VISUALISATION OF FOCAL ADHESION-ASSOCIATED PROTEINS IN THE SKELETAL MUSCLE OF YOUNG AND ELDERLY INDIVIDUALS: EFFECT OF EXERCISE TRAINING

A thesis submitted by

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To the

School of Sport and Exercise Sciences,

University of Birmingham

For the degree of

DOCTOR OF PHILOSOPHY

Exercise Metabolism Research Group

School of Sport and Exercise Sciences

University of Birmingham

September 2013

Acknowledgements

I wish to thank a number of people who, either academically or socially, have made a tremendous contribution towards the completion of this thesis.

Firstly, I wish to thank my supervisors, Prof. Anton Wagenmakers and Dr. Chris Shaw for the opportunity to work in such a well respected research group. Your thoughts, guidance and support have been instrumental in the development of this thesis, in my development as a research scientist and will continue to be invaluable in the future. I have benefited greatly from your enthusiasm, knowledge and the time spent discussing findings, images and reading my manuscripts. I would also like to thank Dr. Gareth Wallis for his help and guidance in my final year and for the stimulating conversations. I must also thank the BBSRC for their generous stipend over the last 4 years.

I would also like to thank the other members of 'Team Anton'; Juliette Clark, Helen Bradley, Sam Shepherd, Matt Cocks and Zhe Song for making research in the histology lab so entertaining and sharing the highs and lows of immunofluorescence microscopy and western blotting. I also wish to thank the research participants who made the skeletal muscle biopsy studies possible.

I made some very strong friendships during my time in SportEx and these were often forged over steaming hot mugs of tea at 'tea club' and then lunch at 'lunch club'. Thanks goes to Dan Crabtree, Adrian Hodgson, Rebecca Randle, Adrian Holliday, Jon Allsop, the ausie John Mahoaney, Matt, and Sam for the banter which provided a welcome relief from all that science. At this time, I would like to say a special thanks to my office mate Adrian Holliday for all the conversations we had about endurance training and adaptation, and for keeping me sane during the final few months of write up. I must also say thanks to our other office mate Carlijn for being patient with us and for putting up with all the senseless conversations.

I must also say thanks to the technicians, particularly Steve Allen and Rob Wheeler for all of your help over the years. Steve, thank you for helping me fix my various bikes even when you had better things to do. The same also goes for Rob who came to sort out my computer with unrivalled enthusiasm no matter how insignificant or inconvenient the problem.

I would like to express my sincere gratitude to my parents and grandmother for supporting me ever since I decided to go back to University all those years ago. I promise no more University courses from now on!

Finally, I wish to thank my wife Caroline for being there during the highs and the lows. Your unwavering love, support, encouragement and patience have been instrumental in getting this thesis completed and would have been extremely difficult without you.

Abstract

Focal adhesion kinase (FAK) and paxillin are proteins implicated in the mechanisms that link chronic alterations in mechanical force within skeletal muscle and its microvasculature to the functional adaptation seen with changes in physical activity. This thesis developed novel immunofluorescence microscopy methods to visualise and measure FAK and paxillin responses in human skeletal muscle and its microvasculature. Chapter 2 reveals high FAK protein content in the (sub)sarcolemma of skeletal muscle fibres and within the microvascular endothelial and vascular smooth muscle cell layers. Chapter 3 demonstrates that FAK protein content is increased at (sub)sarcolemmal and sarcoplasmic regions of skeletal muscle fibres and within the microvascular endothelium following 12 weeks resistance-type exercise training in elderly individuals. Chapter 4 shows that FAK and paxillin colocalise at the (sub)sarcolemma of skeletal muscle fibres and within the microvasculature. Chapter 5 demonstrates that FAK and paxillin are increased at the (sub)sarcolemma and within the microvascular endothelium following 6 weeks endurance- and resistance-type exercise training in young previously sedentary individuals. The novel data generated in this thesis, in combination with recent literature findings, support the hypothesis that FAK and paxillin play an important role in upstream mechanotransduction signals that control skeletal muscle fibre hypertrophy, mitochondrial biogenesis, insulin sensitivity, microvascular function and angiogenesis.

List of conference communications and publications

During postgraduate study at the University of Birmingham, the following papers were published or submitted during postgraduate study:

Oliver J. Wilson, Christopher S. Shaw, Mark Sherlock, Paul M. Stewart and Anton J. M. Wagenmakers. (2012). Immunofluorescent visualisation of focal adhesion kinase in human skeletal muscle and its associated microvasculature. *Histochemistry and Cell Biology*. 2012 Oct;138(4):617-26.

Oliver J. Wilson, Helen Bradley, Christopher S. Shaw, Anton. J. M. Wagenmakers. (2013). Focal adhesion kinase colocalises with paxillin in human skeletal muscle and its microvasculature. *Histochemistry and Cell Biology*. *Revised and in press*.

Juliette A. Clark, Christopher S. Shaw, Helen Bradley, Oliver J. Wilson, James Pilling and Anton J.M. Wagenmakers. Immunofluorescence microscopy of synaptosomal associated protein 23 (SNAP23) in human skeletal muscle reveals colocalisation with plasma membrane, lipid droplets and mitochondria. *BBA-Molecular and Cell Biology of Lipids*. Submitted. (2013).

Oliver J. Wilson and Anton J. M. Wagenmakers. (2012). Sarcopenia. In M.D. Gellman & J.R. Turner (eds.), Encyclopedia of Behavioral Medicine.

The following conference communications were presented:

Oliver Wilson, Mark Sherlock, Gareth Lavery and Paul M. Stewart. Skeletal muscle 11β-HSD1 is modulated by glucocorticoid and pro-inflammatory cytokine treatment, leading to activation of atrophy/ apoptosis pathways. *Obesity and insulin resistance – From cell to whole body: The benefits of exercise,* University of Birmingham, 2009.

Oliver J. Wilson, Kamen Tsvetanov, Gareth Lavery, Mark Sherlock and Paul M. Stewart. Skeletal muscle 11β-hydroxysteroid dehydrogenase type 1 is increased with age. *The British Society for Research on Ageing (BSRA)*, 2009.

Oliver J. Wilson, Christopher S. Shaw, Mark Sherlock, Paul M. Stewart, and Anton J. M. Wagenmakers. Immunofluorescent visualisation of focal adhesion kinase in human skeletal muscle and associated microvasculature. *Physiological Society, Physiology Conference*, 2010.

Oliver J. Wilson, Christopher S. Shaw, Mark Sherlock, Paul M. Stewart, and Anton J. M. Wagenmakers. Immunofluorescent visualisation of focal adhesion kinase in human skeletal muscle and associated microvasculature. *Ageing, sarcopenia and exercise: from cell to whole body*. University of Birmingham, 2010.

Oliver J. Wilson, Verdijk LB, van Loon LJC, Shaw CS and Wagenmakers AJM. Increased focal adhesion kinase in elderly human skeletal muscle after 12 weeks resistance training. *European College of Sport Science*. Liverpool, 2011.

Helen Bradley, **Oliver J. Wilson,** Christopher S. Shaw, Anton JM Wagenmakers. *Imaging of contraction mediated GLUT4 translocation in human skeletal muscle fibres. 15th International Biochemistry of Exercise Congress*. Stockholm, 2012.

Clark JA, Shaw CS, Bradley H, Wilson OJ, Wagenmakers AJM. Visualising the subcellular distribution of SNAP23 in human skeletal muscle. *15th International Biochemistry of Exercise Congress.* Stockholm, 2012.

Christopher S Shaw, Helen Bradley, **Oliver J Wilson**, Anton JM Wagenmakers. Imaging GLUT4 translocation in human skeletal muscle fibres. *Australian Diabetes Society and the Australian Diabetes Educators Association,* 2012.

Oliver J. Wilson, Helen Bradley, Christopher S. Shaw, Anton. J. M. Wagenmakers. Focal adhesion kinase colocalises with paxillin in human skeletal muscle and its microvasculature. *European College of Sport Science*. Barcelona, 2013.

Kinnafick, F. E., Shepherd, S. O., **Wilson, O.J**., Shaw, C. S., Wagenmakers, A. J. M., Thøgersen-Ntoumani C. High intensity interval and traditional endurance training lead to comparable improvents in motivation and well-being outcomes. *European College of Sport Science*. Barcelona, 2013.

List of abbreviations

ACSM	American College of Sports Medicine
AHA	American Heart Association
ALD	Anterior latissimus dorsi
APS	Adaptor molecule containing Pleckstrin homology and Src homology-2 domains
AS160	Akt substrate of 160 kDa
BMI	Body mass index
CAP	c-Cbl-associated protein
Cbl	Casitas B-lineage Lymphoma
C2C12	Murine skeletal muscle cell line
cm	Centimetre
CMV	Cytomegalovirus
CSA	Cross-sectional area
c-Src	Cellular sarcoma
DABCO	1,4-diazobicyclo-[2,2,2]-octane antifade medium
DAPI	4',6-diamidino-2-phenylindole fluorescent dye
DGC	Dystrophin Glycoprotein Complex
DHPR	Dihydropyridine receptor
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDL	Extensor digitorum longus
eNOS	Endothelial nitric oxide synthase
ET	Endurance-type exercise training
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase

F.A.T	Focal adhesion targeting domain
FERM	4.1 protein, erzin, radixin, moesin
FITC	Fluorescein isothiocyanate
FMD	Flow mediated dilation
FRNK	Focal adhesion kinase related non-kinase
GLUT-4	Glucose transporter-4
Grb2	Growth factor receptor-bound protein 2
HbA1c	Glycated haemoglobin A1c
HOMA	Homeostasis Model Assessment
HSfE	Health Survey for England
IGF-I	Insulin-like growth factor-I
IRS-1	Insulin receptor substrate-1
ISI	Insulin sensitivity index
JNK	Jun N-terminal kinase
kDa	Kilodalton
L	Litre
LE	Leg extension
LIM	Lin 11, IsI-1, Mec-3
L-NAME	N-nitro-L-arginine methyl ester
LP	Leg press
MAPK	Mitogen-activated protein kinase
MHC	Myosin heavy chain
mmol	Millimole
mRNA	Messenger ribonucleic acid
MTJ	Myotendinous junction
mTOR	Mammalian target of rapamycin
NHS	National Health Service

nm	Nanometer
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF-1	Nuclear respiratory factor-1
OCT	Optimum cutting temperature
p70S6K	p70 ribosomal protein S6 kinase
PCMV	Plasmid cytomegalovirus
PI3-K	Phosphotidylinositol-3-kinase
PI3P	Phosphatidylinositol-3-phosphate
PKC	Protein kinase C
PGC1α	Peroxisome proliferator activated γ coactivator-1α
Pro	Proline
PTK	Protein tyrosine kinase
1RM	One repetition maximum
RT	Resistance-type exercise training
s	Seconds
SD	Standard deviation
SEM	Standard error of the mean
Ser	Serine
SH	Src homology
Shp2	Src homology phosphatase 2
siRNA	Small interfering ribonucleic acid
Src	Sarcoma
TC10	Teratocarcinoma
Tfam	Mitochondrial transcription factor A
Thr	Threonine
TSC	Tuberous sclerosis

Tyr	Tyrosine
UEA-I	Ulex europaeus Agglutinin I
UK	United Kingdom
μm	Micrometer
UN	United Nations
US	United States of America
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VO_{2peak}	Peak volume of oxygen
VSMC	Vascular smooth muscle cell
WHO	World Health Organisation

Contents

Chapter 1

General Introduction

1.1. Perspective: An ageing global population

We are living amongst an ageing population. By the year 2050, it is expected that the global population of elderly individuals (defined by the UN as aged ≥60 years) will reach 2 billion representing a ~200% increase since 1950 (UN, 2002). As a result, the global population of elderly individuals will outnumber the young (defined by the UN as aged ≤ 15 years) for the first time in the history of humanity (UN, 2002). This feat was already achieved in 1988 by more developed nations and over the following decades, the greatest gains in life expectancy at birth will be in the developing regions (UN, 2002). The growth of the ageing population is partly attributable to the decline in global fertility rates (mean number of children per woman) (UN, 2011). This is particularly prevalent in developed nations where total fertility is below that needed to ensure a population replaces itself in size (UN, 2011). The ageing population is also attributable to improvements in medical care and the prevention and management of disease. These advances enable the continued improvement in life-expectancy despite the increased risk of falls (Campbell et al., 1989; Tinetti et al., 1988) and the development of age-related chronic diseases such as obesity (Flegal et al., 2010), insulin resistance, type II diabetes (Fink et al., 1983; Rowe et al., 1983; Wild et al., 2004) and cardiovascular disease in elderly populations (Najjar et al., 2005). In Great Britain, 66% of individuals aged ≥75 years reported a long-standing illness or disability (www.ons.gov.uk) and an ageing population will present a significant socio-economic challenge to health care provision. Indeed, the estimated cost of UK hospital and community health services in 2009 for people aged ≥65 years was already ~£47 billion, representing ~40% of the annual National Health Service (NHS) budget (www.parliament.the-stationery-office.co.uk).

1.1.1. 'Usual ageing'

The development of disease and the gradual decline in physical function is evidence of 'usual ageing' (Rowe and Kahn, 1997). One of the most visually obvious signs of the ageing process is the progressive involuntary loss of skeletal muscle mass and strength. The former is termed sarcopenia, where *sarx* means "flesh" and *penia* means "poverty" (Rosenberg, 1989) and the latter is termed dynapenia, where *dyna* means "strength" (Clark and Manini, 2008). Sarcopenia is associated with functional impairments and physical disability (Janssen et al., 2002) and dynapenia is associated with an increased risk of falls (LaStayo et al., 2003), dependency (Tinetti and Williams, 1997), self reported disability (Baumgartner et al., 1998; Janssen, 2006) and all cause mortality (Ruiz et al., 2008). Consequently, the deleterious effects of sarcopenia and dynapenia can have a considerable effect on the quality of life in elderly populations.

Ageing is also associated with vascular endothelial dysfunction where blood flow-mediated endothelium-dependent vasodilation of arteries, resistance vessels and skeletal muscle arterioles are impaired with age (Celermajer et al., 1994; Donato et al., 2009; Muller-Delp et al., 2002). The age-associated development of insulin resistance and type II diabetes is also associated with impaired insulin-stimulated vasodilation of the terminal arterioles (Rattigan et al., 2006). Reductions in skeletal muscle microvascular blood flow, capillary recruitment and the capillary surface area available for nutrient (e.g. glucose, fatty acids and amino acids) and hormone (e.g. insulin) delivery may lead to sustained periods of hyperglycaemia and hyperinsulinaemia in elderly individuals (Rattigan et al., 2006; Wagenmakers et al., 2006).

1.1.2. Mechanical loading, physical (in)activity and 'successful ageing'

The maintenance of skeletal muscle mass is dependent on the balance between the rates of skeletal muscle protein synthesis (major contributor to mass are the myofibrillar proteins) and skeletal muscle protein breakdown (Baar et al., 2006). Increasing the mechanical load of a skeletal muscle beyond that of daily living tasks (e.g. resistance-type exercise) can increase muscle protein synthesis in response to feeding so that synthesis rates exceed the rate of muscle protein breakdown, leading to net positive muscle protein balance (Tipton et al., 1999). Performing frequent bouts of resistance-type exercise over at least 4 weeks results in the accretion of myofibrillar proteins, skeletal muscle hypertrophy and increased muscle strength (Tipton and Ferrando, 2008; Woolstenhulme et al., 2006). Conversely, a chronic reduction in the mechanical load of the skeletal muscle has the reverse effect leading to a loss of skeletal muscle mass and strength (Baar et al., 2006; Tipton and Ferrando, 2008). Consequently, the decline in physical activity rates with age (HSfE, 2010) has been suggested as a contributory factor in the development of sarcopenia and dynapenia in healthy individuals (Doherty, 2003). Therefore, frequent exposure to high mechanical loads is required to maintain skeletal muscle mass and strength in ageing.

Performing regular bouts of resistance-type exercise is an effective strategy for combating sarcopenia and dynapenia, even in nonagenarians (Fiatarone et al., 1990). It is also recommended by the American College of Sports Medicine (ACSM) and the American Heart Association (AHA) for its positive effects on insulin sensitivity (Chodzko-Zajko et al., 2009; Haskell et al., 2007). The vasculature is also acutely and chronically sensitive to changes in mechanical stimulation and hemodynamic forces as a result of exercise training (Seals et al., 2009). Resistance-trained older individuals show an absence of the age-related decline in femoral artery blood flow and vascular conductance (Miyachi et al., 2005) and resistance-type exercise training (RT) can prevent the decline in arterial distensibility and increase

resting femoral blood flow and vascular conductance in previously untrained elderly individuals (Anton et al., 2006; Maeda et al., 2006). These studies collectively emphasise the importance of mechanical stimulation through physical activity and exercise training for maintaining skeletal muscle mass, macro and microvascular function and insulin sensitivity with age. Exercise training can therefore reverse and prevent 'usual ageing' and promote 'successful ageing' where elderly individuals show comparatively high physical function, independence, engagement in life and have a lower risk of disease and mortality (Myers et al., 2002; Rowe and Kahn, 1997; Ruiz et al., 2008).

1.1.3. Rationale

Given the rapid growth in the proportion and number of the ageing population and the deleterious nature of sarcopenia on the quality of life, it is imperative that research advances our understanding of how skeletal muscle and its microvasculature adapts to physical (in)activity and the differences that exist between young and elderly individuals leading to anabolic resistance in the elderly. Understanding the molecular responses to exercise may help develop strategies and interventions to counteract sarcopenia and optimise the adaptive response to exercise training. The mechanism(s) that link physical activity to functional adaptation in the skeletal muscle and its microvascular tissues are not fully elucidated. However, mechano-sensitive proteins at the membrane of the skeletal muscle fibres and microvascular endothelial and vascular smooth muscle cells may play an important role (Carson and Wei, 2000; Martinez-Lemus et al., 2003). Integrins are transmembrane proteins that link contractile and hemodynamic forces to intracellular signalling (Carson and Wei, 2000; Hynes, 2002; Martinez-Lemus et al., 2003). Upon mechanical stimulation, integrins signal through two immediately downstream proteins called focal adhesion kinase (FAK) and paxillin (Schaller et al., 1995). These two proteins have been implicated in the hypertrophic

and atrophic response of animal skeletal muscle to periods of increased loading and unloading (Fluck et al., 1999; Gordon et al., 2001) and are essential for normal vascular development (Hagel et al., 2002; Ilic et al., 1995). Consequently, they may play an important role in the adaptation of skeletal muscle and its microvasculature to exercise training.

Currently, little is known about FAK and paxillin in human skeletal muscle and differences that might exist between young and old individuals. Therefore, this PhD thesis will investigate the spatial distribution and content of FAK and paxillin and their response to exercise training in skeletal muscle fibres and their microvasculature in young and elderly individuals. The current chapter aims to describe how skeletal muscle fibres and their microvasculature 'sense' and convert mechanical stimuli into intracellular signalling. The review will conclude by summarising the key messages and highlights several areas in the literature regarded to be worthy of investigation at the start of this BBSRC funded PhD project before outlining the primary research objectives of the PhD thesis.

1.2. Mechanical forces

1.2.1. Tension, compression and shear forces

Force (e.g. gravitational force) is a load applied to a structure. It has both magnitude (measured in Newtons) and direction and can alter the structure's state (Burkholder, 2007) (e.g. the speed, direction or shape). Stress is defined as a force normalised to the surface area it is acting upon (measured in Newtons per square meter), thus stress is the product of force and surface area (Burkholder, 2007). Force applied perpendicular to the cell surface generates tensile or compressive stress and force applied within the plane of surface generates shear stress (Burkholder, 2007). Cells are exposed to internally generated and externally applied forces. Force generated from within a cell and transmitted from the cell interior and across the cell membrane is known as 'inside out' force transmission. Externally applied force is

transmitted across the cell membrane to the cell interior and is known as 'outside in' force transmission. Cellular force transmission is therefore 'bi-directional'.

At rest, skeletal muscles exert both tensile and shear forces on the musculoskeletal system and these forces increase when the skeletal muscle contracts during dynamic exercise. The tensile and shear forces are generated internally by the skeletal muscle and are an example of 'inside out' force transmission. Alterations in blood flow to meet the increased metabolic demand of the contracting skeletal muscle increases the hemodynamic forces acting upon the vascular wall. These forces are also tensile and shear stress forces (e.g. fluid shear stress). The fluid shear stress that acts on the vascular wall is transmitted across the vascular cell membrane to the cell interior and is an example of 'outside in' force transmission.

1.3. Skeletal muscle and force transmission

1.3.1. Skeletal muscle function

Skeletal muscle provides support and movement of the skeleton, stabilises joints and generates heat through metabolism. In young, healthy adults, it represents ~40% of body mass (~32 kg for an 80 kg male and ~24 kg for 60 kg female) and is therefore a substantial reservoir of protein which can be degraded into amino acids and released into the blood stream as precursors for the maintenance of organ and tissue mass such as the brain, heart, skin and liver and as precursors for liver gluconeogenesis (Wolfe, 2006). Skeletal muscle is also a store of lipid in the form of intramuscular triacylglycerols and glucose in the form of glycogen. Indeed, skeletal muscle accounts for >85% of glucose disposal in healthy young individuals during a hyperinsulinemic-euglycemic clamp (DeFronzo et al., 1981). Therefore, minimising the loss of skeletal muscle mass and preventing the loss of metabolic function

with age through high mechanical load exercise training will have a considerable effect on the risk of developing metabolic disorders (Rogers et al., 1990).

1.3.2. Skeletal muscle ultrastructure and force transmission

Skeletal muscle fibres are surrounded by an extracellular matrix (ECM) and have ~500 myofibrils per muscle fibre which account for approximately 85% of the muscle fibre volume (Hoppeler, 1986). Myofibrils give the skeletal muscle fibre a striated appearance due to the abundance of contractile units (sarcomeres) running in series along the length of each myofibril (figure 1.1). Each sarcomere is composed of myosin (measuring 1.6 μm in diameter) and actin (1 μm in diameter) contractile filaments. Other proteins present in myofibrils and essential for the contractile process are troponin, tropomyosin, tropomodulin and nebulin. Actin and myosin slide past each other during contraction through acto-myosin cross bridge formation without a change in filament length (for review see (Huxley, 1974)). The Z-disk is at the boundary of each sarcomere and is an anchor site for actin and myosin (the latter via titin) from opposing sarcomeres (Clark et al., 2002). Consequently, the tensile forces generated by acto-myosin contractile activity are transmitted to and received by the Z-disk. This makes the Z-disk an important component in myofibrillar force transmission (Clark et al., 2002; Faulkner et al., 2001).

The Z-disk is a complex protein-containing structure abundant in α-actinin (Faulkner et al., 2001). α-actinin binds to and cross-links the interdigitating actin filaments and titin from the opposing sarcomeres of a myofibril (Young et al., 1998). In longitudinally orientated skeletal muscle fibres, the number of α-actinin cross-linking layers (called Z-links) determines the Z-disk width (Luther, 2009). Fast twitch animal skeletal muscle fibres have narrow (30 to 70 nm) Z-disks (Luther, 2000) whilst slow fibres have wide (~100 nm) Z-disks (Yamaguchi et al., 1985). This difference between fibre-types is thought to reflect the

differences in mechanical strain that they experience in daily life (Luther et al., 2003). The Z-disk of individual myofibrils align in register with the Z-disk of adjacent myofibrils and are linked at the Z-disk periphery by intermediate protein filaments such as desmin (Lazarides, 1980). Desmin also links the sarcomeres of peripheral myofibrils to the sarcolemma where it plays an important role in muscle fibre integrity (Li et al., 1997b) and the transmission of force from contracting myofibrils to focal adhesions (see 1.3.2. and figure 1.1 and 1.4) and to the sarcolemma (Lazarides, 1980; Li et al., 1997b). The Z-disk is therefore central to the integration of myofibril contractile activity and the lateral transmission of force (shear stress) from the sarcomere to the sarcolemma of skeletal muscle fibres (Burkholder, 2007; Dawson and Hudlicka, 1993).

1.3.3. The vasculature

The skeletal muscle microvasculature includes secondary arterioles (13-18 μm in diameter (i.d.)) terminal arterioles (7 – 13 μm i.d.), capillaries (3.7 μm i.d.), post capillary venules (7 - 13 μm i.d.) and collecting venules (9 - 18 μm i.d.) (Marshall and Tandon, 1984; Wiedeman, 1963). Arterioles regulate blood flow into capillaries and therefore control the delivery of oxygen, hormone and nutrient-rich blood to the muscle fibres. Arterioles are largely comprised of vascular smooth muscle cells (VSMCs), which form a ring around the vessel perimeter, and endothelial cells which form a ring on the luminal side of the vessel. Capillaries and venules are largely composed of endothelial cells and a basement membrane. The major roles of the vascular endothelium are to regulate lumen diameter and vascular tone, control vascular permeability, extend the microvascular network (angiogenesis), produce anticoagulants and control pathological processes such as inflammation and atherosclerosis (Davies, 1995; Michiels, 2003).

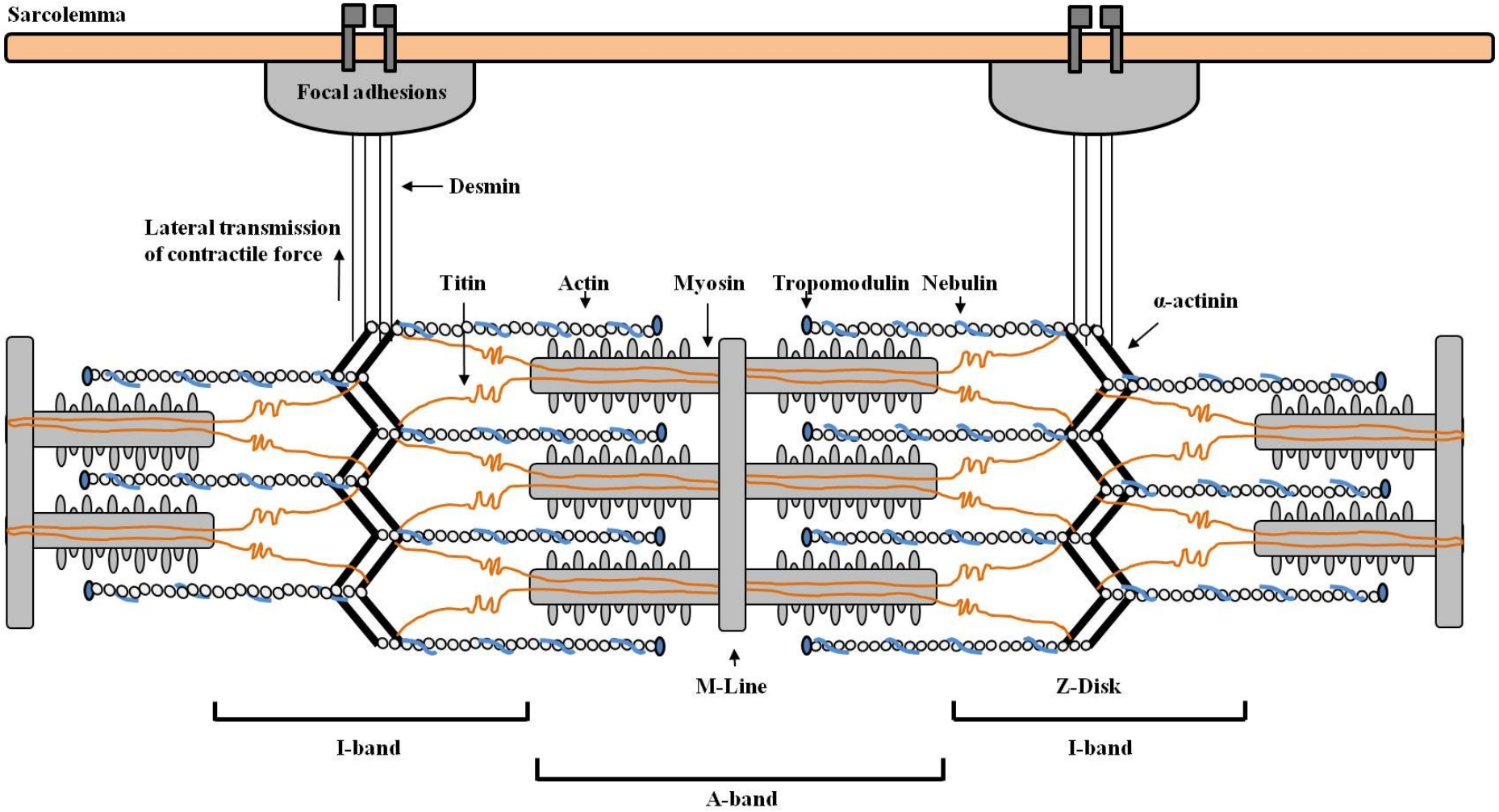

Figure 1.1. Structural composition of the sarcomere and opposing sarcomeres within a skeletal muscle myofibril.

1.3.4. Ultrastructure of the vasculature and force transmission

The luminal surface of the endothelium is covered with membrane-bound molecules such as glycoproteins and proteoglycans. This is known as the glycocalyx layer which extends from the apical surface of the endothelium into the lumen. It is in direct contact with erythrocytes and leukocytes and acts as a lubricating layer for the passage of these cells along the capillaries. The glycocalyx is therefore a barrier between the flowing blood and the apical surface of the endothelium (for review see (Reitsma et al., 2007; Tarbell and Pahakis, 2006; Weinbaum et al., 2007). The thickness of the glycocalyx depends on the luminal diameter. In the mouse carotid artery, the glycocalyx can measure 4.5 μm thick (Megens et al., 2007) and in human sublingual capillaries, it can measure 0.5 to 0.6 μm thick (Nieuwdorp et al., 2008). In the capillaries of hamster cremaster skeletal muscle, the glycocalyx can measure between 0.4 and 0.5 μm thick (Vink and Duling, 1996). The glycocalyx layer is dynamic and rapidly replaced (<1 s) after crushing by leukocytes or by the initial motion of an erythrocyte in a tightly fitting capillary (Weinbaum et al., 2007). However, sustained disruption and degradation of the glycocalyx layer leads to vascular endothelial dysfunction such as impaired flow-mediated dilation (Nieuwdorp et al., 2006) and the development of a pathogenic state (Nieuwdorp et al., 2005). The glycocalyx is therefore thought to be vasculo-protective.

The skeletal muscle microvasculature is constantly exposed to varied magnitudes of pressure, compression, tension (through cyclic stretch and vasoconstriction/dilation) and frictional shear forces (Davies and Tripathi, 1993). The latter is through the frictional shear force of flowing blood per unit of the endothelium, or more specifically, flowing blood per unit of the endothelial glycocalyx layer. As the glycocalyx is in direct contact with the flowing blood, the glycocalyx is also thought to play an important role in hemodynamic force transmission and vasoregulatory responses (Tarbell and Pahakis, 2006). The glycocalyx is

connected to an intracellular cytoskeleton which enables the transmission of force throughout the endothelial cell. The glycocalyx is tethered to multiple sites which include the adherens junctions (via an actin cortical web), the cell nucleus and focal adhesions at the abluminal surface of the endothelium which anchor the endothelial cell to the ECM (Weinbaum et al., 2007) (figure 1.2). This makes the glycocalyx an important component in the transmission and distribution of vascular mechanical forces (e.g. fluid shear stress) and as such, it may play a vital role in the acute stimulation of vasoregulatory processes and the microvascular adaptations to various modes of exercise training.

Because of the glycocalyx, there is negligible fluid shear stress at the apical surface membrane of vascular endothelial cells (Orr et al., 2006). Fluid shear stress is measured in dyne (a unit of force) and is the force needed to accelerate a mass of 1 gram at a rate of 1 cm/s^2. One dyne equals 1 $g/cm/s^2$ and is equivalent to 10 μN (10^{-5} Newtons). Vascular fluid shear stress can range between 10 to 70 $dyne/cm^2$ in the arterial vasculature and from 1 to 6 $dyne/cm^2$ in the venous vasculature (Malek et al., 1999). Arterial fluid shear stress >15 $dyne/cm^2$ is considered atheroprotective, whereas fluid shear stress <4 $dyne/cm^2$ can lead to atherosclerosis (Malek et al., 1999).

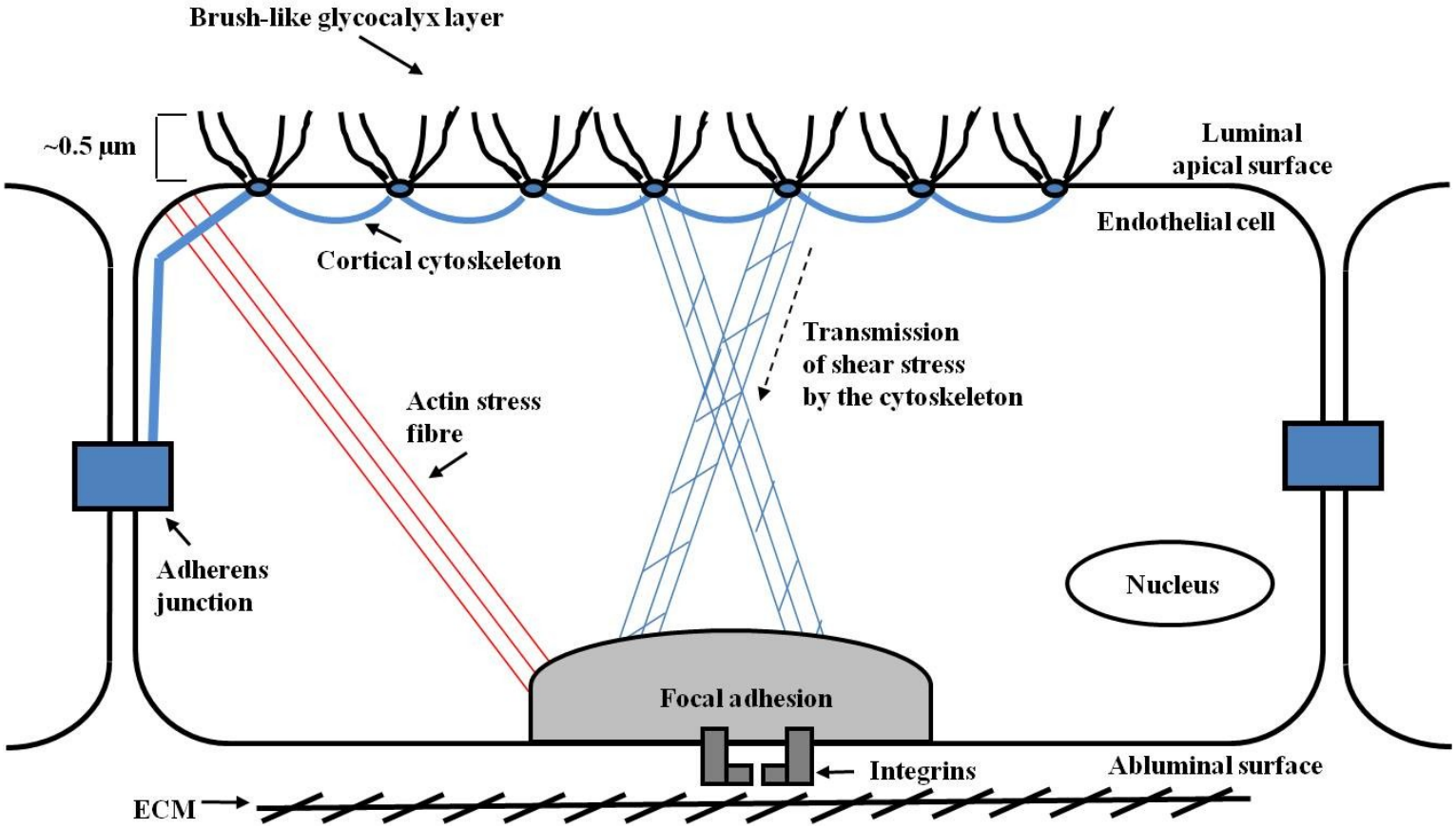

Figure 1.2. Structural composition of the glycocalyx layer on an endothelial cell. The glycocalyx is layer is tethered to focal adhesions through the intracellular cytoskeleton. The glycocalyx is also attached to adherens junctions through the actin cortical web. Stress fibres also connect the apical surface to focal adhesions.

1.3.5. Mechanotransduction

Skeletal muscle and vascular endothelial and smooth muscle cells can convert mechanical forces into biochemical intracellular signals. This is known as mechanotransduction (Ingber, 2006; Schlaepfer et al., 1999) and implies the presence of mechanical sensors that are acutely tuned to the imposed force (Frey et al., 2009; Hornberger et al., 2005). Davies outlined 4 sequential steps in mechanotransduction. These include, 1) the physical deformation of mechano-sensitive cell surface components (e.g. focal adhesions); 2) the extracellular or intracellular transmission of force; 3) conversion of mechanical force into biochemical signals; and 4), stimulation of downstream signalling pathways (Davies, 2009).

Hornberger et al, (Hornberger et al., 2005) showed that skeletal muscle cells could 'sense' and distinguish between different mechanical stresses. Uni-axial (tensile strain) and multi-axial cyclic stretch (non-uniform tensile strain through radial and circumferential axes) of murine C2C12 skeletal muscle cells *in vitro* both increased the phosphorylation of kinases upstream of the protein synthesis signalling pathway. However, multi-axial stretch also increased the phosphorylation of ribosomal S6 kinase (p70S6K) independent of paracrine factors (Hornberger et al., 2005). The authors concluded that mechanical activation of mechanosensory-mechanotransduction signalling pathways in skeletal muscle *in vitro* is specific to the type of mechanical forces applied (Hornberger et al., 2005).

In bovine vascular endothelial cells *in vitro*, both oscillatory (0±15 dyne/cm^2, 60 min) and laminar (15 dyne/cm^2, 60 min) fluid shear stress increased endothelial nitric oxide (NO) production via activation and upregulation of endothelial nitric oxide synthase (eNOS) (Cai et al., 2004). However, this was through separate signalling pathways. Oscillatory flow stimulated eNOS through the calcium/ calmodulin-dependent protein kinase signalling pathway whilst laminar flow stimulated eNOS through cellular Src signalling, independent of calcium/calmodulin-dependent signalling (Cai et al., 2004). These findings demonstrated that

the activation of particular mechanotransduction pathways in vascular endothelial cells *in vitro* is specific to the type of shear stress imposed.

Chronic exposure of cells and tissues to forces above or below those commonly encountered generally disturb cell homeostasis. The cell can respond to this challenge and regain homeostasis with an adaptation perfectly matched to the magnitude of applied forces. For example, the high mechanical loads associated with high-intensity RT can stimulate an increase in skeletal muscle fibre cross sectional area (CSA) in young and elderly individuals (Kosek and Bamman, 2008; Kosek et al., 2006). Conversely, a chronic reduction or loss of mechanical loading leads to skeletal muscle atrophy in young and elderly individuals (D'Antona et al., 2003; Hvid et al., 2010; Suetta et al., 2009). Sustained elevations in vascular shear stress during endurance-type exercise training (ET) are associated with chronic vascular arterial remodelling. To reduce the shear stress, the femoral artery diameter widens, the intima-media thickness decreases and the lumen size increases when compared with untrained individuals (Dinenno et al., 2001). At present, it is incompletely known how applied forces are linked to the activation of gene transcription, translation and adaptation in the skeletal muscle fibres and vascular endothelial and smooth muscle cells. A discussion of the varied mechanical stimuli thought to play a role in mechanotransduction within skeletal muscle and the microvasculature is beyond the scope of this thesis and the reader is referred to the following reviews for a broader insight (Burkholder, 2007; Davies, 1995; Musi et al., 2003; Tidball, 2005; Vandenburgh et al., 1993; West et al., 2010). The focal adhesions which anchor the Z-disks of peripheral myofibrils to the sarcolemma in skeletal muscle fibres and tether the glycocalyx to the ECM at the abluminal side of the endothelium (in 1.3.2 and 1.3.4) have been the focus of much research over the last 2 decades in a variety of cells (Carson and Wei, 2000; Goldmann, 2012a, b; Shyy and Chien, 2002; Zebda et al., 2012). The following

will briefly describe focal adhesions and will focus on the role of the focal adhesion-associated proteins FAK and paxillin in skeletal muscle and microvascular tissues.

1.3.6. Cell focal adhesions and force transmission

Focal adhesions are specialised adhesion plaques or structures that facilitate force transmission in cells. These are localised to the cell membrane (Kanchanawong et al., 2010) and are regulated by chemical as well as mechanical stimuli. Focal adhesions are force-dependent and assemble at points of applied force (Balaban et al., 2001; Riveline et al., 2001). Their growth and size is in proportion to the applied force where a net accumulation of proteins are observed in mechanically-stressed focal adhesions (Balaban et al., 2001; Riveline et al., 2001). Cell focal adhesions are <200 nm in size (Franz and Muller, 2005) and are multi-functional, regulating cell adhesion to the ECM and cytoskeletal organisation and signalling. Focal adhesions therefore facilitate a bidirectional transmission of force between the ECM, cytoskeleton and cytoplasmic contractile machinery (Geiger et al., 2001).

1.3.7. Focal adhesion structure

In response to applied force, immature adhesions (nascent adhesions) rapidly associate with actin and myosin to become a focal complex and mature to focal adhesions after sustained actin and myosin tension generation and force transmission. Focal adhesions are disassembled within milliseconds at sites where the applied force is no longer present (Balaban et al., 2001), a process necessary for endothelial cell migration (Webb et al., 2004).

Focal adhesions are composed of an integrin-signalling layer containing focal adhesion kinase (FAK) and paxillin, an intermediate force transduction layer containing talin and vinculin and an actin regulatory upper-layer that contains α-actinin and a number of other proteins (Kanchanawong et al., 2010). Focal adhesions can contain 156 distinct molecules

and nearly 690 interactions of which 55% are binding interactions and 45% are activation/ inhibition processes (Zaidel-Bar et al., 2007).

Focal adhesions are therefore highly dynamic complex structures and are assembled and disassembled in response to mechanical forces to maintain normal cell function. The following section will now discuss the protein-containing structure in skeletal muscle that contains focal adhesions. It will concentrate on the integrin-signalling layer (Kanchanawong et al., 2010) and its role in skeletal muscle mechanotransduction with a particular focus on FAK and paxillin since this structural protein assembly is regarded to be the principle point of mechano-sensing (Goldmann, 2012a) (figure 1.3, 1.4).

1.4. Force transmission in skeletal muscle

1.4.1. Lateral force transmission

The myotendinous junction (MTJ) and the sarcolemma are the two force-transmitting points in skeletal muscle fibres (Bloch and Gonzalez-Serratos, 2003; Monti et al., 1999). Street (Street, 1983) showed that an electrically-stimulated single fibre (that was still attached to muscle fibres at its distal end) could transmit >75% of the force to a force transducer through its adjacent connections with other muscle fibres, as described in these reviews (Bloch and Gonzalez-Serratos, 2003; Monti et al., 1999). Street (Street, 1983) also found a correlation between markers placed on a stretched sarcolemma and the underlying sarcomeres. This suggested a connection between the sarcomeres and the sarcolemma and these connections kept the sarcolemma in register with the sarcomere (Bloch and Gonzalez-Serratos, 2003; Monti et al., 1999). It is therefore thought that the lateral transmission of force from contracting myofibrils to the sarcolemma and then to adjacent muscle fibres before ending at the MTJ constitutes the greatest avenue of force transmission rather than longitudinally through the sarcomeres (Bloch and Gonzalez-Serratos, 2003; Monti et al., 1999).

1.4.2. Costameres

The sarcolemmal connections were first visualised in avian skeletal muscle and were termed 'costameres' (Greek where *costa* means rib and *mere* means part) (Pardo et al., 1983) based on their rib-like appearance, much like the metal bands around a wooden barrel (Ervasti, 2003; Pardo et al., 1983). Costameres can be defined as subsarcolemmal protein assemblies that circumferentially align in register with the Z-disk of peripheral myofibrils and physically couple force-generating sarcomeres with the sarcolemma (Ervasti, 2003; Pardo et al., 1983). They may also span the I-band and anchor the M-line to the sarcolemma (figure 1.1.) (Mondello et al., 1996). During skeletal muscle contraction, costameres cause the sarcolemma to pleat into small and tolerable folds or 'festoons' which is thought to protect the sarcolemma from developing large festoons and the potential for sarcolemmal damage (Pardo et al., 1983; Street, 1983).

