Hypoxic preconditioning of myoblasts implanted in a tissue engineering chamber significantly increases local angiogenesis via upregulation of myoblast VEGF-A expression, and downregulation of miRNA-1, miRNA-206 and Angiopoietin 1.

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Short Title: Hypoxic pre-conditioned myoblasts significantly increase *in vivo* angiogenesis via upregulation of VEGF-A, and down regulation of miRNA-1, miRNA-206 and Angiopoietin 1

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ABSTRACT

Vascularization is a major hurdle for growing 3 dimensional tissue engineered constructs. This study investigated the mechanisms involved in hypoxic preconditioning of primary rat myoblasts *in vitro* and their influence on local angiogenesis post-implantation. Primary rat myoblast cultures were exposed to 90 minutes hypoxia at < 1% oxygen followed by normoxia for 24 hours. RT PCR evaluation indicated that 90 min. hypoxia resulted in significant downregulation of miRNA-1 and miRNA-206 ($p<0.05$), and Angiopoietin-1 ($p<0.05$) with upregulation of VEGF-A ($p<0.05$). The miRNA-1 and Angiopoietin 1 responses remained significantly downregulated after a 24 hour rest phase. In addition, direct inhibition of miR-206 in L6 myoblasts caused a significant increase in VEGF-A expression ($p<0.05$), further establishing that changes in VEGF-A expression are influenced by miR-206. Of the myogenic genes examined, MyoD, was significantly upregulated, only after 24 hours rest ($p<0.05$). Preconditioned or control myoblasts were implanted with Matrigel™ into isolated bilateral tissue engineering chambers incorporating a flow-through epigastric vascular pedicle, in severe combined immunodeficiency mice, and chamber tissue harvested 14 days later. Chambers implanted with preconditioned myoblasts had a significantly increased percent volume of blood vessels ($p = 0.0325$) compared to chambers implanted with

control myoblasts. Hypoxic preconditioned myoblasts promote vascularization of constructs via VEGF upregulation, and downregulation of Angiopoietin 1, miRNA-1 and miRNA-206. The relatively simple strategy of hypoxic pre-conditioning of implanted cells - including non-stem cell types has broad, future applications in tissue engineering of skeletal muscle and other tissues, as a technique to significantly increase implant site angiogenesis.

1. INTRODUCTION

Tissue engineering represents a mixed medical discipline, combining collective knowledge of physiology, cell biology, scaffold engineering, and genetics to generate clinically applicable tissues to treat a variety of conditions which cannot be cured with conventional therapies.

To achieve blood perfusion throughout a tissue engineered construct, two techniques have been pursued. Extrinsic vascularisation (Lokmic and Mitchell, 2008) relies on the progressive ingrowth of blood vessels from the surrounding recipient bed to invade and nourish the implanted cell seeded scaffold. The size of the construct that can be effectively vascularised by this technique, and support implanted cells, is restricted to small, thin structures (Lokmic and Mitchell, 2008).

Intrinsic vascularisation involves the direct implantation of a vascular pedicle into the centre of a cell/scaffold construct to augment the vascular source and accelerate vascular invasion. When such a vascular pedicle is inserted in a closed plastic chamber space prolific pedicle angiogenic sprouting ensues, that expands in an attempt to replace the chamber dead space (Lokmic *et al*., 2007). This phenomenon can be exploited to support implanted cells/tissues such as adipose tissue (Dolderer *et al*., 2007), and cardiomyocytes (Morritt *et al*., 2007). In a rat chamber model vascular development peaks between 7 and 10 days (Lokmic *et al*., 2007). In the mouse chamber model the peak is at 2-3 weeks, however the chamber models are partially hypoxic until angiogenic development is maximal (Lokmic *et al*., 2007). Consequently cell implantation at the time of construct creation (day 0), results in significant implanted cell death. Previous experiments have relied on implanting extremely high numbers of cells to overcome this loss (Morritt *et al*., 2007), however this strategy is inefficient. Alternatively cell implantation can be delayed, until the vascular bed growth has peaked. Delayed implantation in the rat chamber has been found to increase implanted myoblast survival compared to day 0 implantation; myoblast survival being positively correlated with the degree of construct vascularisation (Tilkorn *et al*., 2010).

An alternative strategy to enhance vascularization is **cellular preconditioning** where a period of hypoxia/ischaemia, heat shock, or a pharmacological treatment is applied to cells prior to their implantation thereby manipulating cells at a transcriptional level to induce a pro-angiogenic/pro-survival response.

Preconditioning is a highly conserved cell response induced by exposure to a sub lethal stress (such as ischemia/hypoxia or a pharmacological agent), causing a transient phenotypic shift in the cells,

allowing them to compensate and therefore be more tolerant of a subsequent lethal stress (Kirino 2002; Bernhardt *et al*., 2007). First described in whole organs by Murry *et al*., (1986) in the heart, the protective potential of preconditioning has been observed in multiple species and a number of different cells and organs. Preconditioning involves a biphasic upregulation of endogenous trophic signals (Murry *et al*., 1991; Bolli 2000), which results in the acquisition of a phenotype conducive to survival following exposure to a subsequent insult of greater intensity or duration (Samoilov *et al*., 2003; Theus *et al*., 2008). Hypoxic preconditioning of stem cells and occasionally other cell types such as blood cells (Kubo *et al*., 2008) increases survival and angiogenic expression *in vitro*, and enhanced angiogenesis and tissue repair when implanted into infarcted heart tissue (Hu *et al*., 2008; Pasha *et al*., 2008), brain (Wei *et al*., 2012) and skin flaps (Hollenbeck *et al*., 2012).

To date successful myoblast pre-conditioning studies have largely involved pharmacological agents (Niagara *et al*., 2007, Tilkorn *et al*., 2012) or heat shock (Susuki *et al*., 2000) methods. In the heart pharmacological pre-conditioned myoblasts show pro-regenerative and pro-angiogenic capabilities in ischaemic myocardium (Niagara *et al*., 2007).

