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Transplantation of Allogeneic PW1^{pos}/Pax7^{neg} Interstitial Cells (PICs) Enhance Endogenous Repair of Injured Porcine Skeletal Muscle

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(Activation of vasculature-associated stem cells and muscle stem cells for the repair and maintenance of muscle tissue). There are no relationships with industry.

Structured Abstract:

Objective: To assess whether delivery of allogeneic skeletal muscle-derived PW1^{pos}/Pax7^{neg} interstitial cells (PICs) enhance the regeneration of injured skeletal muscle in a porcine pre-clinical model, with similarities to human muscle, in terms of anatomical size, tissue biology, and physiology.

Background: An accepted consensus has gained ground that cell therapy protocols exert their beneficial effect by a 'paracrine' mechanism of the transplanted cells through the activation of endogenous reparative processes. PICs can be reproducibly isolated from skeletal muscle, propagated over long-term culture, while maintaining a stable phenotype/genotype.

Methods: We induced injury through a localized injection of cardiotoxin to Tibialis Anterior skeletal muscle of pigs. This was followed by intra-muscular delivery of 20x10^6 allogeneic GFP^{pos} pPICs per pig or saline vehicle control. BrdU was administered to track cell regeneration. Pigs were sacrificed 14 days post-injury. The secretome of transplanted pPICs was assessed using qRT-PCR arrays.

Results: Allogeneic, GFP^{pos} pPIC transplantation significantly accelerated and improved endogenous, GFP-negative skeletal muscle fiber (15±3% vs. 2±1%/total myofiber) and capillary (0.15±0.04 vs. 0.04±0.01/myofiber) regeneration, compared to saline vehicle control. Moreover, pPIC transplantation activated endogenous GFP-negative skeletal muscle progenitor cells (7.9±1.9% vs. 4.6±0.5%/total nuclei). pPICs exhibited a wide range expression profile of regenerative cytokines and paracrine factors, specifically IGF-1 and NRG-1. As would be expected, negligible donor GFP^{pos} pPICs persisted within the host skeletal muscle at 14 days post-injury, suggesting allogeneic GFP^{pos} pPICs were eliminated by the host immune system.

Conclusions: Allogeneic progenitor cell therapy stimulates endogenous repair and regeneration in a pre-clinical muscle model applicable to humans.

Condensed Abstract:

Skeletal muscle-derived PW1^{pos}/Pax7^{neg} interstitial cells (PICs) express and secrete a multitude of pro-regenerative growth factors and cytokines. Utilizing a porcine pre-clinical skeletal muscle injury model, delivery of allogeneic pPICs significantly improved and accelerated myofiber regeneration and neocapillarization, compared to saline vehicle control-treated muscles. Allogeneic pPICs did not contribute to new myofibers or capillaries and were eliminated by the host immune system. In conclusion, allogeneic pPIC transplantation stimulated the endogenous stem cell pool to bring about enhanced autologous skeletal muscle repair and regeneration. This allogeneic cell approach is considered a cost-effective, easy to apply and readily available regenerative therapeutic strategy.

Key Words: Allogeneic progenitor cells, Porcine pre-clinical model, Skeletal muscle, PICs, Regeneration, Growth factors.

Introduction

Skeletal muscle is a dynamic, highly plastic tissue that is capable of undergoing repair and regeneration in healthy individuals (*1*). However, this regenerative capacity can become impaired in muscle diseases, such as muscular dystrophy, and as a result of disuse and ageing (*2, 3*). This leads to decreased proliferation, delayed fusion and reduced differentiation of muscle progenitors, as well as a gradual replacement of muscle fibers by fat and fibrosis with poor vascularization (*4, 5*). The mechanisms that underpin impaired skeletal muscle regeneration are still unclear however recent findings suggest that the microenvironment and/or systemic factors (*6, 7*) affect the response of various endogenous skeletal muscle progenitor cells to repair and regenerate after injury (*8*). This has led us to focus upon therapeutic strategies that target resident muscle progenitors and stimulate endogenous repair mechanisms.