Skeletal muscle costameres are composed of focal adhesions (1.3.2 and 1.3.3) which are known to contain integrins, vinculin, FAK and paxillin (Quach and Rando, 2006). They also contain other protein complexes such as the dystrophin-glycoprotein complex (DGC) (Bloch and Gonzalez-Serratos, 2003) (figure 1.4). As well as dystrophin, the DGC includes the α- and β-dystroglycans, the sarcoglycans, sarcospan, the syntrophins and α-dystrobrevin. Dystrophin binds to dystroglycan and actin and therefore forms a link between actin and the ECM (Chamberlain et al., 1997). The DGC functions to maintain the structural integrity of the muscle fibre and, in addition to focal adhesions, provides an avenue for force transmission. Costameres are dynamic structures and their focal adhesion and DGC protein content is modulated by periods of (un)loading (Chopard et al., 2005; Kosek and Bamman, 2008; Woolstenhulme et al., 2006). Defects in certain costameric proteins such as dystrophin can increase the fragility and susceptibility of skeletal muscle fibres to contraction-induced

damage and lead to severe myopathies such as in Duchenne muscular dystrophy (Bloch et al., 2004).

Costameres therefore play an integral role in lateral force transmission, in maintaining the integrity of the sarcolemma and protecting the sarcolemma from contraction-induced damage. Sections 1.4.3. to 1.5. discuss the principle costameric proteins involved in mechanotransduction.

1.4.3. Integrins

Integrins are αβ heterodimeric transmembrane glycoprotein receptors for ECM proteins (Guan, 1997) and link the ECM to the intracellular actin cytoskeleton (for review see (Guan, 1997; Hynes, 2002). Integrins do not contain an actin-binding site (Roca-Cusachs et al., 2012), but are indirectly attached to the actin cytoskeleton through the structural and signalling protein talin (Calderwood et al., 1999), vinculin (Humphries et al., 2007) and α-actinin (Otey et al., 1990; Pavalko et al., 1991).

The extracellular domain of integrin α-subunit (120 – 180 kDa) is noncovalently bound to the integrin β-subunit (90 – 110 kDa) to form an ECM ligand binding site (Shattil et al., 2010). The large extracellular domain (>100 kDa) therefore binds the ECM and the short intracellular domain (>75 kDa) indirectly binds the actin cytoskeleton (Hynes, 1992) (figure 1.3). A minimum of 18 different α- and 8 β-subunits have been identified and can form 24 separate α- β-integrins in humans (Hynes, 2002). The α7 subunit is the primary α-integrin subunit in adult skeletal muscle and of its three isoforms (A, B, C), α7C is the most abundant (Martin et al., 1996). A spliced variant of the β1-subunit (β1D-subunit) is the most abundant in, and specific to, skeletal and cardiac muscle (van der Flier et al., 1995). The vascular endothelium can express a variety of αβ-subunits with α1β1 and α2β1 (Senger et al., 1997) and αvβ3, β5 integrins being particularly important in angiogenesis (Nisato et al., 2003).

1.4.4. Activation of integrins by force

Integrins can be activated by 'outside in' mechanisms such as ECM ligand binding (known as ligation) and/ or through force (e.g. catch bonds). Integrins can also be activated through 'inside out' mechanisms where cytoskeletal proteins such as talin bind to the β1-subunit (Calderwood et al., 1999; Friedland et al., 2009; Hynes, 2002; Moser et al., 2009; Roca-Cusachs et al., 2012; Tadokoro et al., 2003). These induce a conformational change between the αβ subunits which facilitates bidirectional signalling (Hynes, 2002).

Integrins cluster (known as aggregation) in focal adhesions upon ligand binding or when force is applied. This reduces the amount of force per integrin resulting in increased focal adhesion strength and resistance to force (Choquet et al., 1997). Integrins that are bound to magnetic ligand-coated beads (which impart tensile or twisting forces) demonstrate a high resilience to force which increases in proportion to the applied force (Matthews et al., 2004). Integrins then convert these forces into intracellular signalling through recruitment of intracellular signalling, adaptor and cytoskeletal proteins (Dey et al., 2011; Wang et al., 1993). In murine skeletal muscle, overexpression of the α7β1-subunits increased muscle hypertrophy after a single and multiple bouts of eccentric exercise (Lueders et al., 2011; Zou et al., 2011).

1.4.5. Integrin-mediated recruitment of cytoskeletal proteins

Integrins are without enzyme activity so must recruit signalling proteins for integrin-mediated processes such as cell migration, cell growth and apoptosis (Hynes, 2002). Integrin aggregation and ligation is largely required for binding many cytoskeletal proteins (Miyamoto et al., 1995). Upon integrin activation, the β1-subunit serves as a docking site for focal adhesion kinase (FAK) (Guan and Shalloway, 1992; Guan et al., 1991), talin (along with vinculin) (Calderwood et al., 1999; Horwitz et al., 1986; Humphries et al., 2007;

Tadokoro et al., 2003) and α–actinin (Otey et al., 1990) which are independent of integrin tyrosine phosphorylation (Miyamoto et al., 1995) (figure 1.3 and 1.4). However, aggregation, ligation and the addition of tyrosine phosphorylation of integrins is required for the recruitment of the adaptor protein paxillin, signalling proteins such as cellular Src, members of the mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinase (ERK) 1/2 and other signalling proteins such as phosphotidylinositol-3-kinase (PI3-K) (Miyamoto et al., 1995).

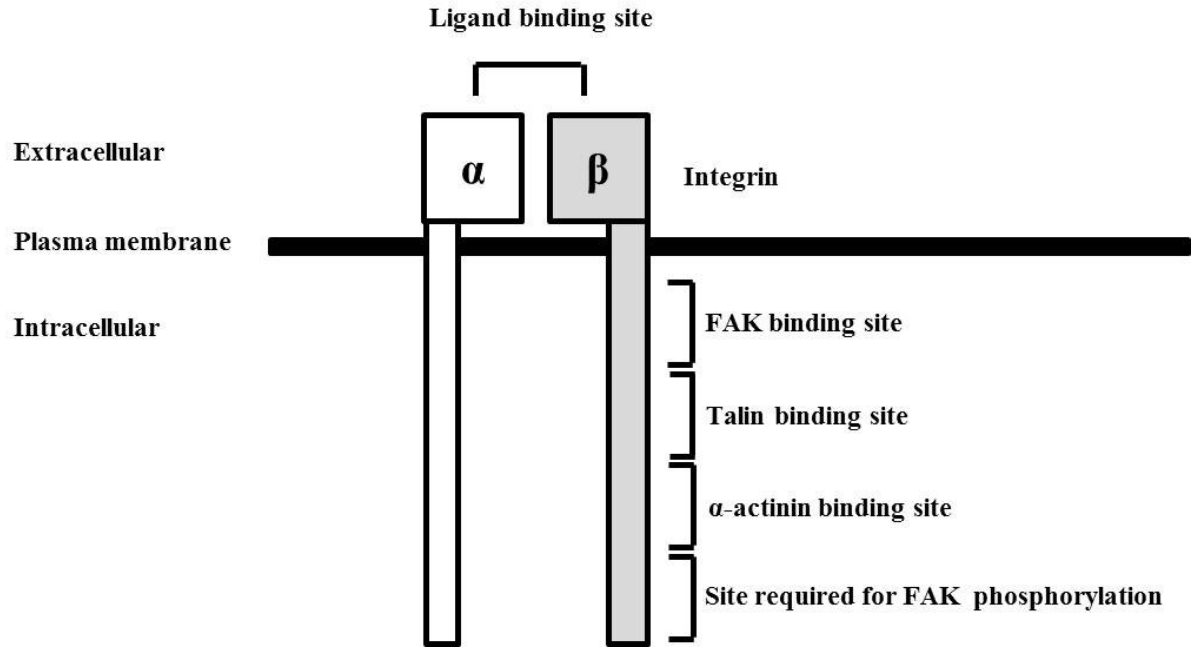

Figure 1.3 Integrin structure. Diagrammatic representation of α- and β-integrins that identifies extracellular matrix (ECM) ligands (e.g. fibronectin) and selected intracellular focal adhesion-associated protein binding sites. FAK, focal adhesion kinase. Adapted from (Schlaepfer et al., 1999).

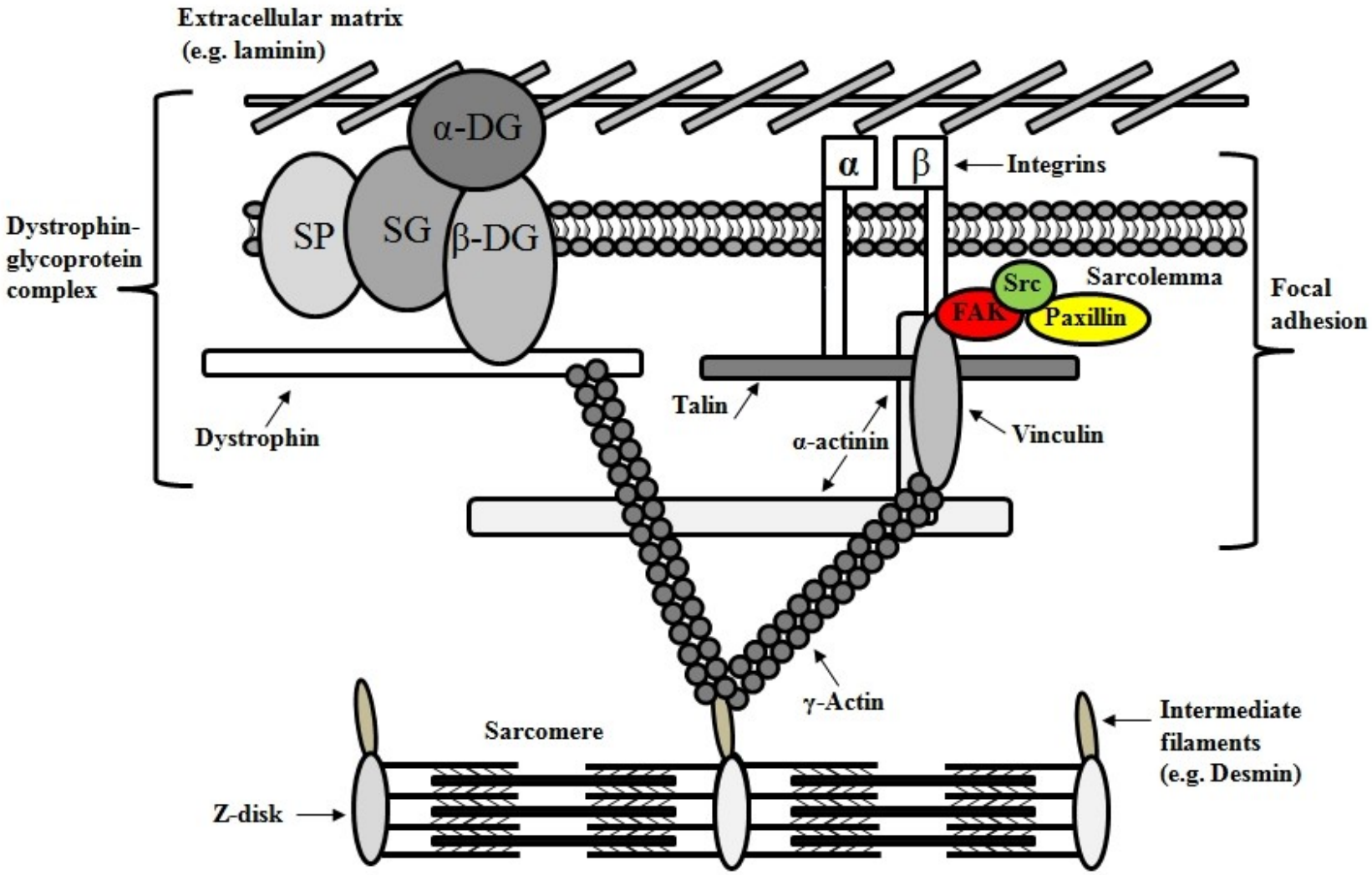

Figure 1.4. Schematic diagram of a skeletal muscle costamere composed of a focal adhesion and dystrophin-glycoprotein complex. α–DG, α-dystroglycan; β-DG, β-dystroglycan; SG, Sarcoglycan; SP, Syntrophin; FAK, focal adhesion kinase.

1.4.6. Focal adhesion kinase (FAK)

FAK is a 125 kDa non-receptor cytoplasmic enzyme (Guan et al., 1991; Schaller et al., 1992) named by Schaller et al, on account of its localisation to and abundance in focal adhesions (Kanchanawong et al., 2010; Schaller et al., 1992). FAK is ubiquitously expressed and evolutionarily conserved across many species sharing ~90% identity (Guan, 1997; Parsons, 2003) and is required for cell adhesion strength and cell stiffness (Fabry et al., 2011). Integrin ligation stimulates the direct recruitment of FAK to the integrin β1-subunits and their interaction is accompanied by increased FAK tyrosine phosphorylation (Guan et al., 1991). Consequently, FAK has been suggested to play a pivotal role in integrin-mediated signalling and force sensing (Guan, 1997; Parsons, 2003; Schlaepfer et al., 1999). This was robustly demonstrated *in vivo* in mice with a knockout of the FAK gene (Ilic et al., 1995). The mice died within 8.5 days of embryo, presenting with poor development of the heart and blood vessels consequent to defects in vasculogenic and angiogenic processes (Ilic et al., 1995).

1.4.7. FAK structure

FAK is composed of 3 distinct regions where the central kinase domain is flanked by N-terminal and C-terminal domains (Parsons, 2003; Schlaepfer et al., 1999). The N-terminal contains an erythrocyte band 4.1, erzin, radixin, moesin (FERM) domain which is linked to the catalytic kinase domain by a linker segment and the kinase domain is linked to the C-terminal domain by a proline rich region (Arold et al., 2002) (figure 1.5). The N-terminal domain regulates the localisation and binding of FAK to the cytoplasmic tail of β1 integrins since deletion of the C-terminal had no effect on FAK localisation (Schaller et al., 1995). FAK is localised to focal adhesions by its C-terminal domain (Hildebrand et al., 1993). This contains a focal adhesion targeting (FAT) domain which is composed of protein binding domains and multiple phosphotyrosine binding sites that facilitate interaction with proteins such as paxillin

(Tachibana et al., 1995). The FAT domain contains two paxillin binding sites (Gao et al., 2004; Scheswohl et al., 2008) where only one functioning site is required for paxillin to target FAK to focal adhesions (Scheswohl et al., 2008). However, two functioning paxillin binding sites are essential for maximal FAK phosphorylation and maximal FAK-mediated downstream signalling (Scheswohl et al., 2008).

Integrin clustering stimulates FAK autophosphorylation on Tyr^{397} (Guan et al., 1991; Parsons, 2003; Schaller et al., 1994) which is situated in the linker segment between the N-terminal and kinase domains. Autophosphorylation creates a high-affinity binding site for proteins containing Src homology (SH) 2 domains. These include the protein tyrosine kinases such as cellular Src and the p85 subunit of PI3-K (Chen et al., 1996) which are both linked to striated muscle hypertrophy (Glass, 2010; Marin et al., 2008). Following the binding of Src at Tyr^{397}, Src then phosphorylates FAK on $Tyr^{576/\ 577}$ which is necessary for maximum FAK activation (Calalb et al., 1995) and can also phosphorylate FAK on Tyr^{925} (Schlaepfer and Hunter, 1996) (figure 1.5). The latter creates an SH2 binding site for growth factor receptor-bound protein 2 (Grb2) which leads to activation of the ERK-MAPK signalling cascade (Webb et al., 2004). In skeletal muscle, FAK can also be phosphorylated on Tyr^{397} by non-integrin stimuli such as insulin (Goel and Dey, 2002), insulin-like growth factor-I (IGF-I) (Baron et al., 1998) and vascular endothelial growth factor (VEGF) in endothelial cells (Abedi and Zachary, 1997; Avraham et al., 2003). FAK phosphorylation and activation is also dependent upon the integrity of the actin cytoskeleton. Inhibition of actin polymerisation by cytochalasin (a toxin that binds to the barbed ends of actin filaments) prevents the activation of FAK by a variety of stimuli such as fluid shear stress (Li et al., 1997a), integrins (Lipfert et al., 1992) and VEGF (Abedi and Zachary, 1997).

1.4.8. Inactive FAK

FAK is not constitutionally active and in the absence of mechanical stimuli, maintains a dephosphorylated and inactive state when FAK is localised to the C-terminal region of myosin heavy chain in cardiomyocytes (Fonseca et al., 2005). Evidence suggests the inactivity of FAK is maintained through the interaction of its N-terminal FERM domain with its kinase domain. (Cooper et al., 2003). Indeed, FAK phosphorylation and activity were increased in experiments that truncated the N-terminal domain and overexpression of the N-terminal domain reduced FAK phosphorylation and activity (Cooper et al., 2003).

It is thought that the FERM domain maintains inactive FAK in a closed conformation through protein to protein and protein to membrane interactions. The FERM domain contains three sub-domain lobes (F1, F2 and F3) which appear to inhibit FAK through two known mechanisms. Firstly, the F1 lobe binds to the linker segment containing the Tyr^{397}autophosphorylation site thereby masking the phosphorylation site. Secondly, the $Tyr^{576/\ 577}$ phosphorylation sites are hidden or "sequestered" between the F1 and F2 lobes (Cooper et al., 2003; Lietha et al., 2007). It is thought that this conformation prevents FAK activation by restricting FAK-associated proteins (e.g. Src) from accessing the phosphorylation and kinase domains (Lietha et al., 2007). Cooper and colleagues (2003) proposed a model whereby proteins such as paxillin bind to inactive FAK's FAT domain thereby stimulating FAKs localisation to focal adhesions. FAK subsequently binds to the cytoplasmic tail of integrin β1-subunits where the integrin cytoplasmic tail is thought to displace the FERM-mediated autoinhibition thereby leading to FAK activation.

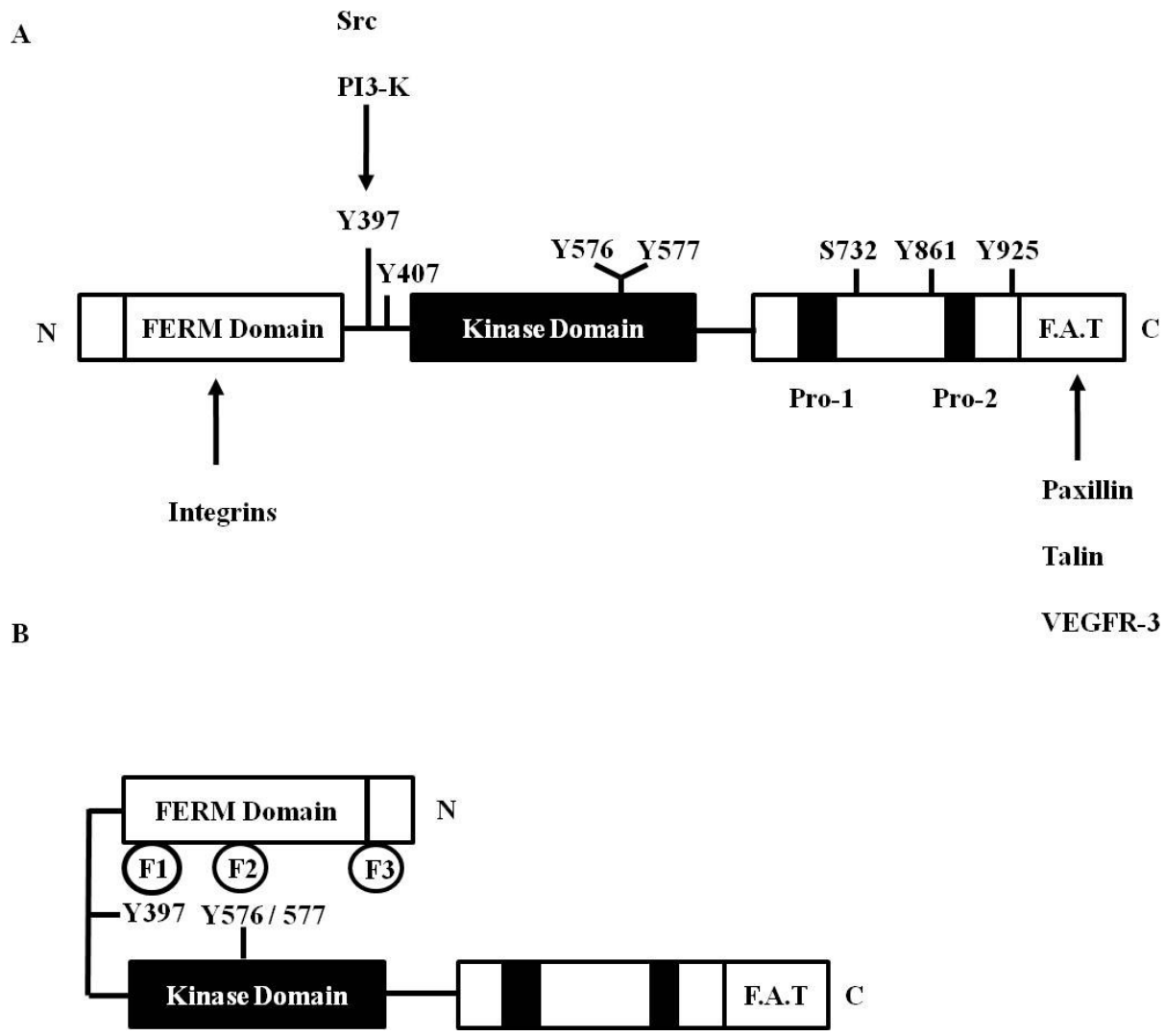

Figure 1.5. Diagrammatic structure of focal adhesion kinase (FAK). A) FAK Tyr^{397} autophosphorylation site is a target of cellular Src (Src) and the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI3-K). Binding of c-Src and p85 stimulates autophosphorylation of FAK which leads to increased $Tyr^{576/577}$ phosphorylation for maximal FAK activation. B) In its inactive state, the FERM domain subdomains F1 and F2 mask the Tyr^{397} and $Tyr^{576/577}$ phosphorylation sites preventing access of Src and PI-3K. FERM, erythrocyte band 4.1, erzin, radixin, moesin; F. A. T, focal adhesion targeting domain; Pro, proline-rich region; VEGFR, vascular endothelial growth factor receptor.

1.4.9. FAK-related non-kinase (FRNK)

FAK is also negatively regulated by its endogenous competitor FAK-related non-kinase (FRNK) (Schaller et al., 1994). FRNK is a separate expression of FAK's 42 kDa C-terminal FAT domain that transiently blocks the formation of focal adhesions, inhibits the binding of FAK and paxillin and their tyrosine phosphorylation (Richardson and Parsons, 1996) and displaces FAK from focal adhesions (Heidkamp et al., 2002). In rat skeletal muscle, FRNK abolished the FAK-mediated upregulation of slow twitch muscle gene expression (Durieux et al., 2009).

1.4.10. Paxillin

Paxillin is a 68 kDa adaptor protein (Glenney and Zokas, 1989) named after the Latin "Paxillus" meaning stake or peg which reflected its role as a scaffold for focal adhesion structural and signalling proteins (Turner et al., 1990). Paxillin is evolutionarily conserved (Brown and Turner, 2004) and binds to β1 integrins (Miyamoto et al., 1995; Schaller et al., 1995). It contains a vinculin- (Turner et al., 1990) and FAK-binding domain (Turner and Miller, 1994) which suggests paxillin may play an important role in integrin-signalling and mechanotransduction. This was critically demonstrated *in vivo* where mice with a paxillin knock out gene died after 9.5 days of embryo consequent to developmental defects in the heart and ex-vivo abnormalities in cell migration (Hagel et al., 2002). The latter was attributed to poor FAK localisation and impaired FAK and MAPK phosphorylation (Hagel et al., 2002).

1.4.11. Paxillin structure

Paxillin is divided into N- and C-terminal domains and has no enzyme activity. It's N-terminal domain contains 5 protein binding sites (LD motifs) of which two are FAK-binding

sites (Brown et al., 1996) which have equal affinity for FAK and are both required for the highest binding affinity of paxillin with FAK (Thomas et al., 1999). Its C-terminal domain contains 4 LIM domains (Lin11, IsI-1, Mec-3) which can mediate protein-protein interaction and are binding sites for proteins such as Src (Weng et al., 1993). LIM domain 3 is the principle determinant for targeting paxillin to focal adhesions (Brown et al., 1996).

Binding of the FAK/Src complex with paxillin phosphorylates paxillin on Tyr^{31} and Tyr^{118} (Richardson et al., 1997; Schaller and Parsons, 1995; Thomas et al., 1999). Paxillin is also phosphorylated on tyrosine by PI3-K (Casamassima and Rozengurt, 1998) and ERK and by p38 MAPK on Ser^{83} (Huang et al., 2004a; Ishibe et al., 2004) and by Jun N-terminal kinase (JNK) on Ser^{178} (Huang et al., 2004b) (figure 1.6). For a more detailed discussion on additional paxillin binding proteins the reader is referred to the following reviews (Brown and Turner, 2004; Deakin and Turner, 2008; Turner, 2000).

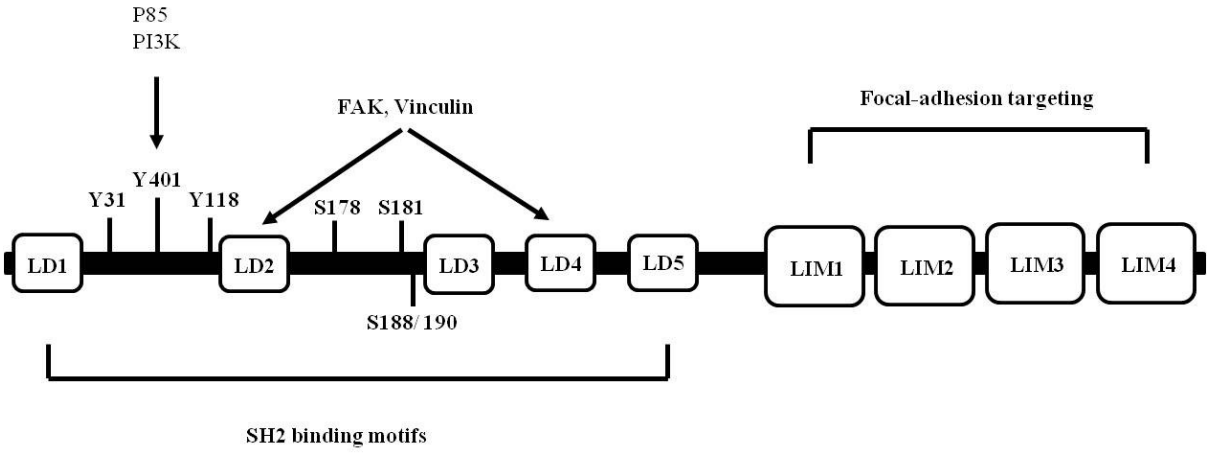

Figure 1.6. Diagram of the paxillin structure and identification of some of the protein binding domains, focal adhesion targeting domains and phosphorylation sites. PI3-K, phosphotidylinositol-3 kinase; LIM, Lin11, IsI-1, Mec-3. SH2, Src homology 2.

1.5. FAK and paxillin responses to physical (in)activity in skeletal muscle

1.5.1. Acute responses of FAK and paxillin to loading

At present, the effect of mechanical loading on skeletal muscle FAK and paxillin phosphorylation is limited. In rodent L6 skeletal muscle cells *in vitro*, FAK $Tyr^{576/577}$ phosphorylation (a marker of maximal FAK activity) was increased after 30 min of uni-axial cyclic stretch and remained elevated for up to 60 min post-stretch (Atherton et al., 2009). The uni-axial stretch also increased Akt kinase activity and ERK 1/2 and p70S6K phosphorylation during the 60 min post-stretch period (Atherton et al., 2009). Fluck et al (Fluck et al., 1999) subjected the rooster *anterior latissimus dorsi* skeletal muscle to stretch-overload by placing a weight equal to 10% of its body mass on the wing. *In vivo* FAK and paxillin tyrosine phosphorylation increased within 1.5 days of stretch-overload and remained elevated for up to 13 days of stretch (Fluck et al., 1999). FAK and paxillin tyrosine phosphorylation also increased at 1 and 8 days of overload of the rat *m. soleus* and *m. plantaris* through surgical ablation of the *m. gastrocnemius* (Fluck et al., 1999; Gordon et al., 2001).

In human *m. vastus lateralis* skeletal muscle, FAK $Tyr^{576/577}$ phosphorylation increased immediately after an acute bout of endurance- or resistance-type exercise in the untrained and trained state (12 weeks ET or RT) and remained elevated for up to 4 hours post exercise. In addition, a higher resting FAK $Tyr^{576/577}$ phosphorylation was reported post-training (Wilkinson et al., 2008). These data suggest FAK and paxillin are rapidly phosphorylated in response to stretch and overload in animal and human skeletal muscle. It also suggests there is no difference in the capacity of endurance- and resistance-type exercise to activate FAK despite the different magnitudes of force generated.

1.5.2. Evidence for a role of FAK and paxillin in skeletal muscle hypertrophy

FAK and paxillin protein concentration increased ~200% in hypertrophied rooster ALD after 7 days of stretch overload as described above in 1.5.1 (Fluck et al., 1999). Overload of the rat *m. soleus* (as described in 1.5.1) increased skeletal muscle mass after 8 days of loading and this was accompanied by an increase in FAK and paxillin protein concentration (>600%) compared with sham-operated controls (Fluck et al., 1999). FAK and paxillin protein content also increased after 8 days of overload of the *m. plantaris* muscle through surgical ablation of the gastrocnemius (Gordon et al., 2001).

These studies do not confirm a causative role for FAK and paxillin in skeletal muscle hypertrophy. However, in cardiomyocytes, an increase in FAK protein expression and phosphorylation was observed in the hypertrophied left ventricle of aortic-banded mice (Clemente et al., 2007). Interestingly, the load-induced hypertrophy of the left ventricle was prevented and reversed by targeting FAK with small interfering RNA (siRNA) *in vivo* (Clemente et al., 2007). In addition, Cre-Lox recombination of the FAK gene of murine cardiomyocytes (cardiomyocyte-specific FAK gene deletion) blunted the aortic-band-induced left ventricular wall thickness *in vivo* (DiMichele et al., 2006). Taken together, these studies suggest a chronic increase in FAK and paxillin protein expression in response to increased mechanical loading in animal skeletal muscle. The changes in protein expression after loading are also accompanied by an increase in skeletal muscle mass. This suggests a potential role for FAK and paxillin in skeletal muscle hypertrophy. However, at the start of this PhD thesis, there was no information on FAK and paxillin protein expression and their response to exercise training in human skeletal muscle. In addition, the studies described above used western blotting analysis of whole skeletal muscle homogenates which does not provide information on fibre- and microvascular-specific responses to exercise training in humans.

1.5.3. FAK-activated downstream signalling pathways *in vivo* and *in vitro*

There is a paucity of information on the downstream targets of FAK-activated signalling in human skeletal muscle *in vivo*. However, there are two *in vitro* studies in rat skeletal muscle that have generated important information. Durieux et al, (2009) induced local overexpression of FAK in the rat *m. soleus* using gene electrotransfer of cytomegalovirus (CMV) promoter-driven expression plasmid which encoded a chicken non-constitutively active FAK gene (pCMV-FAK). The *m. soleus* (primarily slow twitch fibres) was initially subjected to 7 days of unloading before mechanically re-loaded with normal cage activity for 1 day. FAK overexpression increased the expression of genes associated with slow twitch skeletal muscle fibres *in vivo* which included the MHC type I isoform and increased the protein content of nuclear-encoded mitochondrial proteins such as cytochrome oxidase IV, but not mitochondrially-encoded proteins such as cytochrome oxidase I (Durieux et al., 2009). The authors speculated that this was due to a lack of increased energy consumption in the model employed (Durieux et al., 2009).

Using similar techniques, Klossner et al, (2009) showed that after 7 days of unloading by tail suspension *in vivo* mechanical re-loading of the murine *m. tibialis anterior* (primarily fast twitch fibres) led to FAK Tyr^{397} phosphorylation and Akt-independent phosphorylation of p70S6K (Klossner et al., 2009). These studies imply that increases in FAK protein expression and FAK Tyr^{397} phosphorylation are upstream of the molecular adaptation that follows increases in skeletal muscle workload and that the targeted signalling pathways differ between slow oxidative and fast glycolytic muscle fibres.

In vitro studies that have investigated FAK-mediated downstream signalling in skeletal muscle are lacking. However, cyclic uni-axial stretch (15%) of neonatal rat ventricular myocytes increased FAK Tyr^{397} phosphorylation and reduced the high baseline association of FAK with Src homology phosphatase 2 (Shp2) (Marin et al., 2008). Myocyte

depletion of Shp2 by siRNA increased the phosphorylation of FAK Tyr^{397}, Src, Akt, tuberous sclerosis 2 (TSC2), and p70S6K in non-stretched myocytes. In addition, Shp2 depletion also stimulated hypertrophy of non-stretched neonatal rat ventricular myocytes. Conversely, FAK depletion through siRNA blunted cyclic stretch-induced FAK Tyr^{397}, Src, Akt, TSC2, and p70S6K phosphorylation and myocyte hypertrophy (Marin et al., 2008). This study suggests that FAK may play an important role in the protein synthesis signalling pathway and may be an upstream regulator of hypertrophy in neonatal rat ventricular myocytes. Future studies will have to show whether FAK also has this role in human skeletal muscle. A schematic of the proposed role of FAK in the adaptation of skeletal muscle fibres to exercise is presented in figure1.7.

1.5.4. FAK and paxillin responses to skeletal muscle unloading

Mechanical unloading of the skeletal muscle leads to a loss of skeletal muscle mass, cross-sectional area and a reduction in FAK tyrosine phosphorylation (de Boer et al., 2007; Glover et al., 2008; Gordon et al., 2001). FAK total protein content in the *m. soleus, plantaris* and *gastrocnemius* muscles decreased by >40% after 7 days of rat hindlimb unloading via tail suspension (Gordon et al., 2001). This was accompanied by a significant reduction in FAK tyrosine phosphorylation corrected to total FAK protein in only the *m. soleus* muscles (Gordon et al., 2001). Unloading also modulated the FAK and paxillin response in a fibre-type specific manner with their protein concentration increased in the *m. soleus*, but decreased in the *m. plantaris* and *gastrocnemius* muscles. The authors speculated that the increased FAK protein concentration in the *m. soleus* was a compensatory response of the *m. soleus* to the lack of mechanical stimulation (Gordon et al., 2001).

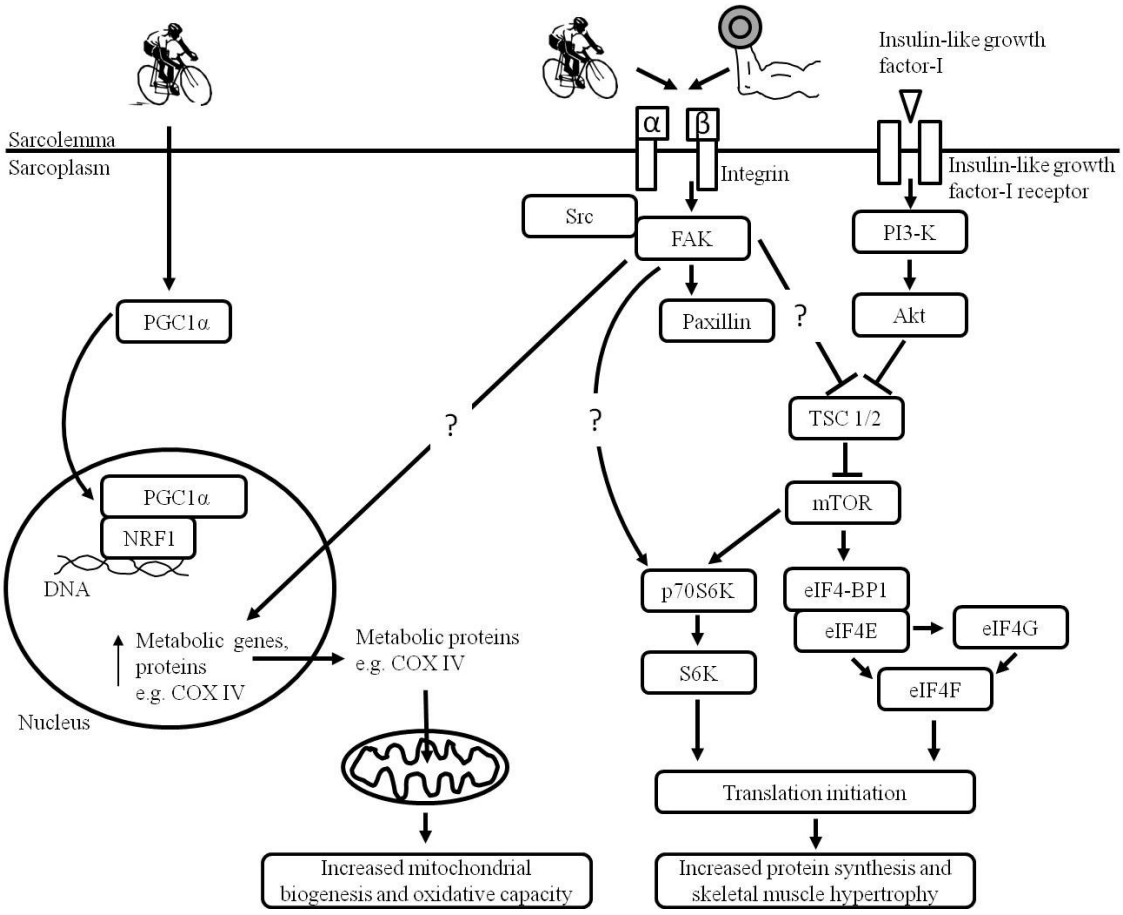

Figure 1.7. Proposed role of endurance-(ET) and resistance-type (RT) exercise training in integrin-mediated activation of FAK and skeletal muscle fibre adaptation. ET leads to the activation and translocation of peroxisome proliferator activated γ coactivator-1α (PGC1α) to the nucleus. The interaction of PGC1α with nuclear respiratory factor-1 (NRF-1) increases the expression of nuclear- (e.g. cytochrome oxidase IV; COX IV) and mitochondrial-encoded genes necessary for increased oxidative capacity and mitochondrial biogenesis (Yan et al., 2011) which may involve FAK (Durieux et al., 2009). RT-induced activation of FAK leads to the Akt-independent phosphorylation of ribosomal protein S6 kinase (p70S6K) (Klossner et al., 2009) possibly through a FAK-mediated phosphorylation and inhibition of tuberous sclerosis complex 1/ 2 (TSC 1/ 2) (Marin et al., 2008). This liberates mammalian target of rapamycin (mTOR) to phosphorylate and inhibit eukaryotic initiation factor 4 binding protein-1 (eIF4-BP1), leading to the formation of the translation initiation complex eIF4F, increased protein synthesis and skeletal muscle fibre hypertrophy.

In humans, FAK $Tyr^{576/577}$ phosphorylation in the *m. vastus lateralis* (mixed fibre types) was reduced by 30% at the mid-phase (10 days) of cast immobilisation (de Boer et al., 2007), and was reduced by ~23% after 14 days knee-braced immobilisation (Glover et al., 2008). Moreover, these studies also reported a decline in the rates of muscle protein synthesis. Taken together, these studies suggest that a reduction of skeletal muscle mechanical load in animals and humans leads to a reduction in FAK tyrosine phosphorylation and muscle-specific alterations in FAK protein concentration in rats. These alterations may also occur in elderly skeletal muscle in response to the reported reductions in physical activity levels and the adoption of a sedentary lifestyle (HSfE, 2010). Consequently, FAK may play an important role in the mechanisms that lead to a loss of skeletal muscle mass and the etiology of sarcopenia. Regulation of FAK by physical (in)activity may therefore be of significance to young and elderly sedentary individuals.

1.5.5. Localisation of FAK and paxillin in skeletal muscle fibres

Immunohistochemistry (Fluck et al., 1999; Fluck et al., 2002) and fluorescence microscopy (Durieux et al., 2009; Klossner et al., 2009; Quach and Rando, 2006) have been used to visualise FAK and paxillin in animal skeletal muscle. In rats, a high FAK protein content was present in the sarcolemma in transversely and longitudinally orientated skeletal muscle fibres of the *m. soleus* and *m. extensor digitorum longus* (EDL) (Fluck et al., 2002). Sarcolemmal FAK was observed in the majority of type I fibres in the *m. soleus*, but only in 50% of EDL muscle fibres (Fluck et al., 2002). This was consistent with the greater total FAK protein content measured with western blots in *m. soleus* compared with *m. plantaris* and *m. gastrocnemius* muscles by Gordon et al., (2001). In addition, <15% of EDL type IIb fibres showed FAK sarcolemmal immunolabeling. These data suggest that in animal skeletal

muscle, a high FAK protein content is present in the sarcolemma primarily in type I muscle fibres.

In the same study, the *m. soleus* and EDL muscles were then explanted and the authors cross-reinnervated the *m. soleus* with EDL nerve fibres. As a consequence, sarcolemmal FAK immunolabeling was reduced in *m. soleus* type I fibres and was increased in EDL type I and type II fibres. The authors suggested that the firing rate of the innervating nerve and the muscle fibres recruited controlled the localisation of FAK at the sarcolemma. As type I fibres are the predominant fibres recruited during free living and standing in rats, the authors speculated that the greater sarcolemmal FAK immunolabeling in type I fibres was an adaptive response to the higher overall mechanical load experienced daily by postural muscles (Fluck et al., 2002).

In addition to the sarcolemmal immunolabeling observed by Fluck et al (Fluck et al., 1999; Fluck et al., 2002), Durieux et al, (2009) also reported an intense FAK intracellular immunoreactivity in type I compared with type II fibres after local overexpression of FAK through electro-gene transfer. In adult mouse transversely orientated EDL skeletal muscle, FAK was distributed along the sarcolemma, but also as striations that spanned the full width and perpendicular to the direction of the fibre (Quach and Rando, 2006). These striations colocalised with α5 integrins, vinculin and α-actinin over the I band and M band and confirmed that FAK was localised to costameres in murine skeletal muscle fibres (Quach and Rando, 2006). There is a paucity of data regarding the localisation of paxillin in mammalian skeletal muscle. In murine myoblasts, paxillin was localised to focal adhesions, and in murine myotubes, was localised to maturing costameres overlying the Z-disk (Quach and Rando, 2006).

These data suggest FAK and paxillin are localised to areas of force transmission corresponding to costameres in rat skeletal muscle. However, the spatial distribution of FAK

and paxillin in human skeletal muscle has not been investigated, nor its protein expression. Furthermore, no studies have investigated whether FAK and paxillin colocalise in human skeletal muscle fibres and their microvasculature.

1.6. Mechanotransduction in the microvasculature

1.6.1. Activation of FAK and paxillin by shear force and cyclic stretch

The brush-like structures of the endothelial glycocalyx layer are thought to have a flexural rigidity which allows the glycocalyx to tolerate fluid shear stress-induced deformations (Weinbaum et al., 2003). It is estimated that the tips of the glycocalyx fibres undergo a maximum deformation of <10 nm at a shear stress of 10 dyne/cm^2 (Weinbaum et al., 2007) (figure 1.7). It is thought that this transmits a bending moment through the glycocalyx to the cytoskeleton below the apical surface (Weinbaum et al., 2007). Consequently, the glycocalyx acts as a force transducer by transmitting fluid shear stress forces through the intracellular cytoskeleton to the abluminal focal adhesions (Florian et al., 2003; Weinbaum et al., 2007). Weinbaum and colleagues (Weinbaum et al., 2007) also discuss the possibility that the glycocalyx is held under an axial tension and changes in fluid shear stress pulls with varying degrees of force on the glycocalyx thereby affecting the state of the underlying cellular cytoskeleton (Weinbaum et al., 2007). In experiments where the glycocalyx is degraded, the apical surface acts as a force transducer by transmitting force through stress fibres to focal adhesions (Weinbaum et al., 2007). It should also be noted that the fluid shear stress forces acting on the luminal membrane are balanced by the forces exerted by focal adhesions to keep the endothelial cell anchored to the ECM and remain in place. Therefore, focal adhesions can still sense fluid shear stress forces independent of the glycocalyx (Tarbell and Pahakis, 2006; Tarbell et al., 2005).

FAK and paxillin are heterogeneously distributed in intracellular regions in static bovine aortic endothelial cells and human umbilical vein endothelial cells (Li et al., 2002; Li et al., 1997a; Mattiussi et al., 2006). Laminar fluid shear stress stimulates their rapid (within ~1 min) tyrosine phosphorylation, maximal activation and their translocation to focal adhesions (Li et al., 2002; Li et al., 1997a; Mattiussi et al., 2006).