Hypoxia has been reported to influence the expression of a number of miRNAs, particularly miRNA-210, which has been implicated in the control of angiogenesis (Fasanaro *et al*., 2008) and the survival and differentiation of stem cells (Kim *et al*., 2009). In addition, the muscle-specific miRNA-1 and miRNA-206 are key modulators of myoblast differentiation (Guller and Russell 2010; Ma *et al*., 2015), and expression of these miRNAs has been reported to be downregulated by hypoxia (Liu *et al*., 2012).

This study describes the effects of a controlled exposure of primary rat skeletal muscle myoblasts to low oxygen (hypoxic) conditions *in vitro.* Cultures were exposed to an initial preliminary hypoxic insult of 90 minutes, and allowed to recover in standard conditions (rest phase). RT-PCR examined up and down regulation of genes associated with angiogenesis and myogenic markers in pre-conditioned myoblasts. In *in vivo* experiments pre-conditioned primary myoblasts were transplanted into a mouse tissue engineering chamber to investigate whether chamber angiogenesis and myoblast survival were influenced by pre-conditioned myoblasts.

2. MATERIALS AND METHODS

Animal experiments were approved by the Animal Ethics Committee of St. Vincent's Hospital Melbourne, Australia. Animals were handled in accordance with the National Health and Medical Research Council (NHMRC) of Australia guidelines for the care and maintenance of animals. All surgical procedures (rats and mice) were performed under general anaesthesia. At the conclusion of experiments, animals were euthanized using Lethabarb® (Virbac Animal Health, NSW, Australia).

2.1 Cell culture

2.1.1 Isolation of primary skeletal muscle myoblasts from neonatal rats

Neonatal (3-week-old) inbred Sprague-Dawley rats were anaesthetized (intraperitoneal injection of 75mg.kg^{-1} ketamine and 10mg.kg^{-1} xylazine, 0.2mL.100g^{-1} rat), and the skeletal muscle from the pectoral, abdomen and hind limbs was dissected under aseptic conditions. Tissue was cleared of

connective tissue and associated fascia, and myoblast cultures generated using an isolation and enrichment technique described previously (Tilkorn *et al*., 2010).

Briefly, the isolated tissue was mechanically minced into fine pieces and cells dissociated using an enzymatic digestion. Cells exhibiting a myogenic phenotype, defined by expression of the myogenic marker desmin, were concentrated in the cultures using a preplating technique that exploited differences in the adhesion kinetics between the desired myogenic cells and the contaminating fibroblastic population. Liberated cells were sequentially cultured in treated tissue culture flasks pre-coated with 0.1mg.mL-1 collagen I (PureCol, Inamed Biomaterials) for increasing durations, after which the supernatant containing poorly-adherent cells was transferred to a fresh flask, representing a subsequent preplate. This technique concentrated the myogenic population due to their lesser affinity for the collagen substrate, such that later preplates (5 to 8) were enriched for the desired myoblast population.

Aliquots of cells at each pre-plate were simultaneously cultured in Lab-Tek 8-well chamber slides (Sigma-Aldrich) during isolation and consequently immunolabelled for desmin using a protocol described by Tilkorn *et al*., (2010). Imaging of the later pre-plates was performed to determine the percentage of cells that were desmin positive and contained a high percentage of myoblasts. All subsequent experiments were conducted using cells pooled from preplates 5 to 8.

2.1.2 L6 myoblast culture

Additional experiments involving transfection of miRNA inhibitors were performed in L6 myoblasts. Cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS). L6 cells were used for these experiments due to the difficulties associated with transfection of primary myoblasts, however confirmatory hypoxia experiments identical to those described for primary myoblasts were also performed to establish that L6 cells had similar responses to hypoxia in terms of miR/growth factor expression (data not shown).

2.2 Hypoxic pre-conditioning conditions for in vitro and in vivo experiments

Hypoxic conditions were achieved by displacing oxygen from the media and local atmosphere. Oxygen tension in F10 media without the 20% Fetal Bovine Serum (FBS), termed 'incomplete media', was reduced by displacing the oxygen through infusion of 5%CO_2/95%N_2 gas delivered using a custom built needle (courtesy of Mr Murray Worner, St Vincent's Hospital, Melbourne) for 30mins. Oxygen tension was measured using an InO_2 Dissolved Oxygen Meter (Innovative Instruments Inc, Fl, USA) and was found to decrease from 25.2 ± 1.96% to 3.4 ± 0.28% following treatment. This now hypoxic media was supplemented with 20% FBS and applied to cultures.

Cultures were placed in a custom-built Perspex chamber which was flushed with a 5%CO_2/95%N_2 gas, until the atmospheric oxygen tension was determined to be 0%, detected by an attached oxygen monitor (Teledyne Analytical Instruments, Ca, USA). The media then equilibrates with the atmosphere in the chamber to an oxygen level close to 0-1%. The chamber was sealed and placed in a standard incubator for 90 mins. This period of hypoxic pre-conditioning for both *in vitro* and *in*

vivo assessments was based on previous hypoxia pre-conditioning experiments by our group on L6 myoblasts (Tilkorn *et al.*, 2010) and preliminary *in vitro* studies with primary rat myoblasts.

2.3 In vitro hypoxia response studies

Multiple culture wells were established with fifty thousand primary rat myoblasts in 12-well tissue culture plates at 60-70 percent confluency. Culture media was replaced with either hypoxic or complete media following two washes with PBS. Cultures were subjected to a 90 minute hypoxic preconditioning insult in the hypoxia chamber, or a 90 minute normoxic incubation under standard conditions in an incubator.

2.4 miRNA inhibition experiments

L6 myoblasts were plated at a density of sixty thousand per well in a 24-well tissue culture plate and 750ng of commercially obtained miRNA inhibitors (anti-rno-miR-1-3p, anti-rno-miR-206-3p or miScript Inhibitor Negative Control; Qiagen, Chadstone, Victoria, Australia) were transfected into the cells using HiPerFect transfection reagent (Qiagen, Chadstone, Victoria, Australia) according to manufacturer's instructions. Cells were incubated for 48 hours without medium change before being harvested for mRNA analysis of VEGF-A expression as described below.