Autologous stem cell approaches are attractive from a theoretical and biological standpoint, however for skeletal muscle where the muscle satellite cells cannot be effectively propagated to large numbers *in vitro* and like other tissue specific stem/progenitor cells, such as cardiac, are affected by age and disease, they are impractical. Moreover, administration of cells can induce therapeutic responses by indirect means, such as secretion of growth factors and interaction with endogenous repair processes, represented by the resident stem/progenitor cells (*9, 10*). This has been termed the paracrine effect. Therefore, there seems to be little advantage in the use of autologous cells because a similar, and perhaps enhanced, effect can be obtained by the administration of a cell type isolated from allogeneic sources, which could be made readily available at reduced costs (*11*). We have previously shown that intracoronary injection of allogeneic porcine cardiac stem cells (CSCs) after myocardial infarction (MI) activates the

endogenous, resident CSCs through a paracrine mechanism resulting in improved myocardial cell survival, function, remodeling and regeneration (*12*).

Skeletal muscle satellite cells although capable of muscle fiber regeneration, have a limited migration capacity with an inability to cross the endothelial wall (*13*). Moreover, cultured satellite cells *ex vivo* have decreased stemness, proliferation, and myofiber differentiation, which has subsequently hampered their translation (*14*). In recent years, skeletal muscle has been shown to harbor other stem/progenitor cells, such as the $PW1^{pos}/Pax7^{neg}$ interstitial cells (PICs), which are bi-potent giving rise to new skeletal muscle fibers and vasculature-associated cell types *in vitro* and *in vivo* (*15, 16*). Unlike the satellite cells, we have shown that porcine PICs (pPICs) can be successfully isolated and propagated *in vitro* up to passage 40, while maintaining a stable phenotype/genotype (*16*). Therefore PICs represent an attractive and alternative cell source for regenerative therapies.

The pig represents an excellent biological model for human biomedical pre-clinical research due to its anatomical, physiological, pathological and genomic similarities to humans (*18*). In terms of porcine skeletal muscle biology considerable correlation between contractile, metabolic, and morphological features to human skeletal muscle exist (*19-21*). Although small animal models are a valuable resource they do not always accurately reflect the clinical manifestation with many cell therapies that have been shown to be highly effective in small animal models (*22, 23*), yielding only modest therapeutic value in human clinical trials (*24, 25*). Combined with the three orders of magnitude difference in mass between the two species there is an obvious need to test cell therapies in more illustrative animal models. Developing a porcine pre-clinical model for skeletal muscle regeneration can provide a useful link between the widely used rodent models

and humans so that an improved prediction of efficacy of therapies can be achieved and successfully translated to patients. Moreover, unlike other organs, the skeletal muscle is an efficient regenerative tissue yielding adequate resident progenitor cells, which give rise to new myofibers and vasculature following injury. Altogether, this makes the porcine skeletal muscle an appropriate model system to test proof-of-concept regenerative therapeutic approaches, such as the allogeneic cell approach described here, for the first time. Allogeneic stem cell therapy is conceptually and practically different from any presently in clinical use and could be applied in the repair and regeneration of other tissues such as the heart.

Here we tested the therapeutic reparative effect of delivering allogeneic porcine PW1^{pos}/Pax7^{neg} interstitial cells (pPICs) to the injured pig skeletal muscle. We hypothesized that allogeneic pPICs transplanted following skeletal muscle injury would supply regenerative bioactive factors capable of activating the endogenous stem/progenitor cells, enhancing and accelerating the regeneration of damaged skeletal muscle tissue. It was also hypothesized that the allogeneic pPICs survive long enough in the allogeneic host to produce their paracrine effect activating the endogenous target cells before being eliminated by the host immune system. Therefore, the cells transplanted are allogeneic but the regeneration is completely autologous.

