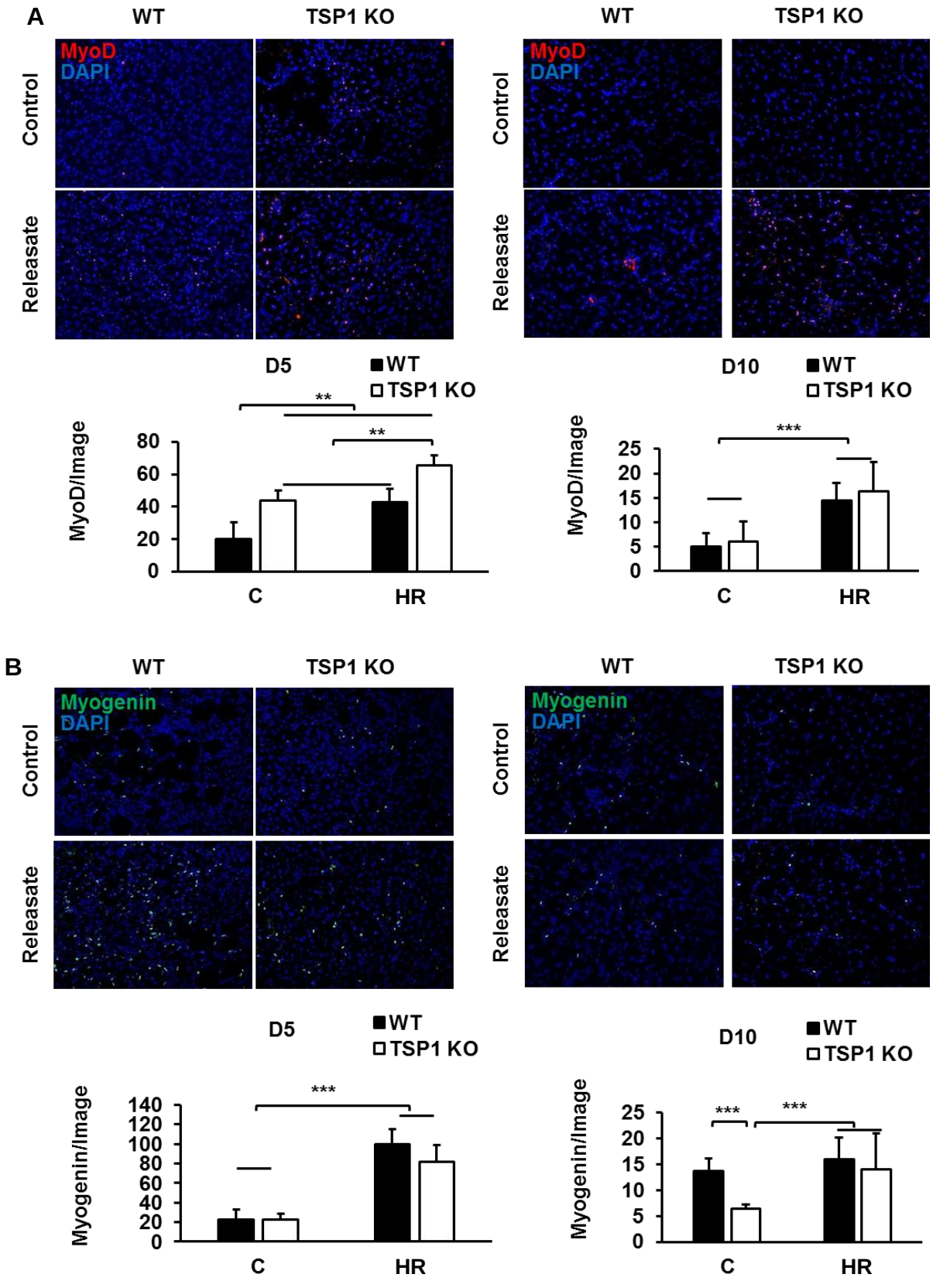

A
WT
TSP1 KO
MyoD
DAPI
Control
Releasate
D5
WT
TSP1 KO
MyoD/Image
**
**
80
60
40
20
0
C
HR
D10
***
25
20
15
10
5
B
Myogenin
Myogenin/Image
140
120
100

Figure 4.9 MyoD^{+ve} satellite cell numbers are increased with both platelet releasate and TSP-1 KO, with a loss of Myogenin on day 10; rescued with platelet releasate. Cardiotoxin (CTX) was injected into tibialis anterior muscles of WT and TSP-1 KO mice, with human platelet releasate (HR) or without HR (control) at time-point 0 (T0), followed by two follow up injections of HR at T24 and T72. (**A**) Representative images and quantitative data for MyoD immunohistochemical staining, conducted on the injured tibialis anterior muscles, co-stained with DAPI to visualise nuclei. (**B**) Representative images and quantitative data for Myogenin immunohistochemical staining, conducted on the injured tibialis anterior muscles, co-stained with DAPI to visualise nuclei. Representative images (x20 magnification, scale bar 50μm). Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test. n=3-5 animals per group. Differences are **p<0.01, ***p<0.001.

4.4.7 The effect of TSP-1 deficiency and platelet releasate on MyoD and Myogenin expression in progenitor cells of injured muscle.

Having shown that MyoD and Myogenin expression have normal expression in TSP-1 KO satellite cells on *ex vivo* myofibres as compared to wild-type, but results in higher satellite cell numbers; we wanted to next measure MyoD and Myogenin expression on day 5 and day 10 after injury in the TA muscle. TSP-1 KO muscle had significantly higher numbers of MyoD^{+ve} cells compared to WT muscle on day 5. Most importantly, administration of platelet releasate increased MyoD expression in both genotypes on day 5 that was also evident on day 10 (**Figure 4.9A**). Furthermore, we did not find any genotype difference on Myogenin expression on day 5 independent of releasate administration. However, platelet releasate resulted in a robust increase of Myogenin^{+ve} cells (**Figure 4.9B**). Interestingly, in line with the primary satellite cell data, there was a significant ($p<0.001$) decrease in Myogenin^{+ve} cells in the TSP-1 KO injured TA muscle that was rescued by platelet releasate for both the extensor digitorum longus muscle and the biceps brachii muscle (**Figure 4.3 and Figure 4.4**).

4.4.8 TSP-1 KO mice have normal fibre type distribution and size

The phenotypes of TSP-1 KO murine skeletal muscle were next assessed. Previous studies have shown increased angiogenesis in heart and skeletal muscles and enhanced physical performance in reduced TSP-1 models (Audet et al., 2013, Frazier et al., 2011). Here, no difference on skeletal muscle weight was found between the TSP-1 KO and wild-type mice (WT) for extensor digitorum longus (EDL), tibialis anterior (TA), gastrocnemius soleus and biceps brachii (BB) (**Figure 4.10**). In addition, there was found to be no differential fibre type distribution between the wild-type and the TSP-1 KO for both the EDL and the superficial tibialis anterior (TA) skeletal muscles (**Figure 4.10B-11**). However, a significant increase was observed for the TSP-1 KO soleus type IIX muscle fibres, therefore an additional staining was conducted for the type IIB where no significance was found, confirming a type IIX significant difference. The EDL and soleus represent fast twitch glycolytic and fast twitch oxidative, respectively. Interestingly, the deep TA showed a decrease in type IIX fibres for the TSP-1 KO (**Figure 10B-11**). Moreover, there was no difference in cross-sectional area (CSA) between WT and TSP-1 KO muscle fibre areas when compared per fibre type (I, IIA and IIB) (**Figure 4.11C**).

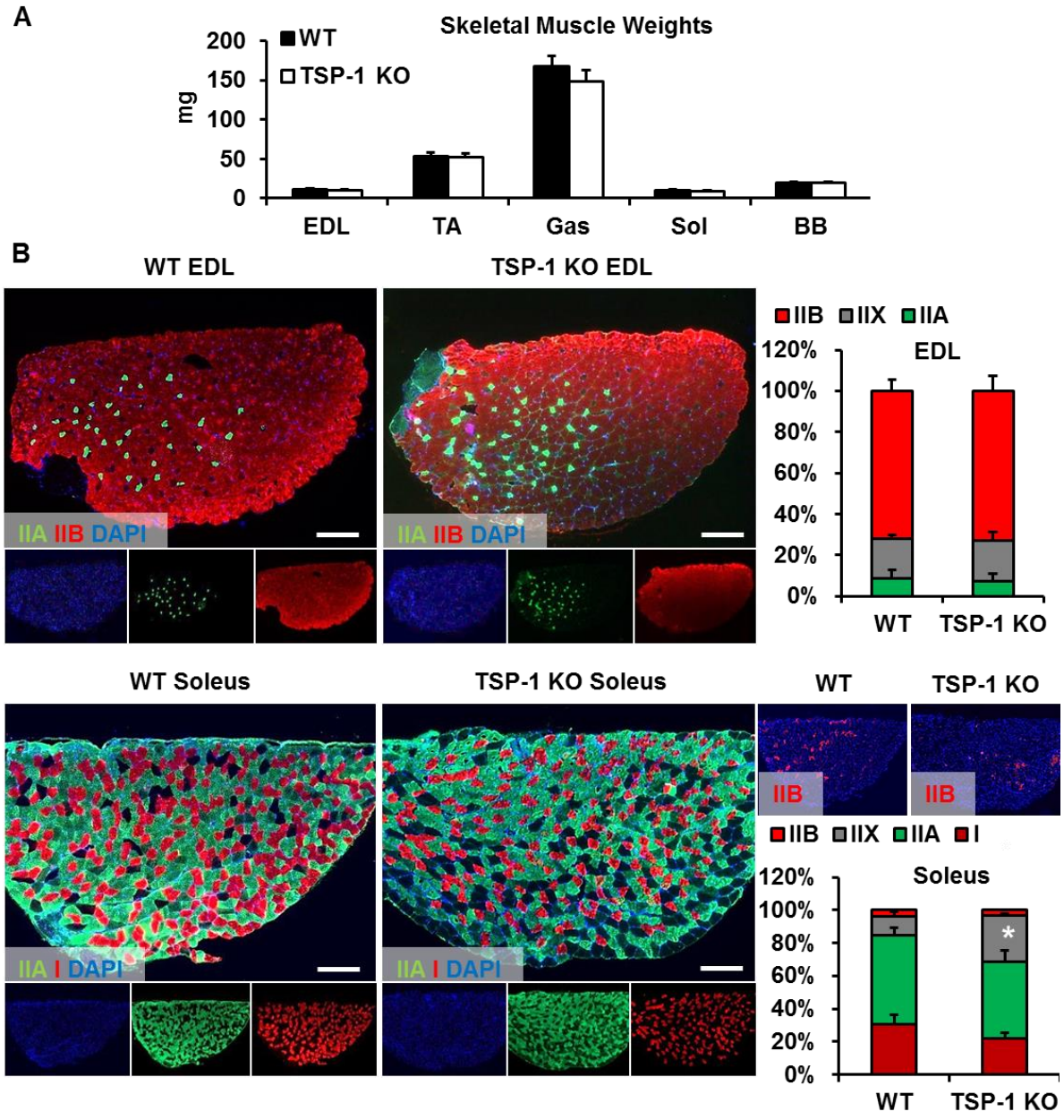


Figure 4.10 TSP-1 KO mice have normal fibre type distribution and size (**A**) Skeletal muscle weights of extensor digitorum longus (EDL), tibialis anterior (TA), gastrocnemius (Gas), soleus (Sol) and biceps brachii (BB). (**B**) Wild-type and TSP-1 KO cryo-section staining for MHC type I, IIA and IIB and DAPI in EDL and Soleus (x5 magnification, scale bar 200 μm). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. n=5 animals per group, 12 technical replicates. Differences are *p<0.05 vs. the type IIX group.

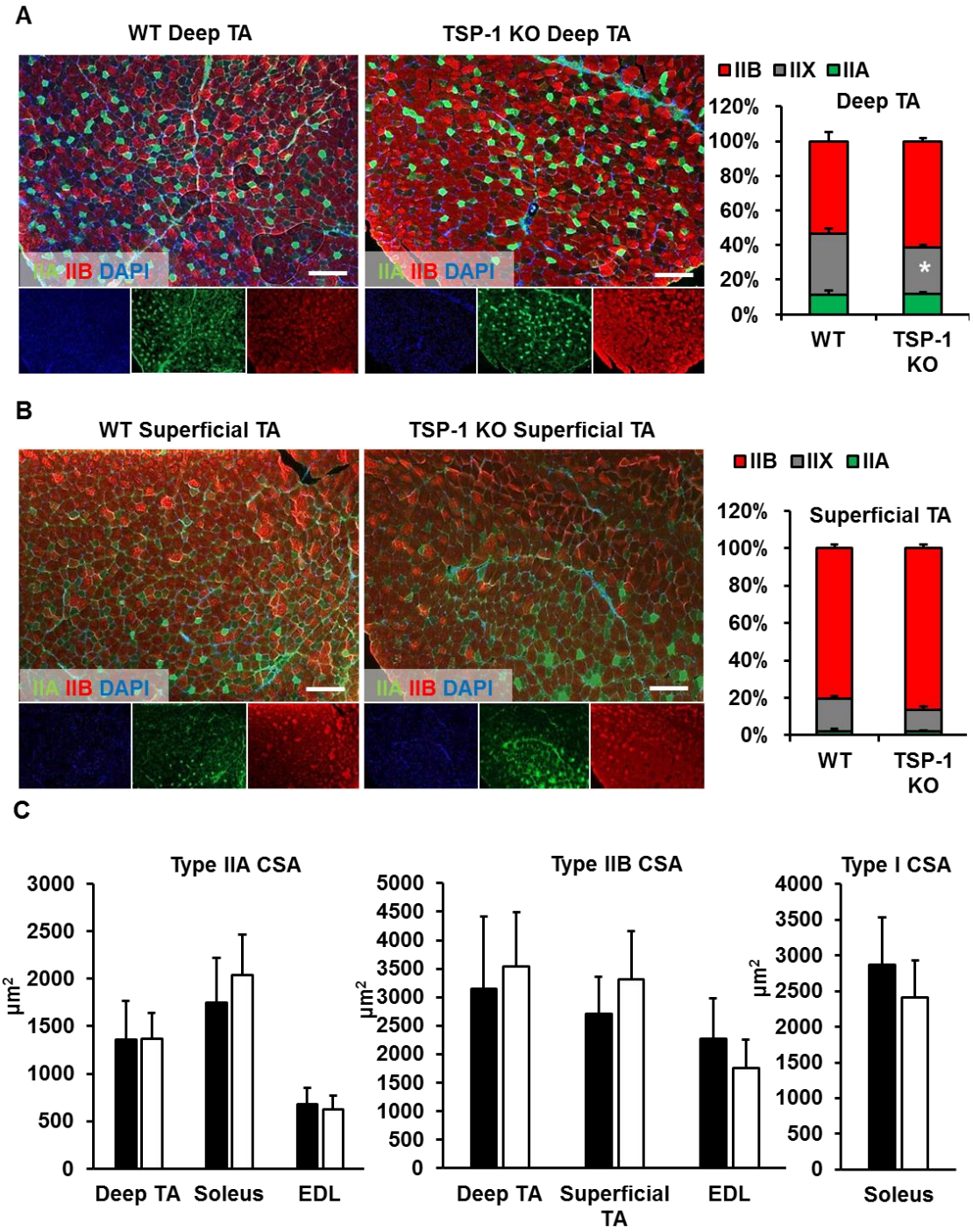


Figure 4.11 TSP-1 KO mice have normal fibre type distribution and size (**A-B**) Wild-type and TSP-1 KO cryo-section staining for MHC type I, IIA and IIB and DAPI in Deep Tibialis Anterior (TA) and Superficial TA sections (x5 magnification, scale bar 200 µm). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. (**C**) Cross-sectional area (CSA) was measured per fibre type (I, IIA and IIB) indicating mean fibre area; on Zen Software. n=5 animals per group, 12 technical replicates. Differences are *p<0.05 vs the type IIX group.

4.4.9 Platelet releasate alters gene expression and capillarisation of non-injured skeletal muscle.

Based on **Figure 4.7B** showing a baseline satellite cell difference between WT mice with or without human releasate, on uninjured skeletal muscle, it was important to explore this finding further. The only research paper experimentally exploring platelet-based applications on uninjured skeletal muscle was Fukuda et al. 2017 showing that an injection of PRP into equine skeletal muscle increases HGF and MHC I after 5 days (Fukuda et al., 2017). We found an increase ($p<0.05$) in number of CD31 positive capillaries in the tibialis anterior muscle after both day 5 and day 10 of platelet releasate injections (**Figure 4.12A**). In addition to this, a Chi-Square test was implemented from **Figure 4.7B** after plotting the frequency distribution of satellite cells per uninjured tibialis anterior muscle fibres showing a significant ($p<0.001$) difference between WT mice treated with platelet releasate versus no platelet releasate (**Figure 4.12B**). It was next important to verify the data from Fukuda et al. on an increased gene expression of in skeletal muscle treated with platelet secretomes. There was an increase in *Hgf* as expected ($p<0.001$). Furthermore, the angiogenesis factor; *Vegfα165*, was increased 10 days after initial platelet releasate injection. There was a significant decrease in *Mhc* IIB and IIX on day 5 but not on day 10. In addition, there was a loss of *Myostatin* gene expression and an increase in *Sirt1* (**Figure 4.12C**). Finally, there was a significant increase in $VEGF^{+ve}$ nuclei in cryo-sections of the tibialis anterior muscles of mice treated with platelet releasate over non-treated (**Figure 4.12D**).

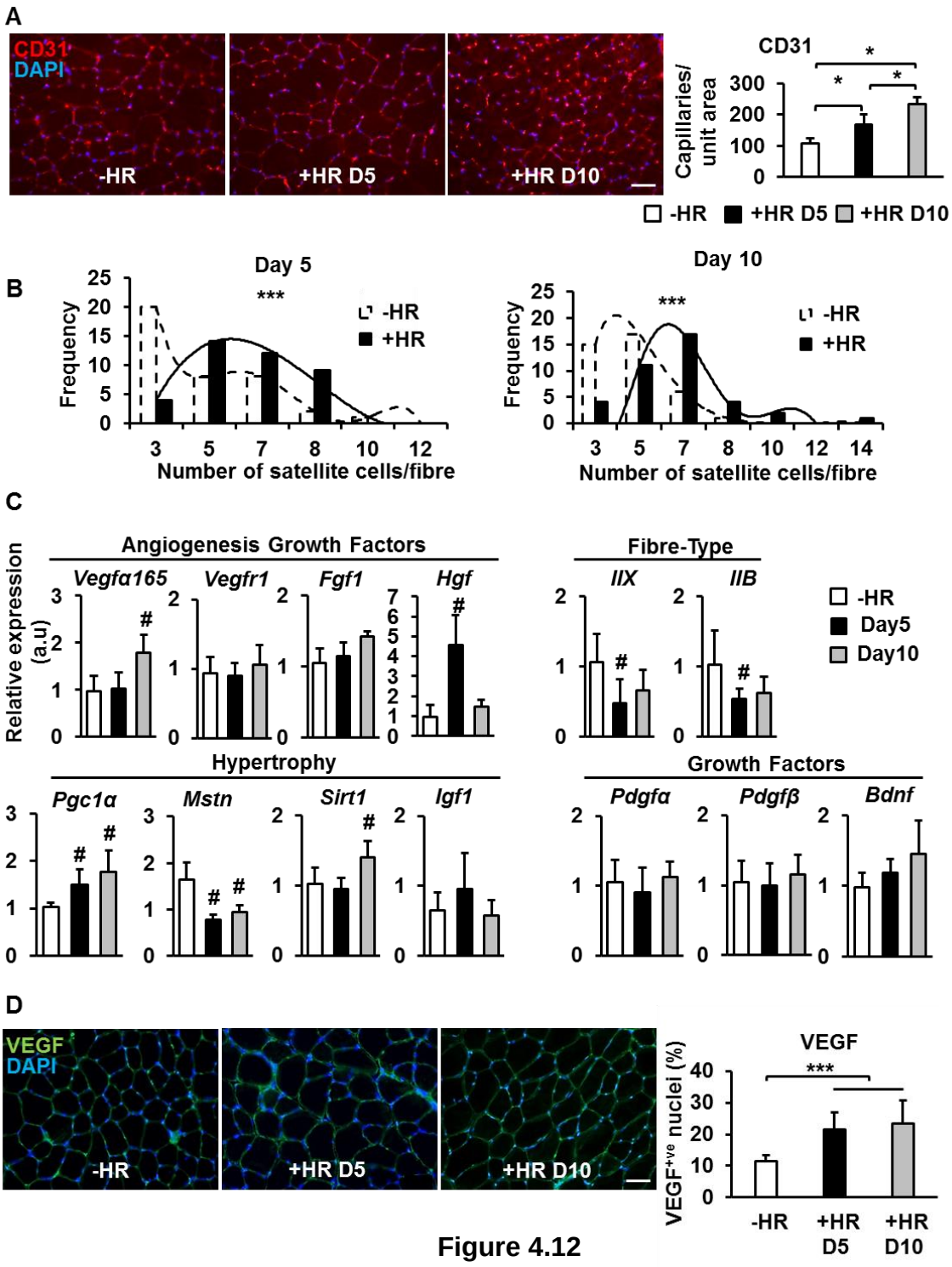


Figure 4.12

Figure 4.12 Platelet releasate alters gene expression and capillarisation of non-injured skeletal muscle (**A**) Wild-type mice treated intraperitoneally with platelet releasate (200µL at 0hours, 24hours and 72hours) Tibialis Anterior cryo-section staining for CD31 and DAPI (x20 magnification). WT with (+) or without (-) human platelet releasate (HR). Representative images and quantitative data for both day 5

and day 10 muscle sections. (**B**) Frequency distribution of single fibre satellite cells *ex vivo* stained Pax7^{+ve} grouped into separate bins for +/-HR. (**C**) Gene expression for WT +/-HR treatment with collection on day 5 (black bars) and day 10 (grey bars) for the relative expression of angiogenesis growth factors (*Vegfα165, Vegfr1, Fgf1, Hgf*), fibre-type myosin heavy chains (*Mhc iix, Mhc iib*), hypertrophy (*Pgc1α, Mstn, Sirt1, Igf1*) and growth factors (*Pdgfα, Pdgfβ, Bdnf*). (**D**) Immunohistochemical staining for VEGF and DAPI on cryo-sections of the tibialis anterior muscle +/- HR on day 5 and day 10. (x20 magnification; scale bar 50μm). Statistical analysis was performed by Student's *t*-test for **A** and **D**, Chi-square for **B** and a one-way ANOVA followed by Tukey's post-hoc test for **C**. Differences are *p<0.05, **p<0.01, ***p<0.001.