2.5 RNA isolation and real-time PCR

RNA was isolated from cultured myoblasts after 90 minutes hypoxia, or after the hypoxic period plus 24 hour rest phase, and at identical points for the control myoblasts using TRIreagent (Life Technologies, Scorsby, Victoria, Australia) following the manufacturer's instructions. RNA concentration was assessed using a Nanodrop ND100 (Thermo Fisher Scientific, Australia). For

growth factor analysis, 100 ng RNA was reverse transcribed using a High Capacity Reverse Transcription kit (Life Technologies) following the manufacturer's instructions. Relative expression of angiogenic growth factors was determined using Taqman® Assays on Demand and Taqman® Fast mastermix with 18S used as the reference gene.

For analysis of mRNA expression of myogenic markers, RNA was reverse-transcribed using a Quantitect Reverse Trancriptase Kit (Qiagen, Chadstone, Victoria, Australia) following the manufacturer's instructions. Relative expression of myogenic markers was determined using the QuantiFast SYBR Green PCR kit (Qiagen, Chadstone, Victoria, Australia) with 18S used as the reference gene (see Table 1 for primers). For analysis of miRNA expression, cDNA was reverse transcribed using a miScript II RT kit (Qiagen, Chadstone, Victoria, Australia). Relative expression of miRNAs was analysed using a miScript SYBR Green PCR kit and miRNA-specific primer assays (Qiagen, Chadstone, Victoria, Australia), using RNU6B as the reference gene.

2.6 Western blot analysis

Total protein from primary myoblast hypoxic and control cultures at 90 mins and after a 24 rest phase was extracted using RIPA buffer (Sigma, Castle Hill, Australia) with 1 µL/mL protease inhibitor cocktail (Roche) and 10 µL/mL Phosphatase Inhibitor Cocktail (Sigma, Castle Hill, Australia). The extracts were incubated for 30min at 4°C with gentle agitation, and then cleared by centrifugation at 13,200g for 15 min at 4°C before total protein content was determined using the D_C Protein Assay Kit (BioRad, Gladesville, NSW, Australia) according to the manufacturer's instructions. Samples were heated at 95°C for 3 min in SDS sample loading buffer consisting of 100µl of 2-Mercaptoethanol (BioRad Laboratories, Gladsville, NSW) per 900µl of 4x Laemmli

Sample Buffer (BioRad Laboratories, Gladsville, NSW) and equal amounts of protein were then separated on Mini-PROTEAN® 4-15% TGX™ pre-cast gels (BioRad Laboratories, Gladsville, NSW). Samples were then transferred to Immuno-Blot® PVDF membranes (BioRad Laboratories, Gladsville, NSW), blocked with 5% milk in TBST and incubated overnight at 4°C with primary antibodies to HIF-1α (1:500; Novus Biological, Littleton, USA). The membranes were then washed in TBST and incubated for 1h with Goat Anti-Mouse antibody conjugated to HRP (1:10,000; BioRad, Gladsville, NSW). The signal was developed using Clarity™ Western ECL Blotting Substrate and imaged using a ChemiDoc XRS with Image Lab software (BioRad, Gladsville, NSW). Following imaging the membrane was stained with Coomassie R250 for confirmation of total protein loading as previously described (Welinder and Ekblad, 2011).

2.7 In vivo tissue engineering experiments

Eighteen adult male severe combined immunodeficiency (SCID) mice received bilateral vascular pedicle chambers under general anaesthesia (chloral hydrate, intra-peritoneal injection, dose: 4mg/g mouse body weight), and were randomly divided into two groups, defined by the time at which cells were implanted into their chambers (at the time of chamber creation - day 0, or 14 days later, N=9 mice/group). Implanted cells were subjected to either a 90 minute hypoxic preconditioning insult (experimental group) or 90 minutes normoxia (control group) period, followed by a 24 hour rest phase in normoxic culture conditions, prior to implantation.

2.7.1 Murine flow-through pedicle chamber model

Bilateral vascularised chambers, first described by Cronin *et al*., (2004) were created around each of the epigastric vascular pedicles in the groin. Briefly, a transverse groin incision was made above the

groin fat pad. The fat pad was dissected free from the underlying muscle facilitating introduction of the chamber around the exposed epigastric vessels which were cleared of connective tissue. The chamber consisted of sterile standard cylindrical silicone non-collapsible laboratory tubing cut to produce chambers 5mm in length with a diameter of 3.35mm and internal volume of 50µL. A longitudinal incision was made along the chamber to facilitate the wrapping of the chamber around the first 8 to 10mm of the superficial epigastric vessels. The chamber end closest to the vessel origin and the longitudinal cut were sealed using melted bone wax. Forty-five 45µL of full Matrigel™ with or without cells depending on the implantation time were injected in the chamber (see below) and the open chamber end closed with bone wax. The skin wounds were closed with metal clips and the mouse allowed to recover.

2.7.2 Implanted myoblast cell labelling

Five hundred thousand myoblasts exposed to the preconditioning hypoxic insult or control conditions were labelled with Cell Tracker CM-DiI (Molecular Probes™, Life Technologies, Scoresby, Victoria, Australia), as described previously (Tilkorn *et al.*, 2010).

2.7.3 Day 0 implantation

Five hundred thousand myoblasts implanted on day 0, hypoxic preconditioned or control (one cell type into each of the bilateral chambers), were evenly distributed in 45µL BD Full Matrigel™ (BD Biosciences, Vic, Australia) which was injected into the bilateral chambers at the time of chamber creation.