Integrins and the FAK/Src complex have been shown to regulate fluid shear stress-activated flow-mediated dilation (FMD) of animal coronary arterioles (Koshida et al., 2005; Muller et al., 1997). The prevailing mechanism is that fluid shear stress-induced eNOS phosphorylation leads to the increased production of NO by eNOS and the diffusion of NO from the endothelium into the VSMC. This leads to the formation of cGMP in VSMCs, VSMC relaxation and vasodilation (McAllister and Laughlin, 2006). In rat coronary arterioles, fluid shear stress increased FAK Tyr^{397} autophosphorylation and this was associated with increased Akt and eNOS Ser^{1179} phosphorylation (Koshida et al., 2005). The latter is equivalent to eNOS Ser^{1177} phosphorylation in the human eNOS sequence which augments eNOS activity and NO production. FMD of rat coronary arterioles was inhibited with the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) and inhibition of FAK Tyr^{397} by a Tyr^{397}–specific blocking antibody abolished eNOS Ser^{1179} phosphorylation and FMD (Koshida et al., 2005). This study suggests that FAK may play an important role in FMD and a lack of FAK protein content and tyrosine phosphorylation may lead to endothelium-dependent vascular dysfunction. A summary of the potential mechanisms are summarised in figure 1.7.

Cyclic stretch of endothelial cells can also stimulate FAK and paxillin tyrosine phosphorylation and their recruitment to focal adhesions *in vitro* (Katanosaka et al., 2008; Naruse et al., 1998; Yano et al., 1996a; Yano et al., 1996b). These studies collectively demonstrate that FAK and paxillin are stimulated and activated by fluid shear stress and

cyclic stretch and these proteins may play an important role in endothelial mechanotransduction.

1.6.2. Role of FAK and paxillin in endothelial cell migration and angiogenesis

Endothelial cell sprouting and migration is one of the principle steps in angiogenesis (Prior et al., 2004; Risau, 1997). Angiogenesis has been reported to occur in response to both ET (Andersen and Henriksson, 1977; Klausen et al., 1981) and RT (Green et al., 1999; Hepple et al., 1997; McCall et al., 1996) and is linked to shear stress and mechanical stretch of vascular endothelial cells (Prior et al., 2004). This implies that FAK may play a potential role in the signalling mechanisms that lead to angiogenesis. In agreement with this suggestion is the observation that overexpression of the FAK gene in mice increased angiogenesis in the ischemic hindlimb (Peng et al., 2004), while conditional knock out of the FAK gene in mice led to a disorganised and apoptotic blood vessel appearance in developing embryos (Shen et al., 2005). In addition, deletion of FAK in endothelial cells from floxed FAK mice reduced endothelial cell survival, proliferation and capillary development *in vitro* (Shen et al., 2005). Increases in FAK protein content and tyrosine phosphorylation within the microvascular endothelium may therefore play an important role in the mechanisms by which exercise training increases angiogenesis and capillary density. On the other hand, decreases in FAK content and phosphorylation may contribute to the rarefaction (reductions in capillary density) that is seen in sedentary elderly individuals (Coggan et al., 1992).

VEGF plays an important role in exercise induced angiogenesis (Bloor, 2005) and circulating plasma VEGF concentration is increased in humans after endurance- (Kraus et al., 2004) and resistance-type exercise (Gavin et al., 2007). In cultured human umbilical vein endothelial cells and porcine aorta endothelial cells, VEGF rapidly phosphorylated FAK and paxillin with similar kinetics in a dose response dependent manner (Abedi and Zachary,

1997; Qi and Claesson-Welsh, 2001). In unstimulated human umbilical vein endothelial cells, FAK and paxillin were localised to intracellular regions and sparsely distributed, but 10 min of stimulation by VEGF potentiated their recruitment and colocalisation with the plasma membrane and focal adhesions (Abedi and Zachary, 1997). Binding of FAK to the C-terminal of the VEGF receptor phosphorylates FAK leading to FAK-mediated recruitment and phosphorylation of PI3-K (Abedi and Zachary, 1997; Qi and Claesson-Welsh, 2001). The latter protein is important for VEGF-mediated cell migration and angiogenesis since defects in the p110α subunit of PI3-K cause embryonic lethality (Graupera et al., 2008). Blocking of FAK through FRNK significantly inhibited focal adhesion formation and the spreading and migration of VEGF-stimulated human brain microvascular endothelial cells (Avraham et al., 2003). This emphasises the importance of FAK and FRNK in VEGF-mediated endothelial cell migration.

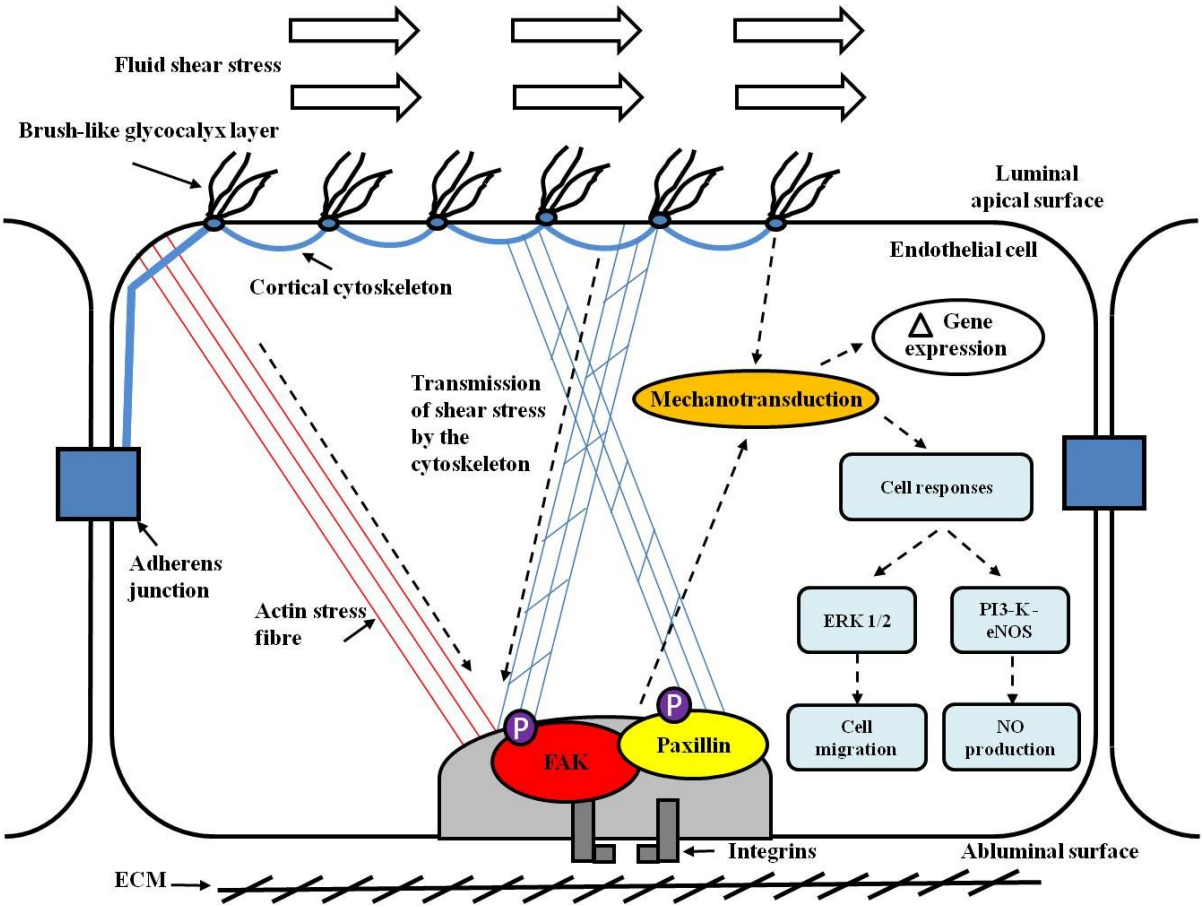

Figure 1.8. Schematic of fluid shear stress-induced deformation of luminal endothelial glycocalyx fibres and the subsequent transmission of force along the intracellular cytoskeleton to abluminal focal adhesions. Mechanotransduction of fluid shear stress by focal adhesions leads to endothelial cell migration and angiogenesis via stimulation of the extracellular signal-regulated kinase (ERK 1/ 2) signalling pathway. Activation and phosphorylation (P) of FAK also leads to activation of endothelial nitric oxide synthase (eNOS) and the subsequent increase in nitric oxide (NO) production. ECM, extracellular matrix; FAK, focal adhesion kinase; PI3-K, phosphotidylinositol-3-kinase.

1.6.3 FAK and paxillin in vascular smooth muscle cells

Information on FAK and paxillin in VSMCs contained within human skeletal muscle are lacking. However, FAK and paxillin are highly expressed in tracheal smooth muscle (Tang et al., 1999; Turner et al., 1991) and FAK is necessary for VSMC growth and migration (Hauck et al., 2000; Koshman et al., 2011; Sundberg et al., 2003). In unstimulated isolated tracheal smooth muscle fibres, FAK and paxillin was distributed throughout the cell as assessed by immunofluorescence microscopy (Opazo Saez et al., 2004). After stimulation by the contractile agonist acetyl-choline, FAK and paxillin were rapidly localised to membrane-associated focal adhesions for increased tension development and contraction (Opazo Saez et al., 2004). FAK and paxillin play an important role in smooth muscle actin cytoskeleton dynamics and force production (for review see (Gerthoffer and Gunst, 2001; Gunst and Zhang, 2008) and paxillin-null cells prevented the acetyl-choline-induced force production (Opazo Saez et al., 2004). FAK and paxillin are also tyrosine phosphorylated in VSMCs by the contractile agonist angiotensin-II (Polte et al., 1994; Turner et al., 1995) and have been proposed to mediate angiotensin-II-stimulated VSMC constriction (for review see (Mehta and Griendling, 2007). Taken together, these studies suggest FAK and paxillin are important in regulating VSMC contractile activity and vascular dilation, but their localisation and distribution in human skeletal muscle had not been investigated at the start of the current PhD research (October 2009).

1.7. FAK and skeletal muscle insulin signalling

There are two known insulin signalling pathways in skeletal muscle that regulate glucose uptake. These include the APS-CAP-Cbl (APS, Adaptor molecule containing Pleckstrin homology and Src homology-2 domains; CAP, c-Cbl-associated protein; Cbl, Casitas B-lineage Lymphoma) signalling pathway and the insulin receptor substrate 1- (IRS-1) PI3-K-

Akt signalling pathway. The role and relative contribution of the APS-CAP-Cbl pathway in insulin-stimulated glucose uptake is poorly understood (Chang et al., 2004), but evidence has been presented that it plays a role in insulin-stimulated actin cytoskeletal remodelling and glucose transporter-4 (GLUT-4) translocation in a TC10-independent manner (Ishiki and Klip, 2005; JeBailey et al., 2004). The most widely researched and understood signalling pathway leading to GLUT4 translocation occurs via IRS-1-PI3-K-Akt signalling and *in vitro* and *in vivo* evidence has been presented that FAK plays a regulatory role in insulin-stimulated glucose uptake and glycogen synthesis via this pathway in skeletal muscle (Bisht and Dey, 2008; Bisht et al., 2007; Bisht et al., 2008; Huang et al., 2006).

1.7.1. Insulin signalling through FAK-IRS-1-PI3-K

Insulin binds to the transmembrane insulin receptor which stimulates autophosphorylation of the IR. This leads to the tyrosine phosphorylation of insulin receptor substrate-1 and 2 (IRS-1/2) and the creation of docking sites for SH2 domain-containing proteins such as PI3-K (Backer et al., 1992; Myers et al., 1992). Binding of IRS-1 to the p85 subunit of PI3-K leads to an increase in PI3-K p110 catalytic enzyme activity (Backer et al., 1992; Myers et al., 1992) which promotes the production of the phosphatidylinositol-3-phosphate compounds $PI(3,4,5)P_3$, $PI(3,4)P_2$ and PI3P within the plasma membrane and leads to the recruitment of phosphoinositol-dependent protein kinase 1 (PDK1) to the plasma membrane. Akt (2 isoforms are expressed in human skeletal muscle) is recruited with PDK1 to the plasma membrane and is phosphorylated by the richtor-mTOR complex on Ser^{473} (Sarbassov et al., 2005) and PDK1 on $Thr^{308/309}$ (Filippa et al., 2000).

Akt then phosphorylates Akt substrate 160 kDa (AS160) which, possibly in combination with APS-CAP-Cbl pathway (Ishiki and Klip, 2005), leads to glucose transporter-4 (GLUT-4) translocation to the sarcolemma and t-tubules . GLUT-4 is the

principle protein in the facilitated diffusion of insulin-stimulated glucose uptake in skeletal muscle fibres (Watson and Pessin, 2001). Protein kinase C zeta (PKCζ) is also activated by the insulin-stimulated PI3-K-PDK1 signalling pathway leading to a remodelling of the actin cytoskeleton and GLUT-4 translocation (Liu et al., 2007).

FAK has been shown to directly interact with the insulin receptor leading to its insulin-stimulated phosphorylation on Tyr^{397} in C2C12 murine skeletal muscle cells (Goel and Dey, 2002). FAK (via its C-terminal domain) then directly interacts with the insulin receptor substrate-1 (Lebrun et al., 1998) leading to FAK-mediated tyrosine phosphorylation of IRS-1 (Bisht et al., 2007; Lebrun et al., 1998). In C2C12 skeletal muscle cells, insulin-stimulated FAK also leads to the phosphorylation of PI3-K and PKC *in vitro* (Bisht et al., 2007; Goel and Dey, 2002). FAK overexpression through transfection of C2C12s with plasmid constructs containing encoding for the wild-type FAK gene increased insulin-stimulated FAK phosphorylation and resulted in a 40% increase in insulin-stimulated glucose uptake compared with control wild-type C2C12s (Bisht et al., 2007). Overexpression of FAK stimulated a FAK-mediated remodelling of the actin cytoskeleton and GLUT-4 translocation following insulin stimulation (Bisht and Dey, 2008).

To test the hypothesis that inhibition of FAK impaired insulin signalling and insulin-stimulated glucose uptake in skeletal muscles, C2C12s were made insulin resistant through the chronic (3 days) presence of insulin (100 nmol/L) in the cell growth medium (Bisht et al., 2007). The authors demonstrated that FAK phosphorylation was reduced in insulin resistant C2C12 skeletal muscle cells in response to 10 min of insulin stimulation, leading to impaired glucose uptake (Bisht et al., 2007). However, the impaired glucose uptake was reversed when insulin resistant cells were overexpressing FAK (Bisht et al., 2007). A reduced expression of FAK via siRNA inhibition in the skeletal muscle of rats *in vivo* after 4 weeks siRNA inhibition (Bisht et al., 2008) was associated with hyperglycaemia, hyperinsulinaemia and

impaired insulin-stimulated glucose uptake *in vivo* (Bisht et al., 2008). The authors attributed the insulin resistance to reductions in the phosphorylation and protein content of IRS-1 and Akt after FAK silencing compared with controls (Bisht et al., 2008).

Insulin also acts on the microvasculature as a means of increasing insulin-stimulated glucose uptake (Barrett et al., 2009; Barrett et al., 2011). Binding of insulin to its receptor on the luminal side of the endothelium leads to phosphorylation of the insulin receptor and the phosphorylation of the IRS-1-PI3-K-PDK-1-Akt-eNOS signalling cascade (Rattigan et al., 2006). The NO produced by eNOS then diffuses into the smooth muscle thereby stimulating vasodilation (Rattigan et al., 2006). Barrett et al (2009) propose a model whereby terminal arterioles regulate blood flow through capillaries and insulin-stimulated vasodilation of the terminal arterioles leads to the recruitment of previously underperfused capillaries. This increases microvascular blood volume and the capillary surface area available for the uptake of insulin and nutrients (glucose, amino acids and fatty acids) into the interstitial fluid surrounding the skeletal muscle fibres (Barrett et al., 2009). A role for FAK and paxillin in insulin-stimulated microvascular recruitment today has not been reported.

1.7.2. Insulin resistance

Insulin resistance is defined as a reduced responsiveness of a target cell (e.g. adipose tissue, liver or skeletal muscle) or a whole organism to the insulin concentration to which it is exposed (Shanik et al., 2008). Chronic insulin resistance can lead to a decline in glucose tolerance with advancing age and can eventually develop into type II diabetes (non-insulin dependent diabetes mellitus), a disease particularly prevalent in elderly populations (Wild et al., 2004). Impaired skeletal muscle insulin-stimulated glucose uptake among others has been linked to the increased availability of circulating free fatty acids and triglycerides (Schenk et al., 2009). Interestingly, FAK tyrosine phosphorylation was reduced in the rat hindlimb

skeletal muscle after a 2 week high fat diet compared with controls (Bisht et al., 2007). A reduction in FAK phosphorylation in response to lipid oversupply to skeletal muscle from high fat feeding, may therefore contribute to obesity-related insulin resistance. On the other hand, activation of FAK through frequent participation in physical activity (walking, cycling, sport) may be a potential mechanism that contributes to the improved insulin sensitivity that has been observed up to 48 hours post exercise (Mikines et al., 1988; Perseghin et al., 1996) and the higher insulin sensitivity observed with exercise training (Dela et al., 1992; Rogers et al., 1990) even in type II diabetic patients (Dela et al., 1995).

1.8. Elderly skeletal muscle

Skeletal muscle is essential for the completion of daily activities and the maintenance of mobility and social engagement in the environment. In a post-mortem study on 43 previously healthy men aged between 15 and 83 years, skeletal muscle area peaked at 24 years of age (Lexell et al., 1988) and declined at a rate of 3-5% per decade before an accelerated loss in the 6th decade. Between the ages of 24 to 50 years, a 10% reduction in muscle area was observed, but this increased to 30% between 50 and 80 years of age. The mean reduction of muscle area from the age of 24 to 80 years was 40% (Lexell et al., 1988). Estimates from longitudinal studies suggest muscle is lost at ~0.6% per year (Janssen and Ross, 2005). In individuals aged >50 years, the speed at which appendicular skeletal muscle mass is lost predicts all cause mortality regardless of age, body composition and health status (Szulc et al., 2010). Lower limb muscle mass is particularly affected compared with upper limbs (Janssen et al., 2000) which may contribute to impaired mobility with age (Janssen et al., 2002). The loss of skeletal muscle mass can be offset by gains in fat mass which may mask the loss of muscle and may lead to sarcopenic obesity (Baumgartner, 2000). At the whole

muscle level, fat and connective tissue may infiltrate the skeletal muscle resulting in a marbled appearance (Goodpaster et al., 2001).

1.8.1. Defining sarcopenia and its epidemiology

Rosenberg (Rosenberg, 1989) coined the term sarcopenia (loss of skeletal muscle mass with age) as a means of attracting attention to a scientifically under-researched area of clinical significance. This definition implies that everybody who loses skeletal muscle mass with age is sarcopenic (100% prevalence) (Janssen, 2010) and makes no attempt to classify how much muscle can be tolerably lost before clinically significant functional impairments manifest.

Attempts have been made to distinguish those individuals with and without substantial muscle loss (sarcopenic) and although no definition is universally accepted (Visser, 2009), the definition of sarcopenia proposed by Baumgartner et al (Baumgartner et al., 1998) as muscle mass/height^2 that is more than 2 standard deviations below the mean reference value for young males and females is often used (Baumgartner et al., 1998; Di Monaco et al., 2011; Iannuzzi-Sucich et al., 2002)

Using this definition, Baumgartner et al (Baumgartner et al., 1998) reported up to 24% of people in New Mexico aged <65 years and 50% of people aged >80 years would fulfil the criteria for sarcopenia as assessed using dual x-ray absorptiometry (DEXA). Sarcopenia was more prevalent in men aged >75 years (58%) compared with women of the same age (45%) (Baumgartner et al., 1998). Iannuzzi-Suchi (Iannuzzi-Sucich et al., 2002) reported similar prevalence rates in New England Caucasians where ~53% of men and 31% women aged >64 years were classified as sarcopenic. Lower rates have been reported in China where ~12% of men and ~8% or women aged >70 years were classified as sarcopenic (Lau et al., 2005). In Italy (Di Monaco et al., 2011), France (Rolland et al., 2003) and Denmark (Tanko et al., 2002), between 58%, 9.5% and 12% of women aged >70 years were

sarcopenic. Although the prevalence is variable depending upon the country of origin, these data suggest the prevalence of sarcopenia increases with age, with elderly men appearing particularly susceptible in later life compared with women.

1.8.2. Muscle strength

The loss of muscle strength (dynapenia) can occur at rates which exceed the loss of muscle mass (Clark and Manini, 2008). Cross-sectional studies have reported a 20 – 40% loss of knee extensor strength in individuals aged between 65 and 90 years (Frontera et al., 1991; Murray et al., 1980; Young et al., 1985). It should be noted, however, that there is great inter-individual variability in the rate of dynapenia. In a 10 year longitudinal study, approximately one third of individuals aged ~60 years at baseline showed no loss of strength at follow-up (Hughes et al., 2001)

1.8.3. Muscle fibre characteristics

The decline in CSA may be partially attributable to a loss of muscle fibre number. Indeed, a post-mortem assessment of the *m. vastus lateralis* muscle recovered from men aged 19-37 and 70-73 years demonstrated fewer muscle fibres in the elderly (~364,000 fibres) compared with the young (~478,000 fibres) (Lexell et al., 1983) with apparently no fibre-type specificity (Klitgaard et al., 1990; Lexell et al., 1986). Atrophy of skeletal muscle fibres is heavily implicated in the loss of skeletal muscle CSA and the response is generally fibre-type specific. Type I fibre CSA is generally well maintained with age even in individuals aged >85 years (Andersen, 2003; Lexell et al., 1988), however, type II fibre CSA is particularly affected by the ageing process (Essen-Gustavsson and Borges, 1986; Klitgaard et al., 1990; Kosek et al., 2006; Larsson et al., 1978; Lexell, 1995; Lexell et al., 1986; Lexell et al., 1988; Verdijk et al., 2007). In individuals aged >85 years, Andersen (Andersen, 2003) reported that

type I fibre CSA atrophied in size by 25% compared with 25 year olds, whereas type II fibres atrophied by 57%. Ageing skeletal muscle also demonstrates fibre type grouping rather than the random distribution observed in the young (Lexell et al., 1986) and a lower muscle capillarisation compared with young individuals (Coggan et al., 1992; Degens et al., 1993). Although it is hypothesized that FAK and paxillin protein content of human skeletal muscle and its associated microvasculature will decrease with ageing and the adoption of a sedentary lifestyle, no studies have measured FAK and paxillin and their response to exercise training in human skeletal muscle and its microvasculature.

1.8.4. Resistance training and impaired mechanotransduction

RT is recommended for young and elderly individuals (Chodzko-Zajko et al., 2009), particularly for its ability to increase skeletal muscle strength (Fiatarone et al., 1990; Frontera et al., 1988; Grimby et al., 1992; Hakkinen et al., 1998; Hakkinen et al., 2001b; Izquierdo et al., 2001; Kosek et al., 2006; Lexell et al., 1995; Verdijk et al., 2009a; Verdijk et al., 2009b) and reduce or prevent progressive type II skeletal muscle fibre atrophy that is seen in many elderly individuals (Fiatarone et al., 1990; Frontera et al., 1988; Kosek et al., 2006; Verdijk et al., 2009a; Verdijk et al., 2009b). The elderly can achieve similar relative gains in skeletal muscle strength as the young after RT (Hakkinen et al., 2001a; Hakkinen et al., 1998; Holviala et al., 2006; Newton et al., 2002) and several studies show no difference between age groups in chronic hypertrophic response to RT (Hakkinen et al., 1998; Ivey et al., 2000), especially when based on percentage gains in muscle CSA per day of training (Narici et al., 2004).

However, these findings are not universally observed (Kosek and Bamman, 2008; Kosek et al., 2006; Lemmer et al., 2000; Martel et al., 2006; Welle et al., 1996). Emerging evidence suggests that the protein synthesis response of ageing skeletal muscle to acute bouts

of endurance and resistance-type exercise is impaired compared with young individuals (Drummond et al., 2008; Kumar et al., 2009; Williamson et al., 2003). For example, there was a blunted post-exercise elevations in p70S6K and eukaryotic initiation factor-4 binding protein-1 (4EBP-1) phosphorylation in elderly individuals following acute bouts of resistance exercise performed at 60-90% 1 RM in the fasted state (Kumar et al., 2009). In addition, Williamson et al (Williamson et al., 2003) reported a fall in ERK 1/2, p38 MAPK and JNK phosphorylation after 70% 1RM knee extensor exercise in the elderly, whereas their phosphorylation increased in the young. These studies suggest an impaired ability for mechanotransduction to stimulate anabolic signalling pathways in senescent tissues and contribute to the development of anabolic resistance with advancing age. The role of FAK and paxillin in skeletal muscle anabolic resistance has not been investigated. Most important, however, is the observation that an increase in load by elevated intraluminal pressure failed to increase FAK Tyr^{925} phosphorylation compared with young rats (Rice et al., 2007). This suggests impairments may also be observed in the activation of FAK by acute increases in mechanical load with age.

Age-associated impairments in endothelium-dependent vasodilation can begin as early as the 4^{th} decade (Gerhard et al., 1996) leading to impaired FMD of the brachial artery (Celermajer et al., 1994; Donato et al., 2007; Eskurza et al., 2004) and forearm resistance vessel (Gerhard et al., 1996) and rat skeletal muscle arterioles (Delp et al., 2008; Muller-Delp, 2006; Muller-Delp et al., 2002; Spier et al., 2007). A reduced NO bioavailability has been proposed to be an important underlying mechanism (for review see (Seals et al., 2011). This suggests impairments in vascular mechanotransduction also develop with age. As discussed earlier, FAK has been shown to regulate fluid shear stress-activated eNOS (and thus NO production by eNOS) (Koshida et al., 2005), therefore, FAK may play a critical role in maintaining vascular function with age.

1.9. Importance of immunofluorescence microscopy

To date, the studies reporting the effect of mechanical (un)loading on FAK and paxillin responses within animal and human skeletal muscle have used western blot analyses on whole muscle homogenates (de Boer et al., 2007; Fluck et al., 1999; Gordon et al., 2001; Klossner et al., 2009; Wilkinson et al., 2008). Western blotting is valid for estimating changes in protein content in whole muscles, but it does not give information on the relative distribution of FAK and paxillin in muscle fibres and within the microvasculature. This is an important gap in the literature that warrants investigation. In addition, Western blotting does not provide information on tissue-specific responses to (un)loading without contamination from other cells and tissues.

Immunofluorescence microscopy allows this distinction and may be an invaluable tool for elucidating the effect of physical (in)activity on FAK and paxillin localisation and changes in content in skeletal muscle fibres and its microvasculature. This is of particular relevance in ageing skeletal muscle and the (micro)vasculature since mechanotransduction to contractile and hemodynamic stimuli are potentially impaired in some elderly populations (Celermajer et al., 1994; Donato et al., 2007; Drummond et al., 2008; Eskurza et al., 2004; Kumar et al., 2009; Williamson et al., 2003). Immunofluorescence microscopy can provide informative images concerning the localisation and distribution of structural proteins (Pardo et al., 1983) and can provide (semi)quantitative data on changes in protein content as a consequence of physical (in)activity (Anastasi et al., 2008) or ageing (Donato et al., 2009). Scientific knowledge regarding the localisation of FAK and paxillin is currently restricted to animal skeletal muscle fibres and there is no information regarding the localisation of FAK and paxillin in the endothelial and smooth muscle layer of the microvasculature of skeletal muscle.

Consequently, there is a need to develop immunofluorescence microscopy assays to investigate the localisation and distribution of these focal adhesion-associated proteins in human skeletal muscle fibres and within the skeletal muscle microvascular endothelial and vascular smooth muscle layers. These assays are necessary to fully understand how mechanical stimuli and exercise training lead to structural and functional adaptations that lead to improved endurance capacity, skeletal muscle hypertrophy and increases in insulin sensitivity in young and elderly individuals.

1.10. Thesis overview and aims

Chapter 1 discussed how cells, specifically skeletal muscle fibres and their microvasculature, 'sense' externally applied or internally generated force, pressure and load and translate these mechanical stimuli into intracellular signalling (mechanotransduction). Two candidate proteins in integrin-mediated mechanotransduction (FAK and paxillin) were specifically identified and their potential role in the mechanotransduction and adaptation of skeletal muscle and the microvasculature to (un)loading was explained. The importance and advantages of immunofluorescence microscopy as a tool for investigating these proteins in human skeletal muscle *in vivo* were also emphasised, yet the literature regarding the visualisation of FAK and paxillin is restricted to cell culture or animal skeletal muscle fibres.

Therefore, the aim of **chapter 2** was to develop reliable and repeatable immunofluorescence microscopy methods for the first investigations of the subcellular localisation and fibre-type distribution of FAK in skeletal muscle fibres of young healthy recreationally active men. The study also aimed to visualise the FAK subcellular distribution in the endothelium and VSMC layer of the skeletal muscle microvasculature.

In rat skeletal muscle, loading is associated with increased FAK protein expression. However, the response of FAK to exercise training in human skeletal muscle and its

microvasculature has not been investigated. The aim of **chapter 3** was to investigate the effect of 12 weeks RT on FAK in the skeletal muscle fibres and the microvascular endothelium of skeletal muscle of previously sedentary elderly men. A second aim was to validate the methods developed in chapter 2 for the investigation of human skeletal muscle adaptation to exercise training. It was hypothesised that RT would increase FAK in the skeletal muscle fibres thus contributing to the increased force production following RT, and within the microvascular endothelium.

FAK and paxillin are direct binding partners and both proteins share similar responses to (un)loading. However, the spatial distribution of paxillin has not been investigated in mammalian skeletal muscle. Therefore, the aim of **chapter 4** was to develop reliable and repeatable immunofluorescence microscopy methods to perform the first investigations of paxillin in human skeletal muscle and its microvasculature as in chapter 2. A second aim was to investigate the colocalisation of FAK and paxillin in these tissues. It was hypothesised that paxillin would be expressed in the plasma membrane region of skeletal muscle fibres and the endothelium and VSMC layer of the skeletal muscle microvasculature and that paxillin would colocalise with FAK in these cells and subcellular locations.

There is only one study in the literature which has reported FAK responses (changes in phosphorylation) to exercise training in human skeletal muscle which was 12 weeks long and used Western blot analyses (Wilkinson et al., 2008). There is no information available in humans on the response of paxillin to exercise training and whether shorter training periods is sufficient to increase the protein expression of these focal adhesion-associated proteins in human skeletal muscle. Therefore, **chapter 5** aimed to utilise the novel immunofluorescence techniques developed in chapter 2 and 4 and build on chapter 3 to investigate the effect of 6 weeks ET and RT on FAK and paxillin in skeletal muscle fibres and their microvasculature

of previously sedentary young men. It was hypothesised that the increased force production during exercise would increase FAK and paxillin protein expression in these tissues.

Chapter 6 (General Discussion) discusses the findings of each experimental chapter in context of the wider literature and suggests potential avenues for future research based on the findings in this thesis.

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Chapter 2

Immunofluorescent visualisation of focal adhesion kinase in human skeletal muscle and its associated microvasculature

2.1. Abstract

Within animal skeletal muscle, focal adhesion kinase (FAK) has been associated with load-dependent molecular and metabolic adaptation including the regulation of insulin sensitivity. This study aimed to generate the first visual images of the localisation of FAK within human skeletal muscle fibres and its associated microvasculature using widefield and confocal immunofluorescence microscopy.

Percutaneous muscle biopsies taken from five lean, active males, were frozen and 5 μm cryosections were incubated with FAK antibodies for visualisation in muscle fibres and the microvasculature. Anti-myosin heavy chain type I was used for fibre type differentiation. Muscle sections were also incubated with anti-dihydropyridine receptor (DHPR) to investigate co-localisation of FAK with the t-tubules. FITC-conjugated Ulex europaeus Agglutinin I stained the endothelium of the capillaries, while anti-smooth muscle actin stained the vascular smooth muscle of arterioles. Fibre type differences in the intensity of FAK immunofluorescence was determined with image analysis software.

In transversely and longitudinally orientated fibres, FAK was localised at the (sub)sarcolemmal regions. In longitudinal fibres, FAK staining also showed uniform striations across the fibre and co-staining with DHPR suggests FAK associates with the t-tubules. There was no fibre-type difference in sarcoplasmic FAK content. Within the capillary endothelium and arteriolar smooth muscle, FAK was distributed heterogeneously as clusters.

This is the first study to visualise FAK in human skeletal muscle microvasculature and within the (sub)sarcolemmal and t-tubule regions using immunofluorescence microscopy. This technique will be an important tool for investigating the role of FAK in the intracellular signalling of human skeletal muscle and the endothelium of its associated microvasculature.

2.2. Introduction

Extracellular matrix proteins interact with cells to generate intracellular signals important in cell growth, survival and migration (Schlaepfer et al., 1999). This interaction is achieved by transmembrane proteins linking extracellular proteins to the actin cytoskeleton (Schlaepfer et al., 1999). In skeletal muscle, these transmembrane proteins form costameres which contain a dystrophin/glycoprotein complex and an integrin associated-complex (Bloch and Gonzalez-Serratos, 2003). Costameres circumferentially align in register with the Z-disk of peripheral myofibrils and connect the sarcomeres with the sarcolemma (Ervasti, 2003; Pardo et al., 1983). At the junction between the integrins and the actin cytoskeleton are focal adhesions which convert integrin- (e.g. mechanical) and non-integrin (e.g. hormonal) stimulation into intracellular signalling (Schlaepfer et al., 1999). Focal adhesions are formed from proteins such as paxillin, talin, vinculin and focal adhesion kinase (FAK) (Kanchanawong et al., 2010).

FAK is a cytoplasmic non-receptor tyrosine kinase (Parsons, 2003; Schaller, 2001; Schlaepfer et al., 1999) implicated in the transduction of mechanical stimuli such as loading and unloading. In skeletal muscle, loading is associated with increased FAK tyrosine phosphorylation (Klossner et al., 2013; Wilkinson et al., 2008) and protein content (Fluck et al., 1999; Gordon et al., 2001), leading to FAK mediated stimulation of the protein synthesis signalling pathway (Klossner et al., 2009) and skeletal muscle hypertrophy in animals (Durieux et al., 2009; Fluck et al., 1999; Gordon et al., 2001) and elderly humans (Flueck et al., 2011; Narici et al., 2011). Conversely, unloading reduced FAK tyrosine phosphorylation in rat soleus skeletal muscle after 7 days of tail suspension (Gordon et al., 2001) and in human skeletal muscle after 10 and 14 days of leg immobilisation (de Boer et al., 2007; Glover et al., 2008).

Non-integrin stimuli such as insulin can also modulate FAK (Baron et al., 1998; Goel and Dey, 2002a, b; Knight et al., 1995) with FAK shown to regulate insulin action in skeletal muscle (Bisht and Dey, 2008; Bisht et al., 2007; Bisht et al., 2008; Huang et al., 2006). Insulin can stimulate the rapid reorganisation of the sub-membrane actin filaments in L6 myocytes leading to the docking of GLUT-4 at the plasma membrane (Tong et al., 2001). Recent evidence in C2C12 skeletal muscle cells suggest this remodelling is FAK mediated (Bisht and Dey, 2008), possibly through direct phosphorylation by the insulin receptor (Goel and Dey, 2002b). FAK also phosphorylates IRS-1 and regulates IRS-1 associated PI3-K activity (Lebrun et al., 1998) leading to GLUT-4 translocation via the reorganised actin filaments and glucose uptake (Bisht and Dey, 2008; Bisht et al., 2007; Huang et al., 2006). Down-regulation of FAK in C2C12 skeletal muscle cells reduced basal and insulin-stimulated FAK tyrosine phosphorylation, prevented insulin-stimulated actin remodelling, impaired insulin signalling and the associated GLUT-4 translocation, glucose uptake and glycogen synthesis (Bisht and Dey, 2008; Bisht et al., 2007; Goel and Dey, 2002a; Huang et al., 2006). Furthermore, *in vivo* FAK inhibition reduced insulin-mediated FAK, IRS-1 and Akt phosphorylation and glucose uptake resulting in the development of hyperglycaemia and hyperinsulinaemia (Bisht et al., 2008).

Immunohistochemistry (Fluck et al., 1999; Fluck et al., 2002) and immunofluorescence microscopy techniques (Durieux et al., 2009; Klossner et al., 2009; Klossner et al., 2013; Quach and Rando, 2006) have been used to visualise the distribution of FAK within cross-sections of animal skeletal muscle and have shown FAK to be more abundant in type I slow oxidative fibres (Fluck et al., 2002; Gordon et al., 2001). FAK is localised in both the sarcolemmal and sarcoplasmic regions in animal skeletal muscle (Durieux et al., 2009; Fluck et al., 1999; Klossner et al., 2009) where it aligns with the Z-disc

in longitudinally orientated muscle fibres (Quach and Rando, 2006). In C2C12 muscle cells, FAK is localised along the actin cytoskeleton (Bisht and Dey 2008; Huang et al, 2006).

FAK is also present within vascular endothelial cells, transmitting the mechanical stimulation of fluid shear stress into biochemical signals (Ishida et al., 1996; Li et al., 2002; Li et al., 1997; Wang et al., 2009). It plays an important role in blood vessel morphogenesis (Ilic et al., 2003) and angiogenesis (Ilic et al., 1995; Peng et al., 2004; Shen et al., 2005) and is a target of vascular endothelial growth factor (Abedi and Zachary, 1997; Avraham et al., 2003). FAK has been visualised using immunofluorescence microscopy in cultured human pulmonary artery and umbilical vein endothelial cells (Shikata et al., 2003; Wu et al., 2003) but not, to the authors' knowledge, in the microvasculature perfusing human skeletal muscle.

Because of the diverse role of FAK in skeletal muscle, endothelial metabolism, and structural adaptations, immunofluorescence microscopy methods to visualise and analyse the content, distribution and association of FAK with other proteins is an important tool in fully elucidating FAK's role(s) in human skeletal muscle and its associated microvasculature. This technique may provide important insights into the regulation of skeletal muscle mass and insulin sensitivity.

Therefore, in the present study we aimed to generate the first visual images of the distribution and localisation of FAK within human transversely and longitudinally orientated skeletal muscle fibres and its associated microvasculature using widefield and confocal immunofluorescent microscopy.

2.3. Materials and Methods

Muscle samples

Muscle samples from the *m. vastus lateralis* of 5 lean recreationally active males (mean ± SD; age 20 ± 2 y; BMI 22.6 ± 1.1 kg/m^2) were obtained using the Bergstrom percutaneous

needle technique (Bergstrom, 1975) and blotted free of excess blood, and dissected free from fat and connective tissue. Samples were embedded in Tissue Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and immediately frozen in liquid-nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK). All tissues were kept at -80°C until required. For each muscle sample, 5µm cryosections were cut at -30°C, placed on room temperature, uncoated glass slides (VWR International Ltd, Leicestershire, UK) and fixed immediately in acetone and ethanol. Muscle cross-sections were cut to visualise FAK within transversely orientated muscle fibres and the associated endothelium and vascular smooth muscle. Longitudinally orientated muscle fibres were used to visualise FAK's arrangement along the sarcomere.

Immunofluorescence staining

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS; 137 mM sodium chloride, 3 mM potassium chloride, 8 mM disodium hydrogen phosphate and 3 mM potassium dihydrogen phosphate, pH of 7.4) was used for dilution of antibodies, reagents and for washing steps.

Sections were fixed in acetone and ethanol (3:1) for 5 min followed by 3 x 5 min wash in PBS. Sections were incubated for 2 h with 1:100 dilution of the rabbit polyclonal N-terminal (A17) and C-terminal C20 region specific FAK antibodies (Santa Cruz, USA). The former has been used successfully in staining rodent skeletal muscle (Durieux et al., 2009; Fluck et al., 2002; Klossner et al., 2009; Quach and Rando, 2006) and the C-terminal specific FAK antibody (C-FAK) was used to validate the localisation of the N-terminal FAK antibody. To assess fibre type differences in FAK content, sections of muscle were cut and stained with FAK antibodies co-incubated with 1:100 dilution of anti-myosin heavy chain type I (developed by Dr. Blau; DSHB; A4.840-c) and 1:400 dilution of anti-dystrophin

(Sigma-Aldrich, UK). The latter was used as a marker for the sarcolemma and identification of individual cell membranes since dystrophin is localised to the sarcoplasmic surface of the sarcolemma (Davies and Nowak, 2006). Type II fibres remained unstained.

Muscle sections were also incubated with 1:25 dilution of anti-calcium channel L type dihydropyridine receptor alpha 2 subunit (DHPR; Abcam ab2864) for visualisation of transverse-tubules (t-tubules). To identify FAK within capillaries, muscle sections were incubated with 1:200 dilution of the FITC-conjugated Ulex europaeus Agglutinin I (UEA-I) lectin (Vector Laboratories, UK) and 1:100 dilution of anti-α-smooth muscle actin for the identification of vascular smooth muscle (α-SMA; ab18460, Abcam, UK). After incubation, sections were washed 3 x 5 min in PBS, blotted dry and then the appropriately targeted fluorescently conjugated secondary antibodies were applied for 30 min. These included 1:200 dilutions of goat anti-rabbit IgG 488 and 594 and goat anti-mouse IgM 350, IgG_{2a} and IgG_{2b} 488 and 594 fluorophores (Invitrogen, UK). For negative controls and the assessment of background fluorescence, the primary antibody was omitted and in all cases, the fluorescence signal was removed. Sections were then washed for 3 x 5 min in PBS before blotting dry and applying cover slips mounted in a glycerol and mowiol 4–88 solution in 0.2 M Tris-buffer (pH 8.5) with the addition of 0.1% 1,4-diazobicyclo-[2,2,2]-octane (DABCO) antifade medium.

A competition experiment for FAK signal specificity was also performed by co-incubating FAK with a FAK-specific blocking peptide (FAK A-17 sc-557 P; Santa Cruz). The concentration of the blocking peptide was 10x higher than the FAK primary antibody. On separate glass slides, sections were stained either with FAK or FAK co-incubated with the blocking peptide using the same protocol as previously described.

Fluorescence microscopy

For the initial visualisation of FAK in skeletal muscle and microvasculature, images were taken using a Nikon E600 microscope coupled to a SPOT RT KE colour three shot CCD camera (Diagnostic Instruments Inc., MI, USA). DAPI UV, FITC and Texas Red excitation filters were used to visualise the Alexa Fluor 350, 488 and 594 fluorophores, respectively. Detailed digital images of the microvasculature and skeletal muscle were acquired using an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) with a 63x oil immersion objective. The Alexa Fluor 488 and 594 fluorophores were excited with a 488 nm line of the argon laser and 594 nm line of the Helium-Neon laser for excitation, respectively. Images used to investigate colocalisation of FAK with DHPR, Lectin and α-SMA were captured sequentially.

All images were processed using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). Two sections per participant were cut and placed on the same slide and duplicated on a second slide for FAK quantitation with five regions of interest (ROI) captured per muscle section on the widefield microscope. ROI's covering areas of all transversely orientated muscle fibres were selected and used to measure FAK immunoreactivity in a fibre-type specific manner. A total of 109 ± 15 transversely orientated fibres were analysed per cross section and fibre type specificity was determined from the mean fluorescence signal intensity of 48 ± 7 type I and 61 ± 10 type II individual whole human muscle fibres, respectively. All fibres that were in the longitudinal plane were excluded from FAK quantitation. Using the appropriate dystrophin image, a participant-specific intensity threshold was selected and used to identify complete individual muscle fibres. The fibre outline was then overlayed onto the raw FAK image for quantitation of sarcoplasmic FAK fluorescence intensity.

Confocal microscopy was used for comparison between sarcoplasmic and sarcolemmal FAK immunofluorescence. Duplicate slides were prepared as above, except only 3 sections were investigated across both slides, with 5 region of interests (ROI's) captured per section. As there was no sarcoplasmic fibre-type specificity determined using widefield microscopy (figure 2.3), muscle fibres captured with confocal microscopy were pooled and a total of 74 ± 19 transversely orientated whole muscle fibres were analysed per person. Sarcoplasmic and sarcolemmal FAK immunofluorescence was quantified using the dystrophin outline surrounding the whole muscle fibres as above.

Western blot

Snap frozen skeletal muscle tissue was powdered in liquid nitrogen using a pestle and mortar and mixed on ice with lysis buffer (RIPA buffer (Cell Signalling) to which a complete mini protease inhibitor tablet was added (Roche Diagnostics, Mannheim, Germany) before homogenisation with a hand held homogeniser. Samples were centrifuged for 20 min at 10,000 g at 4ºC with the supernatant removed and a small aliquot used for protein determination (Pierce BCA assay kit; Pierce, Rockford, IL, USA). Samples were diluted to a total protein concentration of 3 µg/µl and loaded in 15 µl aliquots (45 µg) before separation by electrophoresis on 12% SDS polyacrylamide gels (Thermo Scientific, Rockford, IL, USA). The proteins were transferred to a nitrocellulose membrane for 2 hours at 25 V and incubated for 5 min with Ponceau S to determine transfer efficacy. The membranes were then washed for 1 min in 0.1 M NaOH. After 3 x 1 min wash with deionised water, the membranes were incubated for 60 min in 5% dry non-fat milk (NFM) in PBS and 0.05% Tween (PBST) blocking solution. The membranes were incubated overnight at room temperature with the N-terminal specific FAK antibody (1: 1500) diluted in 5% bovine serum albumin and PBST blocking solution. Membranes were washed 3 x 5 min in PBST and

blocked for 60 min in NFM in PBST. The membranes were then incubated for 60 min with a HRP-conjugated anti-rabbit IgG secondary antibody (FAK, 1:5000). After a 2 x 5 min and 1 x 15 min wash in PBST, membranes were treated for 5 min with enhanced chemiluminescence HRO detection reagent (GE Healthcare, Amersham, UK) and bands were identified and captured.