4.5 Discussion

TSP-1 is a negative regulator of angiogenesis in the heart and skeletal muscles, where its deletion has been shown to enhance exercise capacity and reduce chronic ROS production (Malek and Olfert, 2009, Audet et al., 2013). TSP-1 expression increases with age and can lead to complications such as cardiovascular disease and thrombosis as a process of ageing (Cini et al., 2015). Since TSP-1 is expressed by skeletal muscle and is a constituent of TRAP-6-activated platelet releasate, the effect of TSP-1 KO mouse platelet releasate on myoblast proliferation *in vitro*, *ex vivo* satellite cell progeny rate and fibre type composition and size, as compared to wild-type were assessed.

4.5.1 Identifying potential factors to inhibit in platelets

Customisation of platelet releasate has been used previously (Li et al., 2016b, Miroshnychenko et al., 2017). The data in this thesis suggests a potential benefit to removing TSP-1 from platelet releasate when applying to skeletal muscle. The beneficial effect of TSP-1 KO releasate on myoblast proliferation may be, in part, due to the fact that TSP-1 binds to PDGF and may alter its effectiveness of stimulating myoblast proliferation (Hogg et al., 1997). It has been shown recently that PDGF is a crucial growth factor in platelet-rich plasma for activating and maintaining skeletal muscle satellite cell proliferation (Sugg et al., 2017, Cirri et al., 2005, Jin et al., 1991, Li et al., 2013, Dimauro et al., 2014). Additionally, TSP-1 has been shown to negatively affect phosphorylation of Akt in C2C12 myoblasts potentially leading to impaired cellular function (Matsugi et al., 2016). Since TSP-1 is an angiogenic inhibitor, it interacts negatively with vascular endothelial growth factor (VEGF); one of the main angiogenic factors contained in platelet releasate (Malek and Olfert, 2009). Furthermore, TSP-1 has been shown to inhibit VEGF signalling by targeting VEGF receptor 2 in endothelial cells (Chu et al., 2013). Both PDGF and VEGF are essential components of platelet releasate for myoblast proliferation and differentiation (Li et al., 2013) Therefore, it is not surprising that TSP-1 KO platelet releasate shows an increase in myoblast proliferation significantly over wild-type releasate. FRP-1 and FPR-2 both regulate platelet aggregation and chemo-attraction (Vital et al., 2016,

Czapiga et al., 2005). Previously, FPRs have been identified to play a role in fibrosis and in the fibroblast-to-myofibroblast transition (Brossi et al., 2015). GPR55 (G protein-coupled receptor 55) is a receptor that binds to ligands which can inhibit platelet aggregation (Kargl et al., 2013). GPR55$^{-/-}$ mice have a resistance to inflammation and neuropathic pain (Staton et al., 2008). Interestingly, platelet releasate from both ApoE and FPR2 deficient mice have a significantly lower proliferative effect on C2C12 myoblasts when compared to wild-type platelet releasate. This may be due to their roles in regulating platelet aggregation and potentially altering the proteomic profile, or alpha granule secretions (Riddell et al., 1997, Vital et al., 2016).

4.5.2 Skeletal muscle stem cells treated with human, wild-type and TSP-1 KO platelet releasate

Skeletal muscle satellite cell proliferation and differentiation profiles can be studied on single muscle fibres *ex vivo* (Zammit et al., 2004). Satellite cell lineage progression factors such as Pax7, MyoD and Myogenin are upregulated at certain time-points after fibre isolation, which can be detected immunohistochemically (Zammit et al., 2004). Typically, Pax7^{+ve}/MyoD^{-ve} cells are quiescent satellite cells, while Pax7^{+ve}/MyoD^{+ve} cells are proliferating cells and Pax7^{-ve}/MyoD^{+ve} cells have committed to differentiation (Zammit et al., 2006b). After 72 hours in culture, satellite cells that are Pax7^{-ve}/Myogenin^{+ve} are differentiating cells that become myonuclei and have lost stemness (Zammit et al., 2004). Myogenic regulatory factors such as MyoD have recently been shown to be altered on myofibres from mice *ex vivo* in response to platelet releasate in a dose-dependent manner (Li et al., 2016b).

The relative expression profiles of Pax7, MyoD and Myogenin were similar between WT and TSP-1 KO fibres in response to human releasate. However, the satellite cell progeny at both T48 and T72 was significantly higher in TSP-1 KO fibres compared to WT and the human releasate had similar effects with standard growth medium. Since there were not genotype-specific differences in satellite cell numbers at T0, these findings, indicate a higher cell turn over in the TSP-1 KO fibres. These findings are in line with early research conducted on endothelial cells and their inhibition by TSP-1 (Bagavandoss and Wilks, 1990).

Unexpectedly, when culturing the primary satellite cells off the fibres, this upregulated proliferative effect was not evident between TSP-1 KO and WT mice in response to human releasate. In fact, for satellite cells from both EDL and BB muscles, there was no significant difference in any group for proliferation between the TSP-1 KO and the WT cells. It is worth noting that myoblasts do express and secrete TSP-1 (Adams and Lawler, 1994). Furthermore, skeletal muscle satellite cells, when cultured *ex vivo* off the fibre, are known to be altered when compared to satellite cells fibre located between the basal lamina and the sarcolemma on the fibre. In particular, their expression profiles and regeneration capacity are diminished (Charville et al., 2015, Montarras et al., 2005). Interestingly, TSP-1 deficiency is known to induce mitochondrial alterations of the myofibres (such as a 250% increase in mitochondrial volume), whereas it is currently unknown whether this is apparent in satellite cells (Frazier et al., 2011). Moreover, a study on TSP-1 KO pericytes showed that there was a decreased amount of N-cadherin allowing more migration and proliferation of the cells in their myofibre micro-environmental niche (Scheef et al., 2009). Similarly, M-cadherin is known to stimulate skeletal muscle satellite cell proliferation (Marti et al., 2013). Although our understanding about the role of TSP-1 in satellite cell function and muscle biology is still evolving, novel data is shown that TSP-1 KO may account for the satellite cell proliferation on the myofibre and not *in vitro* culture.

Following from this, the effect of human platelet releasate on satellite cell differentiation was distinguished. It is reported here for first time that isolated satellite cells from TSP-1 mice had compromised differentiation leading to an impaired fusion index and myotube number. CD36 is a TSP-1 receptor is known to be important for the differentiation of skeletal muscle satellite cells (Park et al., 2012). By the addition of human platelet releasate, known to have relatively high amounts of Thrombospondin-1 protein, this TSP-1-CD36 pathway may contribute to the observed rescue of differentiation in this study (Samovski et al., 2015a, O'Connor et al., 2010).

It is shown here that TSP-1 KO platelet releasate drives a faster satellite cell proliferation over wild-type cells, when assessed on cultured single myofibres. This proliferative effect is magnified further when analysing the total cell progeny number at T72 of culture, where TSP-1 KO myofibres have higher total cell progeny numbers over

wild-type. Interestingly, there was no significant difference for the expression patterns between TSP-1 KO and wild-type at T72, irrespective of culture condition. This is consistent with the T48 data. Previous studies have shown an attenuation of proliferation in the absence of TSP-1 in pericytes (Scheef et al., 2009). In contrast, it is worth noting that smooth muscle cells respond positively and proliferate in the presence of TSP-1 through the upregulation of transforming growth factor(TGF)-β2 and Insulin-like growth factor-1 signalling (Stein et al., 2013, Maile et al., 2010). Conversely, TGF-β has been shown to inhibit proliferation of satellite cells (Allen and Boxhorn, 1989). A potential TSP-1-mediated TGF-β downregulation may offer a logical explanation of the observed findings in the present study. This notion is strengthened by recent evidence showing that customised releasate lacking TGF-β improves cell proliferation (Li et al., 2016b, Miroshnychenko et al., 2017).

4.5.3 Skeletal muscle injury in response to platelet releasate treatment for wild-type and TSP-1 deficiency

Our data indicate that TSP-1 KO mice did not show an increase in skeletal muscle weight. This is in line with previous studies showing an increase in capillarisation and not inducing a greater mass overall (Malek and Olfert, 2009). A key finding had shown that critical leg ischemia in TSP-1 null mice has significantly greater regenerating myofibres as compared to wild-type (Brechot et al., 2008). This finding is in line with our data such that necrosis is reduced in TSP-1 KO mice after injury. This reduction in necrotic tissue may, in part be due to the increase in capillarisation observed *via* CD31 immunohistochemical staining of the injured skeletal muscle areas. We show here that 5 days after injury, both the addition of platelet releasate and the absence of TSP-1 independently increase angiogenesis in the injured areas. Interestingly, there was no additive angiogenic effect in the area of injury when injecting platelet releasate into TSP-1 KO mice. This may be explained through the re-introduction of TSP-1 into the KO model *via* platelet releasate. It has been shown that TSP-1 can modulate VEGF signalling *via* the CD36 fatty acid receptor (Chu et al., 2013). Since there is crosstalk between these two factors, this may explain the lack of an additive angiogenesis effect in the skeletal muscle.

This study builds upon previously published data reporting that platelet releasate accelerated wound healing, reduced necrosis and decreased CD68-positive macrophage staining, with an increased tetanic strength of injured muscle (Tsai et al., 2018b). Furthermore, a recent study on rat CTX skeletal muscle injury using an injection of PRP with neutralised TGF-β1 in their platelet preparation and found that fibrosis was reduced, angiogenesis was upregulated and there were a greater number of M2 macrophages recruited to the area of injury (Li et al., 2016b). Importantly, a pioneering study by Dimauro et al. had shown that PRP drives MyoD and Myogenin expression in skeletal muscle 5 days after injury (Dimauro et al., 2014).Therefore in the current study, having identified a TSP-1 as a target to be depleted, the effect of platelet releasate was assessed on the number of both MyoD and Myogenin expressing satellite cells in the area of injury after day 5 and day 10. Here it is shown for the first time that there is an additive effect on the number of $MyoD^{+ve}$ satellite cells when depleting TSP-1 and IP injecting platelet releasate to an injured tibialis anterior muscle after 5 days. Additionally, we show here for the first time that TSP-1 KO mice shown a reduced number of $Myogenin^{+ve}$ satellite cells in the area of injury after 10 days, which is ameliorated by platelet releasate.

It is worth considering the finding that platelet releasate alters gene expression and capillarisation of non-injured skeletal muscle. Until now, only one finding had studied the effects of platelets in uninjured skeletal muscle. Fukuda et al. in 2017 have suggested an altered gene expression of *HGF* and embryonic *MHC* and *MHC I* (Fukuda et al., 2017). In line with this, the data in this thesis suggest downregulated *Mhciix* and *Mhciib* mRNA, which complement the upregulated *Mhx i* expression shown in the Fukuda paper. A novel finding is that there was an increase in *Vegfα165* and a decrease in *Mstn* mRNA potentially indicating a hypertrophic effect of the skeletal muscle fibres.

4.6 Conclusion

This study provides novel insights into the role of platelet releasate in skeletal myogenesis of the TSP-1 null mouse. By depleting TSP-1 from platelet releasate, this may be a way of further optimising the platelet-based applications for clinical use, for

enhanced skeletal muscle recovery. However, it was also shown that TSP-1 is important for myoblast differentiation *via* the addition of human releasate to TSP-1 KO myoblasts. TSP-1 KO platelet releasate is beneficial for myoblast proliferation *in vitro*. TSP-1 KO releasate enhances satellite cells progeny at T48 and T72 in both WT and TSP-1 KO fibres. Furthermore, TSP-1 KO fibres cultured *ex vivo* exhibit higher satellite cell progeny at T48 and T72 independent of platelet releasate. This novel data can be used in future studies as a tool to further optimise platelet-based applications.

Chapter 5

The use of customised platelet releasate to drive cardiac and skeletal myoblast proliferation and differentiation and reduce senescence

5.1 Abstract

Aim: Aiding the regenerative potential of different tissues is particularly challenging, due in part to the unique proliferative capacity of their cells. As platelet-based applications can be customised for tissue-specific efficacy, this makes them strong candidates for developing novel regenerative therapies. Therefore, the aim of this study was to i) determine if platelet releasate could be optimised to promote proliferation and differentiation of cardiomyocytes with varying regenerative capacities and ii) to test the effects of platelet releasate during cellular senescence.

Methods: The effect of physiological and supra-physiological platelet releasate on H9C2 rat cardiomyocytes and C2C12 skeletal myoblasts was analysed. Cellular proliferation and differentiation was assessed through cell proliferation assays, mRNA and protein expression. Multiplex screening was used to profile growth factors in physiological and supra-physiological platelet releasate. In addition, the effect of platelet releasate on cells cultured with a cellular senescence-inducing drug; Doxorubicin, was established.

Results: Physiological platelet releasate induced an increase in C2C12 proliferation rate with no effect on the H9C2s. However, supra-physiological platelet releasate induced a marked increase on the proliferative rate of C2C12 and H9C2 cells. The proliferative effects of skeletal and cardiac muscle cells are in part driven by VEGFα contained in the platelet releasate upregulating endogenous *Vegfα* and *Cyclind1* mRNA and protein levels. Supra-physiological releasate increased the differentiation of H9C2 and C2C12 cells. The proteins contained in platelet releasate that affect myoblast proliferation were not affected by a freeze-thaw cycle, but degrade at 37°C. Platelet releasate does not affect the reactive oxygen species levels of myoblasts, but affect fusion and protein synthesis. Cellular senescence was partially reversed dose-dependently with platelet releasate.

Conclusion: This chapter provides novel insights into the role of platelet releasate on cell lines from the muscle and heart and emphasises the importance of optimisation in a tissue specific manner. These data highlight the benefit of concentrating the composition of the platelet secretome for optimal cell-type targeted applications in regenerative medicine.

5.2 Introduction

Platelet-based applications have been studied in many tissue types *in vitro*, *ex vivo*, *in vivo* and clinically. Although outcomes have been largely successful in terms of improving tissue regenerative capacity, there is controversial evidence in the translational aspect of platelet-based applications from laboratory to clinic (Mosca and Rodeo, 2015). In this thesis, it was found that there was a strong correlation between the platelet concentration used to make platelet releasate and the proliferation of skeletal myoblasts. One potential aspect that may be overlooked in the preparation of platelet-based therapies is the concentration of platelets used; however, this has yet to be established. Importantly, in slow proliferating cell types such as cardiomyocytes, optimised platelet releasate may be used to enhance regeneration (Hargrave et al., 2016, Senyo et al., 2014).

Exercise-induced silent myocardial ischemia increases in prevalence with ageing, with an estimated 3 million people having asymptomatic ischemia in the United States of America (Katzel et al., 1998, Katzel et al., 1999, Stern, 2005). Platelet-rich plasma (PRP) has been studied as an attractive biomaterial for the treatment of myocardial ischemia and cardiac tissue regeneration as it is autologous, inexpensive and easily obtained (Gallo et al., 2013, Hargrave and Li, 2012, Hargrave et al., 2016, Li et al., 2008, Mishra et al., 2011, Morschbacher et al., 2016, Patel et al., 2016, Spartalis et al., 2015, Sun et al., 2014, Tang et al., 2017, Vu et al., 2015). However, concerns have been raised over the efficacy of PRP as an effective method of stimulating cardiac restoration (Morschbacher et al., 2016). One issue of using PRP may be the clotting factors contained in blood plasma such as fibrinogen, as previous studies have described that adding clotting factors to the heart may cause serious negative side-effects (Hargrave and Li, 2012). Additionally, the cardiomyocyte self-renewal rate is known to be low (Senyo et al., 2014), which may offer a plausible explanation for the limited success of platelet-based applications to-date. Removing plasma, cellular debris and clotting factors from activated platelet-rich plasma (i.e. a cocktail of growth factors and cytokines described previously as platelet releasate) may offer an alternative, more effective regenerative platelet-based application for cardiac regeneration.

Platelet storage has previously been shown to be important for growth factor preservation over time. A single freeze-thaw cycle was shown to significantly reduce key growth factors (EGF, VEGF, PDGF-AB, IGF-1 and TGF-β) in PRP (Hosnuter et al., 2017). Therefore, it was essential to see how this potential variation may affect the proliferation rate of myoblasts. Interestingly, PRP has been shown by a different group, to not be affected by differences in temperatures during processing (Amable et al., 2013). Another variable may be the myoblast proliferation rate decreasing during high passages of cells over a prolonged culture. It was shown that in smooth muscle cells, higher passages may have reduced proliferative rates (Erac et al., 2014). Finally, PRP has been shown to reduce oxidative stress in a muscle contusion in rats (Martins et al., 2016).

Cellular senescence is a state of irreversible growth arrest in cells (Collado et al., 2007). Senescence and ageing of vascular, cardiac and skeletal muscle has been studied in laboratories through the implementation of the anti-cancer drug, doxorubicin; a DNA and RNA synthesis inhibitor developed in the 1960s (Bielak-Zmijewska et al., 2014, Chen et al., 2018, Richardson and Johnson, 1997, Sin et al., 2016). Doxorubicin acts by inhibiting the enzyme topoisomerase II, causing DNA damage and induction of apoptosis (Richardson and Johnson, 1997). The global doxorubicin market size was valued at USD 809.6 million in 2015 and is projected to grow to USD 1.38 billion by 2014 (GrandViewResearch, 2016).

The anti-cancer drug doxorubicin has been shown to cause severe hyperglycaemia, insulin resistance and inhibition in 5' adenosine monophosphate-activated protein kinase (AMPK) signalling in skeletal muscle and dysfunction in cardiac and skeletal muscle (de Lima Junior et al., 2016, Hayward et al., 2013). Furthermore, it has been shown that doxorubicin as a chemotherapy drug, can cause skeletal muscle damage and myotube catabolism (Rybalka et al., 2018, Gilliam et al., 2012). Additionally, doxorubicin has been shown to be detrimental for skeletal muscle regeneration when administered 2 days after injury coinciding with the peak satellite cell proliferation period (Nguyen et al., 1998). Similarly, cardiomyocyte damage due to doxorubicin damage has been well documented previously, indicating that Myogenin is a target for doxorubicin-induced cardio toxicity (Liu et al., 2015). Cardiomyocyte proliferation is

inhibited through doxorubicin administration, causing cell cycle arrest and senescence; however, the VEGF pathway has been shown to reverse this effect (Chen et al., 2018). It is shown in **Chapter 3** that VEGF from platelet releasate acts strongly and dose-dependently on C2C12 proliferation. For this reason, the mechanisms behind the anti-senescence effects of platelet releasate on skeletal muscle and cardiac muscle were hypothesised to be VEGF-mediated.