2.7.4 Day 14 – Delayed implantation

Chambers created for delayed implantation were filled with 45µL Matrigel™ without cells at day 0, the chamber sealed and wounds closed and the mice resuscitated. These mice underwent a second surgery 14 days after the creation of the chambers. The chambers were partially exposed and the wax covering the chamber proximal to the femoral vessels was removed to allow 0.5 $x10^6$ hypoxic preconditioned cells to be inserted in one chamber and control myoblasts to be injected in the opposite chamber in a 10 µL volume of Matrigel. (Note: some fluid was lost from the chamber when reopened that allowed another 10 µL of Matrigel to be added). The chamber was re-sealed with wax and the skin wounds closed.

2.7.5 Construct harvest, pedicle patency, construct weight, and volume

Two weeks after cell implantation (that is, at either day 14 for day 0 implantations, or day 28 for day 14 implantations), mice were anesthetized and chambers reopened. Patency of the epigastric vessels was determined by venous outflow after cutting the proximal vessels. The constructs (contents of the chamber) were retrieved, weighed, and volume determined using a standard water displacement technique (Scherle, 1970).

2.8 Tissue processing and histology

Tissue constructs were immersion fixed in 4% paraformaldehyde and processed routinely to paraffin wax. Five µm-thick sections, were mounted onto 3-aminopropyltriethoxy-silane (Sigma-Aldrich, Castle Hill, NSW, Australia) as well as Polysine (Thermo Scientific, Loughborough, UK) slides for morphologic, morphometric, and immunohistochemical assessment. Sections were stained with haematoxylin and eosin for routine morphological assessment.

2.9 Immunohistochemistry

2.9.1 Desmin

Desmin labelling of myoblast cultures at pre-plates 5 and 6, and 7 and 8 to confirm the percentage of myoblasts present in cultures was conducted on 8 well chamber slides as described previously (Tilkorn *et al*., 2010).

For tissue sections of *in vivo* chamber constructs the same desmin antibody (Dako, Carpinteria, Calif., USA) was used for the additional identification of implanted myoblasts, modified for use with a Dako Autostainer. After dewaxing and hydration, sections underwent antigen retrieval in 10mM EDTA (pH 8.0) buffer in a waterbath at 95ºC for 30 min. Endogenous peroxidase was blocked in 3% H_2O_2 for 10 min, and sections were then pre-incubated in 10% normal rabbit serum for 30 min, incubated with primary antibody at 1:100 for 60 min, followed by biotinylated rabbit anti-mouse secondary antibody (Dako) at 1:200 for 30 min and HRP-streptavidin (Dako) at 1:400 for 30 min. Colour was developed with DAB chromagen (Dako) for 3 min and sections then counterstained with haematoxylin, dehydrated, and coverslipped using DPX mounting medium (BDH/Biolab, Auckland, New Zealand). Isotype substituted negative controls (Jackson Immunoresearch, West Grove, Pa., USA) were performed simultaneously.

2.9.2 CD31

CD31 (BD Biosciences, San Jose, CA, USA) was used to label blood vessels in the construct. The method was as described above, with the following changes: antigen retrieval was via proteinase K (Dako) for 4 min, protein blocking via 10% normal rabbit serum, primary antibody was applied at

1:150, followed by rabbit anti-rat biotin and Vector (Burlingame, Calif., USA) ABC elite for 30 min as the enzyme label.

2.9.3 VEGF A

Immunostaining using a rabbit anti human VEGF antibody (Abcam, Cambridge, UK) was performed manually, with the method otherwise as above with the following changes: antigen retrieval was in 10mM tri-sodium citrate buffer, at pH 6.0, for 20 mins, 10% normal goat serum was used for protein blocking, primary was applied at 1:100 and goat anti rabbit - HRP (Dako) at 1:200 was used as enzyme-labelled secondary.

2.10 Photography

Sections were viewed using an Axioscope 2 Microscope and photographed using an attached Zeiss Axiocam MRc5 digital camera using the same microscope and camera, with fluorescence observed using the Epi-fluorescence attachment. The primary filter, with excitation maxima of 320 nm (ultraviolet) and emission maxima of 420+nm (violet/blue-white), was used for visualisation of DAPI. The secondary filter, with excitation maxima of 546 nm (green) and emission maxima of 590+nm (red), was used for visualisation of DiI labelling.

2.11 Morphometry

Morphometric analysis was completed using a Zeiss Axioskop 2 Microscope interfaced with digital video imaging (TKC1480E; JVC, Kingsgrove, Australia) and an automated, systematic random

sampling point-counting system (CAST system, Olympus) at a magnification of 20x. The observer (MW) was blinded to the identity of the sections.

2.11.1 Myoblast density

Counting of desmin-labelled constructs was conducted on two sections, located 250μm apart. The number of desmin-labelled cells per unit area (mm^2) was counted using complete sections that were outlined with their total area recorded. This outlined area was systematically, randomly sampled using the CAST grid-point counting system, such that the analysed fields represented 10 percent of the tissue section area. A 16 point grid was applied and non-nucleated, mononucleated and multinucleated desmin labelled rat myoblasts were counted for each of the randomly sampled fields, using standard forbidden line rules. Desmin-positive rat myoblasts were identified by their intense desmin labelling, large ovoid pale blue nuclei and relatively larger overall size compared to the resident mouse-derived cells. The number of myoblasts was calculated with respect to section area, and myoblast density determined per square millimetre.

2.11.2 Percent volume of myoblasts

The percent volume of implanted desmin-positive myoblasts was also determined using the CAST grid-point counting system. A 16 point grid was applied to systematically, randomly sampled fields representing 10 percent of the tissue sectional area. Desmin-positive rat myoblasts were counted and other tissue points recorded separately. The percent volume of myoblasts is presented as the average of the two sections located 250μm apart in the constructs.

2.11.3 Percent volume of blood vessels

The percentage volume of new blood vessels was determined using one, complete section from the centre of the construct, and similarly determined using the CAST grid-point counting system. A 16 point grid was applied and points located on an endothelial cell or within a luminal structure were recorded, with other tissue points recorded separately. Two distinct groups of vessels were identified and counted during analysis; one group exhibited the characteristic morphology of typical blood vessels, a lumen was present surrounded by flattened endothelial cells, with strong endothelial cell CD31 labelling, while the other group of vessels shared this morphology, but showed only partial CD31 labelling. These counts were recorded independently, but combined to determine the percent volume of blood vessels in the constructs.