Statistical Analyses

Mean fluorescence intensity was used as a measurement of total FAK content at the sarcolemma and across the 2 fibre types. Data are expressed as mean ± SEM and compared using a paired samples t-test (SPSS v15). Significance was set at $P < 0.05$.

2.4. Results

Specificity of the N-terminal specific FAK antibody in human skeletal muscle

Figure 2.1 shows representative widefield fluorescence microscopy images of N-terminal (A) and C-terminal specific antibodies (C-FAK) (B). Both images demonstrate distinct immunofluorescence at the (sub)sarcolemmal regions. Co-incubation of N-terminal FAK with a FAK-specific peptide (C) abolished the strong FAK-specific sarcolemmal immunoreactivity and control experiments omitting the primary antibody also removed sarcolemmal immunoreactivity. Western blot analysis (E) identified a band at ~125 kDa which corresponded to FAK's molecular weight of 125 kDa (Schaller et al., 1992). Two bands at ~90 and ~50 kDa were also detected which have previously been observed in human skeletal muscle when using this antibody (Flueck et al., 2011) and are known FAK cleavage fragments (Carragher et al., 1999). Taken together, these data suggest the FAK A-17 N-terminal specific primary antibody is specific for skeletal muscle FAK protein in agreement

with past research (Durieux et al., 2009; Fluck et al., 1999; Fluck et al., 2002; Klossner et al., 2009; Quach and Rando, 2006).

Localisation of FAK in skeletal muscle fibres

Figure 2.1A shows representative widefield fluorescence microscopy images of skeletal muscle stained with anti-FAK and anti-myosin heavy chain type I. In many transversely and longitudinally orientated fibres, a strong FAK fluorescence was detected at the sarcolemmal area compared with the sarcoplasm (figure 2.2A and B). Indeed, FAK immunoreactivity was 104 ± 4% greater at the sarcolemma compared with the sarcoplasm ($P = 0.001$). Quantitative image analysis from transversely orientated skeletal muscle fibres revealed no fibre type difference in sarcoplasmic FAK content (figure 2.3).

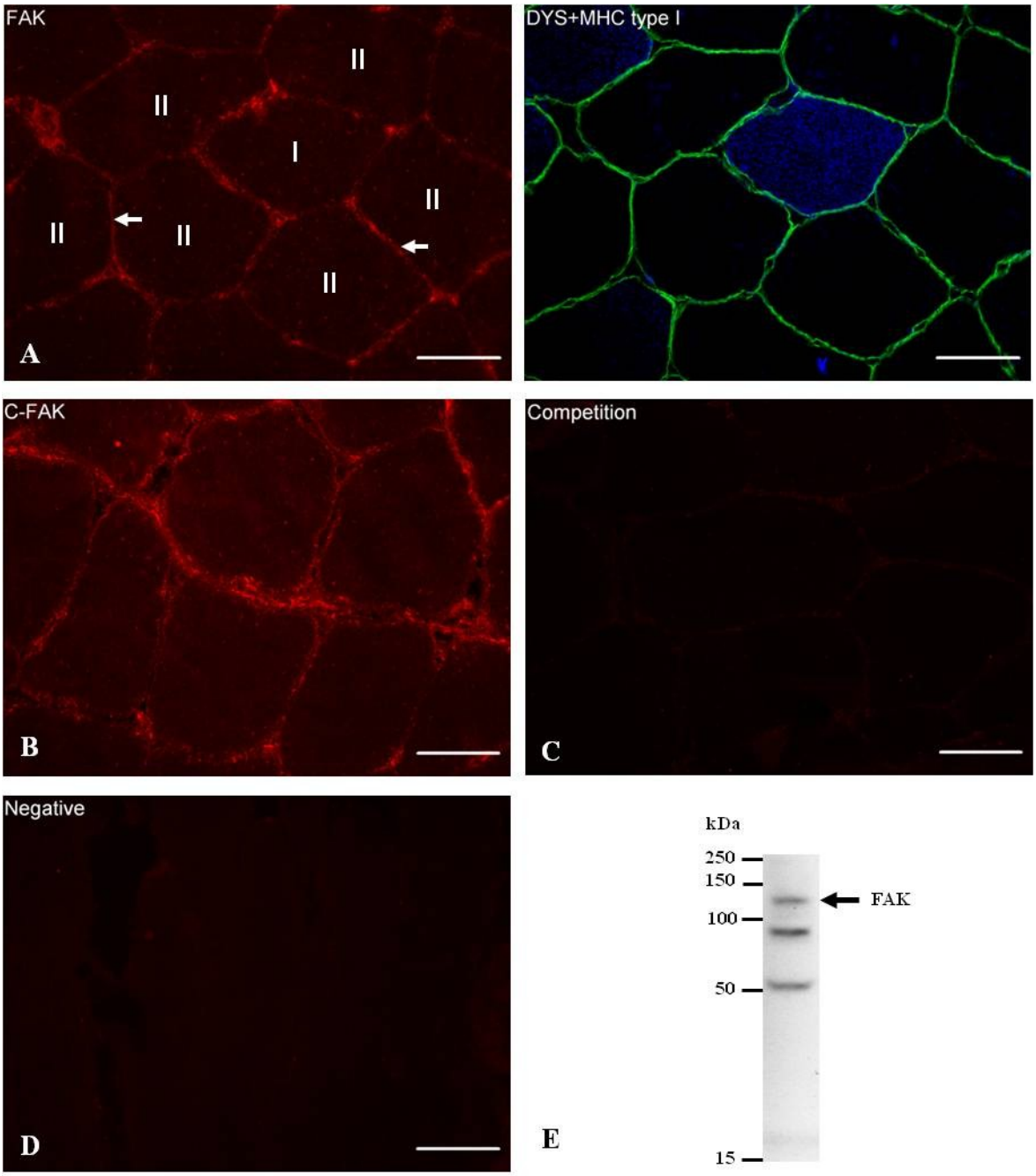

Figure 2.1. Immunofluorescent visualisation of focal adhesion kinase (FAK) in human skeletal muscle. A) Representative widefield microscopy images of FAK in transversely orientated *m. vastus lateralis* type I and type II skeletal muscle fibres. N-terminal region specific FAK (red, *left*) co-incubated with anti-myosin heavy chain type I (MHC type I, blue) for muscle fibre type differentiation and anti-dystrophin (DYS, green) antibodies for visualisation of the fibre membrane (*right*). There was a strong FAK immunoreactivity at the

sarcolemmal and subsarcolemmal regions of the muscle fibre (*arrows*). B) Representative images of C-terminal region specific FAK showed a similar (sub)sarcolemmal FAK immunoreactivity as the N-terminal FAK. C) Representative images of N-terminal FAK co-incubated with a FAK-specific blocking peptide. The inclusion of a specific blocking peptide abolished the FAK-positive signal at the sarcolemma. D) Negative control experiment omitting the N-terminal primary antibody removed the positive signal. E) Western blot of the N-terminal FAK antibody using homogenised *m. vastus lateralis* human skeletal muscle. For all images bars are 50 μm.

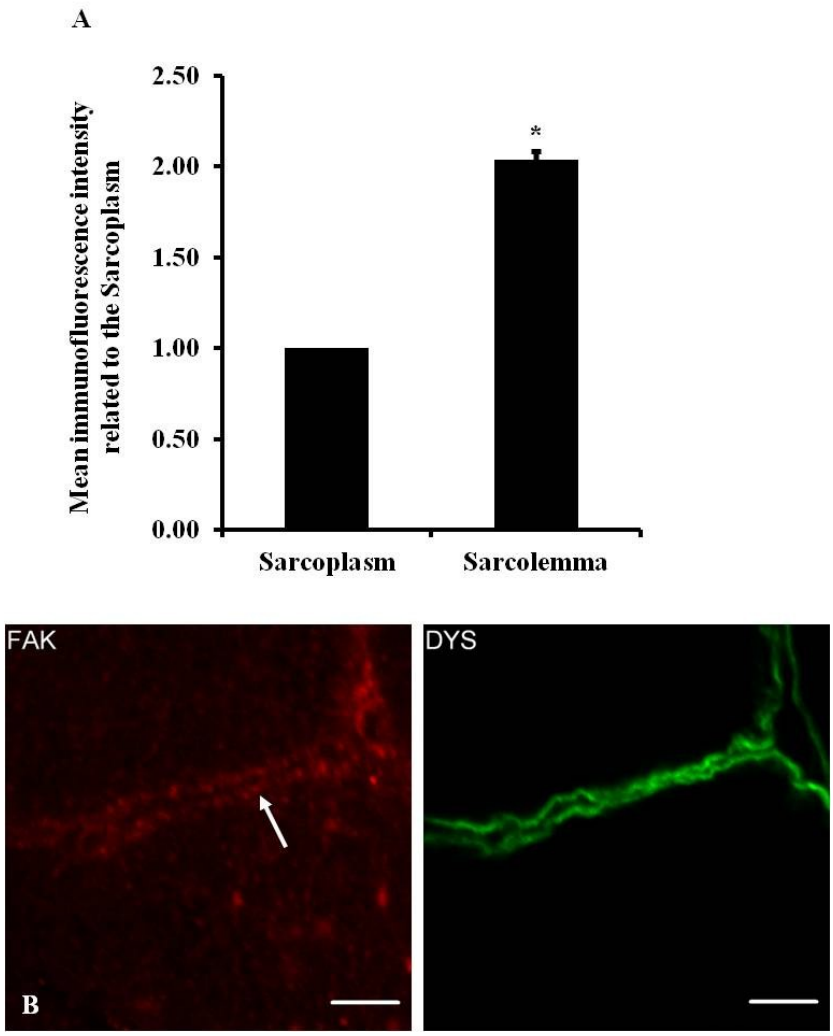

Figure 2.2. Mean sarcolemmal and sarcoplasmic focal adhesion kinase (FAK) immunoreactivity assessed using confocal microscopy. (A) Sarcolemmal FAK immunoreactivity compared to the sarcoplasm. Data was quantified by pooling both fibre types with 74 ± 19 whole muscle *m. vastus lateralis* fibres analysed. Data are means ± SEM. *n*=5. * Significantly different compared with the sarcoplasm (*P*=0.001). (B) Representative images of FAK (red, *left*) and dystrophin (DYS, green, *right*) captured using confocal microscopy. Note that FAK appears in the form of clusters along the sarcolemma (*arrow*). Bars are 5 μm.

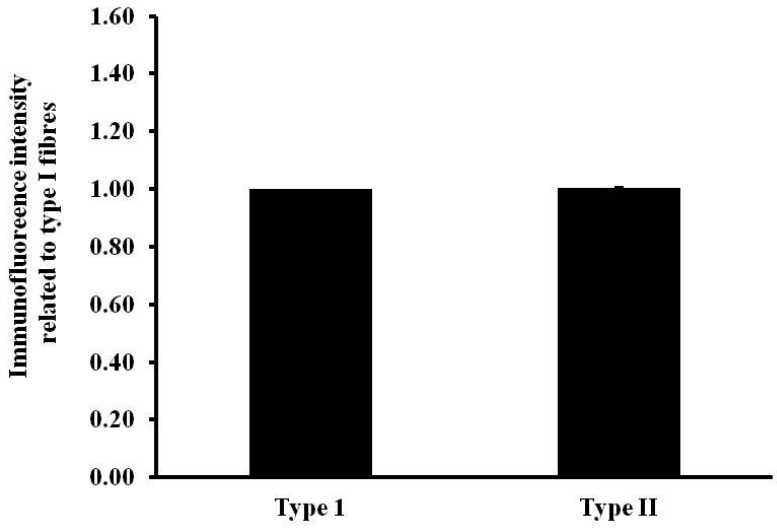

Figure 2.3. Mean focal adhesion kinase (FAK) fluorescence intensity of type I and type II muscle fibres assesed using widefield microscopy. There was no difference in sarcoplasmic FAK content between type I and type II muscle fibres from human *m. vastus lateralis*. Data are related to type I fibres and are mean ± SEM. *n*=5. Data was quantified from 48 ± 7 type I and 61 ± 10 type II whole transversely orientated fibres.

When longitudinally orientated type I and type II fibres were viewed using widefield (figure 2.4A) and confocal microscopy (figure 2.4B), FAK staining revealed an evenly distributed, highly organised structure running perpendicular to the fibre's longitudinal direction and spanning the full width of the fibre. Representative confocal microscopy images of anti-FAK staining in combination with the t-tubule marker, anti-DHPR (figure 2.4B) suggest that FAK colocalises with the t-tubular network.

FAK and the microvasculature

Representative confocal images of the endothelium within skeletal muscle are presented in figure 2.5. FITC-conjugated UEA-I lectin successfully stained the endothelium of capillaries (*far left panel, upper row*) and larger microvessels (likely arterioles) in skeletal muscle (*far*

left panel, bottom row) as evidenced by the intense fluorescent signal surrounding the lumen of each vessel. The FAK antibody successfully stained the same vasculature (*middle panel upper and lower*), with FAK distributed heterogeneously within the endothelium (*lower, arrow*). Merged images for FAK and lectin demonstrate clear colocalisation (*upper and lower far right panels*).

Figure 2.6 represents typical confocal microscopy images of arterioles in human skeletal muscle identified by the positive staining of the vascular smooth muscle (*far left panel*). Anti-FAK staining again demonstrated a ring-like structure around the periphery of the blood vessel (*middle panel*) and merged images clearly illustrate the presence of FAK in vascular smooth muscle (*far right panel*). It should be noted that FAK also appeared to be distributed heterogeneously within the vascular smooth muscle (*lower middle*, arrow).

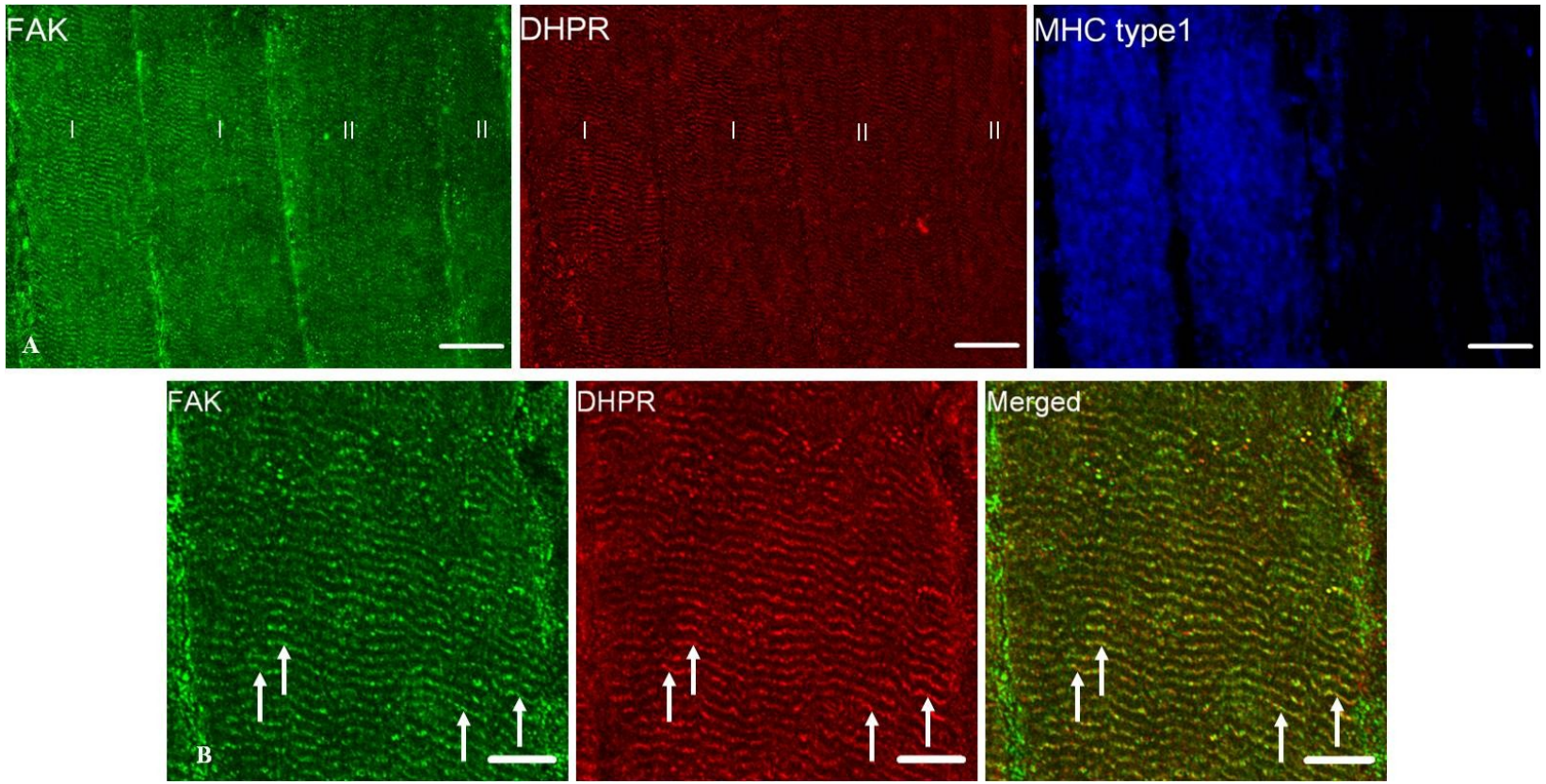

Figure 2.4. Distribution of focal adhesion kinase (FAK) and dihydropyridine receptor (DHPR) in longitudinally orientated skeletal muscle. (A) Representative widefield immunofluorescence microscopy of FAK (green) (*left*), the DHPR (red) (*middle*) and type I muscle fibres (MHC-type I; blue; *right*) in *m. vastus lateralis* muscle fibres. FAK and DHPR staining revealed structures running adjacent to the longitudinal direction and spanning the full width of the muscle fibre. B) Representative confocal image of a type I fibre (8x zoom). Co-staining of FAK with DHPR (*right*) suggests these structures are t-tubules and that FAK may colocalise with the t-tubules. Bars are 25 µm (A) and 2.5 µm (B).

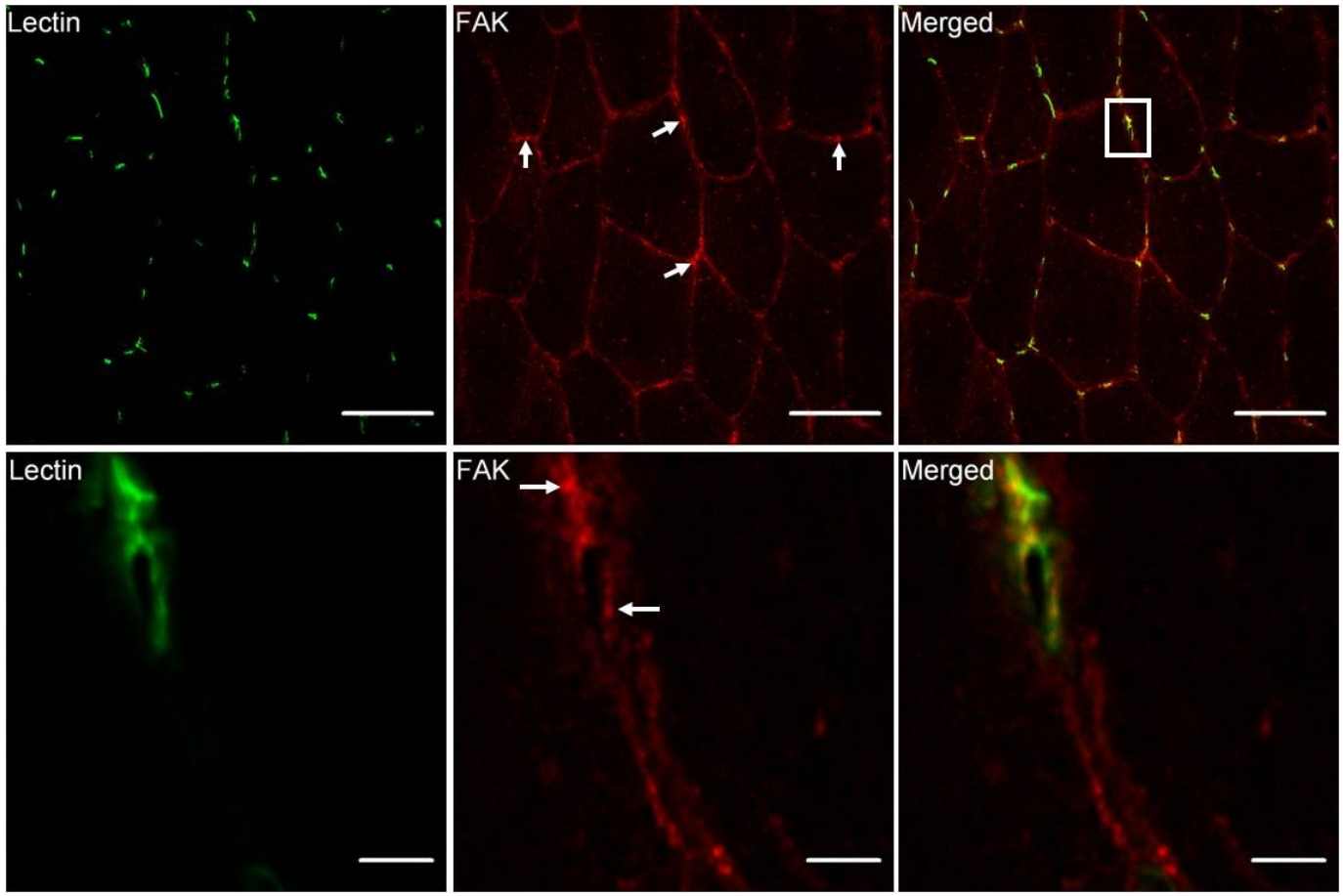

Figure 2.5. Immunofluorescence confocal microscopy to show visualisation of focal adhesion kinase (FAK) in the endothelium of human skeletal muscle microvessels. Microvessels such as capillaries (lectin, green, *left*) are shown amongst transversely orientated muscle fibres (*upper*) and in greater resolution (*lower*). FAK (red, *middle*) colocalises with lectin (merged, *right*). Bars are 50 µm and 5 µm, respectively. Notice the heterogeneous distribution of FAK *(*red*, lower middle*, *arrow*) within the endothelium.

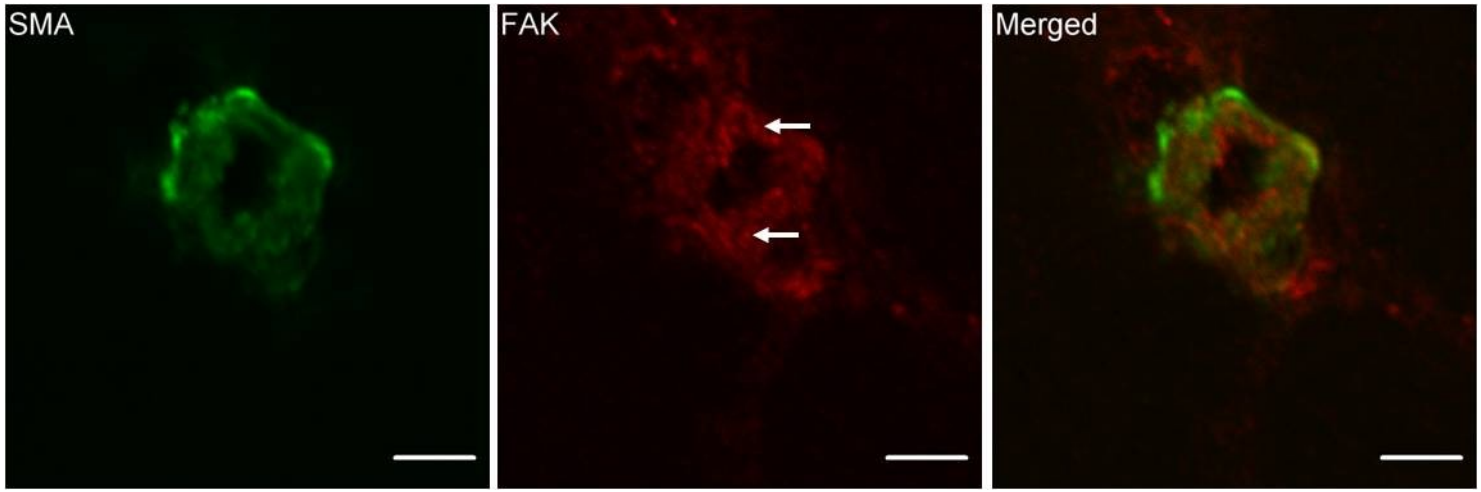

Figure 2.6. Immunofluorescence confocal microscopy to show visualisation of focal adhesion kinase (FAK) in vascular smooth muscle (SMA). Bars are 10 µm. Arterioles were identified using anti-α-smooth muscle actin (SMA) (green, *left*). A strong immnuofluorescent signal for FAK (red, *middle*) (*arrow*) is detected at the periphery of the arteriole forming a ring-like structure (*arrow*). FAK also appeared to be distributed heterogenously in the form of clusters (*arrowhead*). Note that FAK is present within the smooth muscle and the endothelial cell layer (merged, *right*).

2.5. Discussion

This is the first study using immunofluorescence microscopy to report the distribution and localisation of FAK in human skeletal muscle and its associated microvasculature. We also support and enhance previous reports of animal muscle detailing FAK's distribution and localisation in transversely and longitudinally orientated muscle fibres.

In figure 1 and 2, a strong immunoreactivity for FAK was detected at the sarcolemmal regions in both fibre types when compared with deeper areas of the muscle fibre. Our findings are consistent with previous studies where sarcolemmal and subsarcolemmal staining has been observed in the rooster *anterior latissimus dorsi* (Fluck et al., 1999), rat *m. soleus*, *m. extensor digitorum longus* (Durieux et al., 2009; Fluck et al., 2002) and tibialis anterior (Klossner et al., 2009). Although fibre type specific sarcolemmal FAK immunoreactivity was not measured in the present study, observations from animal studies suggest FAK protein content at the sarcolemma is specific to the fibres recruited and is higher in oxidative compared with fast glycolytic muscle fibres (Fluck et al., 2002). More recent data using electrotransfer of a FAK-expression plasmid suggest FAK is a load-dependent governor of type I fibres since rat type I muscle fibres showed greater sarcoplasmic FAK content compared with type II fibres and upregulation of proteins involved in mitochondrial respiration (Durieux et al., 2009). However, in recreationally active human *m. vastus lateralis* skeletal muscle, sarcoplasmic type I fibre specificity was not observed (figure 3) which may be due to differences in the methods and/ or species investigated.

The presence of FAK at the sarcolemma is consistent with FAK's interaction with the transmembrane integrins (Parsons, 2003; Schaller, 2001; Schlaepfer et al., 1999) and it's role in converting mechanical stimuli from loading and unloading into intracellular signalling in animal skeletal muscle (Fluck et al., 1999; Gordon et al., 2001; Klossner et al., 2009) and humans (de Boer et al., 2007; Flueck et al., 2011; Glover et al., 2008; Narici et al., 2011;

Wilkinson et al., 2008). Indeed, rats subjected to 7 days of hindlimb unloading and subsequent reloading increased FAK tyrosine phosphorylation between 1 and 6 h. FAK tyrosine phosphorylation preceded the phosphorylation of p70S6K at 24 h (Klossner et al., 2009), which previously has been shown to correlate with increased muscle mass following resistance exercise (Baar and Esser, 1999). Furthermore, a positive correlation between the gain in muscle thickness and an increase in FAK content has been reported in elderly men and women after 12 weeks of skiing training compared with sedentary controls (Narici et al., 2011). These findings suggest an important role for FAK in increasing skeletal muscle protein synthesis and muscle mass in response to training.

The localisation of FAK at the sarcolemma also supports FAK's role in insulin signalling (Bisht and Dey, 2008; Bisht et al., 2007; Bisht et al., 2008; Huang et al., 2006) since the insulin receptor is also expressed at the sarcolemma (Karlsson and Zierath, 2007) and upon insulin stimulation, directly phosphorylates FAK (Baron et al., 1998; Goel and Dey, 2002b). Defects in human skeletal muscle insulin signalling and glucose uptake have been reported in insulin resistant type 2 diabetic patients, but these studies have typically focused on insulin signalling intermediates such as IRS-1, PI3-K and Akt (Karlsson and Zierath, 2007). FAK may regulate insulin-stimulated glucose uptake since a reduction in basal FAK tyrosine phosphorylation after FAK-silencing *in vivo* was associated impaired insulin-stimulated skeletal muscle IRS-1 and Akt phosphorylation and the development of insulin resistance within 2 weeks (Bisht et al., 2008).

In longitudinally orientated rat skeletal muscle fibres visualised using immunohistochemistry (Fluck et al., 2002), there was little evidence of FAK in the sarcoplasm. In the present study, however, immunofluorescence using both widefield (figure 2.4A) and confocal (figure 2.4B) microscopy revealed a highly ordered structure running perpendicular to the direction of the muscle fibre in both fibre types. These structures

resemble not only costameres as suggested previously (Ervasti, 2003; Pardo et al., 1983; Quach and Rando, 2006), but also the t-tubular network (Lauritzen et al., 2008a; Lauritzen et al., 2008b; Lauritzen et al., 2006). T-tubules are invaginations of the plasma membrane that run deep into the muscle fibre. The combined t-tubules have a surface area up to 3 fold that of the sarcolemma (Ploug et al., 1998; Wang et al., 1996) and after insulin stimulation, have a surface area up to 9 times greater than the sarcolemma, supporting ~94% of GLUT-4 mediated glucose uptake (Wang et al., 1996). FAK has been reported in the t-tubules of vascular smooth muscle, playing an important role in regulating the function of the L-type calcium channel (Wu et al., 2001). The DHPR antibody was used as a marker for t-tubules and for the first time in human skeletal muscle, showed the colocalisation of FAK with DHPR. On the basis of this colocalisation, this may suggest that FAK plays a significant role in insulin receptor and IRS-1 signalling in the t-tubular membranes. The t-tubules have been shown to contain components of the insulin signalling pathway such as the insulin receptor, PI3-K and upon insulin stimulation, by far the largest proportion of GLUT-4 (Lauritzen et al., 2008a; Lauritzen et al., 2008b; Lauritzen et al., 2006).

Within the vasculature, FAK is required for endothelial morphogenesis (Ilic et al., 2003) and angiogenesis (Peng et al., 2004; Shen et al., 2005). Given the similarities in endothelial and skeletal muscle insulin signalling upstream of Akt (Zeng et al., 2000), it is possible that FAK may also play a role in vascular insulin action. The present study demonstrates for the first time that, within the microvasculature of human skeletal muscle, FAK is distributed heterogeneously in the form of clusters within the endothelium and vascular smooth muscle (figure 2.5 and 2.6). This pattern of distribution might reflect large focal adhesions awaiting integrin- (e.g. shear flow) and/or non-integrin- (e.g. insulin) mediated stimuli (Schlaepfer et al, 1999). Therefore, the localisation and phosphorylation

status of FAK within the skeletal muscle microvasculature in the presence of such stimuli warrants further investigation.

In conclusion, this study has used widefield and confocal immunofluorescence microscopy to expand on the findings of previous research on FAK. This study provides the first immunofluorescence images of FAK in human skeletal muscle with FAK's distribution and localisation confirming images previously reported from animal muscle. This study also provides the first images of FAK showing colocalisation with DHPR, suggesting its presence in the t-tubules. Finally, this study provides the first images of FAK within the human skeletal muscle microvasculature. Immunofluorescence staining is a valuable method for visualising FAK in skeletal muscle and the associated microvasculature. This technique may provide important insights into the potential role of FAK in elevating insulin sensitivity up to 48 h after acute exercise (Mikines et al., 1988; Perseghin et al., 1996) and in exercise training adaptation. Future research should investigate how insulin and exercise modulate the protein content, phosphorylation, activity, distribution and localisation of FAK in skeletal muscle in different populations to include comparisons between sedentary and physically active, young and old, and insulin sensitive and resistant.

2.6. Acknowledgements

The antibodies against human slow myosin (A4.840-c) used in the study were developed by Dr. Blau and were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Chapter 3

Increased focal adhesion kinase in the skeletal muscle fibres and microvascular endothelium of elderly humans after 12 weeks resistance-type exercise training

3.1. Abstract

Resistance-type exercise training (RT) can reverse the age-associated decline in skeletal muscle mass, strength and limb blood flow. Focal adhesion kinase (FAK) responds to mechanical forces in skeletal muscle and within the vascular endothelium, but it is unknown how RT may affect the FAK protein content in these tissues. This study tested the hypothesis that a 12 week RT program would increase FAK within the skeletal muscle and its microvascular endothelium of previously sedentary elderly individuals.

Ten healthy elderly males (mean ± SEM) aged 73 ± 2 y completed 12 weeks RT with leg strength assessed and percutaneous *m.vastus lateralis* muscle biopsies collected pre- and post-intervention. Cryosections were incubated with anti-FAK antibodies for visualisation in muscle fibres and co-incubated with anti-dystrophin to identify the sarcolemma and anti-myosin heavy chain type I for fibre-type determination. Ulex europaeus Agglutinin I was used to stain the endothelial layer of the skeletal muscle microvasculature and to investigate its FAK content. Measures of fibre cross-sectional area (CSA) were also made and images were captured using widefield and confocal immunofluorescence microscopy.

RT increased leg extension and leg press strength by 29 ± 3% and 23 ± 3% ($P < 0.001$), respectively. Pre-RT mean type II fibre CSA was smaller than type I fibres ($P < 0.01$), but the difference was abolished after type II fibre CSA increased 24 ± 9% post-RT ($P < 0.01$) with no change in type I fibres. There were no fibre type differences in sarcoplasmic FAK content pre- or post-RT. RT increased type II fibre sarcoplasmic FAK content by 40 ± 13% ($P < 0.01$) with a trend to increase in type I fibres (16 ± 6.5%; $P = 0.057$). RT increased (sub)sarcolemmal FAK protein content by 27 ± 7% ($P < 0.01$) and skeletal muscle microvascular endothelial FAK content by 24 ± 8% ($P < 0.05$). These data suggest FAK protein content is increased in hypertrophied elderly skeletal muscle fibres and within the skeletal muscle microvascular endothelium after 12 weeks RT.

3.2. Introduction

The age-associated loss of skeletal muscle mass (sarcopenia) (Rosenberg, 1989) and strength (dynapenia) (Clark and Manini, 2008) can increase the risk of falls (Janssen et al., 2002; Roe et al., 2009), self-reported disability (Janssen, 2006) and dependency (Tinetti and Williams, 1998). Dynapenia is also an independent risk factor for all cause mortality (Ruiz et al., 2008). Sarcopenia begins in the 4th and 5th decade with the loss of skeletal muscle mass estimated at a rate of ~0.6% per year (Janssen, 2010) or between 3 – 5% per decade (Lexell et al., 1988). It is characterised by a combined loss of type I and type II muscle fibre number (Lexell et al., 1986) and specific type II fibre atrophy (Charette et al., 1991; Lexell et al., 1986; Verdijk et al., 2007). The causes of sarcopenia are likely multifactorial (Doherty, 2003), with the age-associated decline in physical activity rates (Haskell et al., 2007) considered an important contributor (Doherty, 2003).

Resistance-type exercise training (RT) is recommended for older people (Chodzko-Zajko et al., 2009) as it offers an effective treatment for increasing skeletal muscle strength (Fiatarone et al., 1990; Kosek et al., 2006; Lexell et al., 1995; Verdijk et al., 2009b) and inducing type II skeletal muscle fibre hypertrophy in the elderly (Fiatarone et al., 1990; Frontera et al., 1988; Kosek et al., 2006; Verdijk et al., 2009a; Verdijk et al., 2009b). The molecular mechanisms are not fully defined, but proteins proximal in the mechanotransduction signalling cascade may play an important role. Focal adhesion kinase (FAK) is a growth factor- and mechano-sensitive protein localised to regions corresponding to costameres in human skeletal muscle (chapter 2 of this thesis). Costameres are subsarcolemmal protein assemblies that link the Z-disk of peripheral myofibrils to the sarcolemma and facilitate the lateral transmission of force from the contracting myofibrils to the sarcolemma and extracellular matrix (Ervasti, 2003; Pardo et al., 1983). Increased mechanical loading of animal and human skeletal muscle leads to increased FAK

phosphorylation (Fluck et al., 1999; Klossner et al., 2009; Wilkinson et al., 2008) and protein abundance (Fluck et al., 1999; Gordon et al., 2001). FAK has been linked to the skeletal muscle protein synthesis signalling pathway and load-induced hypertrophy (Fluck et al., 1999; Klossner et al., 2009; Wilkinson et al., 2008) but ageing may impair the response of FAK to mechanical load (Rice et al., 2007) and inhibition of FAK can prevent load-induced left ventricle hypertrophy in rats (Clemente et al., 2007; DiMichele et al., 2006). Therefore, FAK may play a role in mechanical load-induced skeletal muscle hypertrophy, but the effect of RT on FAK in elderly human skeletal muscle remains to be explored.

FAK is highly expressed in the human skeletal muscle microvascular endothelium of young recreationally active individuals (chapter 2). The vascular endothelium is exposed to mechanical and hemodynamic forces (Davies and Tripathi, 1993) and the transmission of fluid shear stress forces from the luminal glycocalyx to abluminal focal adhesions (Weinbaum et al., 2007) leads to FAK phosphorylation (Li et al., 2002; Li et al., 1997; Mattiussi et al., 2006). Evidence suggests FAK may play a role in endothelium-dependent vasodilation as inhibition of FAK blocked flow mediated dilation (FMD) of rat coronary arterioles (Koshida et al., 2005). Ageing is associated with impaired endothelial-dependent vasodilation (Seals et al., 2011) leading to blunted FMD in rat skeletal muscle arterioles (Delp et al., 2008; Muller-Delp et al., 2002). RT has been shown to prevent or improve the age-associated decline in limb blood flow (Anton et al., 2006; Maeda et al., 2006; Miyachi et al., 2005; Phillips et al., 2012), but investigations of FAK within the skeletal muscle microvascular endothelium of elderly individuals have not been reported.

The response of FAK to increased mechanical loading of skeletal muscle have been investigated using Western blot analyses of whole muscle homogenates (Fluck et al., 1999; Gordon et al., 2001; Klossner et al., 2009; Wilkinson et al., 2008). This technique is not able to measure the FAK protein content in skeletal muscle without the potential contamination

from the microvasculature and vice versa. The advantage of immunofluorescence microscopy techniques described in chapter 2 of this thesis is that measures of FAK content can be made specifically in these distinct regions without contribution from neighbouring cells.

Therefore, this study aimed to make the first investigations of FAK in elderly skeletal muscle fibres and within the skeletal muscle microvascular endothelium in response to RT. The methods developed in chapter 2 of this thesis were used to test the hypothesis that 12 weeks of progressive RT would increase FAK content in the (sub)sarcolemmal and sarcoplasmic regions of skeletal muscle fibres and within the microvascular endothelium in previously untrained healthy elderly humans and that this would occur alongside hypertrophy of type II fibres and increases in skeletal muscle strength

5.2. Methods

Participants

Ten healthy elderly males (mean ± SEM; 73 ± 2 y; BMI 26.6 ± 1.3) participated in a 12 week RT programme after providing written informed consent. The study was performed according to the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Academic Hospital, Maastricht. The study formed part of a larger project investigating the clinical benefits of exercise intervention in the elderly as described in detail elsewhere (Verdijk et al., 2009b).

Study design

The participants were enrolled in the RT programme subject to health screening with anthropometric measurements (height, body mass) and maximal strength and dual energy x-ray absorptiometry (DEXA) recorded pre- and post-intervention. Blood samples were collected before the training intervention began and 4 days after the final post-intervention

strength assessment for the measurement of plasma glucose and insulin concentrations as described (Verdijk et al., 2009b). Resting, fasted percutaneous needle biopsies were collected from the *m. vastus lateralis* of the right leg 3 days before the training intervention began and 4 days after the final post-intervention strength assessment.

Strength assessment

Maximal leg strength was determined using one-repetition maximum (1RM) strength tests on leg extension and leg press machines (Technogym, Rotterdam, The Netherlands). Participants were familiarised with the proper lifting technique during an initial familiarisation trial and their 1RM estimated using the multiple repetitions testing procedure (Mayhew et al., 1995). At a later date, participants returned for the final pre-training assessment of their 1RM. Subsequent 1RM tests were performed at 4 and 8 weeks of the intervention and 2 days after completion of their final RT session.

Resistance-type exercise training intervention

Participants completed 3 supervised RT sessions per week for 12 weeks comprising 5 min warm-up on a cycle ergometer, followed by 4 sets of leg extension and leg press exercise and a 5 min cool down on a cycle ergometer. Each training session was performed in the morning and at the same time of day. All subjects consumed breakfast 1.5 h before each training session and had lunch 2 h after each training bout. The workload was increased from 60% 1RM (10-15 repetitions per set) to 75% 1RM (8-10 repetitions) on each machine during the first 4 weeks. Starting at week 5, the workload increased to 4 sets at 75% - 80% 1RM (8 repetitions) on each machine. Participants were allowed to rest between sets and exercises for between 1.5 and 3 min. The workload intensity was manipulated according to the 1RM tests

performed in week 4 and 8 and if the participants could lift more than 8 repetitions in 3 of the 4 sets.

Immunofluorescence microscopy

Any visible fat or connective tissue was removed from the muscle samples before being embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and immediately frozen in liquid nitrogen-cooled isopentane. Five μm thick cryosections were cut at -30°C with pre- and post-training sections from a single participant placed on the same room temperature uncoated glass slides. The sections were incubated with N-terminal specific anti-FAK antibody, anti-myosin heavy chain type I (MHC type-I; developed by Dr. Blau; DSHB; A4.840c) and anti-dystrophin before incubating with the appropriate secondary antibodies as previously described (chapter 2). The anti-dystrophin was used as a marker for the sarcolemma as its protein content did not change after 8 and 12 weeks of RT or endurance training in human skeletal muscle (Parcell et al., 2009; Woolstenhulme et al., 2006). To investigate endothelial FAK content, sections were co-incubated with FITC-conjugated Ulex europaeus Agglutinin I (endothelial marker) (Vector Laboratories) as described (chapter 2). Cover slips were applied with a mounting solution containing antifade medium. Background fluorescence was assessed using negative controls where the primary antibody was excluded. Controls were also performed to detect bleed-through of each fluorophore in the respective excitation filters. In all cases the fluorescence signal was abolished.

Image capture and analysis

FAK was visualised using widefield and confocal immunofluorescence microscopy. Widefield images of skeletal muscle fibres and the microvascular endothelium were captured using a Nikon E600 microscope with a 40 x NA objective with a SPOT RT KE colour three

shot CCD camera (Diagnostic Instruments Inc., MI, USA). Because of the intense FAK immunofluorescence intensity in the microvasculature, exposure times were optimised for image collection of each region to prevent pixel saturation. DAPI UV (340 – 380 nm), FITC (465 – 495 nm) and Texas Red (540 – 580 nm) excitation filters were used to visualise the Alexa Fluor 350 (MHC type I), 488 (dystrophin, lectin UEA-I) and 594 fluorophores (FAK). Detailed digital images of FAK and dystrophin were captured with an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) with a 63 x 1.4 NA oil immersion objective. The 488 nm line of the argon laser excited the Alexa Fluor 488 fluorophore (MHC type-I) and the 594 line of the Helium-Neon laser excited the Alexa Fluor 594 fluorophore (FAK).

Image processing was completed using Image-Pro Plus 5.1 (Media Cybernetics, MD, USA). Muscle fibre CSA (μm^2) was determined through an automated method based on individual specific threshold settings or, where necessary, by manually tracing along the dsytrophin-stained border. Only fibres determined as transversely orientated were included in the CSA analysis if the fibre roundness factor was <1.639 (roundness = $perimeter^2/4\pi area$ where a perfect circle = 1.0, pentagon = 1.163, square = 1.266 and equilateral triangle = 0.639) (Kosek et al, 2006). A total of (mean ± SD) 93 ± 39 and 87 ± 37 pre- and post-RT transversely orientated muscle fibres were analysed per individual for CSA.