Thus far, dexrazoxane has been reported to be the only approved drug co-administered with doxorubicin for preventing doxorubicin-induced cardiac damage. Its mechanisms of action involve chelating free iron, to displace iron from doxorubicin-iron complexes, or to prevent doxorubicin from binding to the topisomerase 2β complex (Liu et al., 2015). However, this mechanism removed the effectiveness of doxorubicin as an anti-cancer treatment. Therefore, the need for novel co-administrations is required for the anti-cancer treatment doxorubicin.

Recent evidence has shown that the effect of doxorubicin can be mitigated dose-dependently by platelet lysates (Refolo et al., 2014). Furthermore, platelet-based applications have also been shown to positively affect cardiomyocytes after doxorubicin associated damage *in vivo* (Morschbacher et al., 2016, Zaki et al., 2019). It is shown here that platelet releasates cause a dose-dependent proliferative effect on skeletal myoblasts. Since platelet releasate can modulate proliferation of myoblasts, and ameliorate cellular senescence of hepatocellular carcinoma cell lines; the hypothesis was that platelet releasate may alter cellular senescence and ageing in skeletal muscle (Refolo et al., 2014). The aim was to test if platelet releasate can counter the negative effects of the anti-cancer drug doxorubicin on skeletal muscle.

In light of this, the aim of this study was to determine if customised platelet releasate could be beneficial for aiding the regenerative capacity of different muscle cell types. Cells from tissues with high- (i.e. skeletal muscle) and lower- (i.e. heart) regenerative capacities are focused on here. This article provides methodological insights in optimising the composition of the platelet secretome for cell-type specific applications, and if this optimised platelet releasate could alter cellular senescence. This may also provide an effective intervention for treating injury and trauma such as sports injuries, wound healing and joint disorders.

5.3. Materials and Methods

5.3.1 Ethical standards

The study was approved by the local Ethics Committee of the University. Animal experiments were performed under a project license from the United Kingdom Home Office in agreement with the revised Animals (Scientific Procedures) Act 1986 and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985). Blood sampling from healthy human volunteers was performed with their written informed consent and was approved by the University's Ethics Committee.

5.3.2 Preparation of platelet releasate

Human platelet releasate was prepared as described in section 2.2.1. Briefly, acid citrate dextrose to whole blood at a ratio of 1:5 was centrifuged at 190g for 15 minutes followed by PRP collection and inactivation of platelets using prostaglandin I_2. The PRP was then centrifuged at 800g for 12 minutes and the platelet-poor plasma supernatant was then removed. Modified tyrode's was used to re-suspend the platelet pellet to a concentration of $2.5x10^8$ platelets mL^{-1} (labelled as Physiological Releasate) or $10x10^8$ platelets mL^{-1} (labelled as Supra-physiological Releasate). The platelet preparation was activated using a PAR-1 agonist (TRAP6; 20μM). Platelets were centrifuged at 9500g for 10 minutes, and the releasate supernatant was aliquoted from the cellular debris.

5.3.3 Cell cultures and treatments

Murine C2C12 skeletal myoblasts and rat H9C2 cardiomyocytes were cultured in GM at 37°C. All releasate treatments were cultured in serum-free conditions when not indicated to be in a GM group. To induce differentiation, C2C12 cells were cultured in GM ± releasate until reaching 80% confluence (4 days) before switching to DM further 4 days. H9C2 cells were cultured in GM ± releasate until reaching confluence (4 days) before switching to DM containing sodium pyruvate for a further 4 - 7 days. The myofusion index was calculated as Myogenin-stained nuclei per myotube (n=2

nuclei/myotube) divided by DAPI-stained cells as a percentage. In temperature experiments, cells were treated with platelet releasate that was stored for either 3 hours or 72 hours in 80°C, - 21°C, +4°C or +37°C. Importantly, TRAP6 (20μM), thrombin (0.05-0.1 NIH Units mL^{-1}), or collagen (10μg mL^{-1}) platelet-agonists were applied directly to a myoblast cell culture to verify that they do not cause proliferation, negating the proliferative effect of the platelets. H9C2 cells, C2C12 cells and single EDL muscle fibres were treated with Doxorubicin (0.0625μM, 0.125μM, 0.25μM or 0.5μM Cayman chemical cat. 15007) in order to stimulate a dose-response of cellular senescence.

5.3.4 Cell proliferation analysis

Cell proliferation was evaluated by the pyrimidine analogue EdU incorporation assay using the fluorescent Click-iT® EdU Cell Proliferation Assay according to the manufacturer's instructions. Proliferating cells were measured as EdU divided by DAPI-stained nuclei as a percentage. This was confirmed with monoclonal anti-Ki-67 and with Cyclin D1 immunohistochemistry.

5.3.5 Single fibre isolation and culture

For single-fibre culture, murine C57Bl/6J single fibres were isolated from the EDL muscle as in **section 2.3.6**. In brief, EDL muscles were dissected and subjected to collagenase (0.2%) digestion for 3-4 hours. The digested muscle was then gently triturated and single fibres were then cultured for 48 and 72 hours in single fibre media (FM; 10% horse serum, 0.5% chick embryo extract), or serum free media (SF) with the addition of 10% releasate. Fibres were stained for anti-Pax7, anti-MyoD and anti-Myogenin and with DAPI.

5.3.6 Immunofluorescence

Cells were seeded on coverslips in 1mL of media in 24-well plates. Fixed and permeabilised cells were then treated with primary antibodies for anti-Myogenin, Scrib, anti-VEGF, anti-IL-10, anti-VEGF-C, anti-TLR4 and anti-ICAM-1 in wash buffer overnight. Primary antibodies were removed with 3 washes in wash buffer, followed by the addition of secondary antibodies, see **Appendix I**. Cells were measured by the

intensity of fluorescence per cell divided by DAPI-stained nuclei as a percentage. Dihydroethidium (DHE; ThermoFisher Scientific, cat. D-1168) was used to detect superoxide which displays blue-fluorescence in the cytosol of the cell, until oxidized, where it intercalates within the cell's DNA, staining the nucleus fluorescent red, and used as per manufacturer's instructions.

5.3.7 Luminex Multiplex assay

All releasate samples were analysed by multiplex immunoassay based on Luminex 200 technology and kindly processed by Laura Gutierrez as denoted in the acknowledgements. The ProcartaPlex Human kits were used, which test a panel of 37 molecules, including growth factors, cytokines, chemokines and immune stress markers. The multiplex assay was performed following the manufacturer's instructions and the plates were read using the xPONENT software. The specific factors analysed were: Caspase-3, CD40L, EGF, FGF-2, FGF-23, G-CSF (CSF-3),GM-CSF, GITRL, Granzyme B, GRO-α (KC/CXCL1), HGF, ICAM-1, IFN gamma, IL- 1α, IL-1β, IL-2, IL-6, IL-7, IL-8 (CXCL8), IL-10, MIP-1α (CCL3), MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MIP-1β (CCL4), Osteopontin, PDGF-BB, PECAM-1, P-Selectin, RANTES (CCL5), SDF-1α, Thrombopoietin (TPO), TGFβ, TNFα, VCAM, VEGF-A and VEGF-D.

5.3.8 RNA extraction and real-time qPCR analysis

Quantitative PCR was performed as described previously. In brief, $3x10^4$ H9C2s were seeded per well of a 6-well plate in SF or GM with or without 30% platelet releasate. After 24 hours (proliferation phase) cells were harvested for RNA isolation and qPCR. Details of primers are given in **Appendix I**. Relative expression was calculated using the ΔΔCt method with normalisation to *Gapdh* and *Hprt.* mRNA levels of *Vegfa165, Vegfr1, Igf1*, *Cyclind1*, were measured.

5.3.9 Statistical analysis

Data normal distribution was checked by the D'Agostino-Pearson omnibus test. Data are reported as mean±SD. Statistical differences among experimental groups were determined by one-way ANOVA followed by the Tukey post-hoc test. Differences

between two groups were detected by either using the Student's *t*-test or performed by a one-way ANOVA followed by Tukey's post-hoc test where appropriate. Statistical differences were considered as significant for $p<0.05$. Statistical analysis was performed using SPSS software.

5.4 Results

5.4.1 Physiological releasate is beneficial for myoblast but not cardiomyocyte proliferation

Physiological concentrations of platelets used to make platelet releasate are a powerful inducer of skeletal muscle stem cell proliferation. Therefore, the effect of physiological releasate ($2.5x10^8$ platelets/mL) on cardiomyocyte proliferation was aimed to be determined. To achieve this, H9C2 cardiomyocytes and C2C12 murine skeletal myoblasts were cultured in either serum-free, growth medium (GM; 10% FBS) or 10% platelet releasate (**Figure 5.1A**). H9C2s and C2C12s were stained for the cell-cycle marker Cyclin D1 (immunofluorescence), Ki67 (immunofluorescence) and EdU (live staining). In contrast to the C2C12s, platelet releasate in serum-free conditions failed

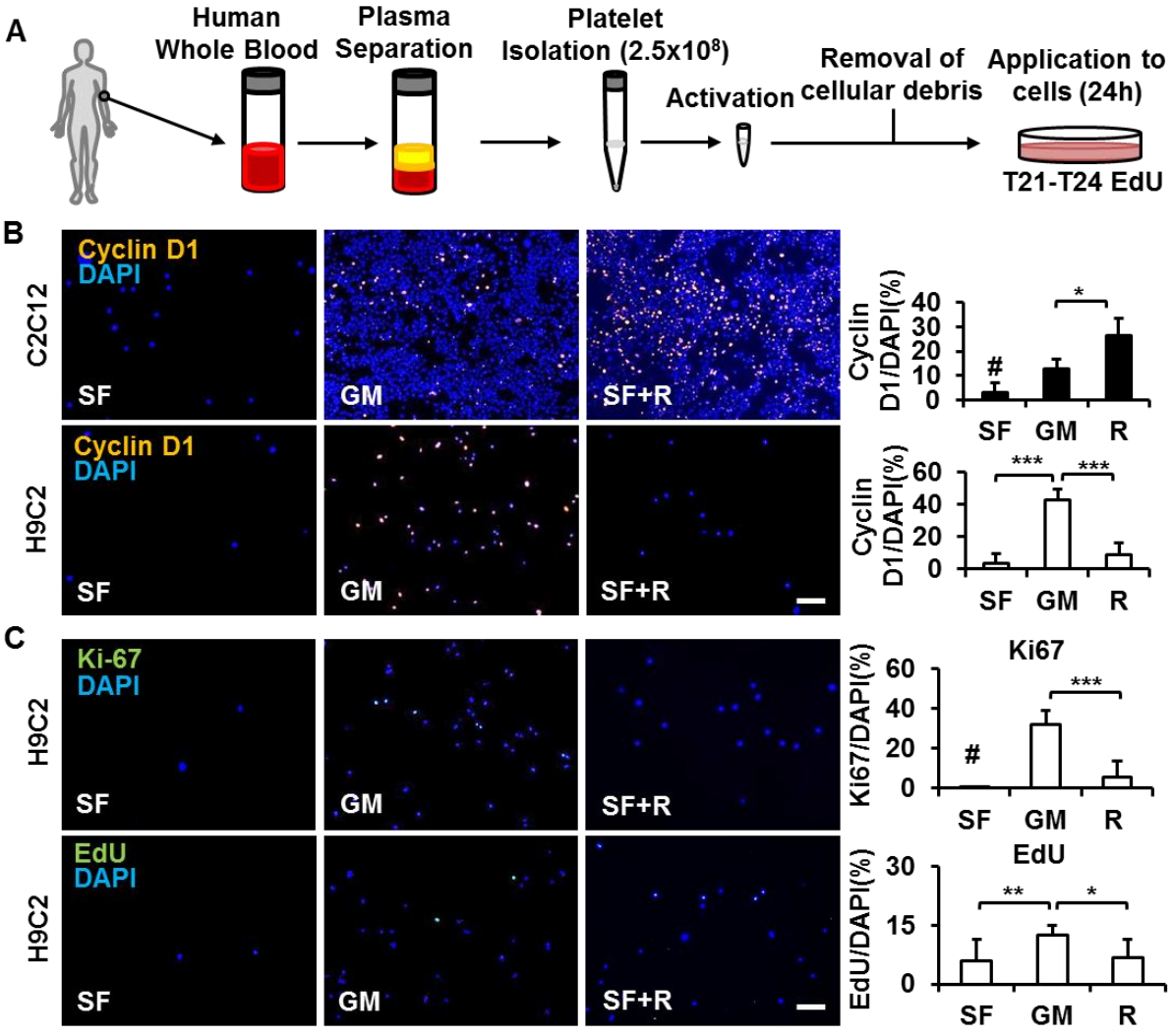


Figure 5.1

to match the growth medium group for H9C2 for all proliferation markers (**Figure 5.1 B-C**).

Figure 5.1 Physiological releasate does not induce cardiomyocyte proliferation. (**A**) A schematic depicting the methodology behind the experimental setup; application of human-physiological releasate to C2C12s or H9C2s for 24 hours. (**B**) Representative images and quantitative data for C2C12 skeletal myoblast and H9C2 cardiomyocyte cellular proliferation staining for Cyclin D1 (Orange) and DAPI. (**C**) Representative images and quantitative data for H9C2 cardiomyocyte Ki-67 expression and EdU proliferative live-staining (Green) was conducted for 3-hours after 21 hour incubation after 24-hours of proliferation in serum-free (SF), growth medium (GM; 10% FBS) or 10% Releasate (R) co-stained with DAPI (Blue). Both cell lines were treated with serum-free, growth media, and 10% releasate. All releasate was made with $2.5x10^8$ platelets/mL for **Figure 5.1**. (x5 magnification, scale bar 200 μm). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *p<0.05, **p<0.01, and ***p<0.001, #P<0.05 vs. every other group.

5.4.2 Growth factor composition of physiological and supra-physiological releasate

A positive dose response was shown in Chapter 3 for C2C12 skeletal myoblast proliferation when treated with platelet releasate made with increasing concentrations of platelets. Therefore, we aimed to profile human physiological and supra-physiological releasate for their growth factor and cytokine constituents, with the hypothesis that more concentrated platelet releasate would yield better proliferation in various cell types by increasing the levels of all components. In this Chapter, we use PAR-1-activated platelet releasate from physiological and supra-physiological platelet concentrations (**Figure 5.2A**). Using multiplex technology, the concentration of 37 molecules, including growth factors, cytokines, chemokines and immune stress markers were assessed in both concentrations of releasate (**Figure 5.2A-B**). Differences in the concentration of growth factors, high concentration analytes and cytokines between physiological and supra-physiological releasate are illustrated in **Figure 5.2C-E**. Of note, PDGFβ and VEGFα were increased in supra-physiological releasate 1.56- and 4.42-fold respectively. VCAM-1 was upregulated in supra-physiological releasate 3.8-fold over physiological releasate. Similarly, growth factors driving such as EGF and FGF-2 were upregulated 1.92- and 3.43-fold respectively.

However not all factors were found at higher levels in the supra-physiological releasate compared to the physiological releasate; TGFβ, a growth factor that can induce fibrosis in muscle regeneration was reduced by 0.5-fold in the supra-physiological releasate. These results imply that the formation of platelet releasate is a regulated process and not one that relies on mass action. The releasates produced under physiological and supra-physiological conditions differ both quantitatively and qualitatively.

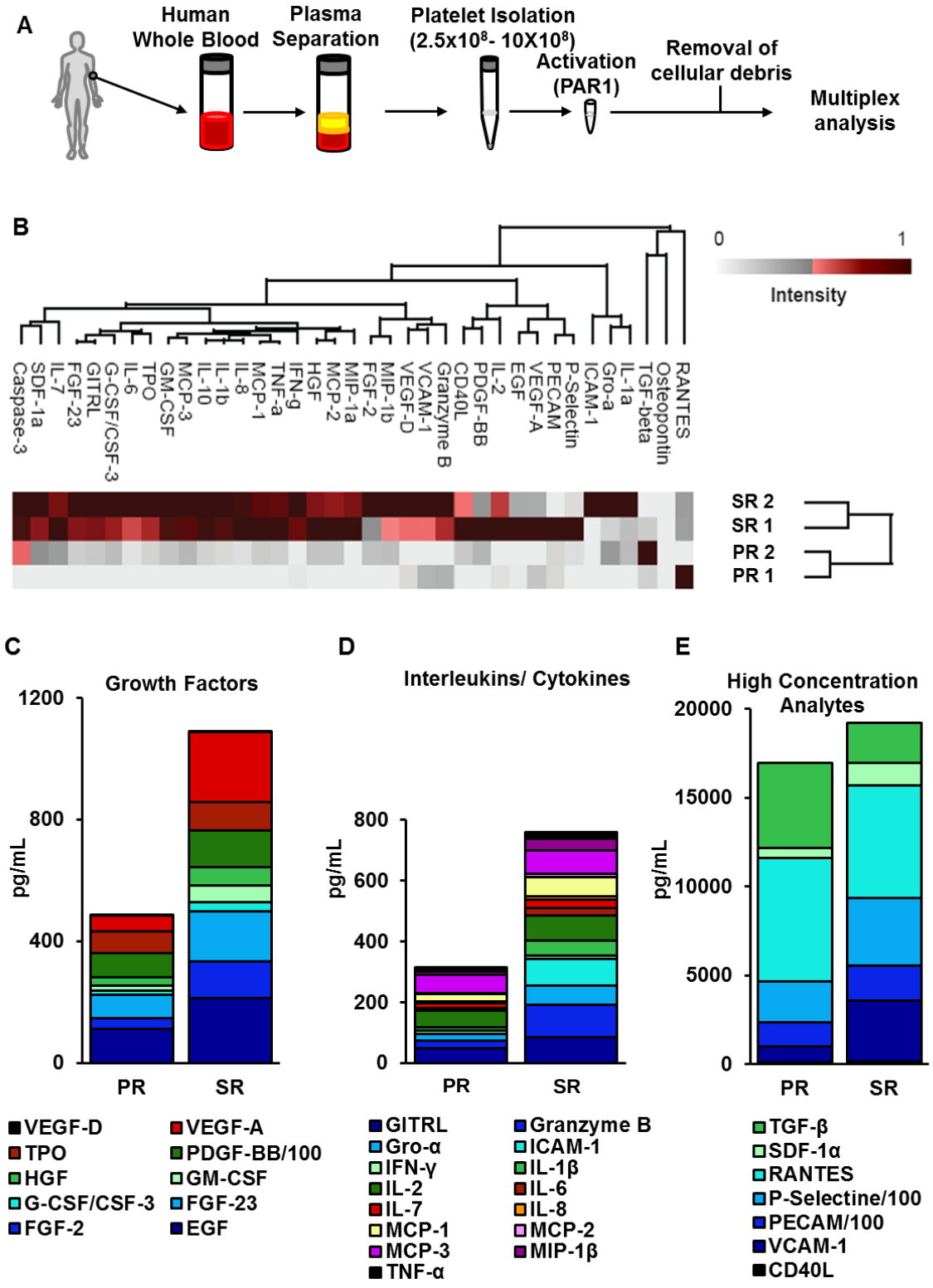


Figure 5.2 Growth factor composition of physiological and supra-physiological platelet releasate. Human platelet suspensions were aggregated using TRAP6 (a PAR1 agonist). Concentrations of specific analytes contained in (ProcartaPlex Human kits) were measured in platelet releasate. (**A**) A schematic depicting the methodology

behind the experimental setup. (**B**) Hierarchical clustering representing the average expression intensity of each analyte (PR; physiological platelet releasate ($2.5x10^8$ platelets/mL), SR; supra-physiological releasate ($10x10^8$ platelets/mL)). (**C**) Concentration of growth factors, (**D**) cytokines and (**E**) analytes in high abundance detected in physiological (PR) and supra-physiological (SR) platelet releasates averaged from both PAR1 and Thrombin-stimulated platelets.