2.11.4 Distance of vessel growth from the vascular pedicle

The distance of furthest blood vessel (capillary) growth from the pedicle was measured in a central longitudinal CD31 immunolabelled section from each construct. The probe was positioned so that it measured the distance of a line positioned perpendicular to the outside of the chamber vascular pedicle to the point of furthest capillary growth from the pedicle. A Mean +/- Standard Error of the Mean (SEM) was determined for each group.

2.12 Statistical analysis

All data are represented as mean ± standard error of the mean (SEM). Data was analysed using unpaired t-tests (*in vitro* data) and two way ANOVA (*in vivo* data) on GraphPad Prism for Windows Version 5.01 (Software MacKiev™), and significance was set at $p<0.05$.

3. RESULTS

3.1 Primary myoblast culture and the in vitro hypoxic preconditioning regime

Imaged cells from preplates 5 and 6 were observed to contain a consistent cell composition, where desmin-positive cells (myoblasts) represented between 50 to 60 percent of the cells in culture (Figure 1A). Preplates 7 and 8 were highly enriched for desmin-positive cells, which represented between 80 to 90 percent of the cells in culture (Figure 1B). All experiments were conducted using cells pooled from preplates 5 to 8. As such, the composition of cultures used in all pre-conditioning and control experiments (*in vitro* and *in vivo*) was 70 percent desmin-positive myoblasts. The preconditioning regime used is illustrated in Figure 1C.

3.2 In vitro: RT-PCR and Western Blot analysis

Preconditioning cells with a 90 min hypoxic insult significantly inhibited expression of miR-1 and miR-206 in myoblasts ($p<0.05$) (Figure 2A). Analysis of myogenic markers indicated that there was no change in expression of Pax7, MyoD or Myogenin following 90 min of hypoxia (Figure 2B). Following 24 hours recovery, myoblasts which had initially been exposed to the 90 min pre-conditioning hypoxic insult continued to display reduced expression of miR-1 ($p<0.05$), and a non-significant trend toward reduced expression of miR-206 ($p=0.076$) when compared to control cells (Figure 2C). Furthermore, analysis of myogenic markers revealed that following 24 hours of recovery, pre-conditioned myoblasts had a significant upregulation of MyoD ($p<0.05$), with no significant changes in Pax7 or Myogenin expression (Figure 2D).

Analysis of angiogenic markers revealed a significant upregulation of VEGF-A and a significant downregulation of Angiopoietin-1 in myoblasts (p<0.05) (Figure 3A), whilst there was no change in FGF-2, PDGF-B and HIF -1α expression between groups at 90 minutes (Figure 3A). Additionally, Western blot analysis demonstrated low expression of HIF-1α in all samples, and specifically no change in HIF-1α protein expression in hypoxic myoblasts compared to normoxic controls at 90 min, or following a further 24 hours rest post-hypoxia (Figure 3C).

After 24 hours rest the pre-conditioned cells no longer had any differences in VEGF-A expression, and no differences in the expression of HIF -1α , FGF-2 and PDGF-B between control and pre-conditioned groups (Figure 3B), however Angiopoietin-1 expression remained significantly downregulated (p<0.05) (Figure 3B).

3.3 miRNA inhibition

Transfection of the L6 rat myoblast line with an inhibitor of miR-1 had no effect on VEGF-A gene expression in L6 myoblasts, however, inhibition of miR-206 significantly increased VEGF-A gene expression (p<0.05) (Figure 3D).

3.4 In vivo implantation experiments

3.4.1 Construct patency, weight and volume at harvest

Preconditioned or control myoblasts were implanted at day 0 or day 14 in a vascularized tissue engineering chamber created around the epigastric vessels (Figure 4). All mice survived the surgical procedure(s) and recovered quickly without any signs of distress after their respective operations.

No surgical complications occurred. However, four chambers were excluded from analysis post chamber harvest due to pedicle occlusion and infection. This left N= 8 in each of the Day 0 control and preconditioned myoblast implantation groups, N=7 in the Day 14 control myoblast implantation group and N=9 in the Day 14 preconditioned myoblast implantation group. 'Constructs' defined as the vascular pedicle and surrounding Matrigel and tissue contents, with the silicon chamber removed, consistently appeared as cylindrical structures, tapering towards the distal end of the construct (Figure 5A). There was no significant difference between groups in overall weight and volume of constructs at harvest (Figure 5B and C).

3.4.2 Histological examination

There were no differences in the general morphological features of constructs that contained preconditioned or control cells implanted at either day 0 or 14. (Figures 6 and 7). . The vascular pedicle was evident running down the posterior length of each construct. Matrigel occupied the large central region and was surrounded by a cellular capsule. The Matrigel region was invaded by a variety of mouse-derived cells including macrophages and fibroblasts, and cells associated with the developing vasculature (endothelial cells, pericytes and vascular smooth muscle cells).

3.4.3 Desmin immunolabelling

Within the Matrigel region implanted rat myoblasts were readily identified by their characteristic intense HRP labelling and larger overall size (Figure 6 A-H), relative to local host (mouse) cells. The distribution of the implanted myoblasts appeared to be influenced by implantation time. Constructs implanted with cells at day 0 contained myoblasts dispersed throughout the Matrigel (Figures 6A and C). Whereas, constructs implanted with cells at day 14 (delayed implantation)

contained a concentrated region of implanted cells some distance from the vascular pedicle (Figure 6E and G). Mononuclear myoblasts and multinuclear myotubes with myofibrillar striations were observed in constructs (Figure 6I). All constructs regardless of myoblast treatment demonstrated striations in myotubes.