For quantitation of FAK (sub)sarcolemmal immunofluorescence intensity, 10 - 15 widefield images were captured across 3 sections for pre- and post-RT and a total of (mean ± SD) 54 ± 16 and 52 ± 21 pre- and post-RT transversely orientated muscle fibres were analysed as before (chapter 2). All longitudinally orientated muscle fibres were excluded from quantitation. Fibre-type specific (sub)sarcolemmal FAK immunoreactivity was not measured, therefore the muscle fibres captured with widefield microscopy were pooled and the mean FAK (sub)sarcolemmal immunofluorescence intensity determined. The same

images were used to quantitate the sarcoplasmic FAK immunofluorescence intensity of complete type I and type II transversely orientated skeletal muscle fibres. Endothelial FAK immunofluorescence intensity was quantitated from a total of 256 ± 40 and 296 ± 60 microvessels pre- and post-RT.

Statistical analysis

Pre- and post-training data were analysed using a paired t-test (SPSS for Windows version 17; Chicago, IL). To investigate changes in muscle fibre CSA, a two way analysis of variance was performed with time (pre vs post) and fibre type (type I vs type II) as within subject factors. Where a significant effect was found, a paired t-test was used to identify the effect of time on fibre-type CSA and/or differences between fibre types at pre- and post-RT. Data are presented as mean ± SEM and statistical significance was set at $P \leq 0.05$.

3.3. Results

Participant characteristics

Participant characteristics are displayed in table 3.1. There was no change in fasting glucose homeostasis (fasting glucose, insulin, HbA1c and HOMA) with RT. Percent body fat and fat mass decreased with RT by 4.3 ± 1.3% and 4.5 ± 1.3%, respectively ($P < 0.01$) without change in total lean mass. Leg strength was significantly increased after 12 weeks RT. Leg extension 1RM increased by 29 ± 3 % from 85 ± 5 kg to 110 ± 6 kg ($P < 0.001$) and leg press 1RM strength increased by 23 ± 3% from 166 ± 9 kg to 202 ± 9 kg, respectively ($P < 0.001$).

Table 3.1 Participant characteristics

Characteristic	Pre	Post
Age (y)	72.2 ± 2	
Body mass (kg)	76.9 ± 3.6	76.7 ± 3.6
Height (m)	1.70 ± 0.01	1.70 ± 0.01
BMI (kg/m^2)	26.5 ± 1.1	26.4 ± 1.1
Fasting glucose (mmol/L)	5.7 ± 0.2	5.6 ± 0.1
Fasting insulin (μIU/mL)	11.1 ± 1.8	11.1 ± 1.5
HOMA Index	2.9 ± 0.4	2.8 ± 0.4
HbA1c (%)	5.8 ± 0.1	5.8 ± 0.1
Body fat (%)	22.4 ± 2.6	21.6 ± 2.7 *
Lean mass (kg)	55.9 ± 1.5	56.4 ± 1.6
Fat mass (kg)	18.0 ± 2.8	17.2 ± 2.7 *
1RM LE (kg)	85 ± 5	110 ± 6 *
1RM LP (kg)	166 ± 9	202 ± 9 *

Data are mean ± SEM. BMI, body mass index; HOMA, homeostatic model assessment; 1RM, one repetition maximum; LE, leg extension; LP, leg press; * Significantly different to pre-training.

Muscle fibre CSA

A significant interaction for 'time x fibre type' was observed ($P < 0.01$) with pre-RT type II muscle fibre CSA significantly smaller than type I fibre CSA (5920 ± 305 type I vs 4678 ± 333 μm^2 type II fibres) ($P < 0.01$). After RT, there was no change in type I fibre CSA, but the mean type II fibre CSA increased by 24 ± 9% from 4678 ± 333 to 5654 ± 359 μm^2 ($P < 0.01$). This abolished the fibre type difference in CSA (5709 ± 359 type I vs 5654 ± 359 μm^2 type II fibres) (figure 3.1).

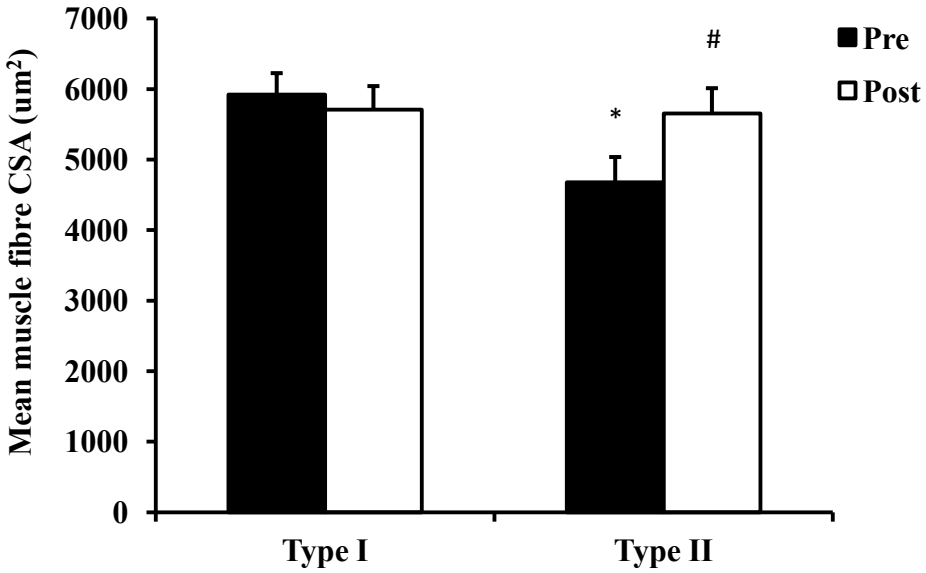


Figure 3.1. Mean type I and type II elderly skeletal muscle fibre cross sectional area (CSA) pre- and post-resistance-type exercise training. Data are mean ± SEM. * Significantly different compared with type I fibres; # Significantly different compared with pre-resistance type training ($P < 0.01$).

Immunofluorescence

FAK immunolabeling appeared strongest at the (sub)sarcolemmal regions of skeletal muscle fibres. The FAK (sub)sarcolemmal immunofluorescence intensity increased 27 ± 7% after 12 weeks RT (figure 3.2) ($P < 0.01$). Higher magnification confocal immunofluorescence microscopy images suggests an increased (sub)sarcolemmal FAK immunofluorescence intensity in type I and type II skeletal muscle fibres (figure 3.3). There was no fibre type difference in sarcoplasmic FAK content pre- and post-RT ($P > 0.05$). However, there was a significant effect of time ($P = 0.001$) where mean type II fibre sarcoplasmic FAK content increased by 40 ± 13% ($P < 0.01$) after RT with a trend for type I fibre sarcoplasmic FAK content to increase after RT (16 ± 6.5%; $P = 0.057$) (figure 3.4). Lectin UEA-I successfully identified the endothelium in 7 of the 10 individuals, therefore, FAK immunofluorescence

intensity in the microvascular endothelial layer is presented for 7 people pre- and post-training. The FAK immunofluorescence intensity was strong in the skeletal muscle microvasculature both before and after RT and increased 24 ± 8% in the endothelial layer after RT ($P < 0.05$) (figure 3.5).

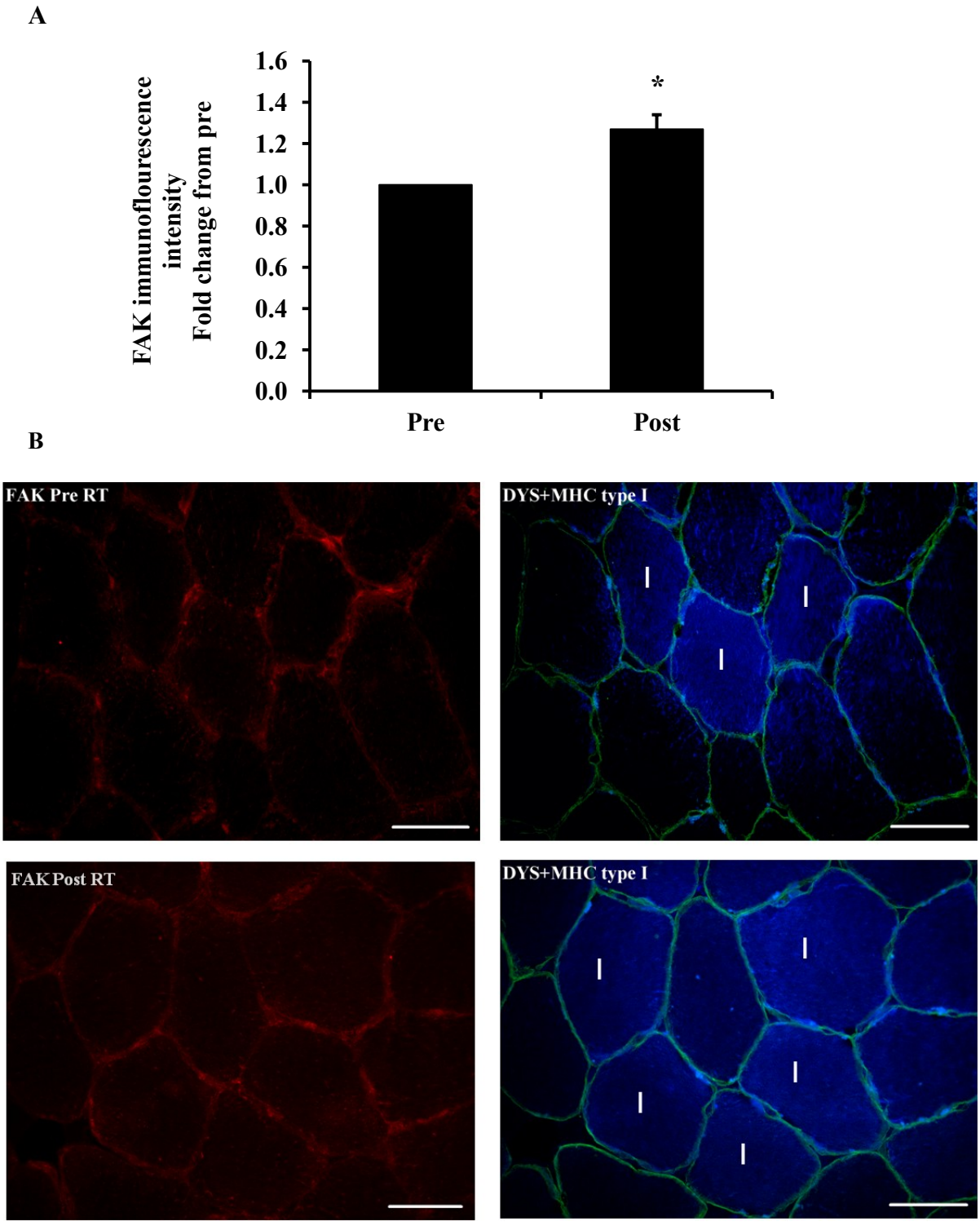


Figure 3.2. Effect of 12 weeks resistance-type exercise training on (sub)sarcolemmal focal adhesion kinase (FAK) in the skeletal muscle fibres of elderly humans. A) Mean of the post-RT (sub)sarcolemmal FAK immunofluorescence intensity expressed as fold-change compared to pre-training. Mean ± SEM (n=10). * Significant effect of training ($P < 0.01$). B) Representative widefield immunofluorescence microscopy images of pre- (above) and post-

(below) intervention skeletal muscle visualising FAK (left; red) and dystrophin (right; green). Note the more intense FAK immunoreactivity at the (sub)sarcolemma post-intervention. Bars are 50 μm.

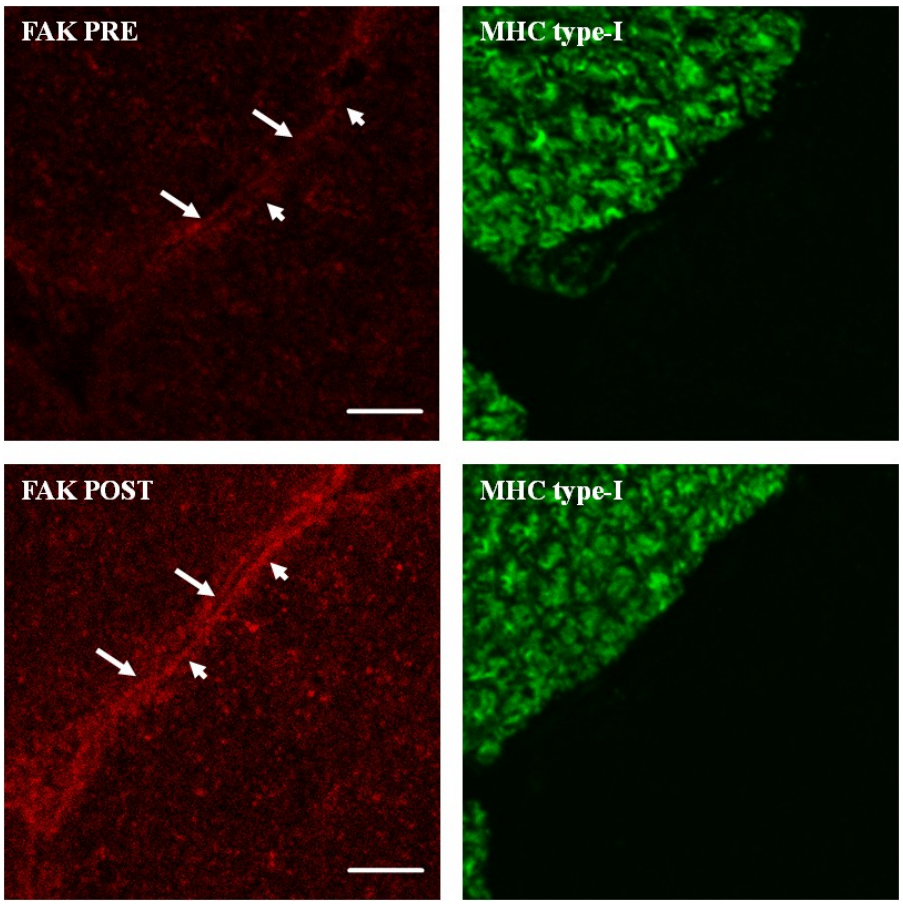


Figure 3.3. Effect of 12 weeks resistance-type exercise training on (sub)sarcolemmal focal adhesion kinase (FAK) in the skeletal muscle fibres of elderly humans. Representative higher magnification confocal microscopy images of FAK (left; red) and myosin heavy chain type I (MHC type-I; right; green) using 8 x zoom. Note a more intense FAK immunoreactivity at the sarcolemma of a type II (*small arrowhead*) compared with type I fibre (*large arrows*). Bars are 10 μm.

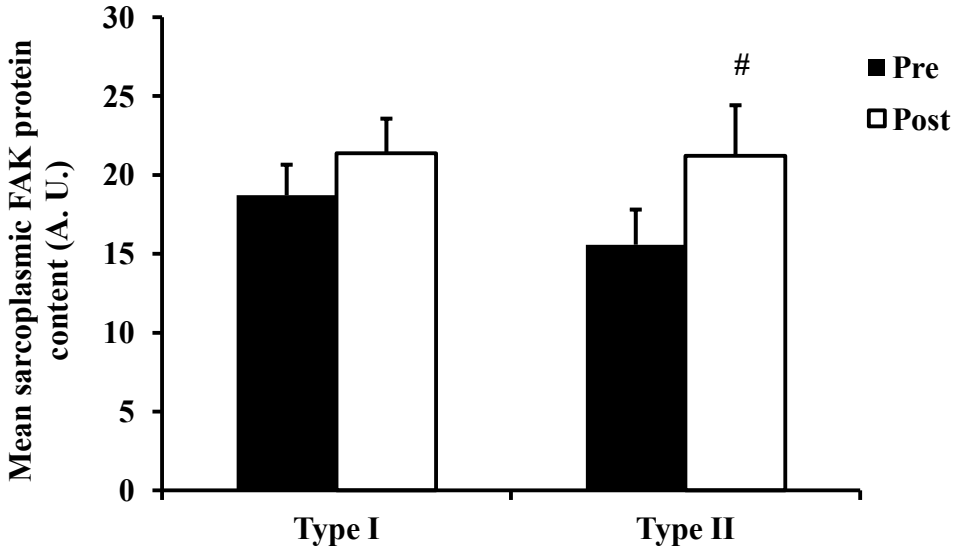
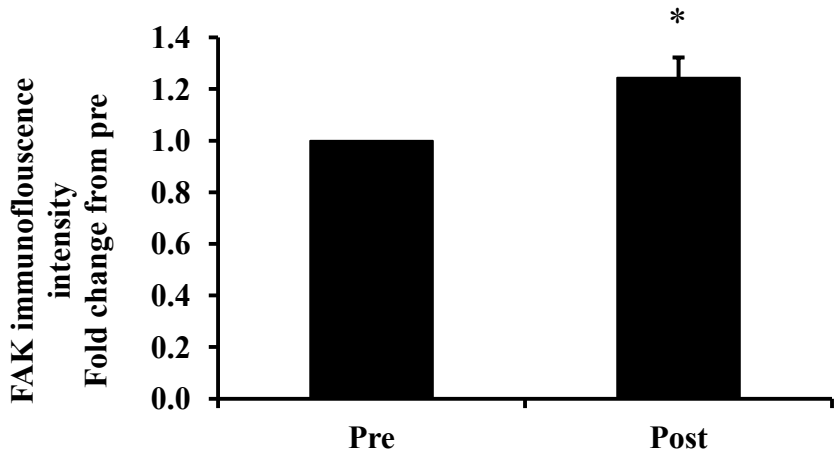


Figure 3.4. Effect of 12 weeks resistance-type exercise training on the sarcoplasmic protein content of focal adhesion kinase (FAK) within the type I and type II skeletal muscle fibres of elderly humans. Mean of the post-RT sarcoplasmic FAK immunofluorescence intensity expressed as fold-change compared to pre-training. Mean ± SEM (n=10). # Significantly different from pre-training ($P < 0.01$).

A



B

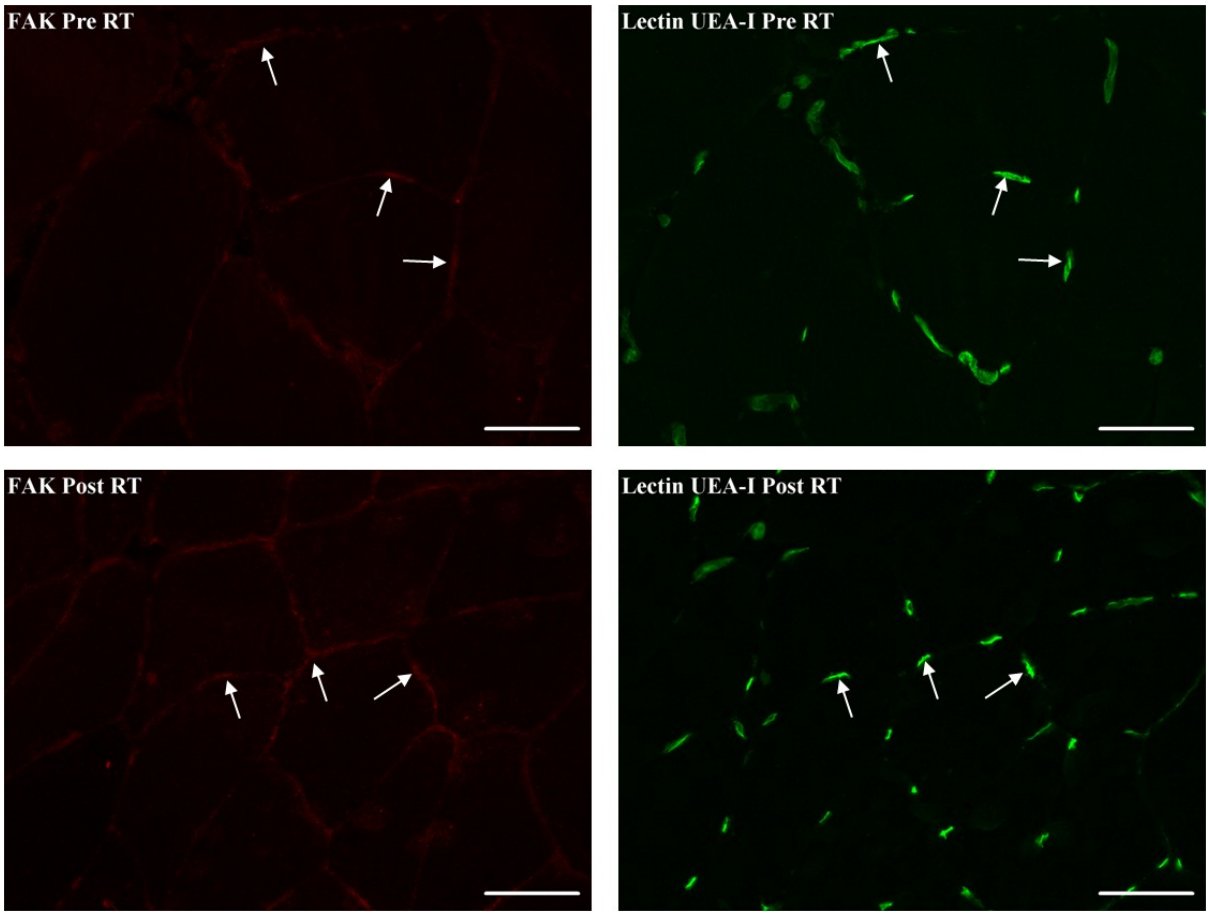


Figure 3.5. Effect of 12 weeks resistance-type exercise training on focal adhesion kinase (FAK) in the microvascular endothelium of elderly human skeletal muscle. A) Mean post-RT FAK immunofluorescence intensity expressed as fold-change of pre-RT. Mean ± SEM (n=7). * Significant effect of training ($P < 0.05$). B) Representative pre- (above) and

post- (below) intervention widefield immunofluorescence microscopy images visualising FAK (left; red) in the skeletal muscle microvascular endothelium (right; green). Microvessels were visualised using Ulex Europaeus-FITC conjugated lectin (Lectin UEA-I). Note the more intense FAK immunoreactivity within the endothelium of post-intervention skeletal muscle (*arrows*). Bars are 50 μm.

3.4. Discussion

This is the first study to investigate FAK responses to 12 weeks RT in the skeletal muscle fibres and microvascular endothelium of previously untrained elderly humans. The novel findings are that FAK immunofluorescence intensity was significantly increased in the (sub)sarcolemmal areas of the muscle fibre. There were no fibre type differences in sarcoplasmic FAK content pre- or post-RT, but RT increased type II fibre sarcoplasmic FAK content with a trend to increase in type I fibres. FAK immunofluorescence intensity also increased in the skeletal muscle microvascular endothelium following RT in previously untrained elderly men. The present study also demonstrates that 12 weeks RT increased skeletal muscle strength and stimulated hypertrophy specifically of the type II muscle fibres. These findings are in agreement with the most recent literature (Narici et al., 2011) showing a positive association between the increase in FAK content and the increase in muscle thickness. This suggests the increased FAK content at the (sub)sarcolemma and sarcoplasm plays a role in the mechanism leading to skeletal muscle fibre hypertrophy. These data confirm the usefulness of the recently developed immunofluorescence microscopy methods (chapter 2) for quantitating exercise-training associated changes in the protein content of human focal adhesion-associated proteins.

The ACSM recommend RT as an effective strategy for increasing skeletal muscle strength and stimulating type II muscle fibre hypertrophy in elderly individuals (Chodzko-

Zajko et al., 2009). In the present study, 1RM leg strength increased after 12 weeks RT (table 3.1). As expected, baseline type II fibre CSA was significantly smaller than type I fibres in line with observations made previously (Andersen, 2003; Charette et al., 1991; Dreyer et al., 2006; Essen-Gustavsson and Borges, 1986; Klitgaard et al., 1990; Kosek et al., 2006; Larsson et al., 1978; Lexell, 1995; Lexell and Downham, 1992; Lexell et al., 1986; Lexell et al., 1988; Orlander et al., 1978; Verdijk et al., 2007), but this difference was abolished post-training as type II fibre CSA increased without change in type I fibres CSA (figure 3.1).

The increased (sub)sarcolemmal FAK content after RT (figure 3.2 and 3.3) suggests a remodelling of the costamere in response to the high forces generated by the contracting sarcomeres during high intensity RT. A remodelling of costamere-associated proteins in response to RT has been reported (Kosek and Bamman, 2008). A novel aspect of this study was to investigate the fibre type specific response of FAK to RT. In confirmation of chapter 2 of this thesis, there were no fibre type differences in sarcoplasmic FAK protein content pre- or post-RT (figure 3.4). However, there was a type II fibre-specific increase in sarcoplasmic FAK content (with a trend to increase in type I fibres) after RT. This is in line with the type II fibre-specific increase in CSA and may be important in the hypertrophic response of type II skeletal muscle fibres to RT.

The present study's findings are consistent with and expand upon earlier studies that reported an increased FAK protein concentration in mechanically-overloaded animal skeletal muscle (Fluck et al., 1999). During the preparation of this chapter, Narici et al, (2011) and Flueck et al, (2011) published data investigating the effects of 12 weeks of skiing training on FAK protein content in the skeletal muscle of elderly individuals as measured using Western blot analyses (Flueck et al., 2011; Narici et al., 2011). Although the training intervention used in the latter studies differed to the present study, it is notable that muscle loading via both skiing training (Flueck et al., 2011; Narici et al., 2011) and RT (in the present study) increased the FAK protein

content in the skeletal muscle of elderly humans. Narici et al, (2011) recently reported the positive correlation between an increased FAK content and increased muscle thickness after skiing training. Furthermore, in rat cardiomyocytes, load-induced increases in FAK protein content and hypertrophy of the left ventricle was prevented and reversed by FAK inhibition *in vivo* (Clemente et al., 2007) with similar findings observed for left ventricle wall thickness after cardiomyocyte-specific gene deletion (DiMichele et al., 2006). These studies suggest that the increased FAK protein content at the (sub)sarcolemma and sarcoplasm may play an important role in the mechanism leading to the type II fibre-specific hypertrophy observed in the present study (figure 3.1).

The microvascular endothelium is exposed to a variety of mechanical and hemodynamic forces (Davies and Tripathi, 1993) and fluid shear stress is transmitted between the luminal glycocalyx and the abluminal focal adhesions by the cytoskeleton (Weinbaum et al., 2007). Fluid shear stress phosphorylates and activates FAK leading to its translocation to focal adhesions (Li et al., 2002; Li et al., 1997; Mattiussi et al., 2006). The latter leads to increased FAK-mediated mechanotransduction, but an increase in FAK protein content as observed in the present study (figure 3.5), may also contribute to the increased adhesion strength of endothelial cells to the extracellular matrix in response to the increased hemodynamic and mechanical forces that are applied to the endothelium during exercise (Balaban et al., 2001; Fabry et al., 2011; Riveline et al., 2001). Activation of FAK by hemodynamic forces also stimulates mechanotransduction pathways that regulate vascular function such as FMD. FAK has been shown to regulate shear stress-activated FMD of coronary arterioles through endothelial nitric oxide synthase (eNOS) phosphorylation (Koshida et al., 2005) and inhibition of FAK phosphorylation blocked shear-stimulated eNOS phosphorylation and FMD (Koshida et al., 2005). Alterations in the protein content and/ or activity of FAK with age (Rice et al., 2007) may contribute to the impaired FMD of arterioles

in rat skeletal muscle (Delp et al., 2008; Muller-Delp et al., 2002) and in the larger blood vessels of elderly humans (Celermajer et al., 1994; Donato et al., 2007; Eskurza et al., 2004). RT can increase plasma nitric oxide concentration (Maeda et al., 2006), basal limb blood flow and vascular conductance in elderly individuals (Anton et al., 2006; Miyachi et al., 2005; Phillips et al., 2012) and the increased FAK content within the microvascular endothelium after RT may be an important mechanism.

The time course for increased FAK protein content in response to exercise training in humans has not been investigated. In animals, loading rapidly increased FAK protein content upto 8 days of overload of the rat *m. soleus,* but returned to baseline after 13 days of stretch overload in the avian *anterior latissimus dorsi* (Fluck et al., 1999; Gordon et al., 2001). Muscle biopsies were only collected prior to training commencement and 4 days following training cessation. Therefore, although the increase in FAK lasts for at least 4 days after the last training bout, it cannot be confirmed whether the increased FAK protein content observed in the present study represents an early adaptation to loading.

In conclusion, this is the first study to report an increased FAK protein content in human skeletal muscle after 12 weeks RT in previously sedentary elderly men. Immunofluorescence microscopy enabled the identification and quantitation of the content of this focal adhesion-associated protein in specific regions of the skeletal muscle, specifically the (sub)sarcolemmal and sarcoplasmic regions of skeletal muscle fibres and within the skeletal muscle microvascular endothelium. FAK immunofluorescence intensity increased in both regions which suggests alterations in costameric and sarcoplasmic FAK content and a remodelling of endothelial focal adhesions in response to the higher overall mechanical load during RT compared with normal daily living tasks. The increased (sub)sarcolemmal and sarcoplasmic FAK content accompanied type II fibre-specific hypertrophy and increased muscle strength after RT. This study contributes to the growing literature implicating FAK in load-induced muscular adaptation.

3.6 Acknowledgements

The antibodies against human slow myosin (A4.840-c) used in the study were developed by Dr. Blau and were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Chapter 4

Paxillin colocalises with focal adhesion kinase in human skeletal muscle and its associated microvasculature

4.1. Abstract

Focal adhesion kinase (FAK) and paxillin are sensitive to hormonal and mechanical stimuli and evidence suggests these proteins are functionally linked. We aimed to describe the localisation of paxillin and test the hypothesis that FAK and paxillin colocalise in human skeletal muscle and its associated microvasculature.

Percutaneous muscle biopsies were collected from the *m. vastus lateralis* of 7 healthy males and 5μm cryosections were stained with anti-paxillin and co-incubated with anti-dystrophin to identify the plasma membrane, anti-myosin heavy chain type I for fibre-type differentiation, anti-dihydropyridine receptor (DHPR) to identify t-tubules, lectin UEA-I to identify the endothelium of capillaries and anti-α-smooth muscle actin to identify vascular smooth muscle cells (VSMC). Colocalisation analysis (Pearson's colocalisation coefficient) was performed using confocal fluorescence microscopy.

Paxillin was primarily localised in (sub)sarcolemmal regions and it colocalised with dystrophin (r=0.414±0.026) in skeletal muscle fibres. The (sub)sarcolemmal paxillin immunofluorescence intensity was ~2.4-fold higher than in sarcoplasmic regions (P<0.001) with sarcoplasmic paxillin immunofluorescence intensity ~10% higher in type I than in type II fibres (P<0.01). In some longitudinally orientated fibres, paxillin was distributed in uniform striations running across the fibre and these colocalised with DHPR. Paxillin immunostaining was highest in endothelial and VSMC and distributed heterogeneously in both cell types. FAK and paxillin colocalised at (sub)sarcolemmal regions and within the endothelium and VSMC (r=0.367±0.036).

The first images of paxillin in human skeletal muscle suggest paxillin is localised to (sub)sarcolemmal regions of muscle fibres and in the microvascular endothelium and VSMC. The spatial colocalisation of FAK and paxillin in these distinct regions supports their suggested role in hormonal and mechano-sensitive signalling.

4.2. Introduction

In skeletal muscle, costameres are specialised structures localised to the sarcolemma which convert mechanical stimuli into intracellular signalling (mechanotransduction) that activate signalling pathways and control gene transcription. Costameres anchor the z-disc of peripheral myofibrils to the sarcolemma (Ervasti, 2003; Pardo et al., 1983) and link the basal lamina with the actin cytoskeleton through its dystrophin/ glycoprotein complex and integrin-associated complex. Stimulation of the integrin-associated complex leads to the formation of a focal adhesion complex (Fluck et al., 2002). Mechanotransduction occurs through integrin-mediated signalling pathways, but non-integrin (e.g. hormonal) signalling can also operate (Schlaepfer et al., 1999). Focal adhesion kinase (FAK) and paxillin are two hormone- and mechano-sensitive proteins (Brown and Turner, 2004; Goldmann, 2012; Schlaepfer et al., 1999; Turner et al., 1990; Turner et al., 1991) present within costameres (Fluck et al., 1999; Fluck et al., 2002; Gordon et al., 2001; Quach and Rando, 2006).

Mechanical stimulation is associated with the phosphorylation and activation of FAK and paxillin in a variety of cell types (Fluck et al., 1999; Lipfert et al., 1992; Shikata et al., 2005; Tang et al., 1999; Yano et al., 1996), and are events that have been implicated in the acute and chronic adaptive response of animal (Fluck et al., 1999; Gordon et al., 2001; Klossner et al., 2009) and human skeletal muscle to increased mechanical loading (Wilkinson et al., 2008). The localisation of FAK to integrins is regulated by its N terminal domain and it's C terminal domain contains two binding sites for paxillin (Scheswohl et al., 2008). Paxillin's N-terminus also contains two binding sites for FAK (Brown et al., 1996) and is a direct binding partner for FAK (Hildebrand et al., 1995; Turner and Miller, 1994). Integrin-mediated activation and subsequent binding of FAK to paxillin leads to the phosphorylation of paxillin at Tyr^{31} and Tyr^{118} residues (Bellis et al., 1995; Schaller and Parsons, 1995). These two paxillin phosphorylation sites are necessary for maximal FAK phosphorylation

(Scheswohl et al., 2008), normal FAK function (Hagel et al., 2002) and the maximal phosphorylation of downstream signalling proteins (Scheswohl et al., 2008).

FAK is localised in the (sub)sarcolemmal regions of human (chapter 2) and animal skeletal muscle (Fluck et al., 1999; Fluck et al., 2002; Klossner et al., 2009), supporting its suggested role in mechanotransduction. FAK and paxillin protein abundance corresponds with muscle loading, with both FAK and paxillin protein concentration increased after chronic stretch-induced hypertrophy/ hyperplasia in rooster *anterior latissimus dorsi* (Fluck et al., 1999) and after 8 days of overload in rat *m. soleus* (surgical ablation of the gastrocnemius) and *m. plantaris* skeletal muscle (Fluck et al., 1999; Gordon et al., 2001). FAK protein content was also increased after 12 weeks resistance-type exercise training (chapter 3) and after 12 weeks ski-training in elderly human *m. vastus lateralis* skeletal muscle (Flueck et al., 2011; Narici et al., 2011). The role of FAK-associated signalling in training adaptation has not been fully elucidated. However, overexpression of FAK through electrogene transfer of a FAK-expressing plasmid has been associated with the increased gene expression for proteins associated with the slow oxidative phenotype (Durieux et al., 2009). FAK has also been associated with the protein synthesis signalling pathway through an Akt-mTOR-independent activation of p70S6K (Klossner et al., 2009).

In the microvasculature of human skeletal muscle, FAK is highly concentrated in endothelial and vascular smooth muscle cells (VSMC) (chapter 2), but little is known about paxillin's spatial distribution in these cells. Both endothelial FAK and paxillin are responsive to mechanical (e.g. fluid shear stress) (Li et al., 2002; Li et al., 1997; Mattiussi et al., 2006) and hormonal stimuli playing a role in vascular endothelial growth factor-mediated signalling (Abedi and Zachary, 1997). They also regulate the motility of endothelial cells (Zaidel-Bar et al., 2007). In VSMCs, paxillin is associated with VSMC spreading (Ishida et al., 1999), regulating actin cytoskeleton polymerisation and tension development and is sensitive to

angiotensin II- and thrombin-mediated stimulation (Brown and Turner, 1999; Ishida et al., 1999). Collectively, these studies suggest paxillin plays an important role in the maintenance of vascular function.

Paxillin has been visualised previously in the developing costameres of murine myoblasts and myotubes using immunofluorescence microscopy (Quach and Rando, 2006). However, the localisation and distribution of paxillin in human skeletal muscle and its associated microvasculature remain unresolved. Therefore, we aimed to generate the first visual images of paxillin and its localisation in human skeletal muscle. As evidence suggests FAK and paxillin are functionally linked, we aimed to test the hypothesis that FAK and paxillin colocalise in human skeletal muscle and its associated microvasculature.

4.3. Materials and Methods

Muscle samples

Before the study commenced, all participants were fully informed of the study demands and provided their written informed consent for participation in the study which was approved by the local Birmingham East, North and Solihull and West Midlands Black Country NHS Research Ethics Committees. Resting percutaneous muscle biopsy samples were obtained from the *m. vastus lateralis* of 7 lean, fasted healthy males (mean ± SD; age 22 ± 4 y; BMI 23.6 ± 0.9 kg/m^2). The biopsies were taken using the Bergstrom needle biopsy technique (Bergstrom, 1975) adapted with suction (Evans et al., 1982). The muscle samples were blotted free of excess blood and any visible fat or connective tissue was discarded. The samples were then embedded in Tissue Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen immediately in liquid-nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK) ready for storage at -80°C. Five µm cryo-sections were cut at -

30°C and placed on room-temperature, uncoated glass slides (VWR International Ltd, Leicestershire, UK).

Antibody specificity and immunofluorescence staining

All chemicals used in this study were purchased from Sigma-Aldrich (UK) unless otherwise stated. The sections were fixed in 3:1 ratio acetone and ethanol for 5 min and washed 3 x 5 min in phosphate buffered saline (PBS; 137mM sodium chloride, 3 mM potassium chloride, 8 mM disodium hydrogen phosphate and 3 mM potassium dihydrogen phosphate, pH of 7.4). Sections were then incubated for 2 h with 5% Normal Goat Serum and N-terminal specific monoclonal anti-paxillin clone 349 targeting amino acids 1 – 557 (BD transduction Laboratories, Oxford, UK) which has been used previously to visualise paxillin in murine myoblasts (Quach and Rando, 2006). The signal specificity of the paxillin antibody in human skeletal muscle was confirmed by western blot (described below) and a peptide competition experiment. The primary antibody was pre-incubated for 2 hours at room temperature with a 10-fold excess concentration of a purified recombinant human paxillin protein (Origene, Rockville, USA; cat. no. TP313811). Sections on separate glass slides were incubated for 2 hours with 1:100 dilution of anti-paxillin or anti-paxillin plus recombinant protein. The recombinant protein eliminated the paxillin immunoreactivity in transversely and longitudinally orientated muscle fibres.

Fibre-type differences in sarcoplasmic paxillin content was assessed by co-staining muscle sections for 2 hours with 1:100 dilution of anti-paxillin and anti-myosin heavy chain type I (MHC type I; developed by Dr. Blau; DSHB; A4.840c) and 1:400 dilution of anti-dystrophin (Sigma-Aldrich, UK). The MHC type I was specific to slow oxidative type I skeletal muscle fibres leaving type II fibres unstained. Anti-dystrophin was used to identify the sarcolemma since dystrophin is localised to the sarcoplasmic surface of the sarcolemma

(Davies and Nowak, 2006). The distribution of paxillin throughout longitudinally orientated muscle fibres was also investigated using anti-dihydropyridine receptor (DHPR) as in chapter 2 of this thesis.

To visualise paxillin within the skeletal muscle microvasculature, muscle sections were incubated with lectin FITC-conjugated Ulex europaeus Agglutinin I (Vector Laboratories) which identified the endothelium and anti-α-smooth muscle actin (α-SMA; Abcam ab7817) which identified the vascular smooth muscle of skeletal muscle arterioles. The incubation period was then proceeded by 3 x 5 min wash in PBS and then incubated for 30 min with the appropriate fluorescently conjugated secondary antibodies including goat anti-mouse 488 and 594 IgG_1 (paxillin), goat anti-mouse 350 IgM (MHC type I) and 488 IgG_{2a} (α-SMA) and IgG_{2b} (dystrophin) fluorophores (Invitrogen, UK). The sections then underwent a 3 x 5 min wash in PBS and cover slips applied with a mounting solution (glycerol and mowiol 4-88 solution in 0.2 M Tris-buffer (pH 8.5) with the addition of 0.1% 1,4-diazobicyclo-[2,2,2]-octane (DABCO) antifade medium). To assess background fluorescence, negative controls were performed where the primary antibody was excluded. Controls were also performed to detect bleed-through of each fluorophore in the respective excitation filters. In all cases the fluorescence signal was abolished.

To investigate colocalisation of paxillin with FAK, cryosections were first incubated with the anti-paxillin antibody as mentioned previously and then sections were washed 3 x 5 min in PBS and the appropriate secondary antibody was applied (mouse 546 or 594 IgG_1). Thirty minutes after the secondary antibody had been applied, sections were washed 3 x 5 min in PBS before incubating sections with an anti-FAK antibody (A17, Santa Cruz, USA) which has been validated for use in human skeletal muscle using methods previously described (chapter 2). Sections were also co-stained with α-SMA as before to investigate colocalisation of FAK and paxillin in the smooth muscle. Sections were then washed 3 x 5

min in PBS and the appropriate secondary antibodies were applied (goat anti-mouse 594 IgG_1 or 546 IgG_1, 633 IgG_{2a} and goat anti-rabbit 488 IgG) before washing and applying the cover slips as described.

Image capture and analysis

Paxillin and FAK were visualised using widefield and confocal fluorescence microscopy. To analyse fibre-type differences in sarcoplasmic paxillin content, widefield images were captured using a Nikon E600 microscope with a 40 x NA objective with a SPOT RT KE colour three shot CCD camera (Diagnostic Instruments Inc., MI, USA). DAPI UV (340 – 380 nm), FITC (465 – 495 nm) and Texas Red (540 – 580 nm) excitation filters were used to visualise the Alexa Fluor 350 (MHC type I), 488 (dystrophin) and 594 fluorophores (paxillin). Detailed digital images of the skeletal muscle and microvasculature were captured with an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) with a 63 x 1.4 NA oil immersion objective. Confocal microscopy images were also used to investigate the colocalisation of paxillin with FAK. The Alexa Fluor 488 fluorophore (dystrophin, lectin UEA-I, paxillin and FAK) was excited by the 488 nm line of the argon laser and the Alexa Fluor 546, 594 (paxillin) and 633 (DHPR; α-SMA) fluorophores were excited by the 543, 594 and 633 nm line of the Helium-Neon laser, respectively. Images were captured sequentially when investigating colocalisation of paxillin with FAK.

Image processing was completed using Image-Pro Plus 5.1 (Media Cybernetics, MD, USA). Two serial sections were cut and placed on the same glass slide and duplicated on a second slide. For quantitation of sarcoplasmic paxillin immunoreactivity in type I and type II fibres, five regions of interest covering only transversely-orientated muscle fibres were captured per muscle section on the widefield microscope. A total of (mean ± SD) 119 ± 36 transversely orientated muscle fibres were analysed per individual and fibre-type specificity

was determined from the mean fluorescence signal intensity of 46 ± 12 type I and 73 ± 33 type II individual whole human muscle fibres, respectively. All longitudinally orientated muscle fibres were excluded from quantitation. The corresponding dystrophin images which identified complete individual muscle fibres were processed using a participant-specific intensity threshold to generate an outline of individual muscle fibres. The outline was then overlayed onto the raw paxillin image so sarcoplasmic paxillin immunoreactivity could be quantitated.

Confocal microscopy images were used for comparison between the intensity of sarcolemmal and sarcoplasmic paxillin immunofluorescence. Duplicate slides were prepared and captured as above. The paxillin immunoreactivity at the plasma membrane of individual fibres was not measured due to limitations in the resolution of the confocal microscope to adequately determine the sarcolemma of one fibre from neighbouring fibres. Therefore, muscle fibres captured with confocal microscopy were pooled and the mean paxillin immunofluorescence at the plasma membrane surrounding a total of 76 ± 26 transversely orientated whole muscle fibres were analysed. Differences in sarcolemmal and sarcoplasmic paxillin immunofluorescence were quantified using the dystrophin outline as above.

Colocalisation analysis

The same images were also used to investigate colocalisation between paxillin and dystrophin. Colocalisation analysis was performed on 14 ± 1 whole images per participant totalling 246 ± 50 muscle fibres per participant. Colocalisation analysis of FAK with paxillin in skeletal muscle and microvessels was performed on a total of 19 ± 2 matched whole confocal microscopy images.

Western blot

Western blots were performed as described in chapter 2. In brief, snap frozen muscle tissue was powdered in liquid nitrogen using a pestle and mortar and mixed on ice with lysis buffer (RIPA buffer; Cell signalling with a complete mini protease inhibitor tablet; Roche Diagnostics, Mannheim, Germany) and homogenised with a handheld homogeniser. The samples were centrifuged for 20 min at 10,000 g at 4ºC with the supernatant removed for protein determination (Pierce BCA assay kit; Pierce, Rockford, IL, USA). The proteins were separated by electrophoresis on 12% SDS polyacrylamide gels (Thermo Scientific, Rockford, IL, USA) and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C with an anti-paxillin primary antibody (1:2000) and then treated for 1 hour to a HRP-conjugated anti-mouse IgG_1 secondary antibody (1:20,000). Bands were identified using enhanced chemiluminescence HRO detection reagent (GE Healthcare, Amersham, UK) and imaged using ChemiDoc XRS+ (Bio-rad, UK).