5.4.3 Supra-physiological releasate induces a strong proliferative response in myoblasts and cardiomyocytes

Given the quantitative and qualitative differences of physiological and supra-physiological releasate reported in this study, it was next hypothesised that supra-physiological releasate would exhibit a stronger proliferative effect on cell types that responded positively with physiological releasate. A higher concentration of platelets used in making releasate correlates strongly with higher proliferation of C2C12 myoblasts. For this reason, a dose response (v/v for percentage of releasate) on H9C2 cardiomyocytes was conducted using a supra-physiological platelet concentration ($10x10^8$ platelets/mL). This releasate was applied in serum-free conditions on the H9C2s showing a v/v dose response. Further to this, both 10% platelet releasate ($10x10^8$ platelets/mL) plus growth media (GM+SR) and releasate at 30% v/v (30% SR) were the highest proliferative groups for the cardiomyocytes based on a 3-hour live staining with EdU (**Figure 5.3A-B**). Supra-physiological releasate on growth medium (GM+SR) had a 1.61-fold increase in H9C2 cell proliferation compared to the GM condition. Of note, 30% supra-physiological releasate in serum-free conditions induced significantly higher H9C2 cell proliferation compared to standard culture conditions with growth medium (**Figure 5.3A-B**). Most importantly, supra-physiological releasate induced a strong proliferative response by 3.1-fold in H9C2 cardiomyocytes as compared to physiological releasate (**Figure 5.3C**). Taken together, platelet releasate made using supra-physiological levels of platelets is able to drive a significantly faster proliferative rate of both skeletal and cardiac myoblasts (**Figure 5.3C**).

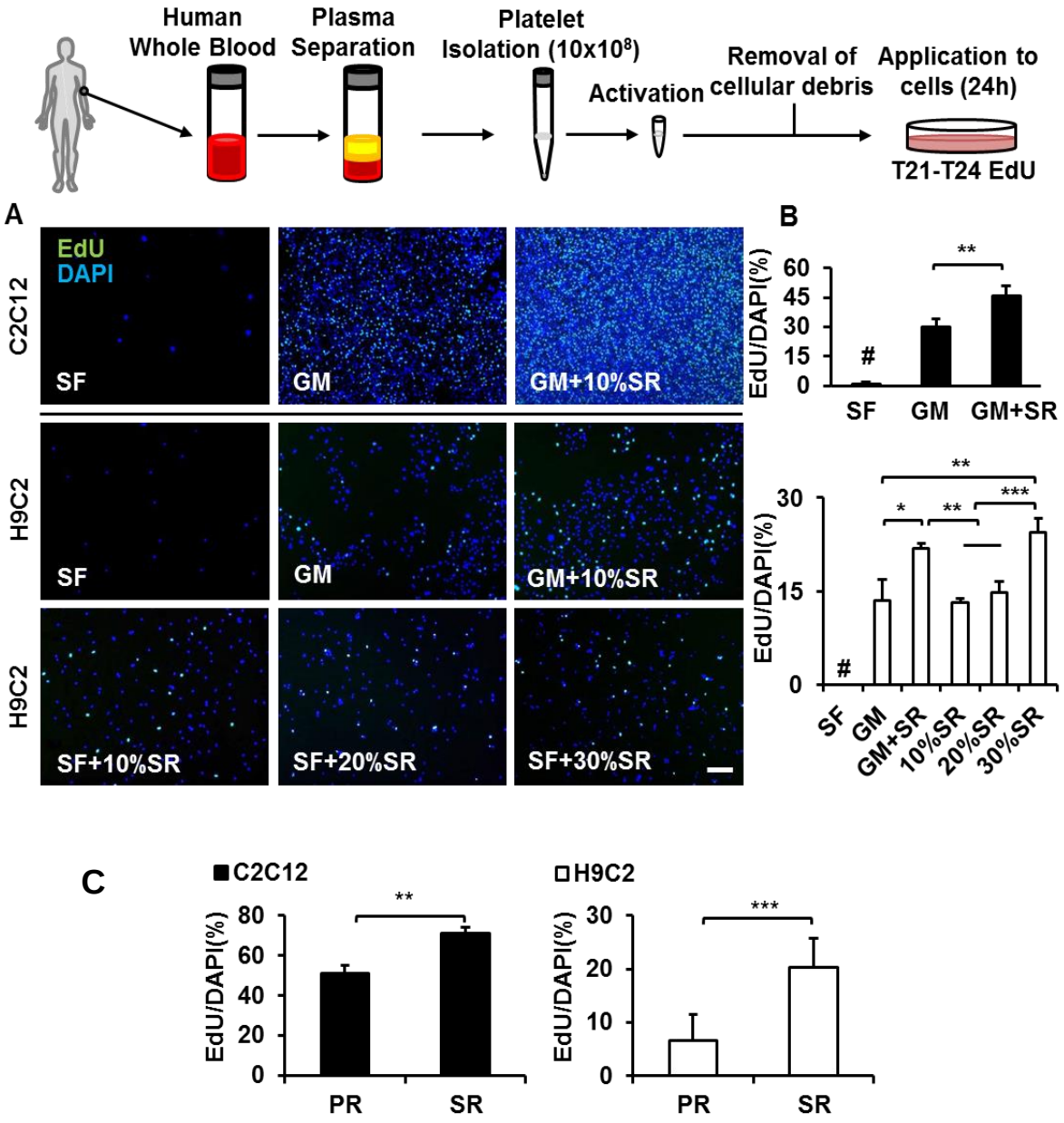


Figure 5.3 Supra-physiological releasate induces a strong proliferative response in cardiomyocytes (**A-B**) EdU proliferative live-staining was conducted on C2C12 skeletal myoblasts (**B**) and H9C2 cardiomyocyte cells for 3-hours after 21 hour incubation for serum-free, growth medium and 10%-30% releasate (SR; $10x10^8$ platelets/ml) conditions. (**C**) Quantitative data for C2C12 and H9C2 comparisons between physiological (PR) and supra-physiological (SR) releasate for live staining with EdU for 3 hours following 21 hours of culture. (x5 magnification, scale bar 200 µm). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test or Student's *t*-test as appropriate. Differences are *p<0.05, **p<0.01, and ***p<0.001, #P<0.05 vs. every other group.

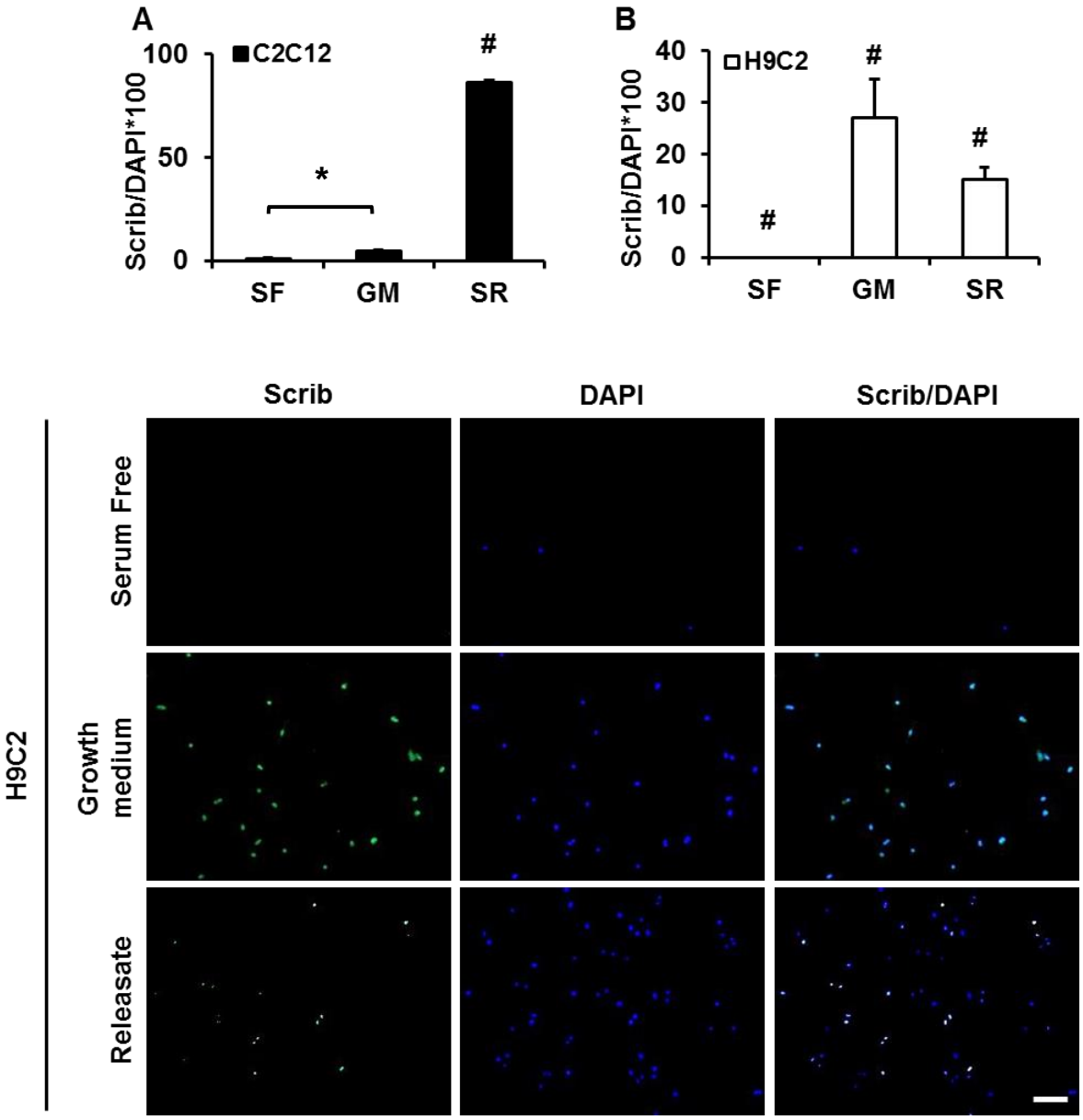


Figure 5.4 Scrib expression in cardiomyocytes exposed to platelet releasate. (**A-B**) Scrib expression of C2C12 skeletal myoblasts and H9C2 cardiomyocytes in serum-free, growth medium and 10% releasate (supra-physiological concentrations $10x10^8$ platelets/mL) conditions (x5 magnification, scale bar 200 μm). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Differences are *p<0.05, #p<0.05 vs. every other group.

5.4.4 Scrib is not a crucial factor in releasate-mediated cardiomyocyte proliferation

Of note, Scrib has been previously shown to be a crucial factor involved in cardiomyocyte development and progression (Boczonadi et al., 2014). C2C12 cell-cycle progression is heavily driven by platelet releasate through Scrib expression. Platelet releasate on H9C2 cells does not show the same Scrib expression that was observed in the C2C12 cells as compared to the serum free and growth medium groups. Interestingly, there was an increase in proliferative capacity which was also observed in H9C2s and C2C12s treated with supra-physiological releasate as compared to physiological releasate (**Figure 5.4A-B**).

5.4.5 Supra-physiological releasate drives cardiomyocyte proliferation at the gene and protein levels

There is a critical role for the vascular endothelial growth factor (VEGF) in the platelet releasate for skeletal muscle stem cell proliferation and differentiation. VEGF has also been shown to be beneficial in H9C2 cells and may potentially drive proliferation (Li et al., 2016a). Releasate drives *Vegfa165, Vegfr1 and Cyclind1* expression of H9C2 cells, independent of FBS. *Igf1* expression was markedly reduced in all proliferating conditions (**Figure 5.5A**). Interestingly, VEGF protein synthesis was increased by supra-physiological releasate in serum-free conditions over growth media for both C2C12s and H9C2 cells (**Figure 5.5B**).

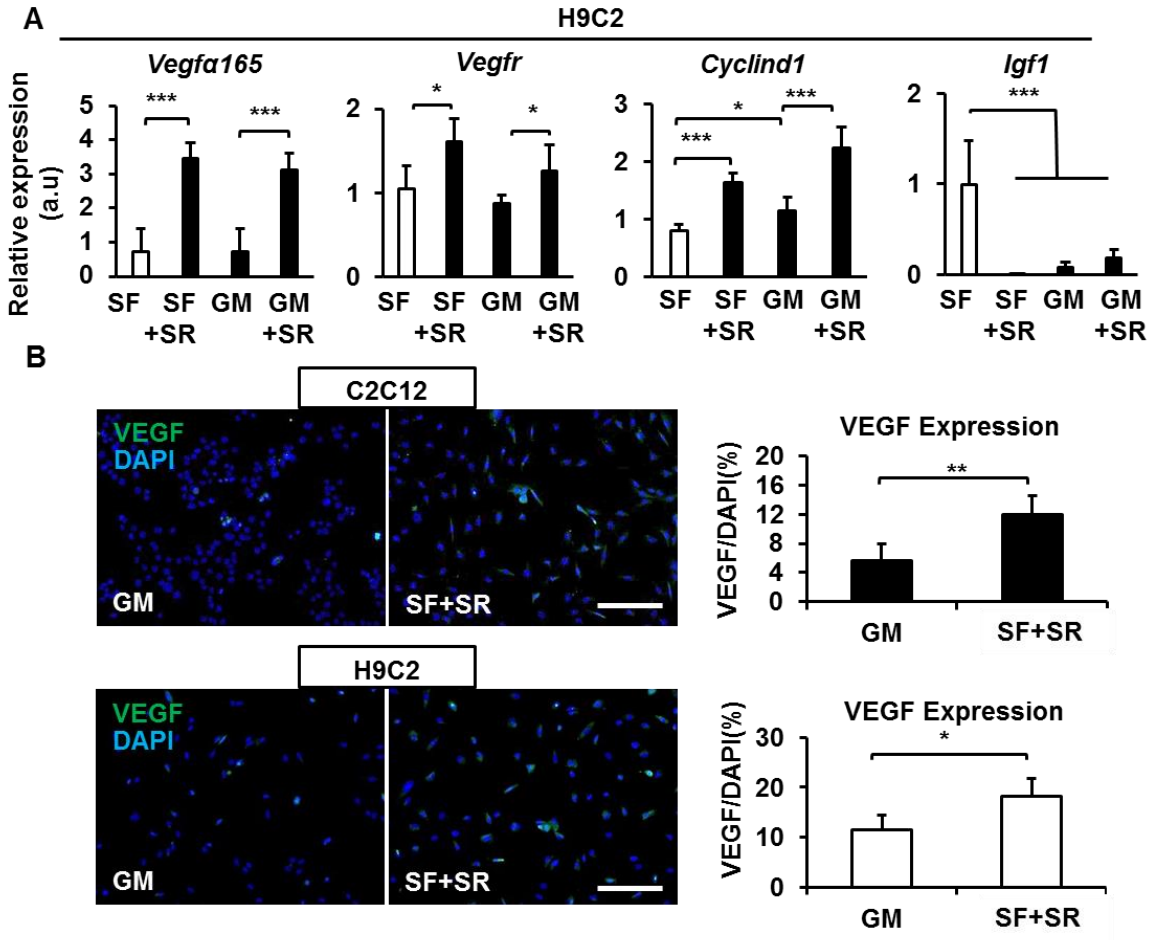


Figure 5.5 Supra-physiological releasate drives cardiomyocyte gene expression and protein synthesis for proliferation markers (**A**) Gene expression for *Vegfa165, Vegfr1, Cyclind1* and *Igf1* were measured for H9C2 cardiomyocytes in serum-free and growth media (10% FBS) conditions with or without 30% platelet releasate. (**B**) C2C12 and H9C2 immunohistochemical staining for VEGF expression during proliferation in growth media (10% FBS) or 10% platelet releasate (SR; $10x10^8$ platelets/mL) in serum-free conditions (x10 magnification, scale bar 200 μm). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test or Student's *t*-test as appropriate. Differences are *p<0.05 and ***p<0.001.

5.4.6 Supra-physiological releasate increases myoblast and cardiomyocyte differentiation

The next aim was to establish the direct effect of supra-physiological releasate on C2C12 myoblast and H9C2 cardiomyocyte differentiation. Cells were grown in serum-free or growth medium conditions with or without 10% supra-physiological releasate before switching to differentiation medium for 4 days. Unlike the C2C12 cells, platelet releasate did not seem to play a significant role in increasing total H9C2 myotube number. However, the trends between cardiomyocytes and skeletal muscle myoblasts remain similar both in terms of increased total cell number, increased Myogenin^{+ve} cell number and no significant increase in both of their myofusion indexes between GM and GM+SR (**Figure 5.6A-B**). It was sought to next determine the effects of platelet releasate in a later stage of differentiation. The same experimental setup was conducted; however, the cells were cultured in differentiation medium for 7 days. Interestingly, with longer incubation in differentiation media, there is a significant increase between GM and GM+SR in cell number, Myogenin^{+ve} cells, myofusion index and myotube number ($p<0.05$) when supplementing H9C2s with releasate in the proliferative phase with the standard growth medium culture (**Figure 5.6C**).

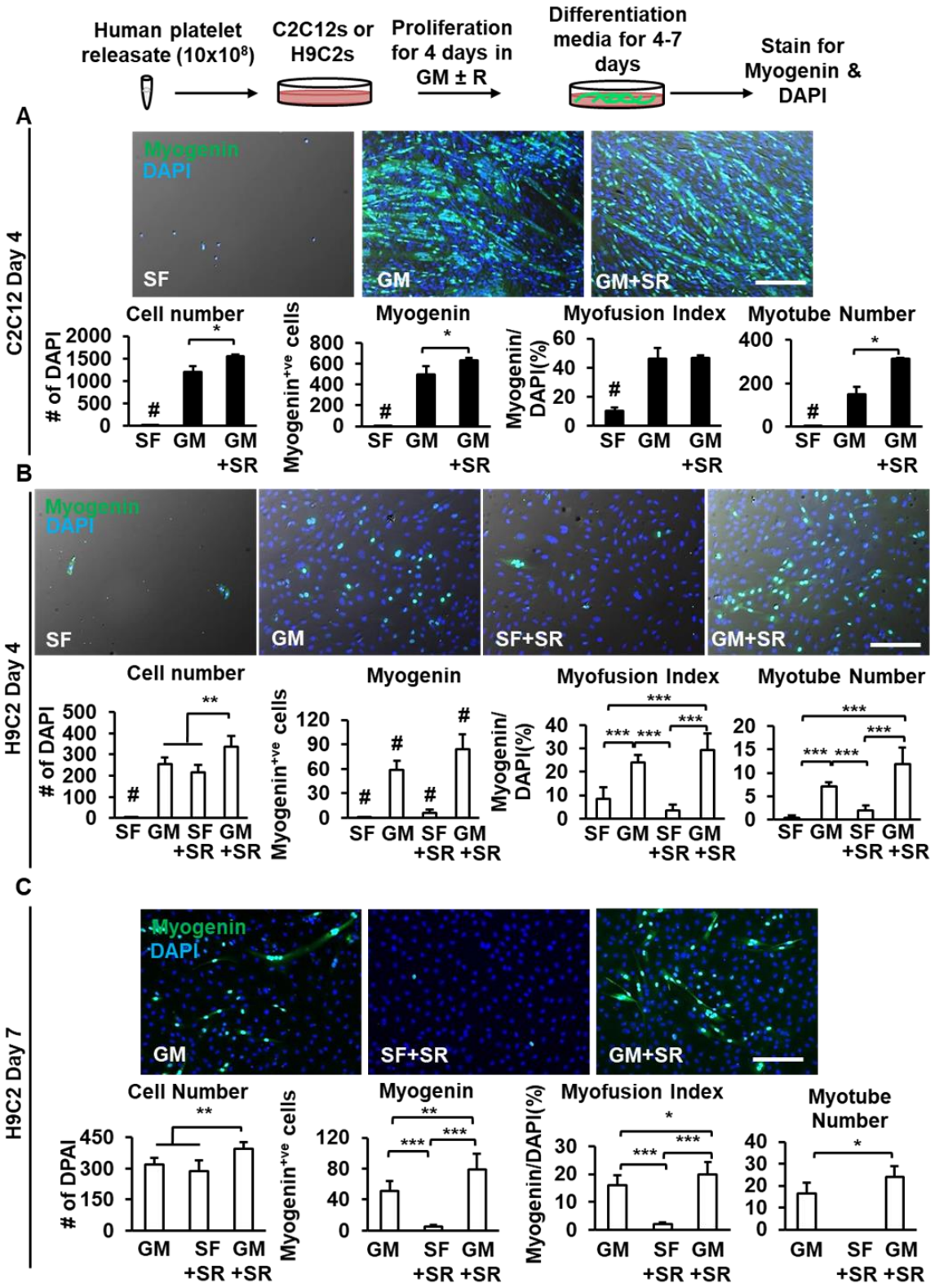

Human platelet releasate (10x10⁸)
C2C12s or H9C2s
Proliferation for 4 days in GM ± R
Differentiation media for 4-7 days
Stain for Myogenin & DAPI
A
C2C12 Day 4
Myogenin
DAPI
SF
GM
GM+SR
Cell number
# of DAPI
Myogenin
Myogenin+ve cells
Myofusion Index
Myogenin/ DAPI(%)
Myotube Number
SF GM GM +SR
B
H9C2 Day 4
SF+SR
C
H9C2 Day 7
Cell Number
# of DPAI
Myogenin/DAPI(%)

Figure 5.6

Figure 5.6 Supra-physiological releasate increases myoblast and cardiomyocyte fusion (**A-B**) C2C12 myoblasts and H9C2 cardiomyocytes were proliferated in serum-free, growth medium conditions with or without 10% platelet releasate (SR; supra-physiological concentrations $10x10^8$ platelets/mL). Differentiation was measured after 4 days in differentiation medium (2% horse serum). Representative images for Myogenin and DAPI (x10 magnification, scale bar 200μm). (**C**) Differentiation was measured after 7 days in differentiation medium (2% horse serum). Representative images for Myogenin and DAPI (x10 magnification, scale bar 200μm). Outcome measures include the nuclei number, number of $Myogenin^{+ve}$ nuclei, myotubes (n=2 nuclei/ myotube). The myotube fusion index was calculated by $Myogenin^{+ve}$ nuclei in myotubes/ DAPI as a percentage. Outcome measures include the nuclei number, number of $Myogenin^{+ve}$ nuclei, myotubes (n=2 nuclei/ myotube). Data are mean±SD (n=3/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #p<0.05 vs. every other group.