3.4.4 DiI fluorescent tracking

Myoblasts and myotubes were elongated cells and could be identified by distinct punctate DiI labelling along the length of their plasma membrane (Figure 6J, K) in constructs at both harvest time points and in Control and Pre-conditioned cells.

3.4.5 VEGF immunolabelling

Both control and preconditioned implanted myoblasts in chambers 2 weeks post-implantation were found to be positive for VEGF labelling indicating both were producing this angiogenic growth factor (Figures 6L and M).

3.4.6 CD31 immunolabelling

The CD31 positive vascular pedicle was observed to run longitudinally along the posterior side of the construct (Figure 7A, B). Capillaries were observed sprouting from the pedicle and extending into the Matrigel region (Figure7C). . Constructs implanted with preconditioned cells contained

more blood vessels compared to those implanted with control myoblasts (compare Figures 7A with Figure 7B, and Figure 7C with Figure 7D).

All constructs were observed to contain strongly CD31 positive new vessels: capillaries, venules and arterioles. Less mature vessels were observed in all constructs at the periphery of the developing vasculature. These structures exhibited the typical morphology of vessels, including one or more cellular layers around a defined lumen containing blood, but demonstrated diffuse to no CD 31 labelling (Figure 7E).

3.4.7 Morphometric Analysis

Myoblasts were measured as either myoblast density (myoblasts/mm^2) or as the percent volume of myoblasts in the constructs. There was no survival advantage to myoblasts conferred by hypoxic preconditioning prior to implantation compared to control myoblasts, as assessed by either measurement (Figure 8A and B). Preconditioned cells implanted into chambers at day 0 appeared to be at a significant survival disadvantage compared to control cells ($p < 0.05$), when assessed by percent myoblast volume (Figure 8B). There was a general tendency for myoblast survival to be reduced when implantation was delayed (to 14 days) regardless of prior myoblast treatment.

Constructs containing preconditioned myoblasts demonstrated a significantly increased percent volume of new blood vessels (Figure 8C, $p = 0.0325$). This indicated that the preconditioned myoblast phenotype induced by hypoxia prior to implantation promoted vascularization of constructs independent of the time of implantation (Figure 8C).

The distance of maximal capillary growth from the pedicle was measured in each group. At both time points (day 0 implantation, 14 days harvest, and Day 14 implantation, day 28 harvest) the preconditioned group at each time point had increased maximal capillary growth length compared to control groups (Day 0 implantation: control myoblast constructs - 0.931+/-0.101 mm (Mean +/- SEM) versus preconditioned myoblast constructs - 1.110+/- 0.063mm; Day 14 implantation: control myoblast constructs – 0.987+/-0.045mm versus pre-conditioned myoblast constructs 1.204+/- 0.115mm). These differences were not significant.

4. DISCUSSION

This study has explored the effects of an *in vitro* pre-conditioning hypoxic insult on primary rat myoblasts in relation to regulation of myoblast angiogenic pathway genes, and their role in influencing local *in vivo* angiogenesis when implanted in a tissue engineering chamber. Tissue engineering constructs implanted with hypoxic pre-conditioned myoblasts had a significantly increased percent volume of new blood vessels compared to control myoblast implanted chambers when explored after 14 days.

Muscle-derived cells are intrinsically tolerant to low oxygen tension, with the natural physiological oxygen tension in healthy skeletal muscle estimated to be approximately 5% (Richardson *et al*., 1998; Brevetti *et al.,* 2003; Yun *et al*., 2005). The oxygen tension in muscle tissues is significantly lower than standard culture conditions. *In vivo,* skeletal muscle is routinely exposed to transient periods of "physiological hypoxia", for example during periods of intense exercise, where the oxygen tension is estimated to fall to 2%. During extreme "pathological hypoxia", such as during arterial occlusion or amputation (Yun *et al*., 2005) oxygen tension is estimated to fall to as low as 0.5-0.01%.

Implantation in the chamber did not demonstrate that preconditioned myoblasts had improved survival at either implantation time point (0 or 14 days). Despite this, hypoxia pre-conditioned myoblasts promoted significant additional new blood vessel growth from the vascular pedicle of the chamber. Myoblasts are known to express VEGF (Germani *et al*., 2003) a potent agent stimulating angiogenesis (Shweiki *et al.,* 1995) and this study confirmed that *in vivo* these cells are producing VEGF. In *in vitro* experiments this pro-angiogenic agent was significantly upregulated whilst another angiogenic agent Angiopoietin 1 was significantly downregulated after 90 mins hypoxic preconditioning, with the Angiopoietin 1 down regulation continuing for another 24 hours, ,whilst FGF-2, PDGF-B and HIF 1-α were relatively unchanged in the same cells after 90 minutes hypoxia and 24 hours later.

The stimulated release of vascular endothelial growth factor (VEGF) in response to a hypoxic insult has been documented in other cells and tissues, for example in the brain, with neural stem/progenitor cells promoting endothelial cell proliferation (Roitak *et al*., 2008), and in the kidney, with hypoxic conditions stimulating the production of VEGF by human renal proximal tubular epithelial cells in culture (Nakamura *et al*., 2006). Hsiao *et al*., (2013) have demonstrated that a single *in vitro* exposure of hypoxia to adipose derived MSC significantly increased the transcription and the production of VEGF-A. Sponges treated with conditioned media from hypoxia preconditioned ASCs demonstrated significantly increased angiogenic growth within the sponge at 2 weeks. The benefits of promoting angiogenesis using cells pre-treated with a hypoxic insult have been observed in other facets of regenerative medicine. Preconditioned stem cells injected into an infarcted heart promoted healing partially through increased angiogenesis (Hu *et al*., 2008; Pasha *et*

al., 2008). Similarly hypoxic preconditioned adipose derived mesenchymal stem cells (MSCs) promote skin flap viability (Hollenbeck *et al*., 2012), whilst hypoxic pre-conditioning of bone marrow MSCs promoted angiogenesis and neurogenesis after cerebral ischaemia (Wei *et al*., 2012). These studies largely used hypoxic preconditioning of stem cells. The study reported here has employed *in vitro* hypoxic pre-conditioning of myoblasts – a progenitor cell – not a stem cell to produce significant increases in vascularization post *in vivo* implantation, this opens a range of possibilities for further skeletal muscle tissue engineering studies.