Statistical analysis

Mean fluorescence intensity was used as a measure of total paxillin content at the plasma membrane and in the sarcoplasm of type I and type II fibre types. Data are expressed as mean ± SEM and compared using a paired samples t-test (SPSS v15). Colocalisation was assessed using Pearson's correlation coefficient. Significance was set at $P < 0.05$.

4.4. Results

Specificity of the paxillin antibody in human skeletal muscle

Figure 4.1 shows a representative widefield fluorescence microscopy image of paxillin (A) in transversely orientated *m. vastus lateralis* human skeletal muscle fibres co-incubated with anti-dystrophin and anti-myosin heavy chain type I. A strong paxillin immunofluorescence

signal was detected at peripheral regions of the muscle fibre (A). This signal was abolished with control experiments excluding the primary antibody (B) and when anti-paxillin was pre-incubated with a recombinant human paxillin protein (C). The antibody specificity and selectivity was confirmed further by western blot analysis (D) which identified a single band at ~68 kDa and this corresponded with paxillin's molecular weight (Turner et al., 1990). These data demonstrate that the paxillin antibody is specific for paxillin in human skeletal muscle using both immunofluorescence and western blotting and supports previous reports in mouse skeletal muscle fibres (Quach and Rando, 2006).

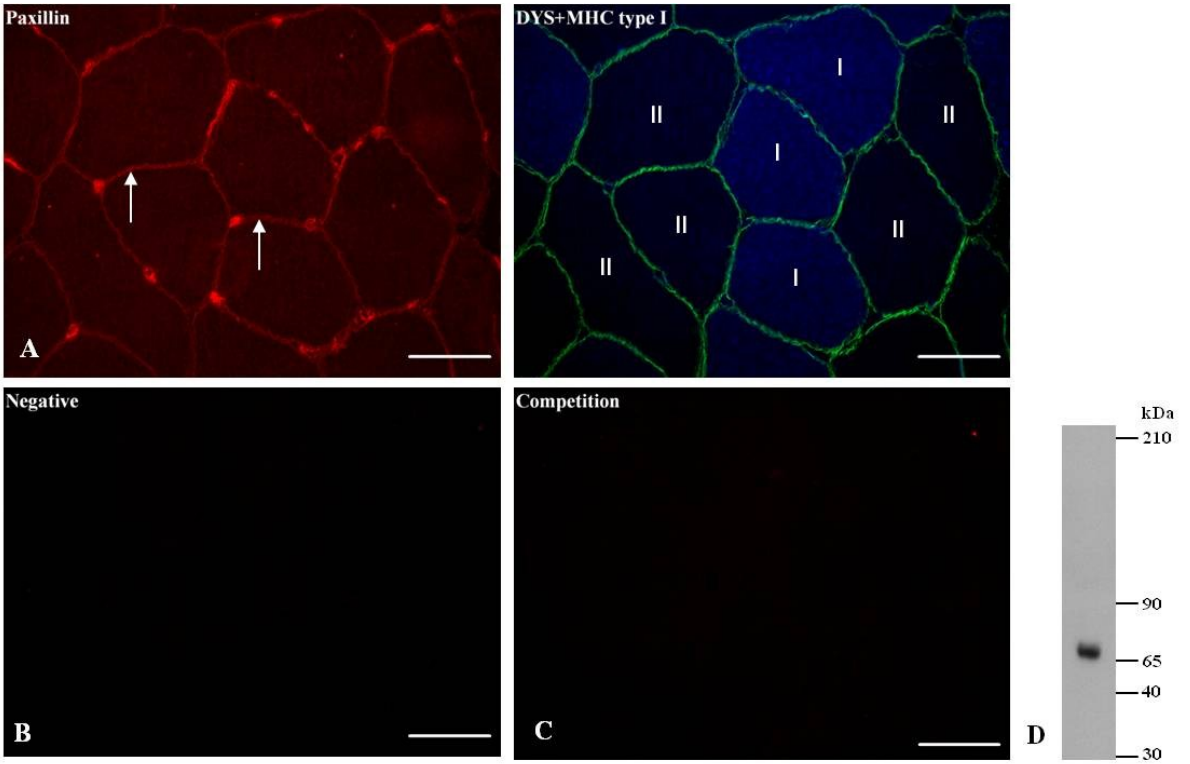

Figure 4.1. Immunofluorescent visualisation of paxillin in human skeletal muscle. **A)** Representative widefield microscopy image of paxillin in transversely orientated human skeletal muscle fibres. Sections were co-stained with anti-paxillin (*red*; left), anti-dystrophin (*green;* DYS) and anti-myosin heavy chain type I (*blue*; MHC type I) (right). Type II fibres were unstained. Paxillin staining exhibited a strong immunofluorescence signal at the

periphery of the muscle fibres in both fibre-types (*arrows*). B) Negative control experiment where the paxillin primary antibody was omitted. C) Representative image of paxillin pre-incubated with a recombinant human paxillin protein which removed the paxillin-positive immunoreactivity. D) Western blot of the anti-paxillin antibody using homogenised *m. vastus lateralis* human skeletal muscle. Bars are 50 μm

The intense paxillin immunoreactivity at the periphery of the fibre strongly suggests that paxillin is localised in (sub)sarcolemmal regions. Co-staining paxillin with dystrophin (used in this study as a marker for the sarcolemma) revealed partial colocalisation (r= 0.414 ± 0.026) (figure 4.2A, B). Sarcolemmal paxillin staining intensity was 139 ± 16 % higher compared with the sarcoplasm (figure 4.2C) (P<0.001).

Using widefield microscopy images of transversely orientated muscle fibres (figure 4.3), quantitative image analysis revealed a small (~10%) but significantly greater sarcoplasmic paxillin content in type I compared with type II fibres (P<0.01). In some longitudinally orientated type I and type II fibres, paxillin was distributed evenly in the form of striations spanning the full width of the fibre and running perpendicular to the fibre's direction (figure 4.4A, B, C). Representative confocal microscopy images of paxillin co-incubated with a t-tubule marker (DHPR) suggests paxillin colocalises with or is in close proximity to the t-tubule network (figure 4.4B, C).

FAK and paxillin interact in a variety of cell types, so we investigated their spatial correlation in human skeletal muscle fibres. Using confocal microscopy, representative whole images (figure 4A, B) show partial colocalisation between FAK and paxillin as denoted by areas of yellow pixels (r = 0.367 ± 0.036) (figure 4C) along the (sub)sarcolemma and in regions between the muscle fibres (e.g. the microvessels). These data suggest both proteins spatially colocalise at sarcolemmal and microvascular regions in human skeletal muscle.

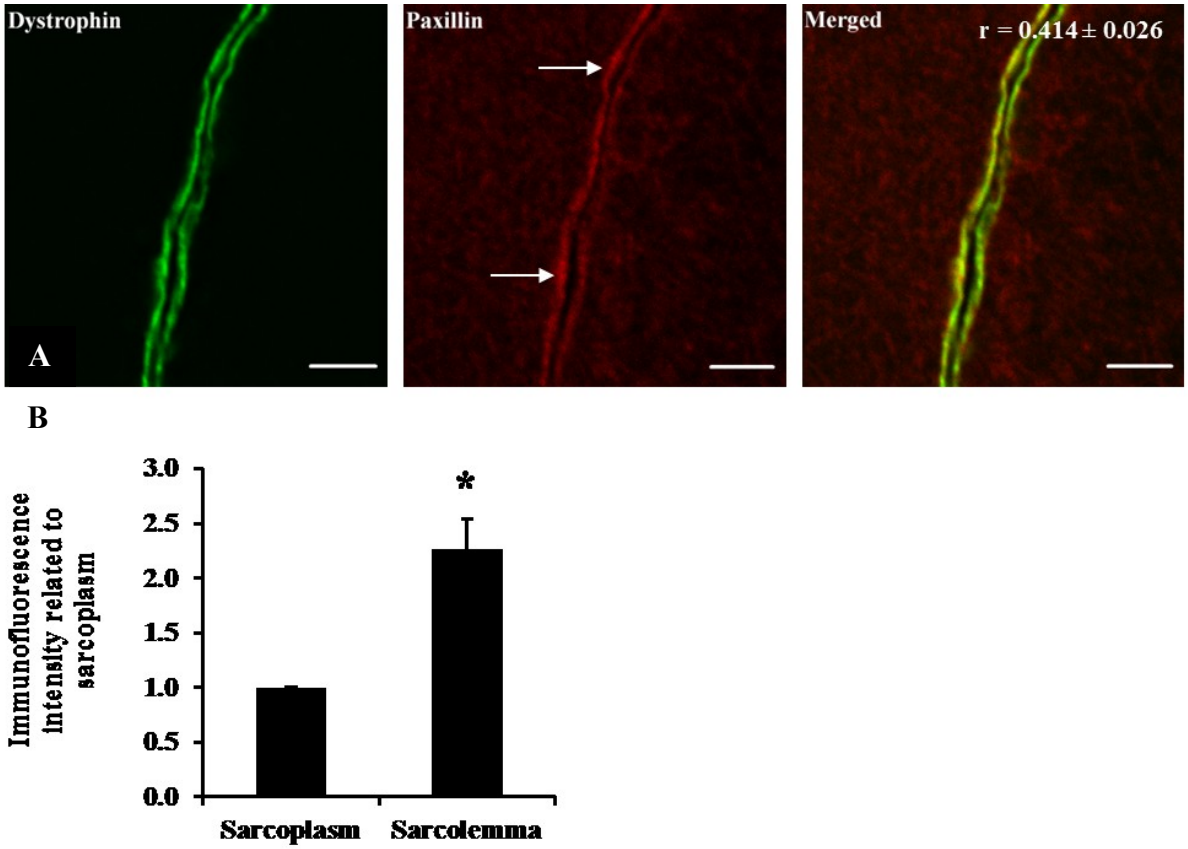

Figure 4.2. Representative higher magnification confocal microscopy images showing colocalisation of paxillin with dystrophin. A) Cross-sections of human skeletal muscle were co-stained with dystrophin, a marker of the sarcolemma (*green*; left) and anti-paxillin (*red*; middle). Note that paxillin is localised to the sarcolemma (*arrows*) and merged images (right) show clear colocalisation. Colocalisation coefficient of paxillin and dystrophin are presented in the merged images. The mean Pearson's correlation was calculated from 14 ± 1 matched whole images. n=7. Bars are 5 μm. Data are mean ± SEM. B) Mean sarcolemmal and sarcoplasmic paxillin immunofluorescence of transversely orientated *m. vastus lateralis* human skeletal muscle fibres assessed using confocal microscopy. Data was quantified by pooling both fibre types with 76 ± 26 whole muscle fibres per participant analysed, respectively. Data are related to the sarcoplasm and are mean ± SEM. *n*=7. * Denotes significant difference ($P<0.001$).

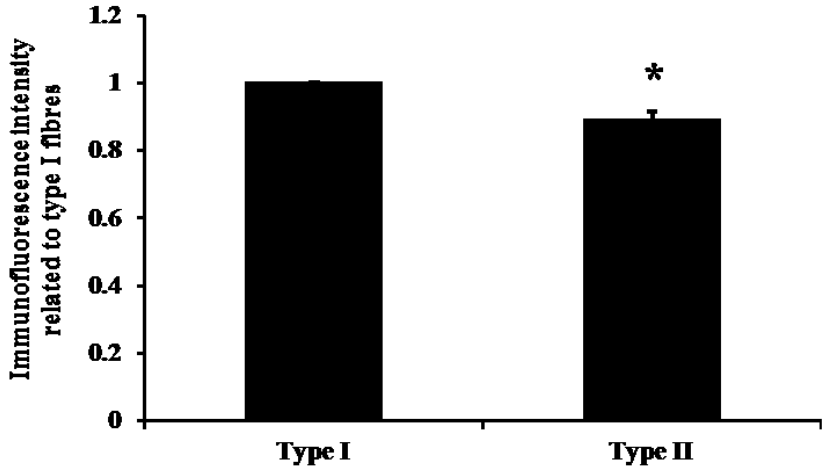

Figure 4.3. Mean sarcoplasmic paxillin immunofluorescence intensity of type I and type II human *m. vastus lateralis* skeletal muscle fibres using widefield microscopy. A total of 46 ± 12 type I and 73 ± 33 type II fibres were analysed per individual. Data are mean ± SEM. * Denotes significant difference (P<0.01).

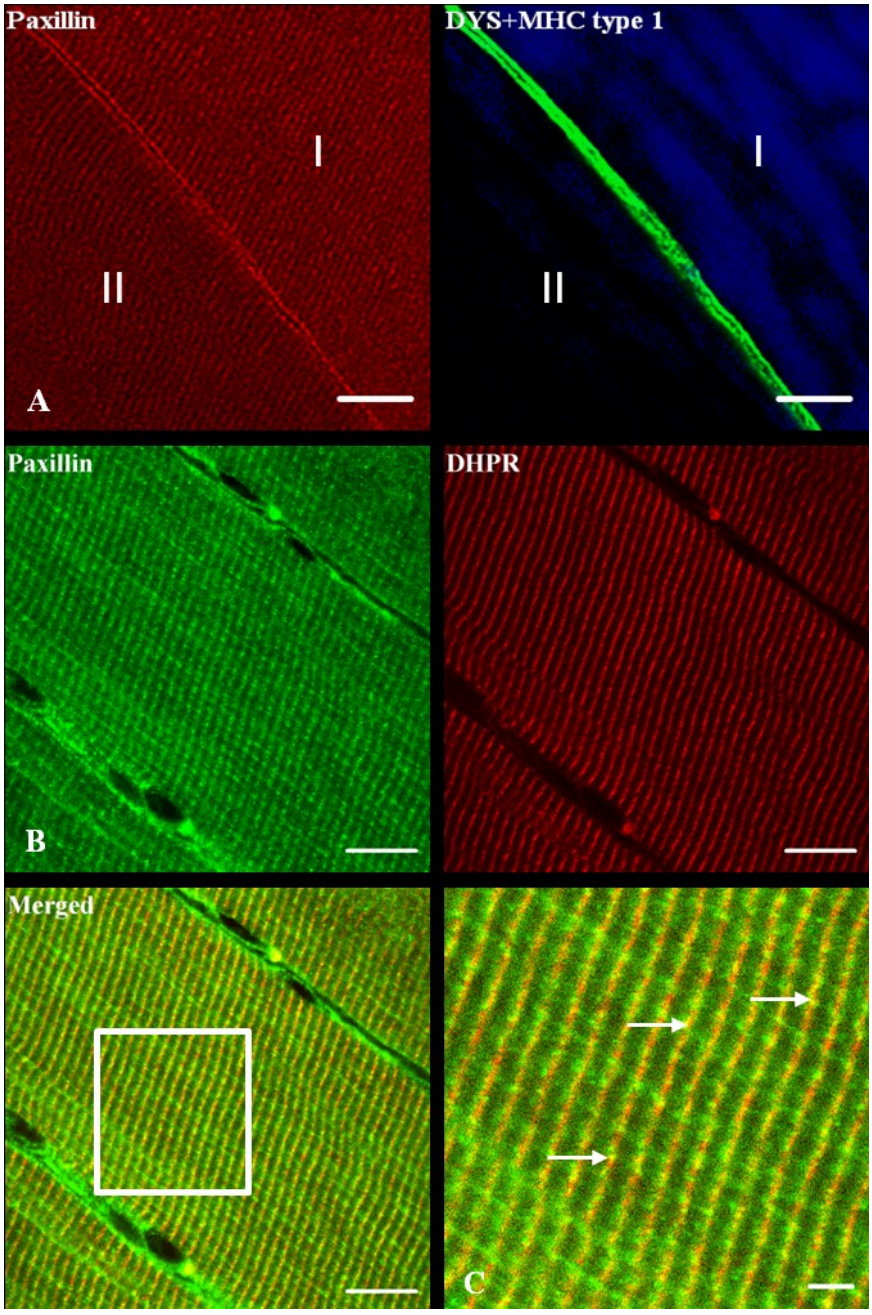

Figure 4.4. Representative distribution of paxillin in longitudinally orientated skeletal muscle fibres. A) Higher magnification widefield microscopy images of paxillin (red; *left*) and merged images (*right*) of dystrophin (DYS; green) and myosin heavy chain type I (MHC type I; blue). B) Confocal microscopy image of a type I fibre detailing paxillin (green) and; C) is an 8x magnification image. In both fibre types, paxillin was distributed along organised structures running perpendicular to the direction of the muscle fibre. C) Merged image of

paxillin with DHPR (a t-tubule marker) suggests paxillin is present at or near the t-tubule network (yellow pixels; *arrows*). Bars are 10 μm (A; B) and 5 μm (C).

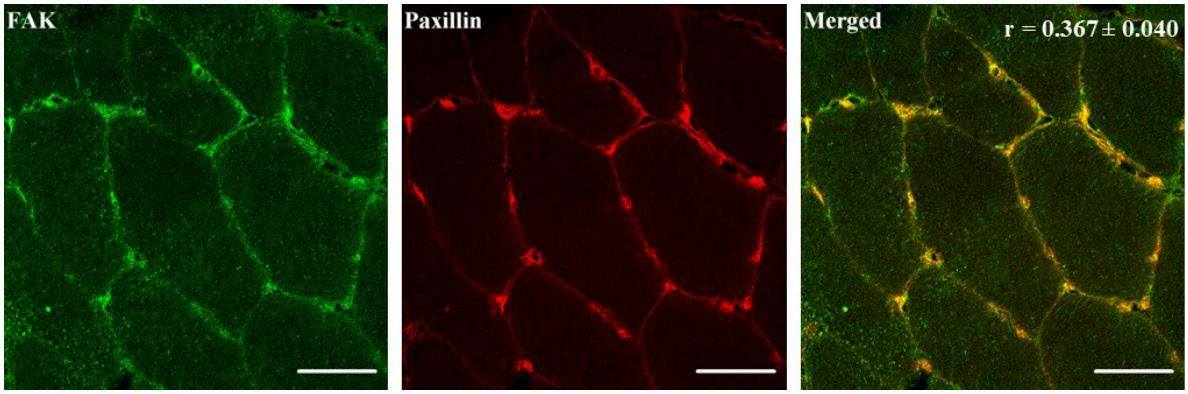

Figure 4.5. Representative confocal immunofluorescent microscopy images of focal adhesion kinase (FAK) and paxillin. FAK (*green*; left) and paxillin (*red*; middle) are present in high concentrations in the sarcolemmal regions and microvessels of transversely orientated human skeletal muscle fibres and the merged image (right) shows colocalisation. The mean Pearson's correlation coefficient analysed from 19 ± 2 whole images using confocal microscopy is presented in the merged image and suggests partial colocalisation between FAK and paxillin. Note also the degree of colocalisation of FAK and paxillin in the microvessels as indicated by the abundance of yellow pixels. Bars are 50 μm. Data are mean ± SEM

Paxillin in the microvasculature

Figure 4.6 shows representative confocal microscopy images of the endothelium surrounding the lumen of microvessels in human skeletal muscle. Lectin UEA-I successfully stained the endothelium of the microvasculature (*left*) and an intense paxillin immunofluorescence was detected in the endothelium (*middle*), with paxillin forming a ring-like structure around the

lumen and distributed heterogeneously in the form of clusters. Merged images (*right*) show clear colocalisation of paxillin with the endothelium. In figure 4.7, α-SMA successfully stained the vascular smooth muscle (*left*), with paxillin again forming a ring-like structure around the lumen and distributed heterogeneously in the form of clusters (*middle*). Merged images (*right*) show colocalisation of paxillin with α-SMA and taken together, suggests this protein is abundantly expressed in endothelial and vascular smooth muscle cell (VSMC) tissues. In figure 4.8, co-incubation of anti-αSMA (*left*), FAK (*middle*) and paxillin (*right*) demonstrate clear colocalisation between FAK and paxillin in the VSMC (*far right*) (figure 4.8A). This suggests the clusters observed in both endothelial cells and VSMCs represent sites of focal adhesions.

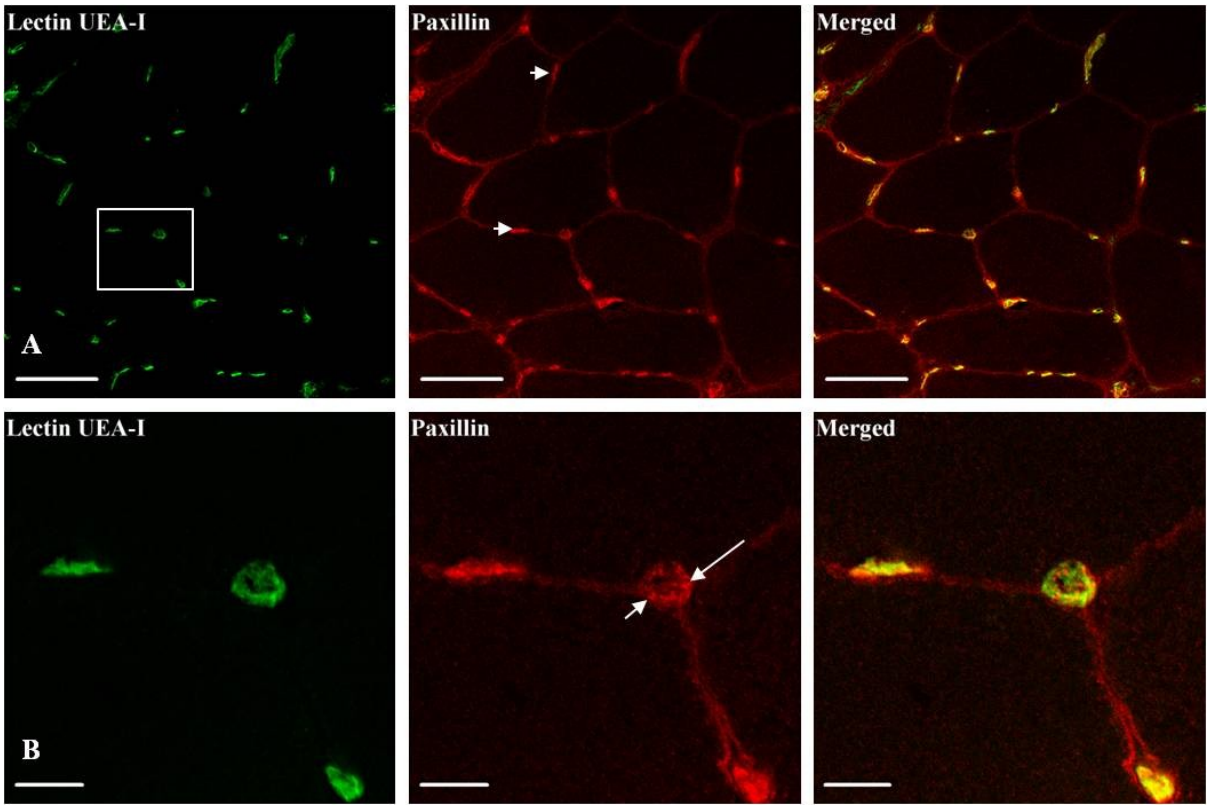

Figure 4.6. Representative immunofluorescence confocal microscopy image illustrating the presence of paxillin within the microvascular endothelium of human skeletal muscle. A) Microvessels (lectin UEA-I) (*green*; left) are presented amongst transversely

orientated skeletal muscle fibres. Sections were co-stained with lectin UEA-I for visualisation of the endothelium and paxillin (*red*; middle) with an intense paxillin immunoreactivity detected within the microvessels (upper; *arrowheads*). Merged images demonstrate clear colocalisation of paxillin with the endothelium. B) Higher magnification images of the region of interest illustrating the heterogeneous distribution of paxillin around the vessel lumen (*arrowhead*) and the ring-like structure (*arrow*). Bars are 50 and 10 μm, respectively.

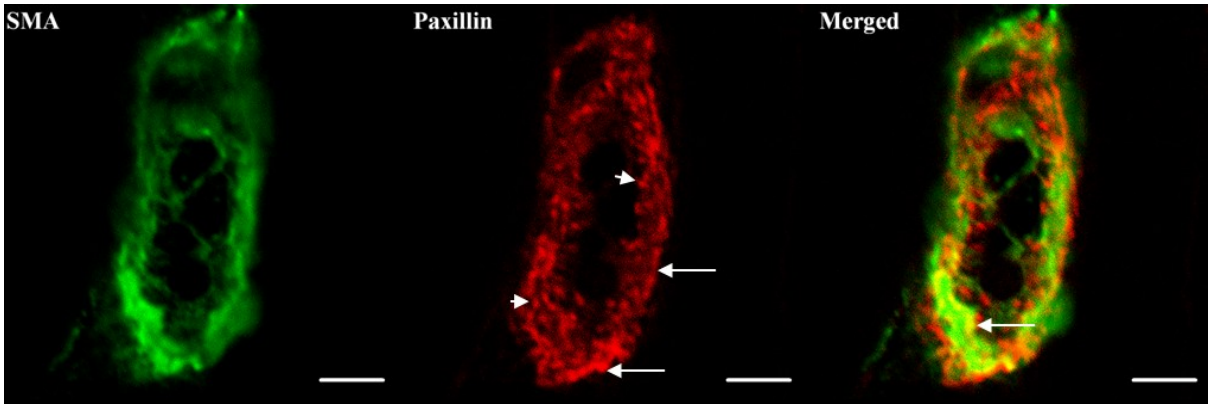

Figure 4.7. Representative immunofluorescence confocal microscopy illustrating the presence of paxillin in the vascular smooth muscle of human skeletal muscle. Sections were costained with anti-α-smooth muscle actin for the identification of arterioles (*green*; left; SMA; α-smooth muscle actin). Paxillin (*red*; middle) was largely punctate and heterogeneous in distribution, appearing to form clusters (*arrowheads*) within a ring-like structure. An intense immnuofluorescent signal for paxillin was detected at the periphery of each arteriole where it appeared to form intermittent straight lines. These may reflect focal adhesion recruitment to areas of applied force (*arrow*). Note also the clear areas of colocalisation of paxillin with the SMA as depicted by the yellow pixels (right; *arrow*). Bars are 5 μm.

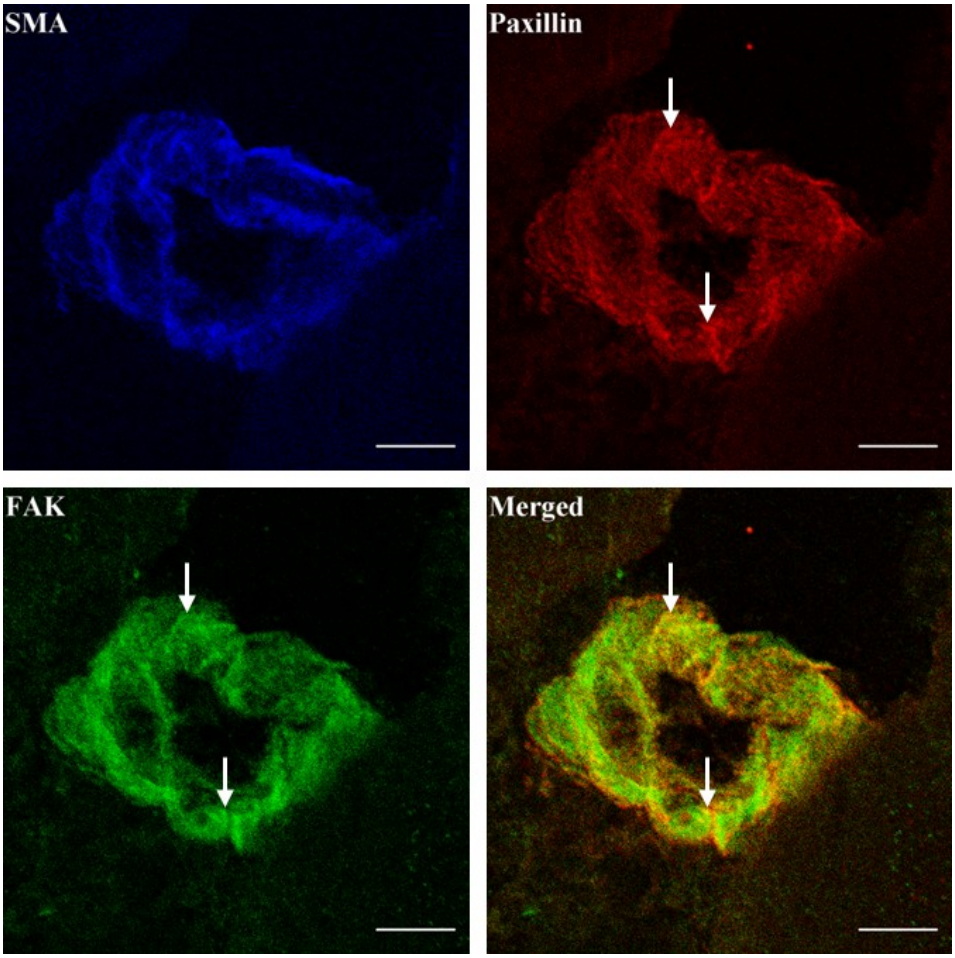

Figure 4.8. Representative confocal immunofluorescent microscopy images of focal adhesion kinase (FAK) and paxillin in the vascular smooth muscle of human skeletal muscle fibres. The anti-α-smooth muscle actin was used to identify arterioles (*blue*; SMA; upper left) and serial staining of paxillin (*red*; upper right) and FAK (*green*; lower left) showed clear colocalisation (*yellow pixels*; arrows) between the two focal adhesion-associated proteins (*merged*; lower right; arrows). Images were captured with a 4x zoom and bars are 10 μm.

4.5. Discussion

This is the first study to report the localisation and distribution of the focal adhesion protein paxillin in human skeletal muscle and its associated microvasculature. Using widefield and confocal microscopy, we support and expand upon the findings previously observed in murine myoblasts (Quach and Rando, 2006) and present novel information regarding the spatial colocalisation of FAK and paxillin in human skeletal muscle fibres and the microvasculature.

A strong paxillin immunofluorescence intensity was observed in the peripheral regions of the muscle fibres (figure 4.1) and colocalisation of paxillin with dystrophin suggests paxillin is localised to sarcolemmal regions (figure 4.2). Based on three-dimensional super resolution confocal microscopy, evidence suggests paxillin is localised <50 nm from the plasma membrane (Kanchanawong et al., 2010), but this observation cannot be confirmed in the present study. Paxillin immunofluorescence intensity in (sub)sarcolemmal regions was ~2.4 fold more intense than in sarcoplasmic regions (figure 4.2) suggesting the majority of paxillin was localised in (sub)sarcolemmal regions with comparatively little sarcoplasmic paxillin content. These data mirrors that of FAK reported in human (chapter 2) and animal skeletal muscle (Fluck et al., 1999; Klossner et al., 2009) and are in agreement with paxillin's presence in costameres (Fluck et al., 1999; Gordon et al., 2001; Quach and Rando, 2006).

There is evidence in rat skeletal muscle that paxillin is expressed in a fibre-type specific manner with an ~50% greater paxillin protein content in the *m. soleus* compared to the *m. plantaris* or *m. gastrocnemius* (Gordon et al., 2001). It was postulated that the greater paxillin content in oxidative skeletal muscle may reflect the habitual loading state of the muscles (Gordon et al., 2001). To test this hypothesis, we investigated the sarcoplasmic paxillin content of human *m. vastus lateralis* type I and type II muscle fibres using widefield microscopy. We observed a slightly (10%) higher sarcoplasmic paxillin fluorescence in type I

compared with type II fibres (figure 4.3) which is in line with previous reports in rodent skeletal muscle (Gordon et al., 2001), but contrast with observations in FAK intracellular immunofluorescence where no fibre-type specificity was observed (chapter 2). We did not measure fibre-type specific sarcolemmal paxillin immunofluorescence intensity due to the resolution required to separate the sarcolemma of one fibre from the sarcolemma of neighbouring fibres, but single fibre analysis may offer a suitable alternative.

In longitudinally orientated type I and type II skeletal muscle fibres, paxillin staining revealed uniform striations which ran perpendicular to the direction of the fibre and these striations spanned the fibre's width (figure 4.4A, B, C). The striations resembled that of FAK (chapter 2) and the t-tubular network (Lauritzen et al., 2008a; Lauritzen et al., 2008b; Lauritzen et al., 2006). Anti-DHPR was used as a marker for the t-tubule network and this study reports the first visual images of paxillin's spatial colocalisation with DHPR in human skeletal muscle. T-tubules contain key insulin signalling proteins such as the insulin receptor and PI3-K, are a docking target of GLUT-4 vesicles and have been suggested to be the major site for insulin-stimulated glucose uptake (Lauritzen et al., 2008a; Lauritzen et al., 2008b; Lauritzen et al., 2006). FAK regulates insulin-stimulated GLUT-4 translocation in L6 and C2C12 skeletal muscle cells (Bisht and Dey, 2008; Bisht et al., 2007; Huang et al., 2006) and given paxillin's close interaction with FAK, and paxillin's sensitivity to insulin signalling in C2C12 skeletal muscle cells (Goel and Dey, 2002), it is possible that FAK and paxillin interact at the t-tubular membrane to collectively regulate the fine tuning of insulin-stimulated glucose uptake, but this warrants confirmation. However, as these striations were observed in comparatively few longitudinally orientated skeletal muscle fibres, it is likely that the striations represent costameres (Anastasi et al., 2008; Ervasti, 2003; Mondello et al., 1996; Pardo et al., 1983; Quach and Rando, 2006) and that paxillin overlies the t-tubule network of peripheral myofibrils.

The colocalisation between FAK and paxillin has been reported in neonatal rat ventricular cardiomyocytes (Heidkamp et al., 2002) and we add to the existing knowledge with the first evidence of FAK and paxillin colocalisation in human skeletal muscle. FAK and paxillin colocalised predominantly in sarcolemmal regions of the muscle fibres and showed substantial colocalisation in the endothelial and smooth muscle layer of the skeletal muscle microvasculature (figure 4.5 and 4.8). Colocalisation at the sarcolemmal regions point at the presence of costameres in human muscle fibres and accumulating evidence suggests these protein complexes play an important role in regulating protein synthesis and the hypertrophic response in animal (Durieux et al., 2009; Fluck et al., 1999; Gordon et al., 2001; Klossner et al., 2009) and human skeletal muscle (chapter 3) (Narici et al., 2011). The adaptive response to loading is likely mediated through a FAK- and paxillin-mediated actin remodelling where actin is anchored to cell membrane focal adhesion complexes which facilitates mechanotransduction (Schaller, 2001; Turner, 2000). These complexes may also be important in insulin-stimulated glucose uptake since cytoskeletal reorganisation is necessary for insulin-stimulated glucose uptake in C2C12 muscle cells (Bisht and Dey, 2008).

We have visualised paxillin in the microvascular endothelium and VSMCs (likely arterioles) with paxillin appearing to form clusters in both tissues (figure 4.6 and 4.7) which might reflect paxillin or focal adhesion complexes in the unstimulated state awaiting mechanical- or hormonal-mediated recruitment. Representative confocal microscopy images of both the endothelium and VSMC suggest some evidence, particularly in the VSMCs, of accumulation of paxillin which appear to partially align along the periphery of the tissue (figure 4.7, *large arrows*). Whether this distribution is associated with vasodilation or constriction warrants further investigation. In unstimulated endothelial cells *in vitro,* paxillin was heterogeneously distributed in intracellular regions (Li et al., 2002; Li et al., 1997; Mattiussi et al., 2006). In response to fluid shear stress paxillin redistributed towards the

plasma membrane where it aligned in the direction of laminar flow (Li et al., 2002; Li et al., 1997; Mattiussi et al., 2006). Analysis of post-exercise skeletal muscle would ascertain whether a distinct and prolonged exercise-induced increase in fluid shear stress affects the distribution of paxillin in human endothelial cells and VSMCs. The heterogeneous distribution of paxillin also mirrors that of FAK as shown in the representative colocalisation images (figure 4.8) and as previously reported (chapter 2 and chapter 3). Colocalisation between FAK and paxillin was strong in the human endothelium and VSMC as evidenced by the abundance of yellow pixels (figure 4.8) which is in line with the observations that FAK and paxillin closely interact in these cell types (Abedi and Zachary, 1997; Zaidel-Bar et al., 2007).

In conclusion, we report the first visual images of paxillin in human skeletal muscle and its associated microvasculature using widefield and confocal immunofluorescence microscopy. We confirmed the specificity of the paxillin antibody, identified paxillin at (sub)sarcolemmal regions along with a higher immunofluorescence in the sarcoplasm of type I fibres. We also observed a high content in the endothelium and VSMCs and significant colocalisation with FAK in these distinct tissues and regions. The newly developed immunofluorescence microscopy methods are expected to be a powerful tool to elucidate the response of FAK and paxillin and their interaction to hormonal and mechanical stimuli.

4.6. Acknowledgements

The antibodies against human slow myosin (A4.840-c) used in the study were developed by Dr. Blau and were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Chapter 5

Six weeks of endurance- and resistance-type exercise training both lead to increases in FAK and paxillin content in human skeletal muscle fibres and its microvascular endothelium

5.1. Abstract

Focal adhesion kinase (FAK) and paxillin are mechano-sensitive proteins highly expressed in skeletal muscle fibres and vascular endothelial cells. FAK and paxillin are modulated by the loading state of the skeletal muscle and through alterations in hemodynamic forces (e.g. shear stress), but little is known about the effect of endurance- (ET) and resistance- (RT) type exercise training on these focal adhesion-associated proteins in young, previously sedentary humans. Therefore, this study aimed to make the first investigations of FAK and paxillin responses to ET and RT in human skeletal muscle fibres and its microvascular endothelium.

Sixteen young sedentary males completed 6 weeks of either ET (5 $d \cdot wk^{-1}$ ~65% VO_{2peak} cycling) or whole body RT (3 $d \cdot wk^{-1}$) and skeletal muscle biopsies were collected pre- and post-training from the *m. vastus lateralis*. Changes in FAK and paxillin protein content in (sub)sarcolemmal regions and the microvascular endothelium was investigated using immunofluorescence microscopy. Measures of cross-sectional area (CSA) were also made.

FAK and paxillin immunofluorescence intensity increased at (sub)sarcolemmal regions by 41 ± 8% and 23 ± 8% (P<0.001) following ET. In the microvascular endothelium, FAK immunofluorescence intensity increased after ET (23 ± 4%) and RT (17 ± 4%) (P<0.001) with no difference between training modes. Paxillin immunofluorescence intensity also increased after ET (21 ± 9%) and RT (16 ± 4%) (P<0.001) within the endothelium with no difference between training modes. RT did not affect type I or type II fibre CSA.

These are the first data to show that human skeletal muscle FAK and paxillin are increased after 6 weeks ET and RT, particularly in (sub)sarcolemmal and microvascular endothelial regions. As skeletal muscle fibre CSA was unchanged by RT, these data suggest the increased FAK and paxillin protein content may precede fibre hypertrophy.

5.2. Introduction

Skeletal muscle demonstrates considerable plasticity in response to mechanical stimulation (Hoppeler and Fluck, 2002) and it is well known that endurance-type exercise training (ET) and resistance-type exercise training (RT) stimulate divergent adaptive responses (Gollnick et al., 1973; Gollnick et al., 1972; Holloszy, 1967; Holloszy et al., 1977). However, it remains unclear how skeletal muscle fibres and the skeletal muscle microvasculature convert exercise-associated mechanical stimuli into the intracellular signalling that regulates their adaptation (mechanotransduction) to exercise training (Coffey and Hawley, 2007).

Focal adhesion kinase (FAK) and its direct binding partner paxillin (Bellis et al., 1995; Hildebrand et al., 1995; Scheswohl et al., 2008) are mechano-sensitive focal adhesion-associated proteins localised to focal adhesions (Kanchanawong et al., 2010). These are protein-dense structures that resist mechanical forces (Balaban et al., 2001; Fabry et al., 2011; Riveline et al., 2001) and stimulate mechanotransduction in which FAK and paxillin are critical components (for review see (Deakin and Turner, 2008; Parsons, 2003; Schlaepfer et al., 1999). In skeletal muscle fibres, focal adhesions are contained in subsarcolemmal protein structures called costameres. These align in register with the Z-disk of peripheral myofibrils, anchor the Z-disk to the sarcolemma and facilitate the lateral transmission of force from the sarcomere to the sarcolemma and extracellular matrix (Ervasti, 2003; Pardo et al., 1983).

FAK and paxillin colocalise and are highly expressed in regions corresponding to costameres in human skeletal muscle fibres (chapter 4 of this thesis). They are also highly expressed in the animal skeletal muscles that bear the higher overall mechanical load (Fluck et al., 2002; Gordon et al., 2001). FAK and paxillin's protein abundance are increased after mechanical loading of animal (Fluck et al., 1999) and human skeletal muscle (Flueck et al., 2011; Narici et al., 2011) (chapter 3 in this thesis) and FAK is implicated in the increased expression of genes associated with the slow-oxidative skeletal muscle fibre type (Durieux et

al., 2009) and the protein synthesis signalling pathway after mechanical loading (Klossner et al., 2009). FAK is also sensitive to insulin-like growth factor-I (IGF-I) *in vitro*, appearing to play a role in IGF-I-mediated increases in mammalian target of rapamycin (mTOR)-dependent signalling and muscle protein synthesis (Crossland et al., 2013).

The positive effects of ET and RT on skeletal muscle insulin sensitivity are well known (Iglay et al., 2007; King et al., 1987; Miller et al., 1994; Miller et al., 1984; Rogers et al., 1990). FAK and paxillin are both phosphorylated by insulin (Goel and Dey, 2002) and FAK plays a regulatory role in skeletal muscle insulin sensitivity in L6 and C2C12 muscle cells *in vitro* and in rat skeletal muscle *in vivo* (Bisht and Dey, 2008; Bisht et al., 2007; Bisht et al., 2008; Huang et al., 2006). Taken together, these studies suggest FAK and paxillin play an important role in the mechanisms that regulate adaptation to mechanical loading.

The vascular endothelium is also exposed to tension, compression and fluid shear stress forces (Davies and Tripathi, 1993). The latter are transmitted from the luminal glycocalyx, via the cytoskeleton, to abluminal focal adhesions at the endothelial membrane (for review see (Weinbaum et al., 2007)). Chapter 2 and 4 of this thesis reported a high FAK and paxillin protein content in the microvasculature of human skeletal muscle. Both focal adhesion-associated proteins are rapidly phosphorylated in response to increased laminar shear stress in endothelial cells *in vitro* (Li et al., 2002; Li et al., 1997; Mattiussi et al., 2006). It is thought that their increased phosphorylation is an important event in endothelial mechanotransduction since FAK and paxillin play a critical role in regulating vascular function, shear stress-induced flow mediated dilation and angiogenesis (Hagel et al., 2002; Ilic et al., 1995; Koshida et al., 2005; Peng et al., 2004; Shen et al., 2005).

To date, no studies have investigated the effect of ET and RT on FAK and paxillin protein content in previously sedentary young individuals. This study aimed to make the first investigations of FAK and paxillin responses at the (sub)sarcolemma of skeletal muscle fibres

and within the skeletal muscle microvascular endothelium using the novel immunofluorescence microscopy methods developed in chapters 2 and 4. It was hypothesised that ET and RT would increase skeletal muscle FAK and paxillin protein content, particularly at the (sub)sarcolemmal regions due to the regular periods of increased force generation by contracting myofibrils. It was also hypothesised that both focal adhesion-associated proteins would increase in the endothelium of the skeletal muscle microvasculature after both training modes due to altered mechanical and hemodynamic stresses acting on the microvasculature during exercise.

5.3. Materials and Methods

Participants and muscle samples

Sixteen previously sedentary male participants (defined as completing less than 1 h per week of organised physical exercise) completed 6 weeks ET (n=8) and RT (n=8) and were well matched according to age, height, BMI and VO_{2max} (table 1). This uses a subset of the participants reported previously (Cocks et al., 2013; Shepherd et al., 2013)(Cocks M, 2012, PhD Thesis; Shepherd S, 2013 PhD Thesis; University of Birmingham). Participants provided their written informed consent. The study was approved by the Black Country NHS Research Ethics Committee which adhered to the Declaration of Helsinki.

Endurance-type exercise testing

The methods for establishing participant peak oxygen consumption (VO_{2peak}) and details of the ET and RT programme have been described in detail previously (Cocks et al., 2013; Shepherd et al., 2013)(Cocks 2012, PhD Thesis; Shepherd 2013, PhD Thesis). In brief, VO_{2peak} was established pre- and post-ET and RT using an incremental exercise test to exhaustion. Participants cycled on an electromagnetically braked ergometer at 95 watts and

the intensity increased by 35 watts every 3 min until volitional fatigue. VO_{2peak} corresponded to the highest value achieved over a 15 s recording period. Repeat tests were performed at week 4 and before the last training session.