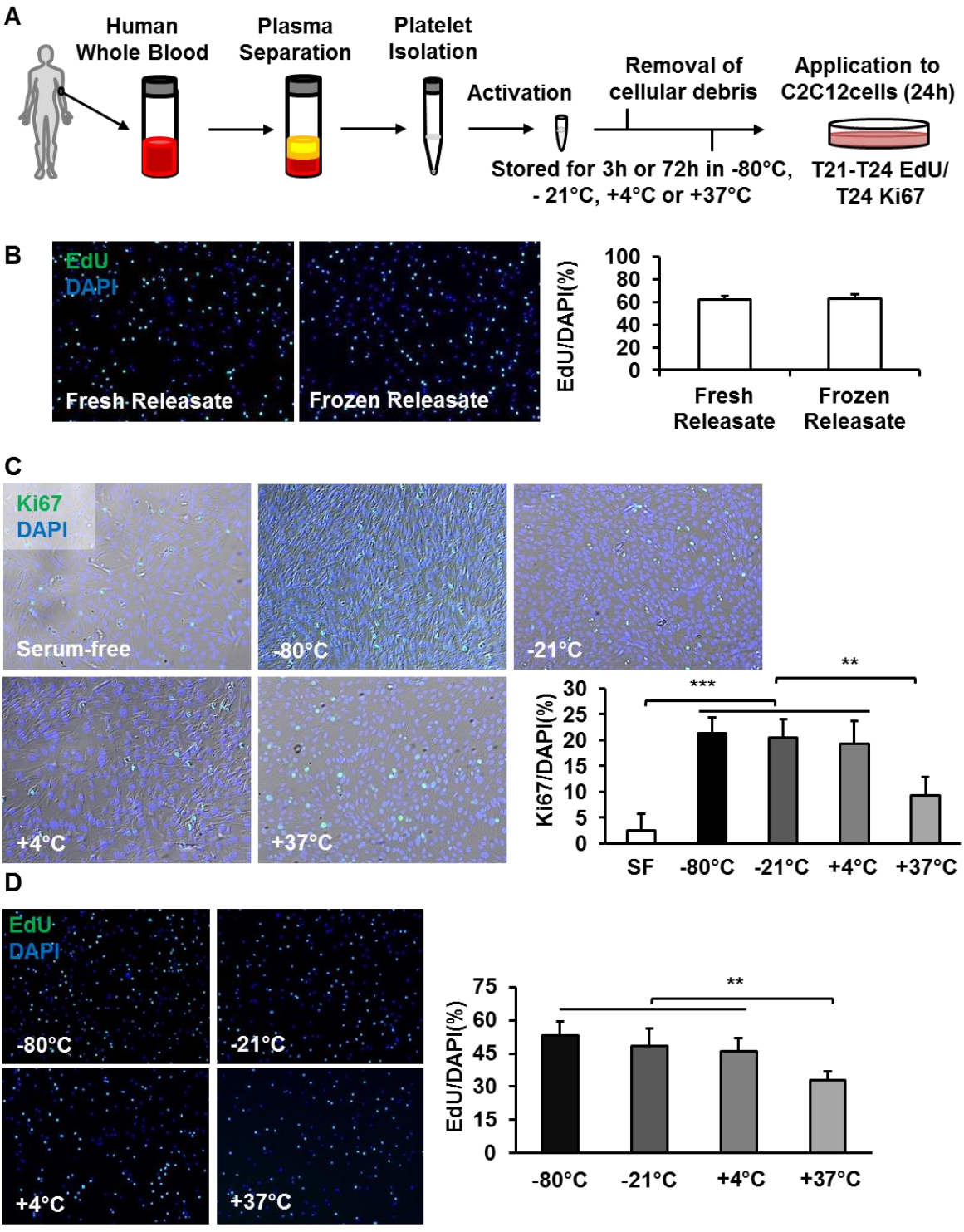


Figure 5.7 A single freeze-thaw cycle of platelet releasate does not remove its proliferative effects on C2C12 myoblasts, however storage at 37°C does (**A**) A schematic depicting the methodology behind the experimental setup. EdU and Ki-67 proliferative staining was conducted after a 24-hour incubation in 10% human platelet releasate that was used fresh releasate or after being subject to a 3-hour incubation at

-80°C. (**B**) Representative images and quantitative data for EdU staining (3hour) and DAPI. (**C**) Representative images and quantitative data for Ki-67 staining and DAPI co-staining with serum-free conditions as a negative control. (**D**) Representative images and quantitative data for EdU staining and DAPI co-staining. Statistical analysis for **B** was performed by a Student's *t*-test, with **C-D** performed by one-way ANOVA followed by Tukey's post-hoc test. n=3 independent experiments with 3 technical replicates per experiment. Differences are **p<0.01, ***p<0.001.

5.4.7 A single freeze thaw cycle does not affect platelet releasate's potential for myoblast proliferation.

It is shown in this thesis that platelet releasate can promote skeletal myoblast proliferation after storing the platelet releasate at -80°C, thawed, and applied to cells. Additionally, enzymatic and protein degradation in blood has been shown to occur after freeze-thaw cycles (Bortolin et al., 2017). Therefore, it was important to see if fresh platelet releasate could out-perform frozen platelet releasate on myoblast proliferation. Fresh platelet releasate did not perform significantly better than frozen platelet releasate over a range of 3 hours of proliferation (**Figure 5.7A-B**). Further to this, the storage of platelet releasate at body temperature (i.e. 37°C) lessens its proliferative effect after 72 hours storage. Therefore, the storage of platelet releasate at different temperatures was tested for their effects on myoblast proliferation. To this aim, both Ki-67 immunohistochemistry and EdU live-staining was used to detect myoblast proliferation with platelet releasate stored for 72 hours in different temperatures (i.e. -80°C, - 21°C, +4°C or +37°C). When stored at 37°C, platelet releasate seemed to have less of a proliferative effect on C2C12 cells than storage in the fridge or freezer (**Figure 5.7C-D**). Therefore, the growth factors contained in the platelet secretome seems to degrade when stored at human body temperature after 72 hours.

Table 5.1 An outline of current evidence of skeletal and cardiac muscle tissues and myoblasts in response to analytes contained in platelet releasate from **Figure 5.2**.

Reference	Factor	Findings
(Choo et al., 2017),	Vascular cell adhesion protein 1 (VCAM-1)	Important for satellite cell fusion and lineage progression, anti-apoptotic in skeletal muscle, and enhances cardiomyocyte proliferation.
(Sonnet et al., 2006) (Salvador et al., 2016)	Intercellular Adhesion Molecule 1 (ICAM-1)	Anti-apoptotic in skeletal muscle and may drive cardiac inflammation, fibrosis and dysfunction.
(Sonnet et al., 2006) (DeLisser et al., 1997)	Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM-1)	Anti-apoptotic in skeletal muscle, pro-angiogenic in cardiomyocytes.
(Baker et al., 2004, Blann et al., 2003)	P-Selectin	Essential for skeletal muscle fibre-regeneration *in vivo*, Increased levels of soluble P-selectin in the plasma have been demonstrated in a variety of cardiovascular disorders.
(Masuda et al., 2018, Sonnet et al., 2006, Wang et al., 2018)	CXCL1 (Gro-α)	Important for skeletal myoblast proliferation and differentiation, anti-apoptotic. May be involved in cardiac dysfunction, hypertrophy and fibrosis.
(Fernando et al., 2002, Putinski et al., 2013)	Caspase-3	Caspase 3 activity is required for skeletal muscle differentiation and apoptosis. May induce cardiomyocyte hypertrophy.
(Leroy et al., 2013, Hammoud et al., 2009)	Epidermal growth factor (EGF)	EGF stimulates skeletal myoblast proliferation and down-regulates differentiation. EGF does not induce cardiomyocyte proliferation.
(Milasincic et al., 1996, Rosenblatt-Velin et al., 2005)	Fibroblast growth factor (FGF)-2	A potent mitogen and inhibitor of myogenic differentiation in skeletal muscle. Upregulates cardiomyocyte differentiation.
(Avin et al., 2018)	FGF-23	No effect on skeletal myoblast proliferation or differentiation. FGF23 can directly tackle cardiac myocytes via FGFR4 thereby contributing to cardiac hypertrophy.
i) (Hara et al., 2011) ii) (Chung et al., 2012, Wright et al., 2014)	Granulocyte-colony stimulating factor (G-CSF)	i) G-CSF stimulates skeletal myoblast proliferation. ii) G-CSF does not affect skeletal myoblast proliferation or differentiation. Anti-apoptotic effects on H9C2s.
(Georgantas et al., 2014)	Granulocyte-macrophage colony-stimulating	GM-CSF has no effect on skeletal myoblast differentiation.

	factor (GM-CSF)	
(Cherin et al., 1996)	Granzyme B	A serine protease that mediates apoptosis in target cells.
(Walker et al., 2015) (Liu et al., 2016)	Hepatocyte growth factor (HGF)	2 ng/ ml promoted cell division but reduced fusion; 10 ng/ml HGF reduced proliferation but increased differentiation of skeletal myoblasts. HGF promotes cell survival in hypoxic conditions.
(Grzelkowska-Kowalczyk and Wieteska-Skrzeczynska, 2010)	Interferon gamma (IFN-γ)	Inhibits differentiation but is necessary for normal cell division in skeletal myoblasts.
(Dagdeviren et al., 2017, Deng et al., 2012, Verma et al., 2012)	Interleukin (IL)-10	Indirectly beneficial for skeletal muscle regeneration through macrophage switch in phenotype. Inhibits H9C2 hypertrophy and may improve heart function.
(Li et al., 2009, Madonna et al., 2005)	IL-1α	Stimulates catabolism and atrophy in skeletal myotubes. Increases inflammation in the heart and nitric oxide synthase in H9C2s.
(Li et al., 2009) (Xu et al., 2015)	IL-1β	Stimulates catabolism and atrophy in skeletal myotubes. Upregulates reactive oxygen species, apoptosis and cytotoxicity in cardiomyocytes.
(Al-Shanti et al., 2014, Zeng et al., 2016)	IL-2	Lymphocytes activated with IL-2 inhibit skeletal myoblast differentiation and induces proliferation. Anti-inflammatory in cardiomyocytes.
(Serrano et al., 2008, Xu et al., 2015)	IL-6	IL-6 regulates skeletal myoblast proliferation and migration. Upregulates reactive oxygen species, apoptosis and cytotoxicity in cardiomyocytes.
(Haneef et al., 2018, Haugen et al., 2010)	IL-7	Increased satellite cell migration and potent inhibition of differentiation with no effect on proliferation in skeletal muscle. Increased cell proliferation, survival and fusion of cardiomyocytes.
(Pedersen and Febbraio, 2008, Kocher et al., 2006)	IL-8	Increases angiogenesis. Potentially induces myocardial neovascularization and protection against cardiomyocyte apoptosis.
(Shireman et al., 2007, Tarzami et al., 2005, Yahiaoui et al., 2008)	Monocyte chemoattractant protein (MCP)-1/ Chemokine (C-C motif) ligand (CCL2)	Important for skeletal muscle regeneration. Directly increases skeletal myoblast proliferation and inhibits differentiation. Protects cardiomyocytes from apoptosis.
(Xiao et al., 2016)	MCP-2 (CCL8)	May potentially play a role in skeletal myoblast differentiation.
(Yahiaoui et al., 2008, Weinreuter et al., 2014)	Macrophage inflammatory	Directly increases skeletal myoblast proliferation. Pro-inflammatory after hypoxic conditions.

	protein MIP-1α (CCL3)	
(Yahiaoui et al., 2008)	MIP-1β (CCL4)	Directly increases skeletal myoblast proliferation.
(Yahiaoui et al., 2008, Medeiros et al., 2009)	RANTES (CCL5)	Directly increases skeletal myoblast proliferation. Increases cardiomyocyte migration and reduced heart tissue damage.
(Barbosa-Souza et al., 2011, Duerr et al., 2014)	Osteopontin	A pro-fibrotic factor in skeletal muscle and myoblasts. Cardio-protective effects with reduced fibrosis.
(Jin et al., 1991, Vantler et al., 2010)	PDGF-BB	Platelet-derived growth factor-BB stimulates growth and inhibits differentiation of skeletal myoblasts. PDGF-BB does not stimulate proliferation or hypertrophy of cardiomyocytes and is anti-apoptotic.
(Brzoska et al., 2015, Liehn et al., 2011)	Stromal cell-derived factor 1 (SDF-1α)/ (CXCL12)	Increased skeletal muscle regeneration through upregulation of CD9-mediated myoblast fusion. Involved in cardiac-homeostasis after injury.
(Bajaj and Sharma, 2006, Zhao et al., 2015)	Tumour necrosis factor (TNF-α)	TNF-α inhibits myogenic differentiation of C2C12 cells through NF-kB. Apoptosis and hypertrophy in cardiomyocytes.
(Baker et al., 2008, Chan et al., 2015)	Thrombopoietin (TPO)	TPO administration upregulates myocardio-protection *in vitro* and *in vivo.* Cardiomyocyte survival.
(Sassoli et al., 2012)	VEGF-A	VEGF induces skeletal myoblast proliferation. VEGF promotes cardiomyocyte migration.
(Rissanen et al., 2003, Wong et al., 2011)	VEGF-D	A potent angiogenic factor. Induces endothelial permeability and is overexpressed in cardiac adipogenic problems.

5.4.8 Cellular stresses have been associated with platelet releasate.

PRP has previously been shown to have the capacity of reducing oxidative stress and increasing the levels of enzymatic antioxidants in skeletal muscle contusions (Martins et al., 2016). There is currently limited data with regards to the levels of reactive oxidation species in skeletal myoblasts after platelet releasate treatment. Therefore it was important to assess the cellular stresses associated with platelet releasate through Dihydroethidium (DHE) (**Figure 5.8A**). Additionally the anti-inflammatory interleukin,

IL-10, was not found to be upregulated at the protein-expression level for platelet releasate treatment in C2C12 myoblasts (**Figure 4B**).

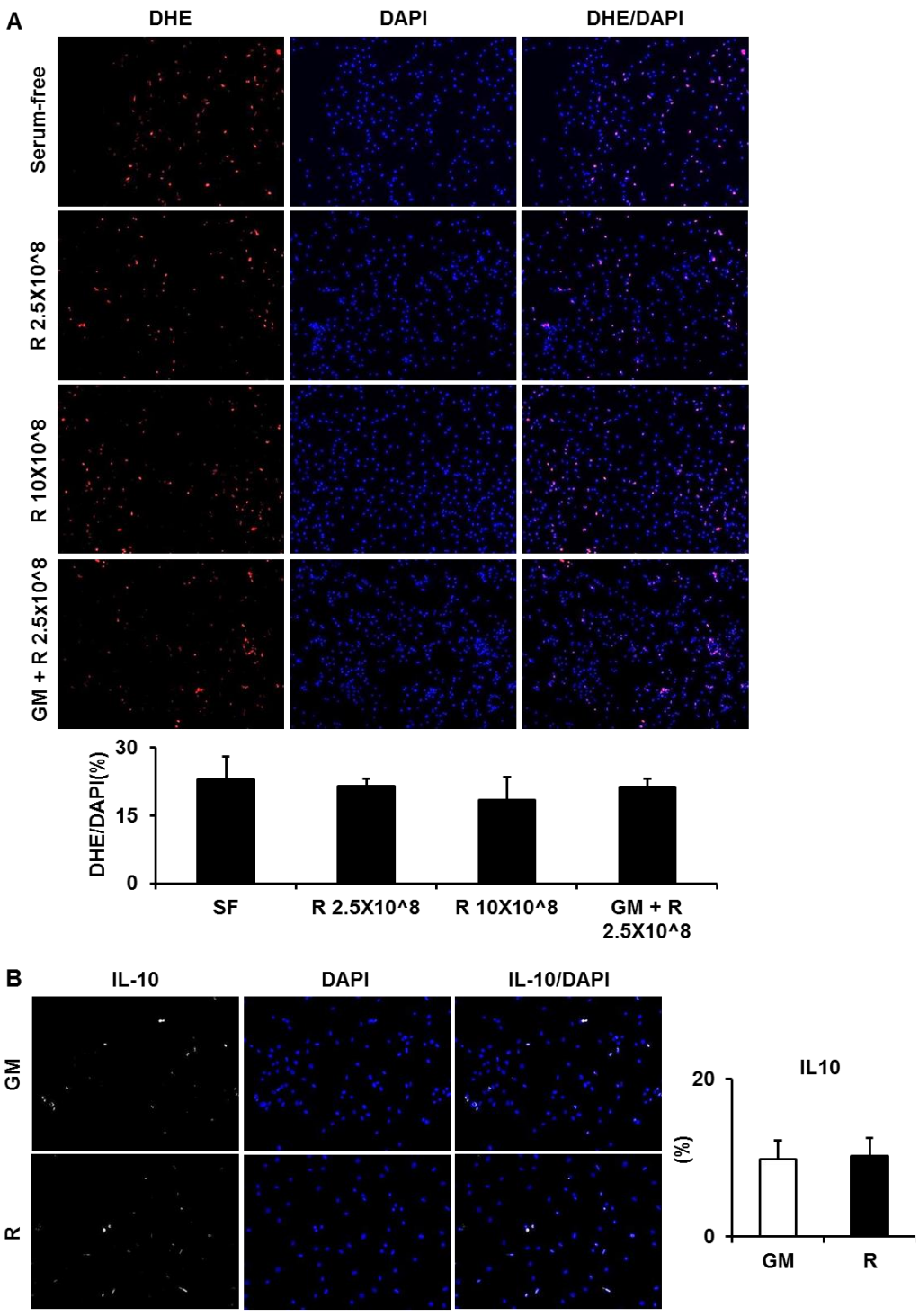
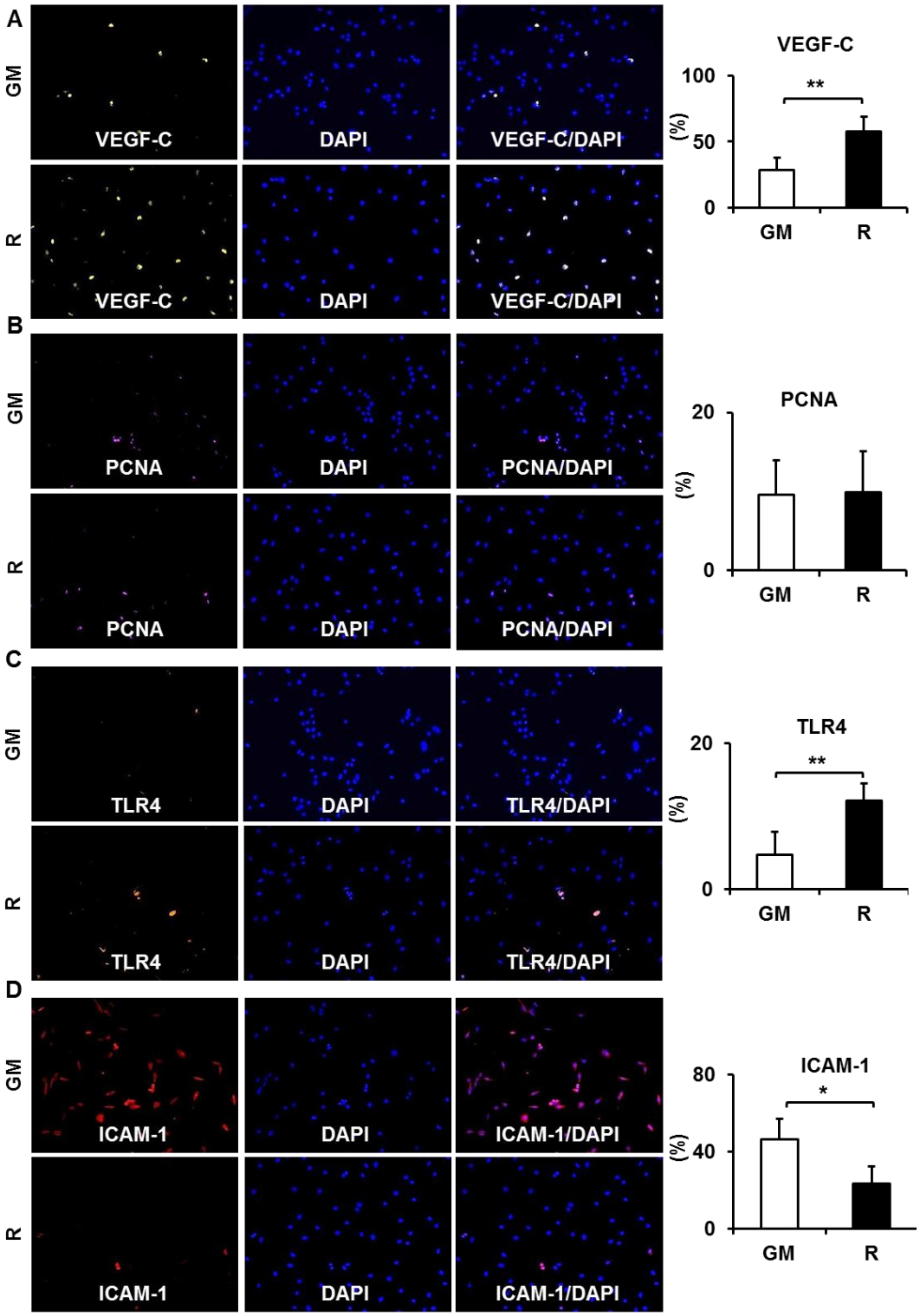