Micro RNAs are known to be involved in the response of cells to hypoxia. PCR analysis of myoblasts *in vitro* indicated that the initial 90 min hypoxic insult significantly downregulated the expression of miR-1 and miR-206, whilst VEGF-A expression was significantly upregulated, and Angiopoietin -1 significantly downregulated. In addition, after a further 24 hrs of normoxia, both miR-1 and Angiopoietin -1 expression remained significantly down regulated. The down regulation of miR-1 and miR-206 agrees with previous studies which have shown that expression of these miRNAs in myoblasts is downregulated by hypoxia (Liu *et al*., 2012), while an upregulation of VEGF expression has been previously demonstrated in many tissues including ischaemic muscle (Wang *et al*., 2011). Importantly, downregulation of miR-1 and miR-206 has been reported to promote angiogenesis in a zebrafish model via an increase in expression of VEGF-A (Stahlhut *et al*., 2012). In addition, the present study further demonstrated that inhibition of miR-1 had no effect on VEGF-A expression, however inhibition of miR-206 significantly increased VEGF-A expression in L6 myoblasts. Taken together, it seems likely that hypoxic pre-conditioning of skeletal myoblasts in the present study triggered a decrease in miR-1 and miR-206, leading to an increase in VEGF expression. Supporting this possibility are previous reports that in addition to increasing VEGF

expression, hypoxia or ischemia result in downregulation of Angiopoietin-1 in both human endometrial stromal cells (Tsuzuki *et al*., 2013) and mouse hindlimb muscles (Wang *et al.,* 2011). Down regulation of Angiopoietin-1 [which is associated with vessel maturation, and decreasing the angiogenic response in proliferating capillaries (Saharinen and Alitalo, 2011)] in the presence of increased VEGF is associated with new blood vessel formation (Visconti *et al*., 2002), and thus may explain the increased vascularisation observed in the constructs implanted with pre-conditioned myoblasts.

The study demonstrated an upregulation of VEGF-A expression after 90 minutes of hypoxia, but not after 24 hours rest when the preconditioned myoblasts were implanted. The timing of the VEGF-A upregulation in pre-conditioned myoblasts would appear to be too early to influence chamber angiogenesis. However it is known that the chamber environment is hypoxic (Lokmic *et al.*, 2007) for several weeks, and it is also known that subsequent hypoxic insults on previously (hypoxia) preconditioned cells dramatically increases their VEGF production and release. Bader *et al*., (2015) found that hypoxic preconditioned cord blood MSCs double their VEGF production compared to non pre conditioned MSCs when exposed to subsequent ischemic conditions. Therefore the hypoxic chamber conditions are likely to further upregulate VEGF-A expression, production and release in previously pre-conditioned myoblasts thereby increasing angiogenesis in chambers receiving pre-conditioned myoblasts.

In addition to affecting the expression of angiogenic factors, a 90 min hypoxic pre-conditioning followed by 24 hrs of normoxia resulted in a significant upregulation of MyoD expression in myoblasts, agreeing with previous studies (Koning *et al*., 2011).

The bidirectional interplay of endothelial cells and myogenic cells in repairing skeletal muscle is well recognised (Abou-Khalil *et al.*, 2010) and VEGF is a significant signalling molecule in this interplay, being expressed as demonstrated by myoblasts *in vitro* and *in vivo* in this study and in other studies (Germani *et al.*, 2003; Bryan *et al.*, 2008). Not only does VEGF stimulate associated angiogenesis it also stimulates the migration of myogenic cells and protects them from apoptosis, and it has been proposed that VEGF secretion is regulated through the Akt pathway (Takahashi *et al.*, 2002). Although FGF-2 is also released by skeletal muscle during ischaemia (Walgenbach *et al.*, 1995) and can stimulate angiogenesis, we could not demonstrate an increase in gene expression of this growth factor in myoblasts under the hypoxic conditions of this study. We could also not detect any changes in HIF1-α expression in hypoxic myoblasts at either the transcript or protein level, suggesting that angiogenic signalling in this context may be HIF1α-independent.

5. CONCLUSION

This study illustrates the positive, tissue regenerative influence that implanted cells including cell types such as primary myoblasts rather than the more commonly transplanted mesenchymal stem cells can have on the local environment. A short period of hypoxic pre-conditioning prior to implantation can significantly increase implantation site angiogenesis, regardless of the ultimate survival rate of the implanted cells. This has important implications for tissue engineering and cell therapies in regenerative medicine where the stimulation of local angiogenesis is desirable. The mechanism by which this was achieved was also examined and implicated the down regulation of miRNA-1, miRNA-206 and Angiopoietin-1 and the concurrent upregulation of VEGF, and is likely to occur in other cell types under similar pre-conditioning regimes.

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Figure Legends

Figure 1: In vitro study

A and B: Representative fields of desmin labelled primary rat myoblast cultures on a 8-well chamber slide prepared for *in vivo* implantation, corresponding to pre-plates 5&6 (**A**) and pre-plates 7&8 (**B**).Scale bars = 50 µm.

C: Diagramatic representation of *in vitro* preconditioning protocol.

Figure 2. mRNA expression of miRNAs and myogenic markers in hypoxic preconditioned and control myoblasts.

Relative mRNA expression of miRNAs (**A and C**) or myogenic markers (**B and D**) following hypoxic preconditioning in primary rat myoblasts either immediately following the 90 min hypoxia (**A and B**) or after a further 24hrs recovery (**C & D**). (N= 6 for A, B, C and D). **p*<0.05.