Dietary controls

The participant's diets were standardised 24 hours before and during the pre- and post-training testing assessments. Participants completed a 3-day food diary and mean energy intake was estimated using diet analysis software (WISP). The standardised diet was matched to the participant's habitual energy intake and had a macronutrient content of 50% carbohydrate, 35% fat and 15% protein.

Bloods

Between 3 and 7 days after the incremental exercise test, participants arrived at the laboratory after an overnight fast for a 2 h oral glucose tolerance test. Participants ingested a 25% glucose beverage containing 75g glucose made up in 300 mL of water. Additional blood samples (10 ml) were collected at 30, 60, 90 and 120 min. Plasma was separated, stored at -80°C and then analysed for plasma insulin (enzyme linked immuno-sorbent assay kit; Invitrogen, UK) and plasma glucose concentrations (IL ILab 650 Chemistry Analyzer, Diamond Diagnostics, USA). Insulin sensitivity was calculated using the Matsuda Index (Matsuda and DeFronzo, 1999).

Endurance-type exercise training

The training programme began 48 h after the pre-training testing. In the first 3 weeks, supervised training consisted of continuous cycling at 65% VO_{2peak} 5 days per week. In the first 10 sessions participants cycled for 40 min, rising to 50 min for sessions 11 – 20 and 60

min for sessions 21 – 30. After 3 weeks of training, the workload was adjusted after the second incremental test to exhaustion.

Resistance-type exercise training

The one repetition maximum (1RM) for each participant was determined on 8 motion guided resistance machines which included the leg extension, leg press, chest press, hamstring curl, lateral pulldown, seated biceps curl and arm extension movements (Cybex International Inc., MA, USA). The 1RM was determined (Kraemer and Fry, 1995) where a warm-up of 6 repetitions at an estimated 50% 1RM, 4 repetitions at an estimated 70% 1RM, and 2 repetitions at ~80% 1RM with 2 min recovery between sets. The 1RM was then estimated and attempted. A lift was valid where the load was lifted through the entire motion without assistance. Subsequent loads were adjusted based on the number of repetitions performed at the estimated 1RM. This was repeated until only 1 repetition was achieved which became their 1RM. Post-RT 1RM was established before the final training bout and was performed on the leg extension, leg press, chest press and shoulder pull-down.

The RT group trained 3 days per week and completed at least 16 sessions and a maximum of 18 sessions over the 6 weeks. In week 1, participants completed 3 sets of 10-12 repetitions each at 50%, 60% and then 70% 1RM. In week 2, participants completed 2 sets of 10-12 repetitions each with set 3 comprising 12 repetitions at 80% 1RM performed to volitional fatigue. Thereafter, once 3 sets of 12 repetitions were successfully completed, the resistance load was increased by 2.5 kg to ensure training progression.

Muscle biopsy

Percutaneous needle muscle biopsy samples from the *m. vastus lateralis* was performed pre- and post 6 weeks ET and RT using the Bergstrom needle biopsy technique (Bergstrom, 1975)

adapted with suction (Evans et al., 1982). Muscle samples were collected 48 h following the last exercise session. The muscle samples were blotted free of excess blood and any visible fat or connective tissue was removed. The samples were then embedded in Tissue Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and immediately frozen in liquid-nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK) for storage at -80°C. Three 5 µm cryo-sections per section were cut at -30°C from the same pre- and post-exercise training biopsies used in the studies published by this research group (Cocks et al., 2013; Shepherd et al., 2013) (Shepherd, 2013, PhD thesis; Cocks, 2012, PhD thesis, University of Birmingham 2013). For staining efficiency and to allow comparisons between ET and RT, muscle sections from pre- and post-training for both training modes were placed on the same room-temperature, uncoated glass slides (VWR International Ltd, Leicestershire, UK) and duplicated on a second slide.

Immunofluorescence staining

All chemicals used in this study were purchased from Sigma-Aldrich unless otherwise stated. At least two pre- and post-training cryo-sections from ET and RT were placed on the same glass slide in duplicate to ensure identical staining efficiency between sections and to allow comparisons between training modes. The sections were fixed in 3:1 ratio acetone and ethanol for 5 min and washed 3 x 5 min in phosphate buffered saline (PBS). Sections were then incubated for 2 h with 5% Normal Goat Serum and polyclonal FAK A17 (Santa Cruz, USA) or monoclonal anti-paxillin (BD transduction Laboratories, Oxford, UK) as described in chapters 2, 3 and 4 of this thesis. Differences in (sub)sarcolemmal FAK and paxillin content pre- and post-exercise training were investigated by co-staining the muscle sections with anti-dystrophin (Sigma-Aldrich, UK). Sarcoplasmic fibre-type differences were investigated using anti-myosin heavy chain type I (MHC type I developed by Dr. Blau;

DSHB; A4.840c) which was specific to slow oxidative type I skeletal muscle fibres leaving type II fibres unstained. After incubation with the primary antibodies, sections were washed 3 x 5 min in PBS and then incubated for 30 min in the appropriate fluorescently conjugated secondary antibodies as described in chapters 2, 3 and 4.

To visualise FAK and paxillin within the skeletal muscle microvasculature, anti-FAK and anti-paxillin were applied to muscle sections as before. The incubation period was then proceeded by 3 x 5 min wash in PBS, blotted dry and then co-incubated for 30 min with lectin FITC-conjugated Ulex europaeus Agglutinin I (Vector Laboratories). To assess background fluorescence and non-specific secondary antibody binding, negative controls were performed where the primary antibody was excluded. In all cases the fluorescence signal was abolished. The sections underwent a 3 x 5 min wash in PBS and cover slips applied as previously described.

Image capture and analysis

FAK and paxillin was visualised using confocal and widefield microscopy. Fluorescence intensity of positively labelled FAK and paxillin was used to assess protein content in (sub)sarcolemmal regions and the microvascular endothelium. To quantify FAK immunofluorescence, detailed digital images of the skeletal muscle were captured with an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) with a 63 x 1.4 NA oil immersion objective. The Alexa Fluor 488 fluorophore was excited by the 488 nm line of the argon laser and the Alexa Fluor 594 fluorophore was excited by the 594 nm line of the Helium-Neon laser, respectively. Widefield microscopy was used to capture and investigate endothelial FAK and paxillin immunofluorescence intensity pre- and post-exercise training as described in chapter 3.

Image-Pro Plus 5.1 (Media Cybernetics, MD, USA) was used to process each image across the duplicate slides. Approximately 5 regions of interest were captured per muscle section using confocal immunofluorescence microscopy. For ET, FAK immunofluorescence intensity at (sub)sarcolemmal regions was quantitated from the mean fluorescence signal intensity from a total of (mean ± SD) 151 ± 56 whole muscle fibres per individual, summed across the duplicate slides. Similarly, paxillin immunofluorescence intensity was quantitated from a total of 176 ± 49 whole muscle fibres for ET. Longitudinally orientated muscle fibres were excluded from quantitation. Quantitation of FAK and paxillin immunofluorescence intensity at (sub)sarcolemmal regions for RT are not presented due to sarcolemmal-specific damage in >50% of RT muscle. Fibre-type specific (sub)sarcolemmal FAK and paxillin content was not investigated due to the limitations of the confocal microscope resolution (200 nm) to sufficiently determine the sarcolemma of one fibre from its neighbouring fibre.

Quantitation of FAK within the microvascular endothelium of widefield-captured images was determined from the mean fluorescence signal intensity from a total of 450 ± 198 microvessels in ET and 269 ± 119 microvessels for RT. For quantitation of paxillin, a total of 364 ± 204 microvessels in ET and 228 ± 104 microvessels in RT were analysed.

Muscle fibre cross-sectional area (CSA) (μm^2) was determined pre- and post-RT through an automated method based on individual specific threshold settings or, where necessary, by manually tracing along the dystrophin-stained border. The images were calibrated using a stage micrometer. Only fibres determined as transversely-orientated were included in the CSA analysis if the fibre roundness factor was <1.639 (roundness = $perimeter^2/4\pi area$ where a perfect circle = 1.0, pentagon = 1.163, square = 1.266 and equilateral triangle = 0.639) (Kosek et al., 2006). A total of 66 ± 15 and 122 ± 20 type I and type II transversely orientated muscle fibres were analysed per individual for CSA.

Statistical analysis

Data were checked for normal distribution. Between group differences in baseline participant characteristics was assessed using an independent t-test. Sarcolemmal immunofluorescence intensity for ET was analysed using a paired t-test. All other data were assessed using a two-way ANOVA with a between factor (group; endurance training versus resistance training) and a within factor (time; pre-training versus post-training). Data are presented as mean ± SEM and statistical significance was set at $P<0.05$.

5.4. Results

Participant characteristics

There were no differences between groups in age or anthropometric values (table 1). ET induced a 15% increase in VO_{2peak} ($P<0.05$) but there was no significant change following RT ($P > 0.05$). With RT, 1RM leg extension, leg press, chest press and shoulder press increased by 32%, 40%, 34% and 39%, respectively ($P<0.05$). Insulin sensitivity increased 27% after ET and 32% after RT, respectively.

Effect of ET and RT on (sub)sarcolemmal FAK immunofluorescence intensity

A strong immunostaining for FAK was observed at the (sub)sarcolemmal regions of the skeletal muscle fibres compared with the sarcoplasm. ET significantly increased FAK immunofluorescence intensity at (sub)sarcolemmal regions by 41 ± 8 % ($P<0.001$) (figure 5.1).

Quantitation of (sub)sarcolemmal FAK in response to RT is not reported due to sarcolemma-specific frost damage in many of the skeletal muscle samples. Representative confocal microscopy images from undamaged skeletal muscle of FAK at the

(sub)sarcolemma are shown in figure 5.2. An intense FAK immunolabeling was detected at (sub)sarcolemmal regions and the immunofluorescence intensity appeared stronger after RT.

Table 5.1. Participant characteristics

	ET		RT	
Characteristics	Pre	Post	Pre	Post
Age(y)	21 ± 1	-	20 ± 1	-
Height (cm)	177 ± 3	-	172 ± 3	-
Body mass (kg)	70.8 ± 4.4	70.8 ± 4.5	71.6 ± 2.4	71.1 ± 2.2
BMI (kg/m^2)	22.6 ± 1.6	22.6 ± 1.2	24.4 ± 0.9	24.2 ± 0.8
ISI Matsuda	3.7 ± 0.5	4.7 ± 0.7*	2.8 ± 0.2	3.7 ± 0.3*
VO_{2peak} (mL/kg/min)	41.7 ± 4.1	48.2 ± 5.0*	41.1 ± 2.0	43.2 ± 2.3
1RM Leg Extension (kg)	-	-	116 ± 5	151 ± 6*
1RM Leg Press (kg)	-	-	189 ± 10	264 ± 15*
1RM Chest Press (kg)	-	-	52 ± 3	69 ± 4*
1 RM Shoulder Press (kg	-	-	45 ± 2	62 ± 3*

Data are mean ± SEM; n = 8 for ET and 8 for RT; ISI, insulin sensitivity index; * P<0.05 main effect of training

Effect of ET and RT on (sub)sarcolemmal paxillin

A strong immunostaining for paxillin was also observed at (sub)sarcolemmal regions of skeletal muscle fibres. ET significantly increased paxillin immunofluorescence intensity at (sub)sarcolemmal regions by 23 ± 8% (P<0.001) (figure 5.3). Representative confocal microscopy images of paxillin in response to ET are shown in figure 5.4.

Representative confocal microscopy images from undamaged skeletal muscle in response to RT showing paxillin at the (sub)sarcolemma are shown in figure 5.5. An intense paxillin immunolabeling was detected at (sub)sarcolemmal regions and the immunofluorescence intensity appeared stronger after RT in this individual. There was no

change in muscle fibre CSA in either type I or type II fibres following 6 weeks of RT (figure 5.6).

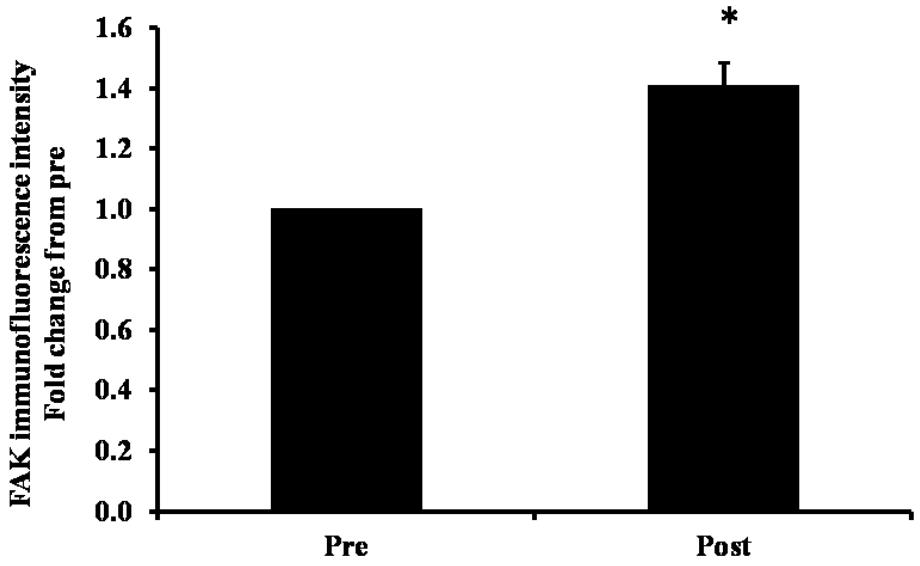

Figure 5.1. Effect of 6 weeks endurance-type exercise training (ET) on FAK content at the (sub)sarcolemma. Mean FAK immunofluorescence intensity at (sub)sarcolemmal regions of skeletal muscle fibres related to pre-training. Data are mean ± SEM and calculated from (mean ± SD) 151 ± 56 whole skeletal muscle fibres. Type I and type II fibre types are pooled in both instances. * Significantly different from pre-training (P<0.001). n = 8.

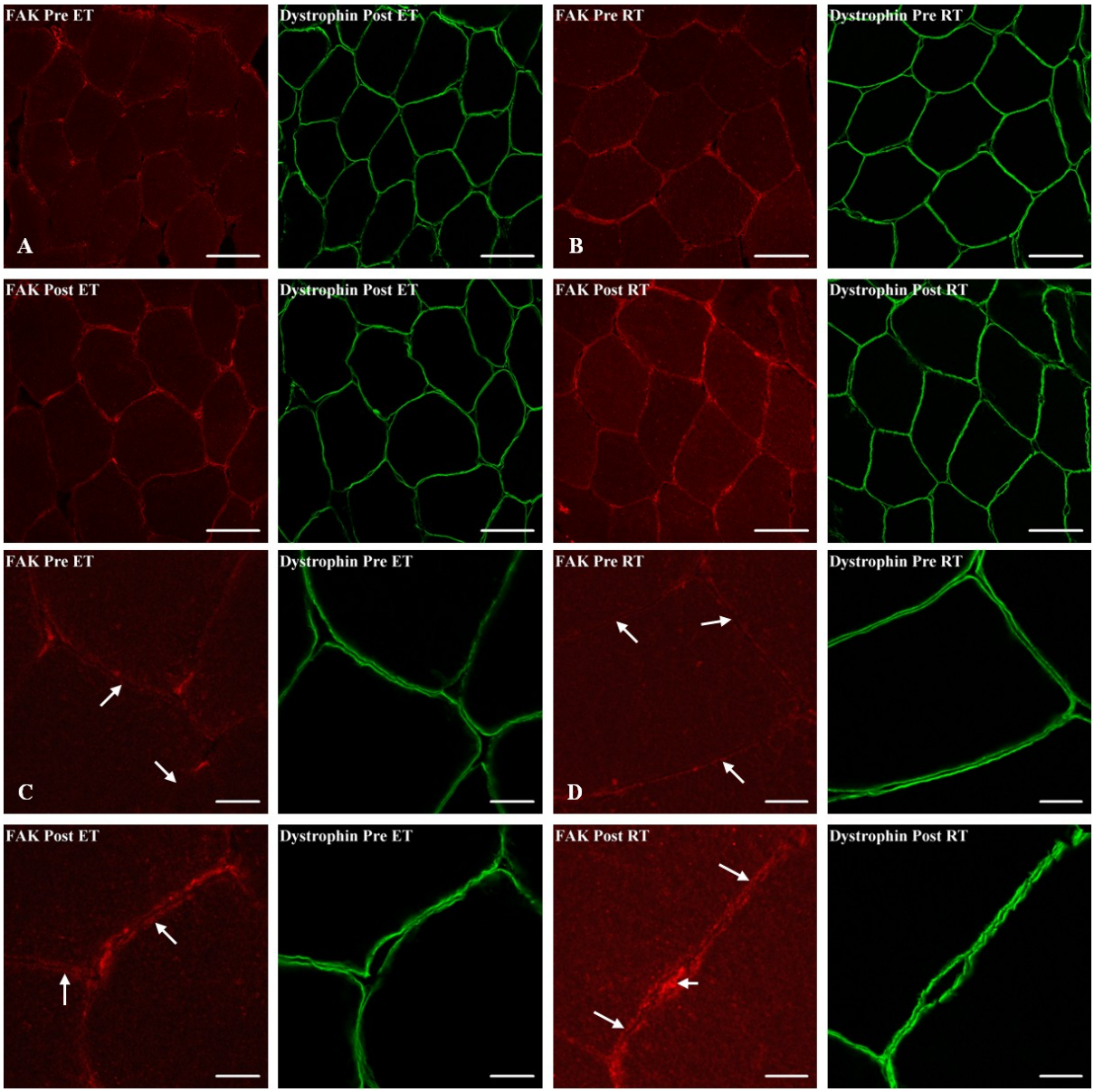

Figure 5.2. Effect of 6 weeks endurance-type exercise training (ET) on (sub)sarcolemmal FAK and paxillin immunofluorescence intensity. Representative confocal microscopy images of transversely orientated human *m. vastus lateralis* skeletal muscle fibres showing FAK (red) at (sub)sarcolemmal regions pre- (*above*) and post- (*below*) ET (A) and RT (B). Dystrophin is shown in green. Bar = 50 µm. (n = 8 for ET and n = 4 for RT). Higher magnification confocal microscopy image of FAK at (sub)sarcolemmal regions of pre- (*above*) and post- (*below*) ET (C) and RT (D) using a 4x zoom. Bar = 10 µm. Note the accumulation of FAK at (sub)sarcolemmal regions after ET (*arrows*) and a potential capillary (D) (*small arrow*).

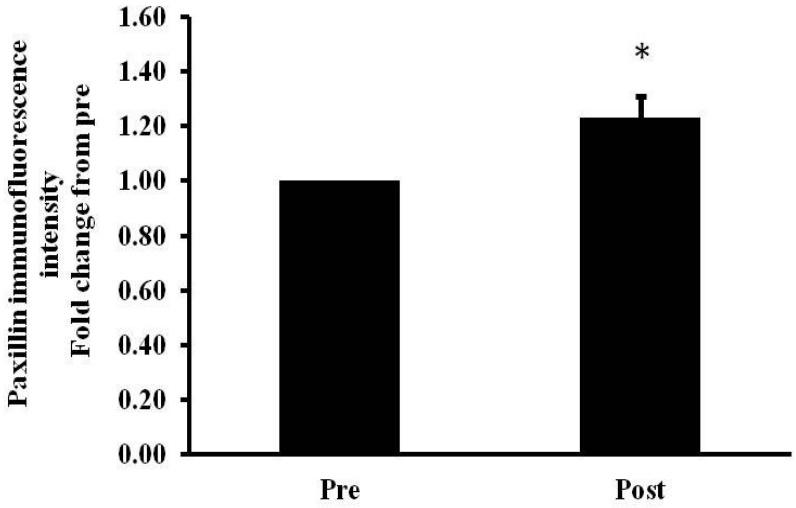

Figure 5.3. Effect of 6 weeks endurance-type exercise training (ET) on paxillin expression at the (sub)sarcolemma. Mean paxillin immunofluorescence intensity at (sub)sarcolemmal regions of skeletal muscle fibres related to pre-training. Data are mean ± SEM and calculated from (mean ± SD) 176 ± 49 whole skeletal muscle fibres. Type I and type II fibre types are pooled in both instances. * Significantly different from pre-training (P<0.05). n = 8.

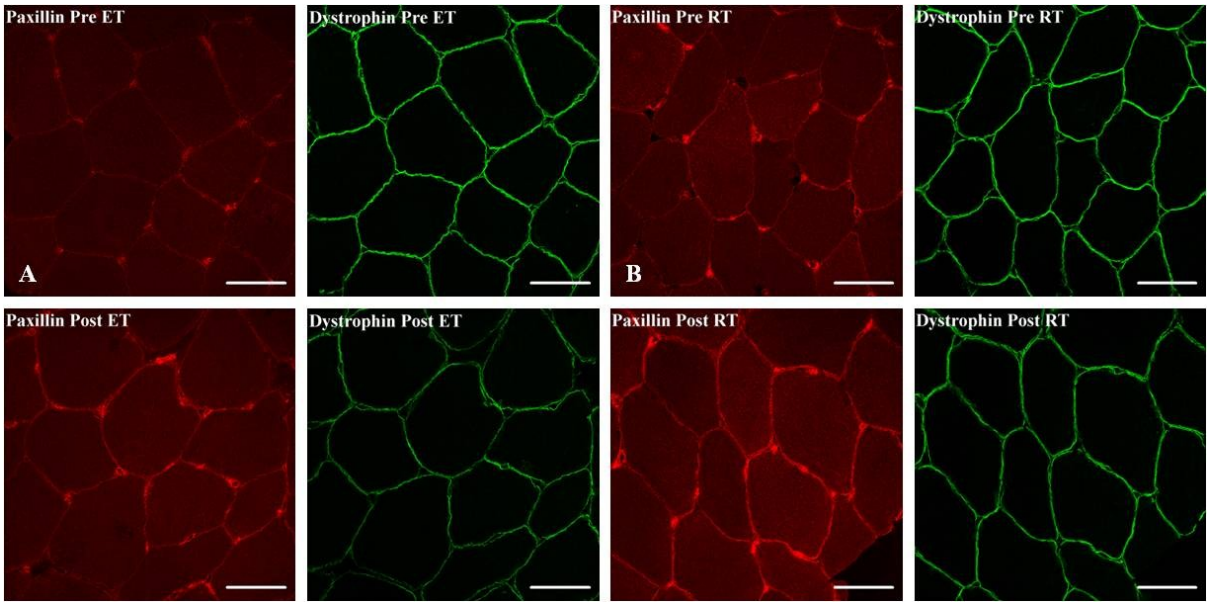

Figure 5.4. Effect of 6 weeks endurance-type exercise training (ET) on paxillin (sub)sarcolemmal expression. Representative confocal microscopy images of transversely orientated human *m. vastus lateralis* skeletal muscle fibres showing FAK (red) at (sub)sarcolemmal regions pre- (*above*) and post- (*below*) ET (A) and RT (B). Dystrophin is shown in green. Bar = 50 µm. *n* = 8 for ET and *n* = 4 for RT.

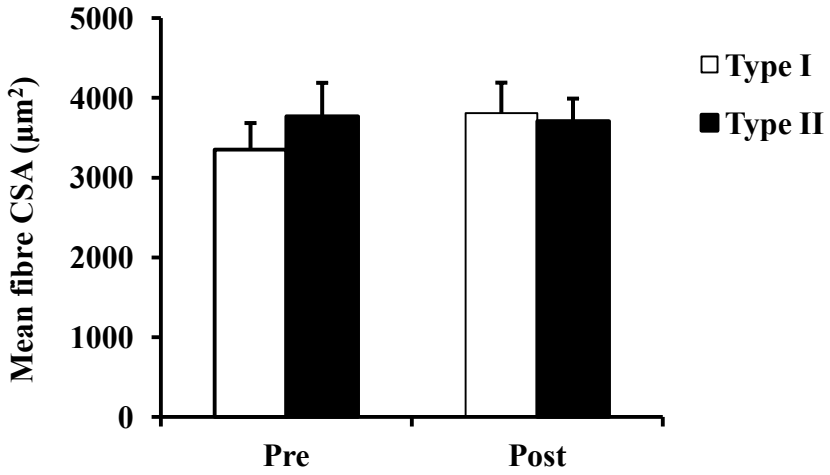

Figure 5.5. Mean type I and type II skeletal muscle fibre cross-sectional area (CSA) after resistance-type exercise training (RT). There was no effect of training on either fibre type. Data are mean ± SEM. *n* = 8.

Microvascular endothelial FAK and paxillin immunofluorescence intensity

FAK immunolabeling was strong in the skeletal muscle microvasculature. There was a main effect of training (P<0.001) on microvascular endothelial FAK immunofluorescence intensity with no difference between training modes (P>0.05). ET significantly increased microvascular endothelial FAK content by 23 ± 4%, (figure 5.6). RT significantly increased endothelial FAK content by 17 ± 4% (figure 5.6). Representative widefield microscopy images are presented in figure 5.7.

Paxillin immunolabeling was also strong in the skeletal muscle microvasculature. There was a main effect of training on microvascular endothelial paxillin content (P<0.001) with no difference between training modes (P>0.05). Paxillin immunofluorescence intensity was 21 ± 9% higher after ET and 16 ± 4% higher after RT (figure 5.6). Representative widefield microscopy images are presented in figure 5.8.

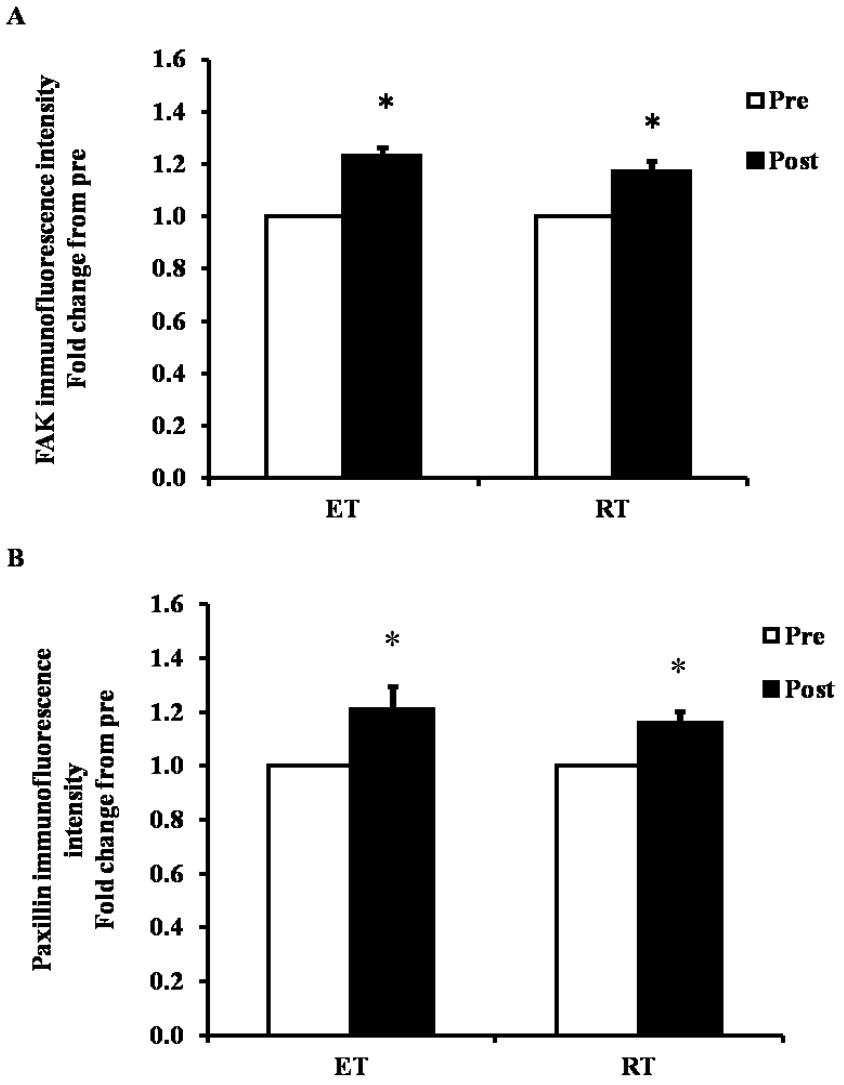

Figure 5.6. FAK and paxillin immunofluorescence intensity in the endothelium of the skeletal muscle microvasculature. A) Mean FAK endothelial immunofluorescence intensity pre- and post-ET and RT calculated from 450 ± 198 microvessels in ET and 269 ± 120 microvessels in RT. B) Mean paxillin endothelial immunofluorescence intensity pre- and post-ET and RT calculated from 364 ± 204 microvessels for ET and 228 ± 104 in RT. Data are mean ± SEM and related to pre-training. *n* = 8/ training mode. Data are mean ± SEM and related to pre-training. * Significantly different from pre-training $P<0.001$.

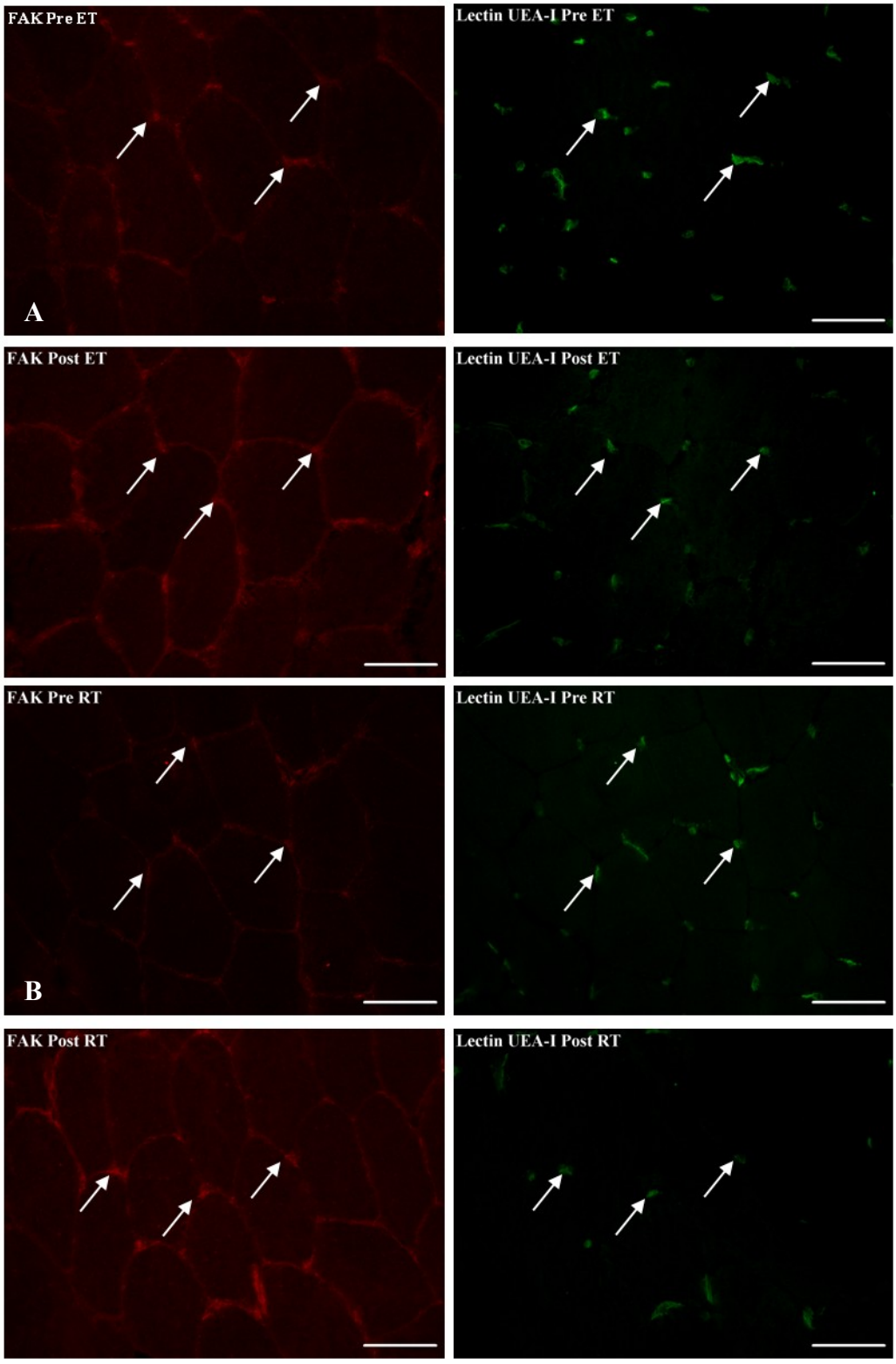

Figure 5.7. Effect of 6 weeks endurance- (ET) and resistance-type exercise training (RT) on endothelial FAK immunofluorescence intensity. Representative widefield microscopy images of FAK (*red*) in the microvascular endothelium (*green*) of human *m. vastus lateralis* skeletal muscle pre- (*above*) and post- (*below*) ET (A) and RT (B). Bar = 50 µm. *n* = 8/group.

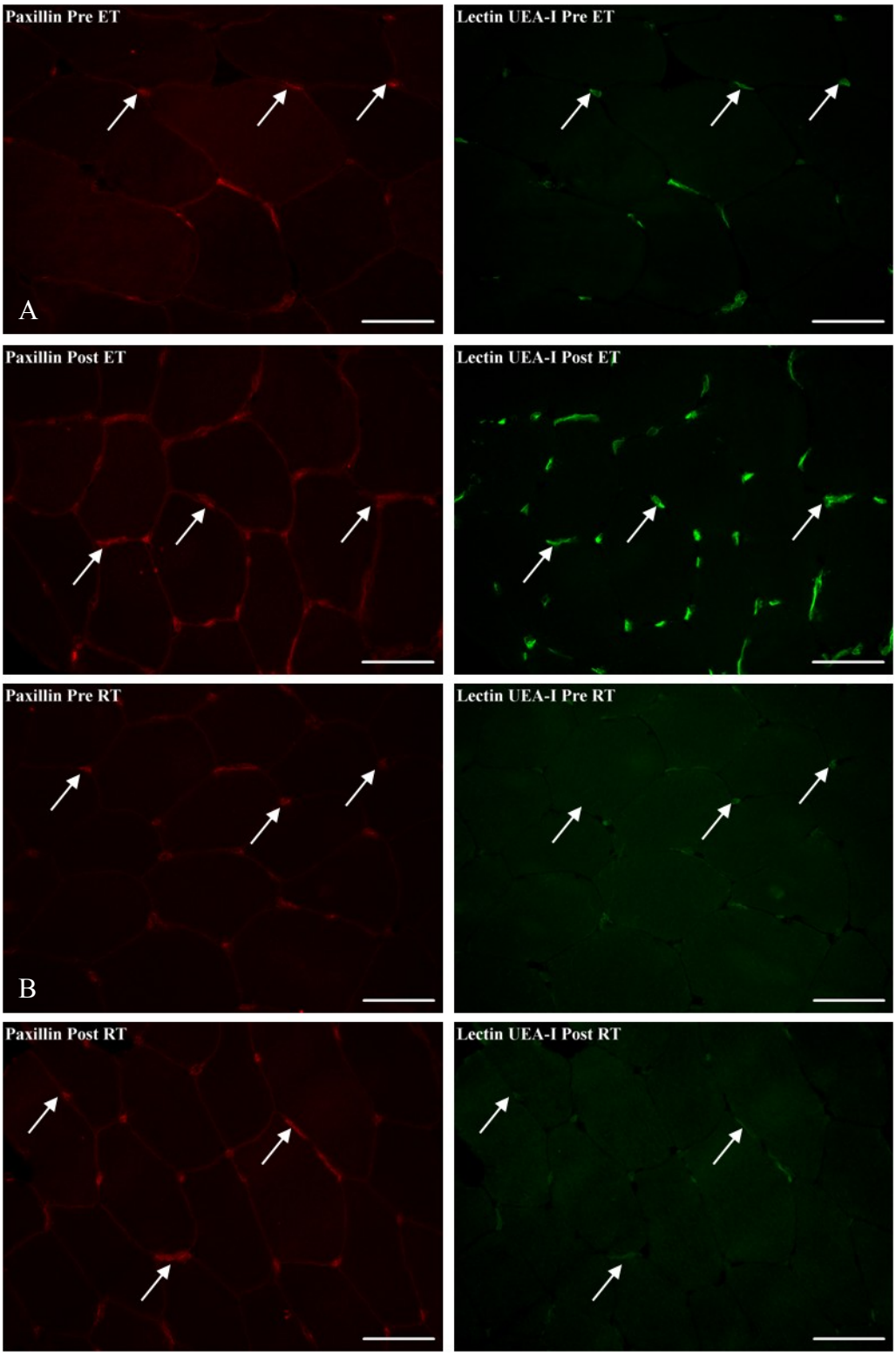

Figure 5.8. Effect of 6 weeks endurance training (ET) and resistance training (RT) on endothelial paxillin immunofluorescence intensity. Representative widefield microscopy images of paxillin (*red*) in the microvascular endothelium (*green*) of human *m. vastus lateralis* skeletal muscle pre- (*above*) and post- (*below*) ET (A) and RT (B). *n* = 8/group. Bar = 50 μm.

5.5. Discussion

This is the first study to investigate FAK and paxillin responses to 6 weeks ET and RT in the skeletal muscle fibres and microvascular endothelium of previously sedentary humans. The novel findings of the study are that the protein expression of both focal adhesion proteins increased significantly in skeletal muscle after both training modes. The highest protein expression of FAK and paxillin in the skeletal muscle fibre was at the (sub)sarcolemma and their immunofluorescence intensity was significantly increased in this location after ET. A high protein expression of both focal adhesion proteins was also present in the endothelial layer of the microvasculature of skeletal muscle. ET and RT also led to significant increases in the protein expression of FAK and paxillin in this location. These data confirm that FAK and paxillin are highly expressed in the (sub)sarcolemmal regions and microvascular endothelium before training and that their abundance is increased after both ET and RT in young previously sedentary men. These data build on chapters 2, 3 and 4 of this thesis to demonstrate that the recently developed immunofluorescence microscopy techniques can be used to quantitate ET- and RT-associated changes in the protein content of human focal adhesion proteins.

The increased FAK and paxillin expression at the (sub)sarcolemma of skeletal muscle fibres after ET are in agreement with and expand upon previous investigations of whole muscle homogenates from mechanically overloaded animal skeletal muscle (Fluck et al., 1999; Gordon et al., 2001) and after 12 weeks skiing training in elderly human skeletal muscle (Flueck et al., 2011; Narici et al., 2011). This implies a significant remodelling of the costamere in response to the higher mechanical forces generated by the contracting sarcomeres during ET compared with common daily living activities (figure 5.1, 5.2). A remodelling of other costamere-associated proteins has been reported after RT (Kosek and Bamman, 2008).

As well as its known role in force transmission (Bloch and Gonzalez-Serratos, 2003), the costamere is also a nexus for mechanotransduction (Burkholder, 2007). FAK overexpression has been linked with the increased expression of genes associated with slow oxidative skeletal muscle and the increased protein content of skeletal muscle mitochondrial enzymes (Durieux et al., 2009). In cardiomyocytes, FAK depletion prevented the stretch-induced elevation in markers of mitochondrial biogenesis such as mitochondrial DNA and mitochondrial enzymes (Tornatore et al., 2011). Paxillin has also been suggested to play a role in lung cell mitochondrial biogenesis (Kawada et al., 2013) which suggests a potential role for both FAK and paxillin in the mechanisms that stimulated the increased mitochondrial density reported by Shepherd et al in the full cohort of subjects after both modes of exercise training (Shepherd et al., 2013)(Shepherd, S. 2013 PhD thesis).

FAK is also necessary for load-induced left ventricle hypertrophy in rats (Clemente et al., 2007; DiMichele et al., 2006) and the change in FAK protein content in human skeletal muscle after exercise training is positively correlated with the change in muscle thickness (Narici et al., 2011). The mechanotransduction pathways that link FAK to load-induced-hypertrophy of striated muscle are not fully elucidated but may include mTOR-dependent (Marin et al., 2008) or –independent pathways (Klossner et al., 2009). However, the present study did not observe a measureable change in type I or type II muscle fibre CSA after high intensity RT in the sampled muscle (figure 5.5). This contrasts with a previous study reporting increased type II fibre CSA after 4 weeks RT in untrained individuals (Woolstenhulme et al., 2006) and hypertrophy of the whole *m. vastus lateralis* muscle after ~3 weeks RT when measured using magnetic resonance imaging (Seynnes et al., 2007). Measures of muscle fibre CSA can vary between 15 – 20% across multiple biopsy sites (Halkjaer-Kristensen and Ingemann-Hansen, 1981) and muscle fibre CSA do not fully account for whole muscle CSA (D'Antona et al., 2006). Therefore, it is possible that

additional measures and methodologies may have detected muscle fibre hypertrophy in the present study. Alternatively, the training duration may have been insufficient to observe measureable hypertrophy in the sampled muscle of these previously sedentary individuals and training durations of 8 weeks or more may have been necessary to observe an affect (Campos et al., 2002; Wilkinson et al., 2006)(chapter 3 of this thesis). Although no direct measures of (sub)sarcolemmal FAK are presented for RT, evidence from ET and the microvascular endothelium after RT suggests that FAK expression in the skeletal muscle fibres after RT would have increased., It is possible, therefore, that costameric remodelling in the present study precedes muscle fibre hypertrophy in these previously sedentary individuals.

The skeletal muscle microvasculature is exposed to tensile, compressive and fluid shear stress (Davies and Tripathi, 1993). Force transmission between the luminal glycocalyx via the cytoskeleton to abluminal focal adhesions (Weinbaum et al., 2007) leads to the rapid phosphorylation and recruitment of FAK and paxillin to focal adhesions in endothelial cells *in vitro* (Li et al., 2002; Mattiussi et al., 2006; Zebda et al., 2012). The latter is thought to lead to a strengthening of the adhesion of endothelial cells to its extracellular matrix during periods of elevated shear stress (Wechezak et al., 1989). An increase in the protein abundance of focal adhesions, including FAK and paxillin (figure 5.7, 5.8), might be an adaptive response to the multiple periods of elevated fluid shear stress and mechanical forces sustained by the endothelium during exercise training. The increased FAK protein content may also play a critical role in the many FAK-mediated mechanotransduction signalling pathways within the endothelium (Zebda et al., 2012). Shear stress stimulates flow-mediated dilation in the vascular endothelium of arterioles through increased nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) (Green et al., 2004). FAK has been shown to regulate fluid shear stress-induced phosphorylation of eNOS and FMD of rat coronary

arterioles (Koshida et al., 2005). The increased FAK expression may therefore contribute to the improved FMD reported after exercise training in rat skeletal muscle arterioles (Spier et al., 2007) and human arteries (Rakobowchuk et al., 2008; Tinken et al., 2010).

The increased content of both focal adhesion proteins may also regulate the angiogenic response to exercise training since they are targets of pro-angiogenic growth factors (Abedi and Zachary, 1997; Kim et al., 2000; Qi and Claesson-Welsh, 2001) and are necessary for angiogenesis (Hagel et al., 2002; Ilic et al., 1995; Peng et al., 2004; Shen et al., 2005). It is well known that skeletal muscle angiogenesis occurs in humans after ET (Andersen and Henriksson, 1977; Klausen et al., 1981) and the two focal adhesion proteins may have played a key role in the mechanisms leading to the increased capillary density previously reported after ET (Cocks et al., 2013). Whereas some studies have reported an increase in capillary density after RT (Green et al., 1999; McCall et al., 1996), others have not (Luthi et al., 1986; Tesch et al., 1990; Wang et al., 1993). There was no change in capillary density after RT in the present study's participants (Cocks, PhD thesis). The authors attributed this to a lack of change in fibre CSA after training and so the diffusion distance between the fibre and surrounding capillaries was maintained (Cocks, PhD thesis) (Green et al., 1999; McCall et al., 1996). This suggests other mechanisms in addition to FAK regulate angiogenesis during RT.

Whole body insulin sensitivity was increased in these participants after both training modes (table 5.1) (Cocks et al., 2013; Shepherd et al., 2013) (Shepherd 2013, PhD thesis). Insulin stimulates FAK and paxillin phosphorylation in C2C12 skeletal muscle cells (Goel and Dey, 2002) and FAK plays a key regulatory role in skeletal muscle insulin-stimulated glucose uptake *in vitro* (Bisht and Dey, 2008; Bisht et al., 2007) and *in vivo* (Bisht et al., 2008). It could be speculated therefore, that the insulin-sensitising effects of both training

modes (Shepherd et al, 2013; Shepherd, S. 2013 PhD thesis) are partly attributable to the increase in the two focal adhesion proteins.