Figure 5.8 Physiological and supra-physiological Platelet releasate do not increase ROS above serum-free conditions. (**A**) Representative images and quantitative data of proliferating cells subject to serum-free, 10% platelet releasate made using $2.5x10^8$ platelets/mL, 10% platelet releasate made using $10x10^8$ platelets/ mL, or 10% FBS and 10% releasate made using $10x10^8$ platelets/ mL for 24 hours stained with DHE and co-stained with DAPI. (**B**) Representative images and

quantitative data of proliferating C2C12 cells in response to growth medium (GM) or platelet releasate (R; $2.5x10^8$ platelets/mL) stained with IL-10 and DAPI. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test for (**A**), and (**B**) was performed by a Student's *t*-test. n=3 independent experiments with 3 technical replicates per experiment.

5.4.9 Platelet releasate drives VEGF-C protein synthesis and alters factors associated with skeletal myoblast differentiation.

As *VEGFα165* gene expression was increased with the addition of platelet releasate, it is important to assess other variations of the VEGF family in which act through separate receptors to VEGFα (i.e. Fms-related tyrosine kinase 4; FLT4). VEGF-C acts through FLT4 and Fetal Liver Kinase 1 (FLK1) or VEGFR3 and VEGFR2 respectively. VEGF-C was increased with platelet releasate in serum-rich conditions as compared to growth medium culture (**Figure 5.9A**). Additionally, PCNA protein expression was found to not be affected by platelet releasate (**Figure 5.9B**). Tole-like receptor 4 (TLR4; associated with decreased myoblast differentiation) had increased expression in C2C12 myoblasts when subject to platelet releasate (Ono and Sakamoto, 2017). Additionally, Intercellular Adhesion Molecule-1 (ICAM-1) has been associated with skeletal myoblast fusion (Goh et al., 2015). Platelet releasate significantly reduced the expression of ICAM-1 which may play a role in the inhibited myoblast fusion seen in **Chapter 3**. Notably, the agonists used in this study did not increase skeletal myoblast proliferation when added directly to serum-free media (**Figure 5.10**).


A
GM
VEGF-C
DAPI
VEGF-C/DAPI
R
VEGF-C
DAPI
VEGF-C/DAPI
VEGF-C
100
50
0
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GM
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PCNA
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PCNA/DAPI
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20
0
(%)
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TLR4
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TLR4/DAPI
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GM
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ICAM-1
DAPI
ICAM-1/DAPI
R
ICAM-1
DAPI
ICAM-1/DAPI
ICAM-1
80
40
0
(%)
*
GM
R

Figure 5.9

Figure 5.9 Proliferation and fusion protein expression in platelet releasate-treated myoblasts. Growth medium- (10% FBS) and platelet releasate- (10% 2.5x10^8 platelets/mL) treated C2C12 myoblasts were stained for (**A**) VEGF-C (**B**) PCNA (**C**) TLR4 and (**D**) ICAM-1 co-stained with DAPI. The percentage of co-stained cells as compared to total cell (DAPI) count was assessed. Statistical analysis was performed by a Student's *t*-test. Differences are *p<0.05, **p<0.01.

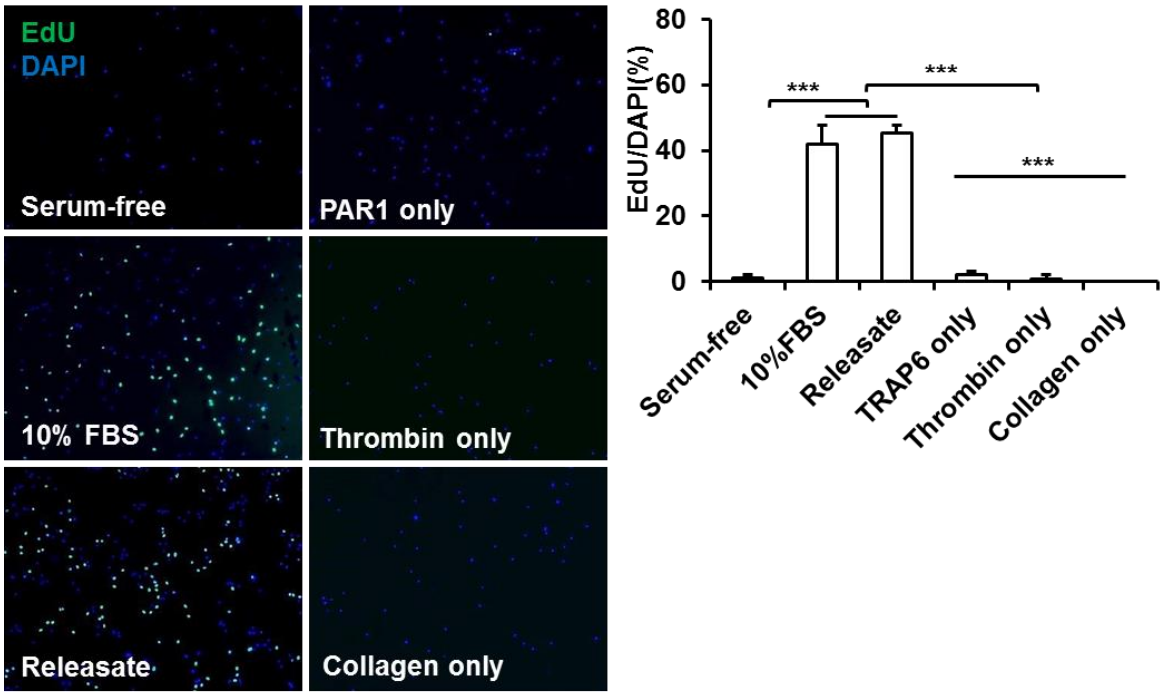

Figure 5.10 Platelet agonists alone do not affect proliferation of myoblasts. Representative images and quantitative data of proliferating C2C12 cells subject to serum-free, 10% FBS, 10% platelet releasate made using 2.5x10^8 platelets/mL or platelet agonists; TRAP6, Thrombin or collagen for 21 hours and stained with EdU for 3 hours, co-stained with DAPI. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. n=3 independent experiments with 3 technical replicates per experiment. Differences are ***p<0.001.

5.4.10 Platelet releasate interferes with Doxorubicin-induced cellular senescence.

Recent evidence had shown that platelet lysates and PRP positively affect various cell types and *in vivo* cardiomyocytes after doxorubicin associated damage (Morschbacher et al., 2016, Zaki et al., 2019, Refolo et al., 2014). In light of this, C2C12 myoblasts and H9C2 cardiomyocytes were subject to 0 – 0.5µM Doxorubicin dosages with or without 10% platelet releasate (10x10^8 platelets/mL). There is a significant loss of proliferation

with higher concentrations of Doxorubicin, and with 0, 0.0625 and 0.125µM; C2C12 cells have more proliferation ($p<0.01$) with platelet releasate than with normal culture medium (**Figure 5.11A**). Similarly, H9C2 cells how a significant increase in proliferation in platelet releasate treated cells when subject to 0 and 0.0625µM doxorubicin ($p<0.001$) (**Figure 5.11B**). These results indicate a loss of cellular senescence for both myoblast types under the influence of platelet releasate treatment.

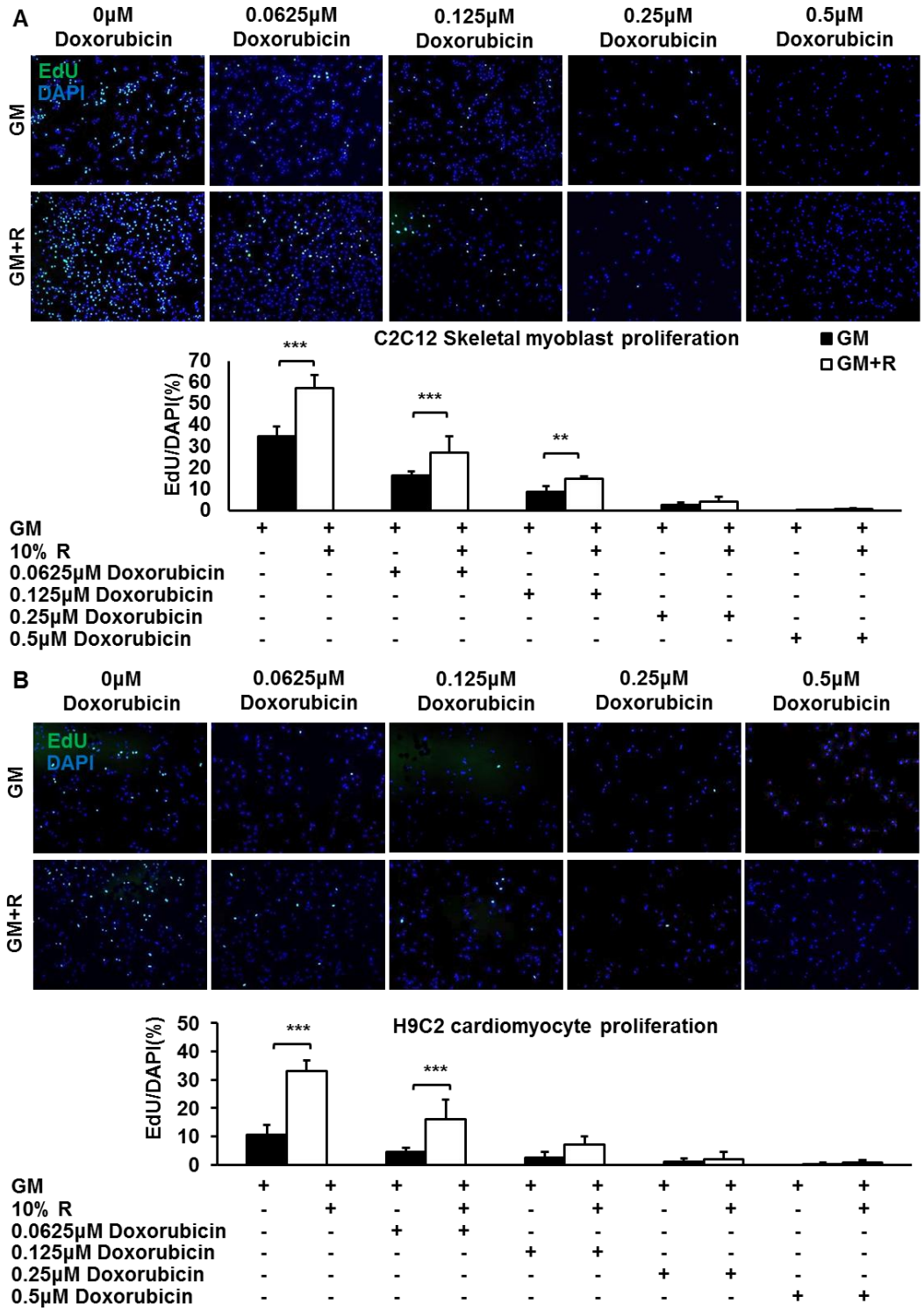

Figure 5.11 Platelet releasate interferes with Doxorubicin-induced cellular senescence. EdU proliferative live-staining was conducted for 3-hours after 21 hour incubation totalling 24-hours of proliferation in growth medium (10% FBS) +/- platelet releasate with 0, 0.0625, 0.125, 0.25 or 0.5μM Doxorubicin. (**A**) Representative images and quantitative data for C2C12 cells stained for EdU and DAPI co-staining. (**B**)

Representative images and quantitative data for H9C2 cells stained for EdU and DAPI co-staining. (x5 magnification). All releasate was made with $10x10^8$ platelets/mL. Statistical analysis was performed by two-way ANOVA followed by Tukey's post-hoc test. Differences are *p<0.05, **p<0.01 and ***p<0.001.

5.4.11 Doxorubicin-induced satellite cell senescence is reversed with platelet releasate treatment.

EDL and BB single muscle fibres were then stained for MyoD and Pax7 in order to profile the function of satellite cells during proliferation during a dose-response of Doxorubicin-induced cellular senescence (48hours). There was a significant increase in the percentage of satellite cells stained positive for Pax7 and negative for MyoD in response to higher doses of Doxorubicin (i.e. quiescent satellite cells). This quiescent response was significantly reversed with platelet releasate treatment (**Figure 5.12A-B**). Similarly, there was a dose-dependent maintenance of satellite cell number per fibre when cultured with platelet releasate when compared to growth medium with 0 to 0.5µM doxorubicin senescent dose-response (**Figure 5.12C**). Doxorubicin is a fluorescent molecule with an emission of 594nm (Karukstis et al., 1998). The intensity of infiltration into the myonuclei of single muscle fibres was analysed for growth medium +/- platelet releasate when cultured with a 0-0.5µM dose-response of Doxorubicin. There was a significantly lower (p<0.001) number of stained myonuclei with a lower intensity of positive nuclei after treatment with platelet releasate (**Figure 4.D**). Taken together, this data indicates that there is a decrease in cellular senescence of satellite cells when treated with platelet releasate, and a protective effect with myonuclei of the muscle fibres.

Figure 5.12 Platelet releasate interferes with Doxorubicin-induced cellular senescence. (**A**) A schematic depicting the experimental setup methodology. (**B**) Expression for quiescence (Pax7^{+ve}/MyoD^{-ve}), proliferation (Pax7^{+ve}/MyoD^{+ve}) and commitment to differentiation (Pax7^{-ve}/MyoD^{+ve}) was assessed for satellite cells after T48 hours of culture with a 0 to 0.5µM Doxorubicin dose-response +/- platelet releasate (10x10^{8} platelets/mL). (**C**) The number of satellite cells per fibre after a 48 hour treatment. (**D**) The percentage of myonuclei with Doxorubicin infiltration per fibre. Statistical analysis was performed by two-way ANOVA followed by Tukey's post-hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #p<0.05 *vs.* GM-R.

5.5 Discussion

To date, platelet-based applications have gained a lot of attention for regenerative purposes in a variety of applications such as musculoskeletal injuries, skin, bone, nerve, liver and other conditions. However, with regards to the use of autologous platelets for the regeneration of skeletal and heart muscle; the literature has been more stringent in accepting its potential benefits. This may be due to clinical trials reporting no advantageous outcomes over current treatment methods (Mosca and Rodeo, 2015). This thesis has shown that potent myoblast proliferation was stimulated by optimising the platelet-preparation method, using platelet releasate devoid of plasma and cellular debris made with the TRAP-6 platelet agonist. For this reason, this method of preparing platelet releasate was applied to additional cell types to test if it was commonly beneficial for skeletal myoblasts and cardiomyocytes.

5.5.1 The effect physiological platelet releasate on skeletal and cardiac myoblasts

First, the effect of physiological platelet releasate on the proliferation of skeletal and cardiac myoblasts was determined. To this end, C2C12 cells were used, with a respectively fast proliferative rate, as a positive control as described in **Chapter 3**, where proliferation was elevated with the application of releasate. However in a slower proliferating cell line; cardiomyocytes, Cyclin D1, Ki-67 and live EdU staining for proliferation were all markedly lower than the growth media group, showing similar levels to serum-free conditions. Indeed, this reinforces the notion that cardiomyocytes are a slow-proliferating cell line and difficult to stimulate. Cardiac conditions such as exercise-induced silent myocardial ischemia have proven difficult to both diagnose and treat (Stern, 2005). However major studies have been conducted to combat cardiac conditions, such as the application of platelet-based applications delivering growth factors to damaged heart tissue (Gallo et al., 2013, Hargrave and Li, 2012, Hargrave et al., 2016, Li et al., 2008, Mishra et al., 2011, Morschbacher et al., 2016, Patel et al., 2016, Spartalis et al., 2015, Sun et al., 2014, Tang et al., 2017, Vu et al., 2015). Promising *in vitro* and *in vivo* data was generated by Hargrave et al., regarding PRP on the ischemic heart; however they held concerns about clotting factors in the platelet

preparation (Hargrave and Li, 2012, Hargrave et al., 2016). Therefore, it was hypothesised that using platelet releasate, without the clotting factors associated with plasma, was an optimal candidate to target the slowly proliferating cardiomyocytes in circumstances such as myocardial ischemia. Previous experimental evidence for the effect of these cytokines and growth factors analysed in human platelet releasate has been studied on skeletal and cardiac muscle as outlined in **Table 5.1**.

5.5.2 Growth factors contained in both physiological and supra-physiological releasate

Supra-physiological releasate had a higher concentration of growth factors, analytes in higher abundance and cytokines compared to physiological releasate. PECAM-1 and P-selectin were the most abundant components of the releasate which have been shown to exhibit anti-apoptotic, pro-angiogenic and regenerative functions in different cell types (see **Table 5.1**). Our data on the composition of platelet releasate revealed that known key growth factors driving myoblast and/or chondrocyte proliferation such as PDGFβ and VEGFα were increased several-fold in supra-physiological releasate (Kieswetter et al., 1997). VCAM-1 was previously shown to increase cardiomyocyte proliferation and skeletal myoblast differentiation and was 3.8-fold more abundant in supra-physiological releasate compared to physiological releasate (Choo et al., 2017, Iwamiya et al., 2016). Similarly, growth factors driving fibroblast proliferation such as EGF and FGF-2 were increased 1.92- and 3.43-fold respectively (Yu et al., 2012). Importantly, TGFβ, a growth factor that can induce fibrosis in muscle regeneration, and inhibits keratinocyte proliferation was reduced by 50% in supra-physiological releasate (Li et al., 2016b, Yang et al., 1996).