Methods

Study Design, Skeletal Muscle Injury and Intramuscular pPIC Administration

Animals were immune-competent, 2-month-old (juvenile), female, large Dalland Landrace pigs (32±5kg). All animal procedures were performed in accordance with the EU Directive guidelines and regulations for animal experimentation by appropriately qualified staff and approved by the institutional animal welfare and ethical review board DEC Utrecht, The Netherlands. Pigs were sedated with 4mg/kg thiopental sodium and anaesthesia maintained with midazolam (0.5mg/kg/hr) via an intravenous catheter placed in a peripheral ear vein before intubation. The animals were subsequently moved to the surgery room and secured to the surgical table with limb bindings. The lower hind limb was shaved and disinfected using iodine prior to surgery.

The first phase of the *in vivo* study utilized 6 pigs. In order to assess two different injury models either an open freeze/crush (n=3) or cardiotoxin injury (n=3) of the tibialis anterior muscle were subsequently performed. Briefly, a 5cm longitudinal incision of the skin and underlying fascia was made along the anterior aspect of the distal limb. The skin and fascia were retracted, the TA muscle located, non-absorbable prolene sutures (Ethicon) were used to mark the injury site (1cm x 1cm^2) and the injury was induced. In the case of freeze/crush injury, the muscle was crushed with forceps (Fine Science Tools), pre-cooled in liquid nitrogen. This procedure was repeated seven times for 5s each in direct continuity with the respective distal crush in the defined area. In the case of cardiotoxin injury induction, a 10µM cardiotoxin solution, Naja mossambica mossambica (Sigma), was filtered and brought to room temperature before injection. The cardiotoxin was dispersed in a series of five intramuscular injections using a 25 gauge needle to deliver a total volume of 500µl to the pre-defined area. The contralateral leg served as a sham-

control and was treated identically however no injury was applied in the case of freeze/crush or PBS alone was injected in animals receiving the CTX injury. The injury area was identified through marking with sutures. After injury-induction the superficial skin was sutured, animals were allowed to recover and then sacrificed by anesthetic overdose at either 14 or 21 days post-injury.

In the second phase of the *in vivo* study 10 pigs were randomly assigned to receive either saline vehicle (PBS) or allogeneic GFP^{pos} pPICs, 15 minutes post-CTX injury. GFP^{pos} pPICs were propagated and cryostored between P3-P12. Prior to transplantation GFP^{pos} pPICs were pre-mixed, brought into suspension, centrifuged, washed twice with PBS, and counted. $20x10^6$ GFP^{pos} pPICs were resuspended in 500µl PBS and injected intramuscularly into the injured TA through a 25 gauge needle, n=5. Control animals were treated identically however 500µl PBS alone was injected, n=5. Both treatments were distributed across five injection sites to the injury site. The contralateral control leg of each animal served as a sham CTRL and received no injury just PBS using the same protocol.

In separate pigs local delivery of human recombinant IGF-1 (8µg) and HGF (2µg) (Peprotech) was achieved by diluting both growth factors in PBS in a total volume of 500µl before being dispersed in a series of five intramuscular injections using a 25 gauge needle to deliver a total volume of 500µl to the pre-defined injured area, n=5. In the case of UPy+IGF-1/HGF treatment, the UPy-hydrogelators were synthesized by SyMO-Chem BV, Eindhoven, The Netherlands, as described previously (*26*). To prepare the hydrogel, polymer solutions were dissolved at 10wt% in PBS by stirring at 70 °C for 1 hour and subsequently cooled to room temperature. To liquefy the polymer solution, the pH was increased to pH 8.5 by adding 2µL aliquots of a 0.1M NaOH stock solution. The hydrogel was then UV-sterilized for 1 hour and human recombinant IGF-1

(Peprotech) and HGF (Peprotech) were added prior to use, yielding a final concentration of 8μg and 2μg respectively. A total volume of 500μl UPy hydrogel+IGF-1/HGF were administered as per the method described above, n=5.

In order to track newly formed cells post-injury we used the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU). In order to deliver BrdU to the animals over the course of the regeneration period we utilised an intravenous (IV) delivery system. This involved making a channel through the pig's neck musculature and feeding an IV line through, which was subsequently connected to the jugular vein. This enabled us to access a cannula situated on the dorsal aspect of the pig's neck, which was directly linked to pig's circulation system. This method allowed daily administration of BrdU at a dose of 10mg/kg/day without the need to sedate the animals. Animals were sacrificed by anaesthetic overdose at 14 days post-injury.