Insulin-stimulated increases in microvascular blood flow is an important response determining insulin and glucose delivery to the muscle fibre and contributes to insulin-mediated glucose uptake (Barrett et al., 2009). Insulin-induced microvascular recruitment is dependent upon NO production by eNOS in the microvascular endothelium (Vincent et al., 2003). As FAK regulates shear stress-mediated increases in NO production by eNOS (Koshida et al., 2005), it is possible that FAK within the microvascular endothelium is an important mechanism in insulin-stimulated vasodilation. Furthermore, the increased FAK protein expression following exercise training may contribute to the exercise training-associated improvements in insulin-mediated microvascular blood flow (Inyard et al., 2007; Rattigan et al., 2001) and improved insulin-mediated glucose disposal (Rattigan et al., 2001).

In conclusion, this is the first study to report an increased FAK and paxillin protein expression in human skeletal muscle after 6 weeks ET and RT. Immunofluorescence microscopy enabled the identification and quantitation of the content of these focal adhesion proteins in specific regions of the skeletal muscle which is a major advantage of this method compared with western blot analyses. FAK and paxillin were increased in the (sub)sarcolemmal regions of skeletal muscle fibres after ET and within the microvascular endothelium of skeletal muscle after both types of exercise training. This suggests a remodelling of the skeletal muscle costamere and endothelial focal adhesions in response the higher overall mechanical load generated during exercise training. As muscle fibre CSA did not change after RT, the accumulation of costameric FAK and paxillin may precede muscle fibre hypertrophy in these participants. FAK may also play a role in the improved insulin sensitivity that was seen in these participants via mechanisms that may both involve increased insulin sensitivity of skeletal muscle fibres and the endothelium of the skeletal

muscle microvasculature. This could be relevant for future research in obese, type II diabetic and elderly individuals. The use of immunofluorescence microscopy techniques is a powerful and informative approach to investigate tissue and region specific responses to exercise training.

5.6. Acknowledgements

The antibodies against human slow myosin (A4.840-c) used in the study were developed by Dr. Blau and were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Chapter 6

General discussion

6.1. Background and aims of the PhD project

Global population ageing is an unprecedented achievement for humanity. However, the age-associated risk of developing cardiovascular disease (Najjar et al., 2005), type II diabetes (Wild et al., 2004) and the functional impairments associated with sarcopenia (Janssen et al., 2002) will be a considerable challenge for future health care provision. The development of sarcopenia has been linked to the age-associated decline in physical activity rates (Doherty, 2003; HSfE, 2010), but the mechanisms by which physical (in)activity and the alterations in contractile and hemodynamic forces that regulate skeletal muscle mass, vascular function and metabolic health are incompletely understood. FAK and paxillin are focal adhesion-associated proteins implicated in the acute and chronic mechanotransduction of (un)loading (de Boer et al., 2007; Durieux et al., 2009; Fluck et al., 1999; Glover et al., 2008; Gordon et al., 2001; Klossner et al., 2009) and hemodynamic forces within the (micro)vasculature (Koshida et al., 2005; Zebda et al., 2012). Age-associated defects in the load-induced activation of FAK (Rice et al., 2007) may underlie the impaired anabolic response of skeletal muscle to acute and chronic contractile activity (Durham et al., 2010; Fry et al., 2011; Kosek and Bamman, 2008; Kosek et al., 2006; Kumar et al., 2009; Williamson et al., 2003) and the impaired FMD of arteries (Celermajer et al., 1994; Donato et al., 2007; Eskurza et al., 2004) and skeletal muscle arterioles with age (Delp et al., 2008; Muller-Delp et al., 2002).

When the research described in this thesis began, knowledge of the spatial distribution of FAK was limited to avian and rat skeletal muscle fibres (Durieux et al., 2009; Fluck et al., 1999; Fluck et al., 2002; Klossner et al., 2009) and there was no information on the spatial distribution of paxillin in mammalian skeletal muscle *in vivo*. In addition, the chronic response of FAK and paxillin total protein content to mechanical overload was restricted to whole muscle homogenates of avian and rat skeletal muscle (Fluck et al., 1999; Gordon et al.,

2001). Consequently, there was no information regarding the spatial distribution of FAK and paxillin in human skeletal muscle fibres and the microvascular endothelium and VSMCs of young and elderly individuals. Furthermore, it was not known how exercise training affected the content of FAK and paxillin within skeletal muscle fibres and the skeletal muscle microvascular endothelium of young and elderly populations.

Therefore, the principle aims of this thesis were to; 1) develop novel immunofluorescence microscopy methods to make the first investigations of the spatial distribution of FAK in skeletal muscle and its associated microvasculature of young, recreationally active men; 2) investigate the effect of 12 weeks RT on FAK protein content and spatial distribution in skeletal muscle and the endothelium of its associated microvasculature in previously untrained elderly men using the novel immunofluorescence microscopy methods developed in chapter 2; 3) develop novel immunofluorescence microscopy methods to perform the first investigations of the paxillin protein content and spatial distribution in human skeletal muscle and the endothelium of its associated microvasculature and to investigate the colocalisation of FAK and paxillin in these tissues; 4) investigate the effect of 6 weeks ET and RT on FAK and paxillin protein content in skeletal muscle fibres and the skeletal muscle microvascular endothelium of young, previously sedentary individuals using the novel immunofluorescence microscopy techniques developed in chapter 2 and 4.

6.2. Immunofluorescence microscopy to investigate focal adhesion-associated proteins in skeletal muscle and its microvasculature

6.2.1. Spatial distribution of FAK and paxillin in skeletal muscle fibres

The highest content of FAK and paxillin was at the (sub)sarcolemma of skeletal muscle fibres in the *m. vastus lateralis* of young and elderly individuals. Both focal adhesion proteins

formed a relatively continuous distribution around the fibre perimeter (chapter 2, 3, 4, 5). These findings are the first to be presented in young and elderly individuals and are in agreement with the distribution of FAK and their localisation to costameres in animal skeletal muscle fibres (Fluck et al., 2002; Klossner et al., 2009; Klossner et al., 2013; Quach and Rando, 2006). However, during the final revisions of this thesis, Li et al (Li et al., 2013) presented images of a distinctly fragmented distribution of FAK at the sarcolemma of *m. vastus lateralis* skeletal muscle fibres of young individuals following immunostaining with the C-terminal-specific FAK antibody (Li et al., 2013). These observations are dissimilar to those presented in chapter 2 following immunolabeling with both N-terminal- and C-terminal-specific FAK antibodies.

There was no difference in sarcoplasmic FAK content of type I and type II skeletal muscle fibres (chapter 2) and the sarcoplasmic paxillin content was only slightly higher in type I compared with type II fibres (chapter 4). These findings are in disagreement with Durieux et al (Durieux et al., 2009) who reported an intense sarcoplasmic FAK immunostaining in type I versus type II rat *m. soleus* skeletal muscle fibres after the combined effects of 10 days mechanical overload with FAK overexpression (gene electro-transfer of a FAK-containing plasmid) (Durieux et al., 2009). The contrasting findings with Durieux et al, (Durieux et al., 2009) may be attributable to the skeletal muscles and species investigated and may also be a phenomenon of the gene electrotransfer methodology employed by Durieux et al, (Durieux et al., 2009).

The high (sub)sarcolemmal content of FAK supports its direct interaction with the transmembrane insulin receptor (Goel and Dey, 2002b). Skeletal muscle is the principle site of insulin-stimulated glucose uptake during a hyperinsulinemic-euglycemic clamp (DeFronzo et al., 1981) and FAK regulates insulin–stimulated glucose uptake in rodent skeletal muscle *in vivo* and in C2C12 and L6 myotubes *in vitro* (Bisht and Dey, 2008; Bisht et al., 2007;

Bisht et al., 2008; Huang et al., 2006). In longitudinally orientated fibres, FAK was distributed as striations which spanned the width of the fibre and ran perpendicular to the fibre's direction (chapter 2). This distribution was also observed for paxillin in longitudinally orientated fibres, but far more rarely. The distribution of both focal adhesion proteins was similar to the rib-like distribution of vinculin (a marker for costameres) (Pardo et al., 1983) and t-tubules (which are deep invaginations of the sarcolemma) (Lauritzen et al., 2006; Ploug et al., 1998). Cryosections were co-incubated with a marker for the t-tubules (anti-DHPR) and both FAK and paxillin appeared to colocalise with the t-tubules, suggesting they are at or in close proximity to the t-tubules (chapter 2 and 4). The t-tubules are the most important site of GLUT-4 docking in mouse skeletal muscle (Lauritzen et al., 2008a; Lauritzen et al., 2008b; Lauritzen et al., 2006) and support the bulk of insulin-stimulated glucose uptake in mice (Wang et al., 1996). In the case of paxillin, the striations which are rarely observed in longitudinally orientated fibres are more likely to be costameres (Mondello et al., 1996; Pardo et al., 1983) and the colocalisation of paxillin with DHPR represents the close proximity of costameres to the underlying peripheral myofibrils.

6.2.2. Spatial distribution of FAK and paxillin in the skeletal muscle microvasculature

FAK and paxillin play a critical role in vascular development and angiogenesis (Hagel et al., 2002; Ilic et al., 1995; Peng et al., 2004; Shen et al., 2005). FAK and paxillin had a high protein content and degree of colocalisation in the endothelium of the skeletal muscle microvasculature of young and elderly individuals both in capillaries and arterioles (chapter 2, 3, 4 and 5). FAK and paxillin were distributed heterogeneously around the lumen in both the endothelium and VSMCs of arterioles and appeared to form clusters which probably reflected the formation of focal adhesions in both cell types in the rested, fasted state. There was also some evidence that FAK (chapter 2) and paxillin (chapter 4) aligned along parts of

the periphery of the VSMC. A similar distribution has also been observed in the monolayers of cultured endothelial cells in response to laminar shear stress (12 dyne/ cm^2) (Li et al., 1997) and in tracheal smooth muscle after acetylcholine-stimulated contraction (Opazo Saez et al., 2004). Human femoral artery blood flow is low in the resting state (~0.3 l/ min) (Saltin et al., 1998) and therefore the fluid shear stress in human skeletal muscle capillaries is also likely to be low (~12 dyne/ cm^2) (Dawson and Hudlicka, 1993). It is yet to be determined if higher rates of blood flow (and fluid shear stress) during an acute bout of endurance- and resistance-type exercise in the untrained and exercise-trained state acutely affect the distribution of FAK and paxillin within the skeletal muscle microvasculature.

6.2.3. Effect of exercise training on FAK and paxillin in skeletal muscle fibres

Chapter 3 and 5 are the first to demonstrate in humans that exercise training of between 6 and 12 weeks leads to increases in FAK and paxillin protein content at the (sub)sarcolemma of skeletal muscle fibres in young and elderly individuals. The chapters also demonstrate that FAK and paxillin are increased in the skeletal muscle microvascular endothelium after exercise training. These data provide a greater insight into the findings previously reported in whole muscle homogenates from animal skeletal muscle after loading (Fluck et al., 1999; Gordon et al., 2001) and elderly individuals after exercise training (Flueck et al., 2011; Narici et al., 2011).

The accumulation of FAK and paxillin after both training modes (chapter 3 and 5) likely reflects a remodelling of mature costameres in response to the higher forces repeatedly generated by the contracting myofibrils during exercise compared with normal daily living activities. The remodelling may also be FAK mediated since local overexpression of FAK (gene electrotransfer) in the rat *m. soleus* increased costamere-associated protein levels for

β1-integrins and meta-vinculin (Klossner et al., 2013). The increased FAK content may also represent the formation of new costameres (Quach and Rando, 2006) which may be required to link the Z-disk of newly generated myofibrils to the sarcolemma following RT.

The role of FAK and paxillin in skeletal muscle mechanotransduction and training adaptation is not fully understood. An increase in the aerobic capacity (chapter 5) and mitochondrial density was reported in the previously sedentary individuals after ET (Shepherd 2013, PhD thesis). In the rat *m. soleus,* local overexpression of FAK was achieved through gene electrotransfer of a FAK-containing plasmid (Durieux et al., 2009). FAK protein content was 2.6 fold higher after 8 days of overexpression compared with contralateral controls transfected with an empty plasmid and led to the upregulation of genes and proteins associated with mitochondrial oxidative metabolism (Durieux et al., 2009). The effect of FAK overexpression was muted after unloading the hindlimbs through tail suspension and were absent after co-expression with FRNK (Durieux et al., 2009).

The mechanism that links FAK with mitochondrial biogenesis is not fully resolved. In neonatal rat ventricular myocytes, cyclic stretch (2-12 h) increased important mediators of mitochondrial biogenesis such as peroxisome proliferator activated γ coactivator-1α (PGC1α), nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Tfam). Prolonged stretching also led to increased mitochondrial biogenesis (as measured by mitochondrial DNA and increased protein content of the oxidative enzyme cytochrome oxidase IV (Tornatore et al., 2011). Stretching increased FAK Tyr^{397} phosphorylation after 2 h and this was accompanied by the nuclear translocation and association of FAK with PGC1α (Tornatore et al., 2011). FAK was shown to predominantly interact with nuclear PGC1α via its C-terminal domain and depletion of FAK protein through siRNA inhibited basal levels of PGC1α and the stretch-associated upregulation of PGC1α, NRF-1, mitochondrial DNA and cytochrome oxidase IV (Tornatore et al., 2011). FAK depletion in the left ventricle of mice

also suppressed the upregulation of PGC1α, NRF-1 and mitochondrial DNA by pressure-overload *in vivo* (Tornatore et al., 2011). These experiments suggest a critical role for FAK in the regulation of mitochondrial biogenesis by mechanical load. It is expected that the age-associated decline in physical activity rates leads to a reduction in the phosphorylation and protein content of FAK (de Boer et al., 2007; Glover et al., 2008; Gordon et al., 2001; Li et al., 2013). This will lead to a decline in mitochondrial protein synthesis rates, mitochondrial biogenesis, mitochondrial density, reduced exercise capacity and glucose tolerance with age (Rooyackers et al., 1996; Short et al., 2005).

The increased FAK content in elderly individuals after 12 weeks RT was accompanied by specific type II fibre hypertrophy (chapter 3). This is in agreement with two companion studies published during this thesis (Flueck et al., 2011; Narici et al., 2011). In these studies, 20 – 22 elderly males and females aged ~67 years skied 3.5 hours per training session 2-3 times per week over 12 weeks. The authors reported a 1.9 fold increase in total FAK protein content in the *m. vastus lateralis* of males without change in females after skiing training (Flueck et al., 2011). The change in FAK content after training was also positively correlated with the change in muscle thickness (r=0.59) (Narici et al., 2011). Interestingly, load-induced hypertrophy of the left ventricle of mice was prevented and reversed by FAK siRNA *in vivo (*Clemente et al., 2007) and FAK deletion prevented pressure-overload-induced hypertrophy of the left ventricle in mice (DiMichele et al., 2006). In addition, IGF-I-stimulated C2C12 myotube cell growth (based on myotube diameter and protein content) was suppressed in FAK knockdown-containing myotubes (Crossland et al., 2013). The time course association between load-induced changes in FAK content and muscle fibre CSA are unknown, but chapter 5 demonstrated an increase in FAK and paxillin protein content without a measureable change in type II fibre CSA following 6 weeks RT in the sampled muscle (chapter 5). It is possible, therefore, that FAK may play an important role in load-

induced skeletal muscle hypertrophy and the accumulation of FAK at (sub)sarcolemmal regions after 6 weeks RT may be an adaptation that precedes skeletal muscle hypertrophy.

The molecular pathways that link load-induced activation of FAK and paxillin to skeletal muscle hypertrophy are poorly understood. In neonatal rat ventricular myocytes, FAK is thought to signal through an mTOR-dependent signalling pathway (Marin et al., 2008). siRNA-mediated FAK depletion led to a blunted cyclic stretch-induced phosphorylation of FAK, Src, Akt, TSC2, and p70S6K and stretch-induced myocyte hypertrophy (Marin et al., 2008). In EDL skeletal muscle fibres, local overexpression of FAK by gene electrotransfer in combination with contractile activity also stimulated the activation of p70S6K in an Akt-independent in EDL muscle fibres (Klossner et al., 2009).

Recently, Crossland and colleagues showed that FAK was required for normal and IGF-I-stimulated C2C12 cell growth (measured using cell diameter) *in vitro* (Crossland et al., 2013). Lentiviral-mediated knockdown of FAK resulted in 87% depletion of FAK and a 35% smaller myotube diameter at day 4 of myotube differentiation when compared to a negative control (C2C12 cells with scrambled shRNA).

When scrambled shRNA cells were treated with IGF-I for 24 h, the increased cell diameter (41%) and total protein content (44%) were attenuated in FAK knockdown C2C12 cells (Crossland et al., 2013). At the molecular level, FAK knockdown was associated with attenuated IGF-I-mediated increases in FAK Tyr^{397}, TSC2 Thr^{1462}, 4EBP1 $Thr^{37/46}$ and p70S6K phosphorylation (Thr^{389} and Thr^{421}/ Ser^{424}). IGF-I-mediated increases in Akt Ser473 and Thr308 were relatively unaffected by FAK knockdown. FAK knockdown was also associated with impaired IGF-I-mediated binding of eIF4E to eIF4G to form the translation initiation complex eIF4F. Formation of eIF4F and phosphorylation of p70S6K are important events that lead to increased protein synthesis and IGF-I-stimulated muscle protein synthesis (incorporation of puromycin into newly synthesised peptide chains) in scrambled shRNA

cells was attenuated after FAK knockdown. When scrambled shRNA C2C12 cells were treated with an inhibitor of FAK Tyr^{397} phosphorylation, IGF-I-stimulated FAK Tyr^{397}, Akt Ser^{473}, p70S6K Thr^{389} and 4EBP1 $Thr^{37/46}$ phosphorylation were all suppressed.

Taken together, these data suggest an important role for FAK in IGF-I-mediated stimulation of the mTOR-p70S6K and -4EBP1 signalling pathways and the upregulation of protein synthesis in cell culture. The role and importance of IGF-I in load-induced skeletal muscle hypertrophy *in vivo* has been challenged (Miyazaki et al., 2011; Philp et al., 2011; Spangenburg, 2010; Spangenburg et al., 2008), but these data are in line with those presented in load-induced cardiomyocytes (Marin et al., 2008) and imply an important role for FAK in regulating load-induced hypertrophy. A summary of the potential signalling pathways though which load-induced activation of FAK leads to mitochondrial biogenesis and stimulation of skeletal muscle protein synthesis is presented in figure 6.1.

Chapter 5 showed that an increased FAK protein content following 6 weeks ET and RT was accompanied by increased insulin sensitivity. FAK has been shown to regulate insulin-stimulated glucose uptake in rat skeletal muscle *in vivo* and in C2C12 and L6 myotubes *in vitro* (Bisht and Dey, 2008; Bisht et al., 2007; Bisht et al., 2008). Depletion of C2C12 skeletal muscle cells of FAK protein by siRNA inhibited insulin-stimulated cytoskeletal remodelling *in vitro* leading to impairments in GLUT4-translocation and glucose uptake both *in vitro* and rats *in vivo* (Bisht and Dey, 2008; Bisht et al., 2008). A reduction in insulin-induced FAK phosphorylation was also observed in insulin resistant skeletal muscle cells which led to impaired glucose uptake (Bisht et al., 2007). Conversely, overexpression of FAK increased insulin-stimulated glucose uptake in C2C12 skeletal muscle cells (Bisht and Dey, 2008) and restored skeletal muscle insulin sensitivity and glucose uptake in insulin resistant C2C12 muscle cells (Bisht et al., 2007). These studies collectively demonstrate the importance of FAK in regulating insulin-stimulated skeletal muscle glucose uptake.

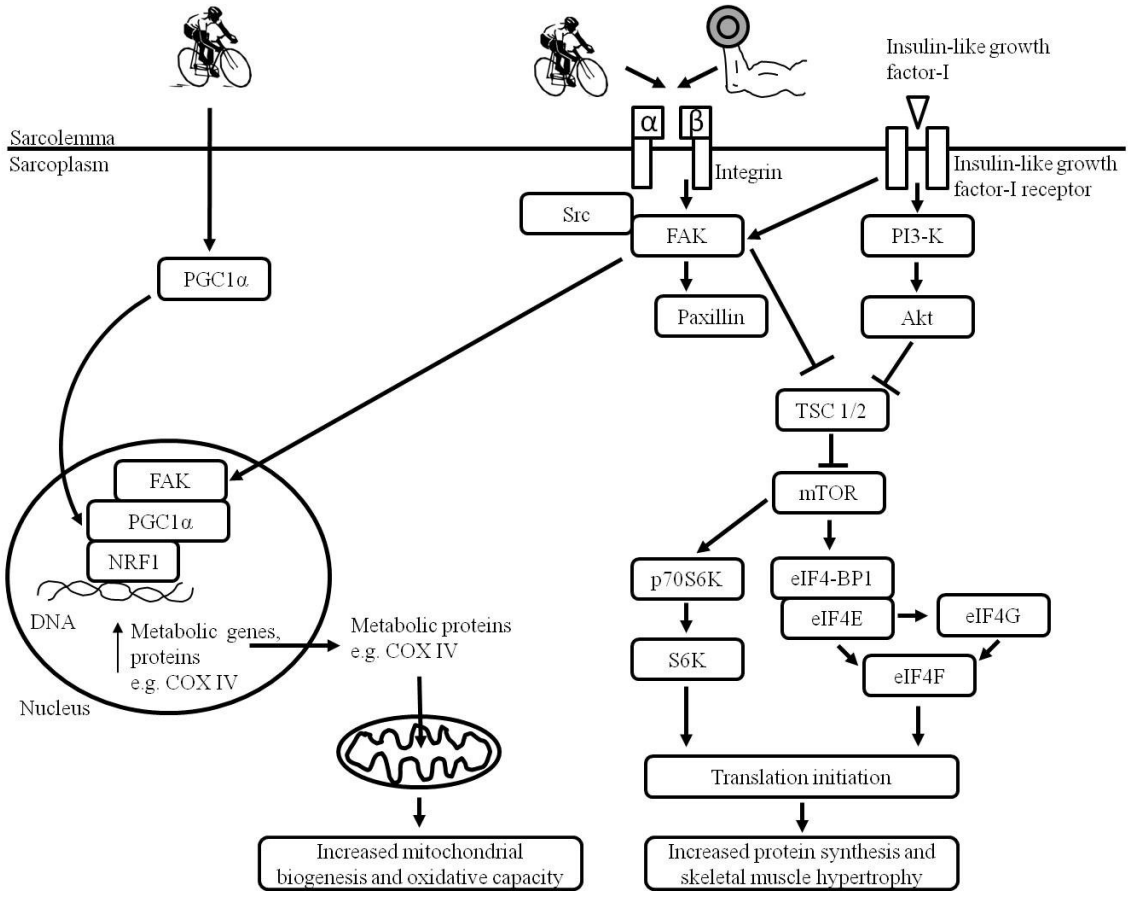

Figure 6.1. Proposed FAK-mediated regulation of load- and growth factor-induced skeletal muscle adaptation. Endurance- (ET) or resistance-type exercise (RT) activates integrins leading to FAK autophosphorylation and the binding of FAK with Src and paxillin. Evidence suggests FAK may increase oxidative capacity and mitochondrial biogenesis through its nuclear translocation where it directly interacts with peroxisome proliferator activated γ coactovator-1α to increase the transcription of nuclear-encoded genes (e.g. cytochrome oxidase IV; COX IV) by nuclear respiratory factor-1 (NRF-1) (Tornatore et al., 2011). Recent evidence suggests FAK may increase skeletal muscle protein synthesis and skeletal muscle hypertrophy through a FAK-mediated phosphorylation and inactivation of tuberous sclerosis complex 1/ 2 (TSC 1/ 2). This leads to the subsequent activation of mammalian target of rapamycin (mTOR) and the phosphorylation of downstream intermediates (Crossland et al., 2013).

They also demonstrate how impairments in insulin-stimulated glucose uptake can be averted or overcome by maintaining or increasing the FAK protein content and phosphorylation within the skeletal muscle cells. The hypothesis is that endurance- and strength-trained individuals are protected from insulin resistance because they have a high FAK protein content and FAK phosphorylation status compared with untrained individuals. This enhances skeletal muscle insulin sensitivity and glucose disposal in trained individuals compared with those who lead a sedentary lifestyle (Clevenger et al., 2002; Fujitani et al., 1998; King et al., 1987; Seals et al., 1984).

6.2.4. Effect of exercise training on FAK and paxillin in the microvasculature

The frequent exposure of microvascular endothelial cells to periods of elevated hemodynamic and mechanical forces during exercise training seems to require an increase in the content of focal adhesion-associated proteins. This may increase the adhesion strength of endothelial cells to the ECM during periods of elevated fluid shear stress (Balaban et al., 2001; Fabry et al., 2011; Riveline et al., 2001; Wechezak et al., 1989) since abluminal focal adhesions are exposed to the same level of shear stress to that which is applied at the apical surface, independent of the presence of the luminal glycocalyx, due to the force required to maintain the position of the endothelial cell (Tarbell et al., 2005).

Laminar fluid shear stress rapidly phosphorylates and activates FAK (Li et al., 2002; Li et al., 1997) and inhibition of FAK phosphorylation prevented shear-stress-induced eNOS phosphorylation (and thus NO production) and FMD of rat coronary arterioles (Koshida et al., 2005). Load-induced FAK phosphorylation may be impaired with age (Rice et al., 2007) which could be central to the age-associated decline in NO bioavailability (Maeda et al., 2004; Taddei et al., 2000) and the FMD of arteries (Celermajer et al., 1994; Donato et al., 2007; Eskurza et al., 2004; Jensen-Urstad et al., 1999) and skeletal muscle arterioles (Delp et

al., 2008; Muller-Delp et al., 2002; Spier et al., 2007). The increased FAK content after both training modes may be among the principle mechanisms that lead to increased NO bioavailability and improved FMD after exercise training (Clarkson et al., 1999; Maeda et al., 2004; Sciacqua et al., 2003; Watts et al., 2004). This may also explain why physically active and exercise trained elderly individuals are protected against the age-associated decline in FMD (Jensen-Urstad et al., 1999; Rywik et al., 1999) and the development of atherosclerosis and cardiovascular disease (Myers et al., 2002).

Given FAK's direct interaction with the insulin receptor, IRS-1 and PI3-K signalling intermediates (Baron et al., 1998; Goel and Dey, 2002a, b; Lebrun et al., 1998), it is possible that increased FAK protein content may also facilitate improvements in insulin-stimulated skeletal muscle glucose uptake and insulin-stimulated microvascular blood flow through increased NO production by eNOS. This may be an important mechanism contributing to the higher insulin sensitivity reported after exercise training (chapter 5) (Henriksen, 2002; Wojtaszewski and Richter, 2006; Zierath, 2002) and in young and elderly individuals who habitually engage in ET and RT (King et al., 1987; King et al., 1990; Lanza et al., 2008; Miller et al., 1994; Rogers et al., 1990).

RT is recommended for older people as it is an effective intervention for reversing the effects of sarcopenia and dynapenia and can improve insulin sensitivity (Chodzko-Zajko et al., 2009; Haskell et al., 2007). The findings in chapter 3 are consistent with the literature in that 12 weeks RT increased 1RM leg strength and reversed the specific type II fibre atrophy observed at baseline (Andersen, 2003; Charette et al., 1991; Klitgaard et al., 1990; Kosek et al., 2006; Larsson et al., 1978; Lexell, 1995; Lexell and Downham, 1992; Lexell et al., 1986; Lexell et al., 1988; Verdijk et al., 2007). A blunted chronic hypertrophic response of type I and type II skeletal muscle fibres to RT has been reported in the elderly compared with younger individuals (Hakkinen et al., 2001; Kosek and Bamman, 2008; Kosek et al., 2006;

Mero et al., 2013). FRNK is an endogenous inhibitor of FAK and is present in elderly skeletal muscle, particularly in females (Flueck et al., 2011). FRNK, FAK and paxillin protein content was higher in the aged rat aorta compared with young rats (Rice et al., 2007), but the phosphorylation of FAK and paxillin by increased intraluminal pressure overload of the aorta was inhibited in elderly compared with young rats (Rice et al., 2007). This suggests FRNK may play a role in the blunted adaptation to exercise training in the elderly and warrants further research in human skeletal muscle.

6.3. Future research

6.3.1. Role of FAK and paxillin in insulin-stimulated glucose uptake

The novel findings in this thesis, coupled with the recently published complimentary literature, have led to the hypothesis that maintaining high rates of physical activity and habitual exercise training lead to an increase in FAK and paxillin protein content and phosphorylation status. This will lead to a chronic enhancement of the insulin sensitivity of skeletal muscle and its associated microvasculature (as observed in chapter 5) and may contribute to the increased rates of insulin-stimulated glucose uptake observed in the 48 h period after an acute bout of exercise (Mikines et al., 1988; Perseghin et al., 1996).

Conversely, insufficient mechanical stimulation through immobilisation (de Boer et al., 2007; Glover et al., 2008; Gordon et al., 2001; Li et al., 2013) or chronic adoption of a sedentary state, particularly in the elderly, would lead to a loss of FAK protein content and impaired insulin- and exercise-induced FAK Tyr^{397} phosphorylation. In the short term, this will lead to progressively higher fasting and post-prandial plasma glucose concentrations and in the long term, will lead to glucose intolerance, the development of insulin resistance and overt pathology such as type II diabetes and cardiovascular disease. The time course of such changes is only partially known. However, the observation that just two weeks of reduced

physical activity leads to impairments in glucose tolerance in healthy elderly individuals (Breen et al., 2013) and uninterrupted sitting leads to excursions in post-prandial plasma glucose concentrations (Dunstan et al., 2012) suggest that these adaptations may occur in periods as brief as 4-5 h of sitting behind a desk. Incredibly, interrupting sitting behaviour with just 2 min of moderate intensity exercise every 20 min is enough to reduce meal induced glucose excursions (Dunstan et al., 2012). Future research should investigate whether FAK and paxillin are the upstream signals that control both the acute metabolic and health benefits of exercise and the molecular and metabolic adaptation during exercise training regimens last 6 weeks or more.

The novel immunofluorescent microscopy methods developed in this thesis could be used to investigate the role of FAK and paxillin in the regulation of insulin sensitivity in human skeletal muscle and its microvasculature in greater detail. In particular, information on the time course of FAK activation and downstream signalling is currently lacking. Future research should focus on FAK Tyr^{397} (autophosphorylation site) and $Tyr^{576/577}$ phosphorylation (necessary for maximum FAK activation) following insulin and exercise stimulation. Such measures should be made alongside parallel measurements of downstream targets (e.g. Akt, mTOR, p70S6K and PGC1α) and on differences that occur between sedentary and trained individuals.

The response of FAK and paxillin to insulin and exercise stimulation should be investigated at all the principle sites of insulin action within skeletal muscle that have been identified in this thesis; namely the (sub)sarcolemma and t-tubules of skeletal muscle fibres, and the microvascular endothelium and VSMCs of terminal arterioles. An important strength of immunofluorescence microscopy is that it allows simultaneous measures of FAK responses to insulin at focal adhesion sites in all these tissues. Consequently, the novel methods developed in this thesis, coupled with new assays to investigate the phosphorylation

status of FAK and paxillin and their downstream targets, are expected to generate a wealth of new information concerning the acute and chronic adaptive response to exercise and the adoption of a sedentary state. T-tubules are the principle site of skeletal muscle glucose uptake in mice (Lauritzen et al., 2006; Wang et al., 1996). Three-dimensional reconstruction of the t-tubules using confocal microscopy (Jayasinghe and Launikonis, 2013) could be used to confirm the observations made in chapter 2 that FAK is at or in close proximity to the t-tubules of skeletal muscle. Intravital imaging of the skeletal muscle of living animals in combination with investigations of GLUT-4 trafficking (for review (Lauritzen, 2013)) would be expected to provide a considerable insight into the functional relevance of FAK at the t-tubules and the role and temporal response of FAK Tyr^{397} and $Tyr^{576/577}$ phosphorylation in insulin- (Lauritzen et al., 2006) and contraction-induced GLUT-4 trafficking (Lauritzen et al., 2010).

As an acute bout of exercise potentiates microvascular insulin action (Rattigan et al., 2001). Future studies should therefore investigate the functional consequences of acute or chronic exercise-induced increases in FAK and paxillin protein content and phosphorylation status in insulin- and exercise-induced microvascular blood flow and capillary recruitment. Contrast enhanced ultrasound could be used (Vincent et al., 2004) to test the hypothesis that a high FAK and paxillin protein content and phosphorylation status leads to superior insulin-stimulated microvascular capillary recruitment and higher rates of skeletal muscle glucose disposal relative to sedentary individuals (Rattigan et al., 2001).

Immunofluorescence microscopy and contrast enhanced ultrasound could also be used to investigate the role of FAK and paxillin in the mechanisms leading to the improved insulin sensitivity, mitochondrial biogenesis and (micro)vascular function in untrained and type II diabetic patients after high-intensity interval training (HIT) involving repeated short (~30 s) exercise bouts (Babraj et al., 2009; Cocks et al., 2013; Little et al., 2011; Richards et al.,

2010; Shepherd et al., 2013). Despite the considerably lower energy expenditure and time spent exercising with HIT, the improvements are comparable to the more traditional endurance-type exercise training (Burgomaster et al., 2008; Cocks et al., 2013; Gibala et al., 2006; Shepherd et al., 2013). The contractile demands of both exercise modes are considerably different yet FAK is responsive to low and high contractile forces (chapter 3 and 5). It would be interesting to see if the high contractile forces generated during each sprint lead to increases in FAK and paxillin protein content and phosphorylation. Moreover, future research should compare the effect of acute exercise bouts and exercise training interventions on FAK content and phosphorylation to better understand why the adaptation to ET and HIT are similar but RT is not.

6.3.2. Role of FAK and paxillin in the adaptation of skeletal muscle to (un)loading

The role of FAK and paxillin in the adaptation of skeletal muscle to mechanical (un)loading is still largely unexplored. Several studies have linked the load-associated increase in FAK and paxillin with increased skeletal muscle mass (Fluck et al., 1999; Gordon et al., 2001), muscle thickness (Narici et al., 2011) and type II fibre hypertrophy (chapter 3 of this thesis). However, it is obvious from these studies that a major limitation in the literature is the lack of evidence for cause and effect.

Because knockout of either FAK or paxillin genes are lethal (Hagel et al., 2002; Ilic et al., 1995), future investigations should take inspiration from a study by Clemente et al., (2007) who used siRNA to deplete the cardiomyocytes of FAK whilst subjecting the heart to an aortic band. After 12 weeks of increased load, siRNA inhibition of FAK prevented the load-induced hypertrophy of the left ventricle (Clemente et al., 2007). These experiments should be complemented with studies of FAK overexpression where gene electrotransfer a FAK-containing plasmid into the skeletal muscle may provide an important insight into the

mechanisms of FAK-mediated hypertrophy (Durieux et al., 2009; Klossner et al., 2009; Klossner et al., 2013).

These studies will clearly provide an insight into the impact of increases in FAK and paxillin protein content and activity in skeletal muscle hypertrophy and will also provide the ideal opportunity to study further the interaction between FAK and paxillin and other FAK-paxillin binding proteins. It will be of great interest to investigate the response of FAK- and paxillin-activated downstream signalling intermediates which may be involved in stimulating skeletal muscle protein synthesis and skeletal muscle hypertrophy. At present, load-activated FAK in skeletal muscle has been linked to the Akt-independent phosphorylation of p70S6K (Klossner et al., 2009), but studies in cardiomyocytes (Clemente et al., 2012; Marin et al., 2008) and in C2C12 skeletal muscle cells after IGF-I stimulation (Crossland et al., 2013) suggest FAK may also signal through mTOR-dependent pathways.

The immunofluorescence methodology developed in this thesis could be used to investigate FAK and paxillin at the (sub)sarcolemma and t-tubules of skeletal muscle fibres in response to siRNA inhibition, overexpression and increased mechanical loading. In addition, it should be investigated whether the gene electrotransfer technique introduces FAK overexpression into the microvasculature. It would be of great interest to see whether overexpression of FAK in the microvasculature leads to increased FAK-mediated angiogenesis (Peng et al., 2004) and improved microvascular function in insulin-resistant rats.

To date, chapter 5 is the only study that has reported the response of FAK protein content after ET but very little is known about the functional relevance and downstream signalling. FAK overexpression in the *m. soleus* was associated with the upregulation of nuclear-encoded mitochondrial enzymes such as cytochrome oxidase IV (Durieux et al., 2009) and FAK was linked to mitochondrial biogenesis following cyclic stretch in

cardiomyocytes (Tornatore et al., 2011). Little is known about the role of paxillin in mitochondrial biogenesis. Moreover, the mechanistic link between FAK and changes in oxidative capacity and mitochondrial biogenesis with (un)loading are lacking. It has been shown that cyclic stretch stimulates the nuclear interaction of FAK with PGC1α in cardiomyocytes (Tornatore et al., 2011). This was associated with the cyclic stretch-induced increase in mitochondrial DNA and inhibition of FAK by siRNA abolished the stretch-induced increase in mitochondrial DNA (Tornatore et al., 2011). These studies suggest FAK is heavily involved in load-induced increases in oxidative capacity and mitochondrial biogenesis but this requires further investigation with immunofluorescence microscopy, especially in human skeletal muscle. These techniques may reveal the colocalisation of FAK and PGC1α in skeletal muscle and confirm that load-induced activation of FAK (and paxillin) is the common factor that leads to comparable increases in oxidative capacity and mitochondrial biogenesis following ET and HIT (Burgomaster et al., 2006; Burgomaster et al., 2008; Gibala et al., 2006; Shepherd et al., 2013) and after RT (Frontera et al., 1990; Jubrias et al., 2001; Tang et al., 2006) despite the comparatively different contractile demands of each training regimen.

6.3.3. Ageing and anabolic resistance

Ageing is associated with an attenuation of the chronic hypertrophic response of skeletal muscle to mechanical loading in rats (Degens and Alway, 2003) and humans (Kosek and Bamman, 2008; Kosek et al., 2006). This is of great clinical significance since periods of immobilisation (e.g. bed rest or cast immobilisation) also lead to a loss of skeletal muscle mass and a resistance to load-induced hypertrophy (Hvid et al., 2010; Suetta et al., 2013; Suetta et al., 2009) will have important ramifications for the accretion of myofibrillar proteins and the accelerated development of sarcopenia. There is evidence that the post-exercise

increases in mTOR, p70S6K and ERK 1/2 phosphorylation are blunted in healthy elderly individuals compared with the young (Drummond et al., 2008; Fry et al., 2011; Kumar et al., 2009) leading to impaired elevations in post-exercise muscle protein synthesis rates (Drummond et al., 2008; Durham et al., 2010; Fry et al., 2011; Kumar et al., 2009). This may contribute to the development of sarcopenia and suggests impairments in contraction-stimulated mechanotransduction. Whether there are defects in signalling upstream of mTOR remains unclear.

The hypothesis is that the decline in physical activity rates with age will reduce the mechanical load upon the skeletal muscle and its associated microvasculature. As FAK and paxillin are mechano-sensitive proteins, this will result in reduced FAK and paxillin protein content and basal phosphorylation status in the skeletal muscle and its microvascular tissues (de Boer et al., 2007; Glover et al., 2008; Gordon et al., 2001; Li et al., 2013) when compared to a physically active young cohort. This would be observed predominately at the (sub)sarcolemmal and intracellular regions of skeletal muscle fibres and within the endothelium and VSMC of the skeletal muscle microvasculature. In the skeletal muscle fibres, the reduced FAK and paxillin protein content and basal phosphorylation status may attenuate the response of FAK to subsequent periods of loading in elderly individuals when compared to a young cohort (Rice et al., 2007). This would lead to the blunted activation of mTOR and p70S6K in response to RT (Drummond et al., 2008; Fry et al., 2011; Kumar et al., 2009) and an attenuated rise in post-exercise skeletal muscle protein synthesis (Drummond et al., 2008; Durham et al., 2010; Fry et al., 2011; Kumar et al., 2009). The net consequence of a chronic reduction in FAK and paxillin basal and post-exercise phosphorylation status and protein content would be type II fibre atrophy and a loss of skeletal muscle strength in elderly individuals when compared with a young cohort.

The association of FAK with PGC1α (Tornatore et al., 2011) in skeletal muscle fibres may also be impaired post-exercise leading to impairments in the post-exercise stimulation of protein synthesis and mitochondrial biogenesis compared with young individuals (Durham et al., 2010). This would manifest as a lower oxidative capacity and reduced mitochondrial content of skeletal muscle and a lower aerobic capacity in elderly compared with young individuals (Coggan et al., 1992; Rooyackers et al., 1996).

The decline in mechanical forces (e.g. fluid shear stress) that act on the microvasculature and the subsequent loss of endothelial FAK and paxillin with age may lead to a reduction in the FAK-associated activation of eNOS (Koshida et al., 2005). This would be associated with a decline in NO bioavailability (Maeda et al., 2004; Taddei et al., 2000) and impaired fluid shear stress-induced endothelial-dependent vasodilation in elderly compared with young individuals (Delp et al., 2008; Muller-Delp et al., 2002; Spier et al., 2007). The reduced content and activation of FAK and paxillin may also impair endothelial cell migration and angiogenesis (Ilic et al., 1995; Shen et al., 2005) leading to a loss of capillaries and a decline in capillary density when compared to a young cohort (Coggan et al., 1992; Degens et al., 1993).

Cross-sectional studies should therefore investigate age-associated differences in the protein content and basal phosphorylation status of FAK and paxillin at (sub)sarcolemmal and intracellular regions of skeletal muscle in young and elderly individuals alongside age-matched elderly master athletes. The differences in physical activity levels and protein content and phosphorylation of both focal adhesion-associated proteins could then be related to and compared against the molecular (e.g. mTOR and p70S6K) and protein synthetic response to an acute bout of endurance- and/ or resistance-type exercise. There is also evidence for gender-specific differences in the FRNK content (endogenous inhibitor of FAK)

of elderly skeletal muscle (Flueck et al., 2011). It would be interesting to investigate the effect of age on FRNK and whether this plays a role in anabolic resistance.

There is evidence that age-associated insulin resistance also leads to impairments in the insulin-stimulated blunting of skeletal muscle protein breakdown (Wilkes et al., 2009). In this study, basal rates of leg protein breakdown were not different between the young and elderly, but physiological elevations in the insulin concentration (15 microIU/mL) reduced the rates of leg protein breakdown by 47% in the young, but only 12% in the elderly (Wilkes et al., 2009). Consequently, an impaired inability to increase skeletal muscle protein synthesis rates after exercise, coupled with the reduced ability of insulin to suppress rates of skeletal muscle protein breakdown, may explain the gradual decline in skeletal muscle mass with age. If future studies confirm a role for FAK in human skeletal muscle insulin signalling, then FAK may play a critical role in the maintenance of skeletal muscle mass through inhibition of protein breakdown as well as activation of protein synthesis.

6.4. Conclusions

An important contribution of this PhD thesis is the development of novel immunofluorescence microscopy methods. These methods have subsequently been used to generate the first information on the spatial distribution, relative protein content and colocalisation of FAK and paxillin in human skeletal muscle fibres and within the skeletal muscle microvascular endothelium and vascular smooth muscle cell layers. The experimental chapters have generated important new information on the effect of exercise training on their protein content in the skeletal muscle and its microvasculature of young and elderly individuals and suggest an important role for both focal adhesion-associated proteins in the adaptive response of these tissues to ET and RT. Future studies should aim to investigate the hypothesis that the focal adhesion-associated proteins are key components in the mechano-

sensing mechanisms that act upstream of the insulin signalling cascade and mTOR and are upregulated by various modes of exercise training and downregulated in the sedentary state, obesity, metabolic syndrome and ageing. In trained and healthy young physically active individuals, this will lead to a high FAK and paxillin content within the skeletal muscle and powerful activation of both focal adhesion-associated proteins by insulin and exercise within the skeletal muscle and its microvasculature. As a result, both tissues will be insulin sensitive, type II skeletal muscle fibres will hypertrophy in response to RT, and the rate of mitochondrial biogenesis and angiogenesis will increase considerably in response to ET. In sedentary and especially elderly sedentary or bed-ridden individuals, the FAK and paxillin protein levels will fall and the ability of insulin and exercise to activate the focal adhesion proteins will be impaired. As a result, both muscle and its microvasculature become insulin resistant, anabolic resistance to RT will lead to sarcopenia, rates of mitochondrial biogenesis and angiogenesis will fall and rarefaction and endothelial dysfunction of the muscle microvasculature will limit the supply of insulin, fuels and oxygen to the skeletal muscle fibres. The novel methods developed in this PhD thesis, in combination with previously developed microscopy methods and complementary methods to measure FAK and paxillin activation and functional measures, will provide a unique tool to investigate these hypotheses and the effect of exercise, diet and pharmacological interventions.

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