Figure 3. mRNA expression of angiogenic markers in hypoxic preconditioned and control myoblasts

Relative mRNA expression of angiogenic markers following hypoxic preconditioning in primary rat myoblasts either immediately following the 90 min hypoxia (**A**) or after a further 24hrs recovery (**B**). (N=6 for both A and B). (**C**) Western blot for HIF-1α protein following 90 min hypoxia (lane 2) or after a further 24hrs recovery (lane 4) and their respective normoxic controls (lanes 3 & 5)

(**D**) VEGF-A mRNA expression following inhibition of miR-1 or miR-206 in L6 myoblasts. (N=4). **p*<0.05.

Figure 4: Diagrammatic representation of the *in vivo* chamber model.

Figure 5: *In vivo* tissue engineering construct weight and volume

A: Construct after removal of silicone chamber and associated wax. The epigastric pedicle is evident (thick black arrow) running longitudinally along the length of the construct, and new blood vessels are branching of the pedicle (smaller arrows) to supply the developing construct.

B: The mean weight (g) (±SEM) of constructs seeded with control or preconditioned cells in the day 0 implantation and day 14 (delayed) implantation groups.

C: The mean volume (µL) (±SEM) of constructs seeded with control or preconditioned cells in the day 0 implantation and day 14 (delayed) implantation groups.

N numbers for each counted group: Day 0 implantation (both groups) N=8. Day 14 implantation, Control myoblasts N=7, Preconditioned myoblasts N=9.

Figure 6 Desmin and VEGF immunohistochemical labeling, and DiI fluorescence tracking of myoblasts in the *in vivo* tissue engineering construct

A, C, E and G: Low power (1.5xobj) view of constructs (arrows: myoblast), and high power view of myoblasts: **B, D, F, and H** (40xObj). A and B: Construct containing control (non-preconditioned) myoblasts implanted at day 0, harvested at day 14. C and D: Construct containing preconditioned myoblasts implanted at day 0, harvested at day 14. E and F: Construct containing control (non-preconditioned) myoblasts implanted at day 14, harvested at day 28. G and H: Construct containing preconditioned myoblasts implanted at day 14, harvested at day 28. Myoblasts characterised by their irregular organization and intense desmin immunolabelling (brown) (B, D, F and H).

I: enlargement of myoblasts in F. Note: myofibrillar striations (arrows) evident in myotubes.

J and K: DiI fluorescence labelling rat-derived myoblasts. Positive DiI labelling of implanted cells in a chamber at day 28, left, control (J); right, preconditioned (K). Myoblasts demonstrated distinct punctate labeling (specific labelling in elongated cells which are surrounded by white box).

M and L: VEGF immunolabelling of control, (L) and preconditioned (M) myoblasts (arrows) in the chamber at 14 days.

Scale bars: A, C, E and G: 1,000 µm; B, D, F, H, I, J, K, L and M=50 µm

Figure 7 CD31 immunohistochemical labelling of blood vessel development in constructs implanted with hypoxic pre-conditioned myoblasts and control myoblasts

A, B: Low-power microscopic (1.5xObj) view of constructs (arrows: new CD31+ blood vessels; p: pedicle). **A**: Construct containing control (non-preconditioned) myoblasts implanted at day 14, harvested at day 28. **B**: Construct containing preconditioned myoblasts implanted at day 14, harvested at day 28. Note area surrounded by black line in A and B indicates the area of the chamber infiltrated with CD31+ blood vessels. The area of infiltration is larger in the construct implanted with pre-conditioned myoblasts (B) compared to the chamber that received control

myoblasts (A). **C and D**: Higher-power microscopic view illustrating an increase in number of CD31+ blood vessels in chambers receiving pre-conditioned myoblasts (D) compared to control myoblasts (C). New micro-vessels (arrows, CD31+ immunolabelling – brown labelled structures), with typical vessel morphology and strong CD31 labelling. Thicker arrow in C indicates a vessel branching directly off the epigastric vein (the pedicle: p).

E: Higher power of new blood vessels that are showing very little CD 31^{+} immunolabelling (arrows) in the outer regions of the chamber, furthest from the pedicle.

Scale bars: A, B:1,000 µm; C and D:200 µm; E: 50 µm.

Figure 8 Morphometric analysis of the *in vivo* tissue engineering construct

A: Effects of *in vitro* hypoxic preconditioning and time of cell implantation on number of myoblasts/mm^2. Overall, there was no significant effect for either parameter (2-way ANOVA). Post-hoc tests (Bonferroni) indicated a significant difference in control myoblast survival when myoblasts were implanted at day 14 (delayed) compared to implantation at day 0 (* $p < 0.01$).

B: Effects of *in vitro* hypoxic preconditioning and time of cell implantation on percent volume of myoblasts. Overall, there was no significant effect in either parameter (2-way ANOVA). Post-hoc tests (Bonferroni) indicated a significant difference in percent volume of myoblasts in constructs with preconditioned myoblasts compared to control myoblasts, in the day 0 implantation group *(* $p < 0.05$).

C: Effects of *in vitro* hypoxic preconditioning and time of cell implantation on percent volume of new blood vessels in the chamber at 14 and 28 day harvest. Hypoxic preconditioning of myoblasts prior to implantation overall was found to significantly increase construct vascularization ($p = 0.0325$) (two-way ANOVA). Bonferroni's multiple comparison post-hoc test was subsequently used to test individual group differences, without significance.

N numbers for each group (counted constructs): Day 0 implantation (both groups) N=8. Day 14 implantation, Control myoblasts N=7, Day 14 implantation, Preconditioned myoblasts N=9.

Table 1 – PCR primer sequences for myogenic markers

Target	Forward -5’	Reverse- 5’
18S	GGTGCATGGCCGTTCTTA	TCGTTCGTTATCGGAATTAACC
Pax7	GAACCGTCTGGATGAGGGCTCAGA	GCTCCTCCAGCTGCTCGGCTGTGA
MyoD	CGCTCCAACTGCTCTGATG	GACACAGCCGCACTCTTC
Myogenin	CTGCCACAAGCCAGACTC	GACTCCATCTTTCTCTCCTCAG