Cell culture

Porcine PICs were isolated and maintained as previously described (*15*) in growth media; Dulbecco's MEM/Ham's F12 (DMEM/F12; Sigma) medium containing 10% embryonic stem cell qualified-fetal bovine serum (ESQ- FBS) (Invitrogen), leukemia inhibitory factor (LIF) (10ng/ml; Millipore), bFGF (10ng/ml; Peprotech), EGF (20ng/ml; Peprotech), insulin-transferrin-selenite (ITS; Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.1% gentamicin (10 mg/ml; Invitrogen). Myogenic differentiation was induced by replacing growth media with DMEM/F12, 2% horse serum for either 24 hours or 5 days. Human myoblasts were isolated and maintained as previously described (*16*). Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in endothelial cell growth medium (EBM) supplemented with 2% fetal bovine serum (FBS) and growth factors (Lonza).

GFP transduction of pPICs

To generate GFP lentivirus HEK293T cells were cultured overnight in dishes pre-coated with 0.1mg/ml collagen solution (Sigma) in DMEM, 10% FCS, 2% glutamax and 1% pen/strep until 70% confluent. The following day, a mix of 6.5µg pCMV Δ8.9 packaging plasmid, 3.5µg VSV-g envelope plasmid and 10µg GFP expression construct were diluted in 500µl OptiMEM-1 without FCS or antibiotics. A second mixture, containing 30µl Lipofectamine 2000 (LifeTechnologies) in 500µl OptiMEM-1 without FCS or antibiotics, was added to the plasmid mix solution and incubated at room temperature for 20 min inverting the tube every 5 min. The plasmid/lipofectamine mixture was added dropwise to the HEK293 cells and incubated at 37 °C for 4h. After 4h an equal volume of OptiMEM-1 with the addition of 10% FCS and 1% pen/strep was added. The supernatant was collected 48h post-transfection and filtered with a 0.45 mm filter. The viral supernatant was subsequently concentrated using a lenti-X-concentrator kit (Clontech) according to manufacturer's specification and viral titre measured using GoStix (Clontech). The pseudovirus was subsequently used for transduction or stored at -80°C. Target pPICs at passage 2 were transduced by adding 30µl/ml pseudovirus in DMEM, 10% FCS containing 12µg/ml polybrene. After 24h media was changed to DMEM with the addition of serum and antibiotics. The infected target cells were further cultured and analyzed at 24h post-transduction for GFP expression, using fluorescent microscopy and flow cytometry. Porcine PICs transduced with the GFP reporter gene were propagated for allogeneic transplantation experiments in pPIC growth media.

Flow cytometry

Flow cytometry was performed as previously described (*14*) Immunophenotyping was performed using the following antibodies; PW1 (antibody kindly gifted by D. Sassoon), CD34 (ThermoScientific), CD45 (AbD Serotec), Pax7 (DSHB). Isotype controls were used to define the specific gates and analysis was performed using a FACSCantoII with FACSDiva software (BD BioSciences).

Tissue processing and Histology

Whole tibialis anterior muscles were excised, washed in PBS, weighed and fixed in 10% formalin with gentle agitation for 4 days. Muscles were processed for paraffin embedding using a Leica TP1020 tissue processor as previously described (*15*). To measure myofiber diameter and identify fibrosis, muscle sections were stained with hematoxylin and Van Gieson (HVG), respectively, according to standard procedures (*25*). Cross sectional area (CSA) of connective tissue was determined from five fields of view at x20 magnification per muscle using ImageJ analysis. To determine the mean myofiber diameters, measurements were performed on transverse sections with 100 myofibers analyzed per section.