5.5.3 The effect of supra-physiological platelet releasate on skeletal and cardiac myoblasts

As physiological platelet releasate failed to stimulate H9C2 proliferation, supra-physiological releasate was used to stimulate proliferation. This observation was made previously on skeletal myoblasts; where increased platelet concentration in the releasate preparation correlated strongly with enhanced proliferation. Here it is shown

for the first time, novel evidence that supra-physiological releasate indeed enhanced H9C2 proliferation to the same levels as growth medium, with a v/v dose-dependent response. An increase in proliferative capacity was also observed in C2C12s treated with supra-physiological releasate as compared to physiological releasate.

5.5.4 How platelet releasate affects cardiac myoblasts on a genetic and protein level

H9C2 cellular proliferation is in part mediated through VEGF signalling (Chen et al., 2018). For this reason, qPCR was conducted to show the transcription levels of *Vegf* and translation into protein through immunohistochemistry in cardiomyocytes after application of platelet releasate. Supra-physiological releasate increased *Vegfα165*, *Vegfr* and *Cyclind1* mRNA expression in both serum-free and serum-rich conditions, indicating an increase in H9C2 cellular proliferation. Additionally, there was increased VEGF protein expression in both C2C12 and H9C2 cells. Notably, *Igf1* mRNA was reduced in all groups versus serum-free, indicating a cell cycle progression and a subsequent differentiation suppression at the proliferative stage of growth (Smith et al., 1999). Therefore, it is surmised that cardiomyocyte proliferation is being driven, at least in part, through the VEGF pathway.

5.5.5. Effects of temperature on platelet releasate

A single freeze-thaw cycle of platelet releasate did not diminish the proliferative effects it had on C2C12 myoblasts. Although a single freeze-thaw cycle was shown to significantly decrease growth factors in PRP, it is shown here that, much like FBS, platelet releasate can retain its proliferative properties on myoblasts under short-term storage over a variety of temperatures (Hosnuter et al., 2017). This data suggests that platelet releasate is a robust cocktail of growth factors in which can maintain its effectiveness after a freeze-thaw cycle.

5.5.6 Platelet releasate is not involved in cellular reactive oxygen species during proliferation

Platelet-based applications have previously been shown to have the capacity of reducing oxidative stress and increasing the levels of enzymatic antioxidants in skeletal muscle contusions (Martins et al., 2016). IL-10 has previously been shown to be beneficial for skeletal muscle regeneration through the macrophage switch in phenotype and had inhibited H9C2 hypertrophy which may improve heart function (Dagdeviren et al., 2017, Deng et al., 2012, Verma et al., 2012). However, there was not shown to be an altered myoblast oxidative stress in response to platelet releasate in this thesis. Further to this, platelet releasate was found to drive VEGF-C protein synthesis, and factors associated with skeletal myoblast differentiation are altered with platelet releasate; supporting the inhibited differentiation and fusion found in **Chapter 3**.

5.5.7 Platelet releasate interferes with Doxorubicin-induced cellular senescence

It has previously been suggested that age-related alterations in the capillary bed may negatively affect muscle mass (Degens and Korhonen, 2012). Additionally, a recent study reported that the receptor of IL8; CXCR2, has a senescence-associated phenotype of injured tissue in wound healing. It is shown in this thesis that IL-8 is increased in supra-physiological releasate. The recent study reported a blockade of CXCR2 using SB265610 accelerated wound healing (Wilkinson et al., 2019). It would be interesting to see if this molecule could accelerate skeletal muscle regeneration in the presence of supra-physiological platelet releasate.

Proliferation was previously shown to be inhibited through doxorubicin administration, causing cell cycle arrest and senescence; however the VEGF pathway has been shown to reverse this effect (Chen et al., 2018). It was shown in **Chapter 3** that VEGF from platelet releasate acts strongly and dose dependently on C2C12 proliferation. Furthermore, Doxorubicin's effect on myoblast proliferation has been shown to act through several key pathways such as NAD-dependent deacetylase sirtuin-1 (SIRT1), Mitogen-activated protein kinase (MAPK), the aforementioned VEGF pathway and ROS (Sin et al., 2016, Jian et al., 2017, de Lima Junior et al., 2016, Gilliam et al., 2012,

Chen et al., 2018). Importantly, platelet-based applications have been shown to affect cellular signalling in all of these pathways (Liu et al., 2014, Martins et al., 2016, Milasincic et al., 1996, Chen et al., 2018).

Platelet releasate does not increase the differentiation of C2C12 myoblasts at human-physiological levels. However, in the current study, it was shown that there was a beneficial effect of supra-physiological releasate on C2C12 differentiation (total cell number, Myogenin^{+ve} cells and the total myotube number). This study was first to show that H9C2 cells treated with supra-physiological releasate have increased total cell numbers and Myogenin^{+ve} cell counts. Additionally, when H9C2 cells are treated for a longer period of time in differentiation media; the additive effect of platelet releasate with 10% FBS was more pronounced resulting in greater Myogenin expression, higher cell numbers, a greater myofusion index and myotube number. One can speculate that this additive effect is due to a higher total cell number in the proliferation period, leading to more cells to undergo differentiation as a whole.

5.6 Conclusion

Aiding the regenerative potential of different tissues is particularly challenging, due in part to the unique proliferative capacity of their cells. As platelet-based applications can be customised for tissue-specific efficacy, this makes them strong candidates for developing innovative regenerative therapies. This study provides novel insights into the role of platelet releasate on C2C12s, and H9C2s and suggests an optimised preparation method to maximise the proliferative/differentiative response for potential regeneration. This study highlights the benefit of concentrating the composition of the platelet secretome for optimal cell-type targeted applications in regenerative medicine. Here, it is shown for the first time that supra-physiological releasate either significantly improves proliferation and/ or differentiation of various cell types. Finally, platelet releasate was shown to interfere with Doxorubicin-induced cellular senescence. In summary, the data in this thesis shows that standardising the concentration of platelets for therapeutic use may be a key factor determining varied results surrounding clinical success.

Chapter 6

General Discussion

6.1 Overview

Platelet-based applications have recently gained attention as an effective way to promote skeletal muscle regeneration (Navani et al., 2017, Andia and Abate, 2018). However, clinical data are inconsistent and there is currently a limited understanding of the molecular events that govern skeletal myogenesis using platelet-based products as biomaterials due to largely varying protocols in platelet preparation (Li et al., 2016b, Mosca and Rodeo, 2015, Tsai et al., 2018a). In this thesis, the presented data suggest that platelet releasate from collagen and TRAP6, two widely used platelet agonists, have a stronger effect on myoblast proliferation as compared to thrombin or mechanically lysed platelets by sonication, independent of the final centrifugation speed used. This finding may potentially be explained by possible detrimental effects of proteases on releasate growth factors, as thrombin itself is a serine protease, as well as digestive enzymes released in the ablation of sonicated platelets (Duvernay et al., 2013, Zhao et al., 2014).

6.2 Variability in platelet releasate preparation

The concentration of platelets in platelet-based applications was previously recognised as a potentially important variable for the diverse and conflicting data of PRP or releasate on tissue regeneration (Tschon et al., 2011). The presented data in **Chapter 3** firstly indicated that there was a linear relationship between platelet concentrations used for the production of releasate and myoblast proliferation. Physiological platelet releasate stimulated skeletal myoblast proliferation, however in **Chapter 5**; data indicate that H9C2 cardiomyocytes did not proliferate when cultured with physiological releasate. Cardiac conditions such as exercise-induced silent myocardial ischemia have proven difficult to both diagnose and treat (Stern, 2005). However major studies have been conducted to combat cardiac conditions, such as the application of platelet-based applications delivering growth factors to damaged heart tissue (Gallo et al., 2013, Hargrave and Li, 2012, Hargrave et al., 2016, Li et al., 2008, Mishra et al., 2011, Morschbacher et al., 2016, Patel et al., 2016, Spartalis et al., 2015, Sun et al., 2014, Tang et al., 2017, Vu et al., 2015). Promising *in vitro* and *in vivo* data was generated by Hargrave et al., regarding PRP on the ischemic heart; however they held concerns

about clotting factors in the platelet preparation (Hargrave and Li, 2012, Hargrave et al., 2016). Importantly, supra-physiological platelet releasate had a higher concentration of growth factors, analytes in higher abundance and cytokines compared to physiological releasate. As physiological platelet releasate failed to stimulate H9C2 proliferation, supra-physiological releasate was used to stimulate proliferation. There was an enhanced rate of H9C2 proliferation to the same levels as growth medium, with a v/v dose-dependent response when cultured with supra-physiological releasate. An increase in proliferative capacity was also observed in C2C12s treated with supra-physiological releasate as compared to physiological releasate.

Collectively, despite a positive role of platelet releasate on myoblast proliferation, the data in this thesis suggests that methodological variations such as: i) platelet agonists, ii) amount of releasate administered, iii) species of platelet origin and iv) platelet concentration may account for some of the discrepancies among clinical studies.

6.3 Cardiomyocyte and skeletal myoblast mRNA and protein alterations during proliferation in response to platelet releasate

Gene and protein expression data from proliferating C2C12 myoblasts revealed a consistent significant increase in PDGF and VEGF in response to human releasate as compared to serum free conditions. The physiological effect of PDGF on cell growth and C2C12 myoblast proliferation has been recognised previously (Smith et al., 1999, Yablonka-Reuveni and Rivera, 1997). It has been shown that PDGF-BB promotes muscle stem cell proliferation but inhibits differentiation (Yablonka-Reuveni et al., 1990, Jin et al., 1991, Arsic et al., 2004). In turn, VEGF165α has been shown to enhance C2C12 myoblasts migration and exhibit anti-apoptotic effects (Germani et al., 2003). Higher mRNA levels of *Pax7, Myf5* and *Myod* in proliferating C2C12 myoblasts in response to releasate treatment may be part of a coordinated response of myogenic regulatory factors that are known to promote myogenic population expansion (Collins et al., 2009). This is in line with previous evidence showing failure to withdraw from the cell cycle and commit to differentiation in response to releasate use (Tsai et al., 2017). Similarly, H9C2 cellular proliferation is in part mediated through VEGF signalling (Chen

et al., 2018). For this reason, qPCR was conducted to show the transcription levels of *Vegf* and translation into protein through immunohistochemistry in cardiomyocytes after application of platelet releasate. Supra-physiological releasate increased *Vegfα165, Vegfr* and *Cyclind1* mRNA expression in both serum-free and serum-rich expression indicating an increase in H9C2s cellular proliferation. Additionally, there was increased VEGF protein expression in both C2C12 and H9C2 cells. Notably, Igf1 mRNA was reduced in all groups versus serum-free, indicating a cell cycle progression and a subsequent differentiation suppression at the proliferative stage of growth (Smith et al., 1999). Platelet-based applications have previously been shown to have the capacity of reducing oxidative stress and increasing the levels of enzymatic antioxidants in skeletal muscle contusions (Martins et al., 2016). IL-10 has previously been shown to be beneficial for skeletal muscle regeneration through a macrophage switch in phenotype and had inhibited H9C2 hypertrophy which may improve heart function (Dagdeviren et al., 2017, Deng et al., 2012, Verma et al., 2012). However, there was not shown to be altered myoblast oxidative stress in response to platelet releasate in this thesis. Further to this, platelet releasate was found to drive VEGF-C protein synthesis. In addition, factors identified to be associated with skeletal myoblast differentiation were altered with platelet releasate, supporting the inhibited differentiation and fusion found in **Chapter 3**. Therefore, it is surmised that both cardiomyocyte and skeletal myoblast proliferation is being driven, at least in part, through the VEGF pathway.

6.4 Growth factor composition of platelet secretomes

In **Chapter 3** and **Chapter 5** of this thesis, the growth factors in the human TRAP6-activated platelet secretome were quantified. The heatmaps of these factors suggests that their amount increases several-fold in platelet releasate resulting in two clusters of moderate and higher intensity respectively. Of note, the factors SCF, FGF-β, HGF, HER2, Follistatin, VEGFR-1, EGFR, PDGF-AB and PDGF-BB (known to regulate proliferation (Deasy et al., 2001, Milasincic et al., 1996, Walker et al., 2015, Kocamis et al., 2004, Wright et al., 2014, Bryan et al., 2008, Thornton et al., 2015, Leroy et al., 2013, Jin et al., 1991, Yablonka-Reuveni et al., 1990) and VEGFR2, IL-6, TIE-2 and

HB-EGF (known to induce differentiation (Bryan et al., 2008, Serrano et al., 2008, McClung et al., 2015, Horikawa et al., 1999, Chen et al., 1995) showed a 4-20-fold increase in the TRAP6-activated platelet releasate as compared to unstimulated platelets. EGF, FGF, VEGF and PDGF-BB have been shown to promote myoblast proliferation and consistently inhibit myogenic differentiation, while VEGFR2 expression is increased during differentiation (Wallaschofski et al., 2004, Bryan et al., 2008, Sassoli et al., 2012, Leroy et al., 2013, Milasincic et al., 1996, Jin et al., 1991). Moreover, SCF increases skeletal muscle stem cell number, IL-6 regulates myoblast proliferation and migration, while TNF-α inhibits myogenic differentiation in C2C12 cells through NF-κB and promotes myotube atrophy (Deasy et al., 2001, Serrano et al., 2008). Conversely, IGFBP-1, Angiopoietin-2 and uPA have been shown to promote myogenic differentiation (Milasincic et al., 1996, Mofarrahi and Hussain, 2011, Suelves et al., 2002). Of note, it cannot be ruled out that other growth factors (not included in the Bioplex kits used in this study) contained in the platelet releasate may potentially affect myogenesis. For this reason, a second growth factor analysis (Luminex kits) was implemented in **Chapter 5** on supra-physiological releasate. Supra-physiological releasate had a higher concentration of growth factors, analytes in higher abundance and cytokines compared to physiological releasate. PECAM-1 and P-selectin were the most abundant components of the releasate which have been shown to exhibit anti-apoptotic, pro-angiogenic and regenerative functions in different cell types (see **Table 5.1**). Our data on the composition of platelet releasate revealed that known key growth factors driving myoblast and/or chondrocyte proliferation such as PDGFβ and VEGFα were increased several-fold in supra-physiological releasate (Kieswetter et al., 1997). VCAM-1 was previously shown to increase cardiomyocyte proliferation and skeletal myoblast differentiation and was 3.8-fold more abundant in supra-physiological releasate as compared to physiological releasate (Choo et al., 2017, Iwamiya et al., 2016). Similarly, growth factors driving fibroblast proliferation such as EGF and FGF-2 were increased 1.92- and 3.43-fold respectively (Yu et al., 2012). Importantly, TGFβ, a growth factor that can induce fibrosis in muscle regeneration was reduced by 50% in supra-physiological releasate (Li et al., 2016b). Taken together this data suggests that

the numerous growth factors, cell cycle regulators and transcription factors present in platelet releasate can be used to promote skeletal myogenesis.

Two growth factors that are important in skeletal muscle function and maintenance that were found in high abundance in the platelet releasate secretome were PDGF and VEGF. An independent dose-dependent decrease of skeletal myoblast proliferation and differentiation for both PDGFR and VEGFR inhibitors was found with the use of platelet releasate. These findings suggest that the powerful proliferative effect of platelet releasate on C2C12 myoblasts was, at least in part, mediated through PDGF and VEGF signalling. The proliferative effect of releasate is abolished by the highest dosage of PDGF or VEGF inhibitors. This may possibly be due to a crosstalk with other growth factor receptors present in the releasate such as EGFR when used in high concentrations (Manley et al., 2002). This concept is strengthened by previous studies showing that PDGF and VEGF inhibition resulted in diminished myoblast proliferation and recombinant PDGFs improved skeletal muscle recovery after injury (Sugg et al., 2017, Jin et al., 1991, Yablonka-Reuveni et al., 1990, Sassoli et al., 2014, Arsic et al., 2004, Bryan et al., 2008, Germani et al., 2003, Li et al., 2013).

6.5 Platelet releasate affects cardiac and skeletal myoblast differentiation

In contrast to myoblast proliferation, the use of platelet releasate during myogenic differentiation seemed to be more complex. It is shown here that on one hand; treatment of myotubes with releasate during terminal differentiation promotes myogenic differentiation (i.e. Myogenin^{+ve} cells and myotube number). On the other hand, administration of platelet releasate throughout both proliferation and differentiation of C2C12 myoblast culture resulted in attenuated differentiation based on largely impaired *Myogenin, Mhc1, Mhc2a, Mhc2b, Acta1, Tmem8c, Bex1, Igf-1* mRNA, Myogenin protein expression, myotube number, myofusion index, myotube length and thickness. This finding was in line with recent studies using platelet-rich plasma on similar cultures (Miroshnychenko et al., 2017, Saury et al., 2018, Kelc et al., 2015). This is indicative of a failure to withdraw from the cell cycle, supported by the increased nuclei number. Importantly, evidence is provided that platelet releasate is beneficial for myogenic differentiation leading to higher Myogenin expression and

larger myotubes, when it is administered after the early differentiation phase, where myoblast fusion into myotubes has started. Taken together, these data suggest that the timing of application of releasate to C2C12 myoblasts or myotubes is crucial and may account for discrepancies in the literature over whether releasate obstructs or improves differentiation (Denapoli et al., 2016, Dimauro et al., 2014, Mosca and Rodeo, 2015). Similarly, Platelet releasate did not increase the differentiation of C2C12 myoblasts at human-physiological levels. However, it was shown in **Chapter 5** that there was a beneficial effect of supra-physiological releasate on C2C12 differentiation (total cell number, Myogenin^{+ve} cells and the total myotube number). In line with this, H9C2 cells treated with supra-physiological releasate had increased total cell numbers and Myogenin^{+ve} cell counts. Additionally, when H9C2 cells were treated for a longer period of time in differentiation media; the additive effect of platelet releasate with 10% FBS was more pronounced resulting in greater Myogenin expression, higher cell numbers, a greater myofusion index and myotube number. One may speculate that this additive effect is due to a higher total cell number in the proliferation period, leading to more cells to undergo differentiation as a whole.

6.6 How platelet releasate effects primary satellite cells on single muscle fibres

It was important to study the proliferation and differentiation effects of platelet releasate administration on satellite cells of single myofibres *ex vivo* after having established the *in vitro* effects. The application of platelet releasate resulted in a significant increase of Pax7^{-ve}/MyoD^{+ve} muscle stem cells in a dose-dependent manner. These findings represent an increased commitment of proliferating muscle stem cells to differentiation via MyoD^{+ve} cells per total myonuclear number. Platelet releasate increased the committed muscle stem cells to differentiation which was evident for both serum free and standard fibre growth conditions, indicating that releasate may act as an appropriate substitute for the regular fibre growth medium. To this end, several studies have aimed to replace bovine serum by autologous platelet-based applications (Saury et al., 2018, Li et al., 2013).

Upregulation of MyoD mRNA and protein in rat skeletal muscle stem cells by PRP has been shown previously on muscle sections, however novel evidence is provided in this

thesis that this upregulation is taking place with the concomitant downregulation of Pax7 which results in higher number of stem cells following the differentiation pathway to support myofibre regeneration (Zammit et al., 2006b, Dimauro et al., 2014). Notably, the effect of platelet releasate on MyoD is reversed by PDGFR and VEGFR inhibition, possibly indicating a common functional pathway between these factors. Importantly, the addition of PDGF-BB decreases myoblast fusion, however it upregulates MyoD during myogenesis (Yablonka-Reuveni and Rivera, 1997). Additionally, the application of platelet releasate resulted in a robust increase of Cyclin D1 and Scrib expression on muscle stem cells. Cyclin D1, driven by platelet releasate, is known to regulate the cell cycle and is important for cell proliferation (Tsai et al., 2017). Scrib is a significant regulator of myogenic progression after commitment to differentiation that dictates muscle stem cell fate and is indispensable for muscle repair (Ono et al., 2015).
It has been claimed that the beneficial effects of platelet-rich plasma on myogenesis can be brought about by the plasma alone (Miroshnychenko et al., 2017). However, this possibility can be ruled out, since the data in this thesis indicates the use of platelet-poor plasma does not mimic the findings on muscle stem cells observed with platelet releasate. Beyond that, platelet preparations were conducted with washed platelets in modified tyroid's buffer devoid of plasma throughout this study.