Immunohistochemistry/immunocytochemistry

To identify regenerating muscle fibers, muscle sections were stained with anti- human neonatal myosin heavy chain (nMHC) (DSHB). Sections were also stained with anti- laminin (Abcam) to identify the basal lamina of individual fibers and centralized nuclei identified through DAPI staining (Sigma). All newly formed cells were identified by BrdU staining (Roche). The number of centralized nuclei was determined by counting 5 fields/section at x20 magnification. A total of 5 slides/ animal were assessed using fluorescent microscopy (Carl Zeiss, ApoTome).

Capillary density was evaluated by staining with an antibody against von-willebrand factor (vWF) (DAKO). The number of capillaries (defined as 1 or 2 endothelial cells spanning the vWF-positive vessel circumference) was determined by counting 5 fields/ section at x 20 magnification. A total of 5 slides/ animal were assessed and the amount of capillaries was expressed per no. of myofibers. Double staining for BrdU/ vWF was performed to identify newly formed capillaries and counterstained with DAPI to detect nuclei using fluorescent microscopy (Carl Zeiss, ApoTome). Tracking of donor GFP^{pos} cells was achieved by staining with anti-GFP (Rockland). GFP^{pos} cells were quantified by counting 5 fields/ section at x20 magnification. A total of 5 slides/ animal were assessed and number of GFP^{pos} cells quantified. To identify whether transplanted cells retained their PIC phenotype *in vivo*, sections were stained against PW1 (antibody kindly gifted by D. Sassoon), Pax7 (DSHB) and images of were acquired using a confocal microscope (Nikon, A1R). Bi-potent myogenic differentiation was assessed by staining for MHC (DSHB) and smooth muscle actin (Sigma) as previously described (*15*). Macrophages were identified by staining with anti-IBA-1 (Wako). IBA-1^{pos} macrophages were quantified by imaging 5 fields/ section at x 20 magnification using fluorescent microscopy (Carl Zeiss, ApoTome). Staining intensity was subsequently quantified using ImageJ analysis. All secondary antibodies were purchased from ThermoFisher.

Gene expression profiling

To identify growth factors and cytokines expressed by undifferentiated and early myogenic differentiated pPICs, gene array analyses were performed (n=3/group). RNA was extracted from pPICs that were either maintained in an undifferentiated condition in standard growth media or placed in a myogenic permissive environment (DMEM/F12, 2% horse serum) for 24 hours.

Total RNA was isolated using the Qiagen RNeasy Mini Kit and reverse transcribed using the RT2 first strand kit (Qiagen) according to the manufacturer's recommendations for the RT2 Profiler PCR Array. Quantitative RT-PCR was then performed using RT2 SYBR Green (Qiagen) and the RT2 Profiler PCR Array (Qiagen), which was custom-designed for the identification of porcine growth factors and cytokines of interest. Briefly, samples were denatured for 10 minutes at 95°C, cycled 40 times at 95°C for 15 seconds, followed by 40 cycles at the annealing temperature of 60°C for 1 minute on a MyIQ thermocycler (BioRad). All reactions were carried out in triplicate; data were normalized to five housekeeping genes (ACTB, B2M, GAPDH, HPRT1, RPL13A) and analyzed using BioRad IQ software.

Western blotting

Immunoblots were carried out using protein lysates obtained from undifferentiated pPICs and pPICs that had undergone early myogenic differentiation (n=3/group). Approximately, 50 µg of protein were separated on gradient (10-15%) SDSpolyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked with 5% dry milk, then incubated with antibodies against IGF-1 (Santa Cruz), HGF (Santa Cruz), TGF-β1 (Santa Cruz), NRG-1 (Santa Cruz) at dilutions suggested by the manufacturers. GAPDH (Millipore) was used as a loading control. Proteins were detected by chemiluminescence using HRP-conjugated secondary antibodies (Santa Cruz) and visualized using ECL Plus Western Blotting Detection Reagents (Amersham Ltd.) and a Chemidoc imaging system (Bio-Rad Ltd.).

BrdU proliferation assay *in vitro*

pPICs and human myoblasts were plated in 24-well plates at a density of 5 x 10^3 per well and were serum starved for 6 h in 0% serum DMEM/F12 medium. To investigate the effect of pPIC conditioned media on proliferation, wells were either supplemented with standard growth media, unconditioned media, pPIC conditioned media or heat inactivated conditioned media. Each well was supplemented with BrdU (1ug/ml) every 8 h, fixed after 24 h and BrdU incorporation was assessed using the BrdU detection kit (Roche) (n=3/condition).