As seen in **Figure 3.9** and in **Figure 5.2**; there are particularly high levels of PDGF in platelet releasate produced by using TRAP6 as an agonist. Despite previous evidence on compromised myogenic differentiation in response to PDGF administration; it is found here that platelet releasate was beneficial for muscle stem cell differentiation (Yablonka-Reuveni and Rivera, 1997). It is reported in this thesis that platelet releasate on single muscle fibres leads to significantly higher expression levels of Myogenin in differentiated muscle stem cells, significantly higher stem cell progeny and total stem cell numbers as well as number of stem cell clusters per fibre. Isolated primary skeletal muscle stem cells have an increased number, fusion index and myotube number when co-cultured with platelet releasate and growth medium simultaneously; mitigating the negative effects that releasate has on differentiation alone. Altogether these findings provide novel evidence on a powerful effect of platelet releasate on muscle stem cell

proliferation and commitment to differentiation through the PDGF/VEGF signalling pathways (**Figure 3.18D**).

6.7 Bioenergetic effects of platelet releasate on skeletal myoblasts

In order to obtain insights in the metabolic homeostasis of proliferating myoblasts treated with platelet releasate, the oxygen consumption rate was measured. It was found that administration of releasate resulted in significantly higher spare oxidative capacity in isolated muscle stem cells under either serum free or serum rich conditions. This finding indicates that platelet releasate may alter metabolic homeostasis of muscle stem cells, which is important for myogenesis and most importantly for muscle regeneration. This observation is supported by previous studies showing that cardiac myoblasts (i.e. H9C2 cells) had an increased respiratory reserve capacity in response to platelet-rich plasma (Hargrave et al., 2016).

6.8 Cardiotoxin-induced skeletal muscle injuries in WT and TSP-1 KO mice treated with platelet releasate

After delivering an acute damage to skeletal muscle tissue, regenerating fibres are reliant on a number of cell types that coordinate their activity to reconstitute a working muscle (Harris, 2003). Macrophages and angioblasts are important in skeletal muscle regeneration as well as primarily the resident stem cell (satellite cell) population which regenerate most, if not all, the muscle cells that eventually fuse to form muscle fibres (Collins et al., 2005, Latroche et al., 2017, Munoz-Canoves and Serrano, 2015). In **Chapter 3**, it is shown that platelet releasate promotes muscle regeneration following cardiotoxin injection, evidenced by the presence of larger newly formed muscle fibres. This outcome is in keeping with *in vitro* data in this thesis showing that platelet releasate impacts on the proliferation and differentiation of myoblasts.

Thus far, the observed accelerated regeneration is postulated to be a consequence of direct impact of the releasate on satellite cells. What's more, an additional possibility is that angiogenic factors in the releasate could act on satellite cells indirectly such that they firstly promote angiogenesis in the regenerating tissue. Angiogenesis is a major factor in muscle regeneration and can even compensate to a large decrease in muscle

stem cells and still deliver robust regeneration (Omairi et al., 2016). It is shown here that the number of dying fibres was greatly reduced by platelet releasate administration; a process controlled by macrophages.

After observing that platelet releasate administration had increased skeletal muscle angiogenesis after injury in **Chapter 3**, an ideal way of further testing the application of platelet releasate was on the TSP-1 null mouse model – a negative regulator of angiogenesis (Audet et al., 2013, Isenberg et al., 2007a). Previously, TSP-1 deletion enhanced exercise capacity and reduced chronic ROS production (Malek and Olfert, 2009, Audet et al., 2013). Notably, TSP-1 is expressed by skeletal muscle and is a constituent of TRAP-6-activated platelet releasate. Critical leg ischemia in TSP-1 null mice has significantly greater regenerating myofibres as compared to wild-type (Brechot et al., 2008). Data presented in this thesis is in line with Brechot et al. such that necrosis is further reduced in TSP-1 KO mice after injury potentially due to the increase in capillarisation of the injured skeletal muscle. Both the addition of platelet releasate and the absence of TSP-1 independently increase angiogenesis in the injured areas. Interestingly, there was no additive angiogenic effect in the area of injury when injecting platelet releasate into TSP-1 KO mice. This may be explained through the re-introduction of TSP-1 into the KO model *via* platelet releasate. Such that the crosstalk between TSP-1 and VEGF signalling *via* the CD36 fatty acid receptor may provide clarity on this observation (Chu et al., 2013). Importantly, when comparing between human and mouse platelet secretome proteomics, TSP-1 seems to be more abundant in mouse platelets when normalised to total proteins (Maynard et al., 2007, O'Connor et al., 2010).

Platelet releasate has been shown to accelerate wound healing, reduce necrosis and decrease CD68-positive macrophage staining, with an increased tetanic strength of injured muscle (Tsai et al., 2018b). PRP had also been shown to drive MyoD and Myogenin expression in skeletal muscle 5 days after injury (Dimauro et al., 2014). This thesis builds upon this data as it is shown for the first time that there is an additive effect on the number of $MyoD^{+ve}$ satellite cells in the injured TSP-1 KO mouse skeletal muscle after administering platelet releasate. Finally, TSP-1 KO mice had shown a

reduced number of Myogenin^{+ve} satellite cells in the area of injury after 10 days, which is ameliorated by platelet releasate.

Platelet releasate alters gene expression and capillarisation of non-injured skeletal muscle. Until now, only one finding had studied the effects of platelets in uninjured skeletal muscle (Fukuda et al., 2017). In line with this, the data in this thesis suggest downregulated *Mhciix* and *Mhciib* mRNA, which complement the upregulated *Mhxi* expression shown in the Fukuda paper. A novel finding is that there was an increase in *Vegfα165* and a decrease in *Mstn* mRNA potentially indicating a hypertrophic effect of the skeletal muscle fibres. By depleting TSP-1 from platelet releasate, this may be a way of further optimising the platelet-based applications in clinical use, for enhanced skeletal muscle recovery. However, it was also shown that TSP-1 is important for myoblast differentiation *via* the addition of human releasate to TSP-1 KO myoblasts. TSP-1 KO platelet releasate is beneficial for myoblast proliferation *in vitro*. TSP-1 KO releasate enhances satellite cells progeny at T48 and T72 in both WT and TSP-1 KO fibres. Furthermore, TSP-1 KO fibres cultured *ex vivo* exhibit higher satellite cell progeny at T48 and T72 independent of platelet releasate administration. This novel data is important as there is a need for an autologous medium when culturing skeletal muscle stem cells where they solely proliferate and not differentiate (Ito et al., 2016).

6.9 TSP-1 Single fibres

Unexpectedly, when culturing the primary satellite cells off the myofibres, the aforementioned satellite cell proliferative effect was not evident between TSP-1 KO and WT mice in response to human releasate. Moreover, a study on TSP-1 KO pericytes showed that there was a decreased amount of N-cadherin allowing more migration and proliferation of the cells in their myofibre micro-environmental niche (Scheef et al., 2009). Similarly, M-cadherin is known to stimulate skeletal muscle satellite cell proliferation (Marti et al., 2013). Although our understanding about the role of TSP-1 in satellite cell function and muscle biology is still evolving, novel data is shown that the TSP-1 KO phenotype may account for the satellite cell proliferation on the myofibre and not *in vitro* culture.

It is reported here for first time that isolated satellite cells from TSP-1 KO mice had compromised differentiation leading to an impaired fusion index and myotube number. CD36 is a TSP-1 receptor is known to be important for the differentiation of skeletal muscle satellite cells (Park et al., 2012). By the addition of human platelet releasate, known to have relatively high amounts of Thrombospondin-1 protein, this TSP-1-CD36 pathway may contribute to the observed rescue of differentiation in this study (Samovski et al., 2015b, O'Connor et al., 2010).

Platelet releasate from TSP-1 KO mice drives a faster satellite cell proliferation over wild-type cells, when assessed on cultured single myofibres. This proliferative effect is magnified further when analysing the total cell progeny number at T72 of culture, where TSP-1 KO myofibres have higher total cell progeny numbers over wild-type. In contrast, it is worth noting that smooth muscle cells respond positively and proliferate in the presence of TSP-1 through the upregulation of transforming growth factor (TGF)-β2 and Insulin-like growth factor-1 signalling (Stein et al., 2013, Maile et al., 2010). Conversely, TGF-β has been shown to inhibit proliferation of satellite cells (Allen and Boxhorn, 1989). A potential TSP-1-mediated TGF-β downregulation may offer a logical explanation of the observed findings in the present study. This notion is strengthened by recent evidence showing that customised releasate lacking TGF-β improves cell proliferation (Li et al., 2016b, Miroshnychenko et al., 2017).

6.10 Senescence

Doxorubicin administration causes cell cycle arrest and senescence; however the VEGF pathway has been shown to reverse this effect (Chen et al., 2018). It was shown in **Chapter 3** and **Chapter 5** that VEGF from platelet releasate acts strongly and dose dependently on both C2C12 and H9C2 proliferation. Previously, Doxorubicin's effect on myoblast proliferation has been shown to act through several key pathways such as NAD-dependent deacetylase sirtuin-1 (SIRT1), Mitogen-activated protein kinase (MAPK), the aforementioned VEGF pathway and ROS (Sin et al., 2016, Jian et al., 2017, de Lima Junior et al., 2016, Gilliam et al., 2012, Chen et al., 2018). Importantly, platelet-based applications have been shown to affect cellular signalling in all of these pathways (Liu et al., 2014, Martins et al., 2016, Milasincic et al., 1996, Chen et al.,

2018). The data in this thesis back up previously published data and add to the field with novel information indicating that senescence may be reversed with platelet secretome administration.

6.11 Concluding remarks

This thesis provided robust evidence that methodological variability may account for discrepancies among studies on the role of platelet releasate in skeletal muscle regeneration. Platelet releasate devoid of plasma and cellular debris promoted skeletal and cardiac myoblast proliferation and terminal differentiation both *in vitro* and *ex vivo*. However, the timing of releasate application appeared to be a critical factor due to the inhibitory role of releasate on myoblast fusion as shown here and elsewhere (Saury et al., 2018). Most importantly, it was shown for the first time that platelet releasate increased skeletal muscle stem cell commitment to differentiation and promotes skeletal myogenesis through the PDGF/VEGF-Cyclin D1-MyoD-Scrib-Myogenin axis and accelerated skeletal muscle regeneration after acute injury. Collectively, the present findings can be exploited in future studies to pinpoint the role of platelets as biomaterials in skeletal muscle regeneration.

The data in this thesis suggests a potential benefit to removing TSP-1 from platelet releasate in applications to skeletal muscle. As TSP-1 is important for skeletal myoblast differentiation, the novel data in this thesis is important as there is a need for an autologous medium when culturing skeletal muscle stem cells where they solely proliferate and do not differentiate. Both PDGF and VEGF are essential components of platelet releasate for myoblast proliferation and differentiation. As TSP-1 interacts with both PDGF and VEGF, it is not surprising that TSP-1 KO platelet releasate shows an increase in myoblast proliferation significantly over wild-type releasate.

As platelet-based applications can be customised for tissue-specific efficacy, this makes them strong candidates for developing innovative regenerative therapies. This thesis provides novel insights into the role of platelet releasate on C2C12 and H9C2 cells and suggests an optimised preparation method to maximise the proliferative/differentiative response for potential regeneration. This thesis also highlights the benefit of concentrating the composition of the platelet secretome for

optimal cell-type targeted applications in regenerative medicine. It is shown for the first time that supra-physiological releasate either significantly improves proliferation and/ or differentiation of various cell types. Finally, platelet releasate was shown to interfere with Doxorubicin-induced cellular senescence. In summary, the data in this thesis shows that standardising the concentration of platelets for therapeutic use may be a key factor determining varied results surrounding clinical success.

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Appendix I

Comprehensive list of materials

Materials	Supplier	Cat. Number
Amphotericin B	Sigma-Aldrich, UK	A2942-20ML
Bio-Plex Pro™ Human Cancer Biomarker Panel 1 and 2	BioRad, UK	171AC500M & 171AC600M
Chick embryo extract	APS, UK	MD-004D-UK
Click-iT® EdU Cell Proliferation Assay	BaseClick, Germany	BCK-EdU488
Collagen	BioData, UK	101562
Collagenase	Sigma Aldrich, UK	C2674
Dihydroethidium (DHE)	Thermo Scientific, UK	D-1168
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, UK	67-68-5
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate	Thermo Scientific, UK	10569010
Doxorubicin	Cayman chemical	15007
Eosin	Sigma, UK	E.4382
E.Z.N.A. Total RNA kit I	Omega Bio-Tek, USA	R6834-01
Foetal bovine serum (FBS)	Sigma Aldrich, UK	F0804
Hematoxylin Solution, Harris Modified	Sigma Aldrich, UK	HHS32
Horse Serum - Gibco	FischerScientific, UK	16050122
HyClone™ Dulbecco's High Glucose Modified Eagles Medium (DMEM)	FischerScientific, UK	SH30022.01
Hydrophobic PAP pen	Sigma Aldrich, UK	Z377821
Matrigel	Corning, UK	354234
Microscopy glass slides	Thermo Scientific, UK	J2800AMNT
***Naja pallida* cardiotoxin**	Latoxan, Valence France	56574-47-1
Optimal cutting temperature compound (OCT)	VWR, UK	00411243
Paraformaldehyde	Sigma Aldrich, UK	P6148-500G
PCR grade 96-well plates	Thermo Scientific, UK	AB-1400
PDGFR Inhibitor (Tyrphostin AG 1295)	Santa Cruz, UK	71897-07-9
Penicillin/streptomycin	Thermo Scientific, UK	15140122
PermaFluor™ Aqueous Mounting Medium	Thermo Scientific, UK	TA-030-FM
Phosphate buffered saline	Oxoid, UK,	BR0014
Prostaglandin I_2	Cayman Chemical	18220
RiboZol™ RNA Extraction Reagent	VWR Life Science AMRESCO, UK	N580-30ML
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo Scientific, UK	K1632
SYBR Green	Thermo Scientific, UK	4364344
TC-Treated 24-Well Plates	Corning® Costar®	CLS3527-100EA
Thrombin	Sigma Aldrich, UK	9002-04-4

TRAP6	AnaSpec, UK	AS-60679
VEGFR Inhibitor (AAL-993)	Merck, UK	16351

PCR primer sequences

Primer	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
Acta1	CCCAAAGCTAACCGGGAGAAG	GACAGCACCGCCTGGATAG
Bdnf	TCATACTTCGGTTGCATGAAGG	AGACCTCTCGAACCTGCCC
Bex1	ATGGAGTCCAAAGATCAAGGCG	CTGGCTCCCTTCTGATGGTA
Cyclind1	TTGTGCATCTACACTGACAACTC	AGGGTGGGTTGGAAATGAACT
Cyp	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT
Fgf1	GAAGCATGCGGAGAAGAACTG	CGAGGACCGCGCTTACAG
Gadd45g	AAAGTCCTGAATGTGGACCCT	AACGCCTGAATCAACGTGAAA
Hgf	AAAGGGACGGTATCCATCACT	GCGATAGCTCGAAGGCAAAAAG
Hprt	GCTCGAGATGTCATGAAGGAGAT	AAAGAACTTATAGCCCCCCTTGA
Igf1	GTGAGCCAAAGACACACCCA	ACCTCTGATTTTCCGAGTTGC
Igfr1	CTTCTACAACTACGCACTGGTC	TCGGCGTTCTTCTCAATCCTG
Mhc1	AGTCCCAGGTCAACAAGCTG	TTCCACCTAAAGGGCTGTTG
Mhc2a	AGTCCCAGGTCAACAAGCTG	GCATGACCAAAGGTTTCACA
Mhc2b	AGTCCCAGGTCAACAAGCTG	TTTCTCCTGTCACCTCTCAACA
Mhc2x	AGTCCCAGGTCAACAAGCTG	CACATTTTGCTCATCTCTTTG
Mstn	AAGATGACGATTATCACGCTACC	CCGCTTGCATTAGAAAGTCAGA
Myf5	GCCTTCGGAGCACACAAAG	TGACCTTCTTCAGGCGTCTAC
Myod1	CGCCACTCCGGGACATAG	GAAGTCGTCTGCTGTCTCAAAGG
Myogenin	GAGACATCCCCCTATTTCTACCA	GCTCAGTCCGCTCATAGCC
Ngf	CCAGTGAAATTAGGCTCCCTG	CCTTGGCAAAACCTTTATTGGG
Ntf3	AGTTTGCCGGAAGACTCTCTC	GGGTGCTCTGGTAATTTTCCTTA
Ogg1	CAACAACATTGCTCGCATTACTG	TCAAGCTGAATGAGTCGAGGT
Parp1	GGTCTTTAAGAGCGACGCTTAT	TTCTGTGTCTTGACCATGCAC
Pax7	GCTGCTGAAGGACGGTCACT	CTGAGCACTCGGCTAATCGAA
Pdgfα	TGGCTCGAAGTCAGATCCACA	TTCTCGGGCACATGGTTAATG
Pdgfβ	TCCGGCTGCTGCAATAACC	GGCTTCTTTCGCACAATCTCAAT
Pdgfrα	GGAGACTCAAGTAACCTTGCAC	TCAGTTCTGACGTTGCTTTCAA
Pdgfrβ	CAAGAAGCGGCCATGAATCAG	CGGCCCTAGTGAGTTGTTGT
Pgc1α	AACCACACCCACAGGATCAGA	TCTTCGCTTTATTGCTCCATGA
Prmt1	TACTACTTTGACTCCTATGCCCA	ATGCCGATTGTGAAACATGGA
Sirt1	TGATTGGCACCGATCCTCG	CCACAGCGTCATATCATCCAG
Tmem8c	GTGATGGGCCTGGTTTGTCT	GCATTGTGAAGGTCGATCTCTG
Trka	GCCTAACCATCGTGAAGAGTG	CCAACGCATTGGAGGACAGAT
Trkb	CTGGGGCTTATGCCTGCTG	AGGCTCAGTACACCAAATCCTA
Trkc	CTCTACACGGGACTCCAGAAG	GGTGAGCCGGTTACTTGACA
Vegfa165	TGCAGGCTGCTGTAACGATG	GAACAAGGCTCACAGTGATTTTCT
Vegfr1	CACTGACATACCCAAACTTGTGC	GTCCCATGTTATTCTTTGCCCAT

Primary and secondary antibodies

Antigen	Species	Dilution	Supplier, Catalogue
	Primary		
MYH3	Mouse	1:200	Santa Cruz, sc-53091
MhcI	Mouse	1:1	DSHB, A4.84
MhcIIA	Mouse	1:2	DSHB, A4.74
MhcIIB	Mouse	1:1	DSHB, BF.F3
Pax7	Mouse	1:200	Santa Cruz, sc-81648
MyoD	Rabbit	1:200	Santa Cruz, sc-760
Myogenin	Rabbit	1:200	Santa Cruz, sc-570
Cyclin D1	Mouse	1:200	Santa Cruz, sc-450
Scrib	Mouse	1:200	Santa Cruz, sc-374139
PDGF-B	Mouse	1:200	Santa Cruz, sc-365805
VEGF	Mouse	1:200	Santa Cruz, sc-7269
IGF-1Rα	Rabbit	1:200	Santa Cruz, sc-271606
Ki-67	Rat	1:200	ThermoFisher, 14-5698-80
CD31	Rat	1:40	AbD serotec MCA2388
CD68	Mouse	1:200	Santa Cruz, sc-20060
IL-10	Mouse	1:200	Santa Cruz, sc-8438
VEGF-C	Mouse	1:200	Santa Cruz, sc-374628
PCNA	Mouse	1:200	Santa Cruz, sc-56
TLR4	Mouse	1:200	Santa Cruz, sc-293072
ICAM-1	Mouse	1:200	Santa Cruz, sc-8439
	Secondary		
Alexa fluor 488 anti-rat	Goat	1:200	Life Technologies A11006
Alexa fluor 488 anti-mouse	Goat	1:200	Life Technologies A11029
Alexa fluor 488 anti-rabbit	Goat	1:200	Life Technologies A11034
Alexa fluor 594 anti-rabbit	Goat	1:200	Life Technologies A11037
Alexa fluor 633 anti-mouse	Goat	1:200	Life Technologies A20146

Antigen	Excitation	Emission
DAPI	358nm	461nm
Alexa fluor 488	499nm	520nm
Alexa fluor 594	590nm	619nm
Alexa fluor 633	631nm	647nm