In order to investigate the effect of growth factors on pPIC proliferation, wells were serum-starved for 6h and then switched to basal media (control), or supplemented with IGF-1 (100ng/ml; Peprotech), HGF (100ng/ml; Peprotech), neuregulin-1 (NRG-1; 100ng/ml; R&D) or TGF-β1 (5ng/ml; Peprotech). Each well was supplemented with BrdU (1ug/ml) every 8h, fixed after 24h and BrdU incorporation was assessed using the BrdU detection kit (Roche) (n=3/growth factor). Nuclei were counterstained with DAPI. Cells were evaluated using fluorescence microscopy (Carl Zeiss, ApoTome). The percentage of BrdU positive cells relative to the total number of cells was determined by counting 5 random fields at x20 magnification for each dish, and then expressed as fold change over un-supplemented control.

Myogenic differentiation assay *in vitro*

To assess the effect of different growth factors in the induction of myogenic differentiation *in vitro*, 1 x 10^4 pPICs were plated in 24- well plates on gelatin-coated coverslips in basal media (control). Individual growth factors (concentrations stated above) were supplemented (n=3wells/growth factor). 3 wells acted as controls, with no growth factors added to the medium. Cells were fixed after 5 days and myogenic differentiation was quantified by staining for MHC (DSHB). Nuclei were counterstained with DAPI. Cells were evaluated using fluorescence (Carl

Zeiss, ApoTome), 5 random fields at x20 magnification were quantified for each well and fusion index was calculated by counting the number of nuclei inside myotubes per total nuclei.

Matrigel angiogenesis assay

To study angiogenesis, 150 µl of Matrigel substrate diluted with DMEM (1:10) was pipetted into each well of a 24-well plate and allowed to solidify for 1hr at 37°C. Thereafter, $2x10^4$ HUVECs were seeded into each well in either EBM (see details above), unconditioned media (serum-free), pPIC conditioned media or heat inactivated conditioned media. Cells were imaged at 24h using a light microscope (Olympus). Five fields of view at 20x magnification were imaged for each condition. Parameters of angiogenesis (capillary area, number of tubes per field of view, tube length and branching points) were analyzed using Wimasis Image Analysis software.

Cytotoxicity assay in vitro

Peripheral blood mononuclear cells (PBMCs) were isolated from whole pig blood using density gradient centrifugation with Ficoll-Paque (Sigma). Briefly, whole blood was diluted at the ratio of 1:1 with PBS and carefully layered over 6 mL of Histopaque solution. Tubes were centrifuged at 400g for 30 min at room temperature. PBMCs were then carefully collected from the buffy coat layer, washed twice with PBS and frozen at -80 until ready to use. PBMCs were resuscitated and cultured for 3 days in RPMI 1640 GlutaMAX medium (Gibco), 10% FBS and 1% pen/strep (Gibco). PBMCs were either maintained in media alone or activated with phytohemagglutinin (PHA) (Sigma) (5 µg/ml) for 3 days. PICs (passage 6) were plated at $1x10^5$ per well in a 24 well plate, 1 day before co-culture. PBMCs were harvested and co-cultured with PICs in RPMI medium as follows; no PBMCS, 1:10, 1:20 and 1:40. After 4 h of co-culture, PBMCs were discarded, PICs were harvested and subsequently labelled with anti-CD45 antibody

(BD Biosciences)/Annexin V-PE Apoptosis Detection Kit I (BD Pharmigen) and analysed using flow cytometry.

Statistical analysis

Data are reported as Mean ± SD. Significance between 2 groups was determined by paired samples T-test and in multiple comparisons by analysis of variance (ANOVA). Holm-Šídák method was used to locate the differences. A probability of less than 5% ($P < 0.05$) was considered to be statistically significant. Data were analyzed using SPSS V22.

Results

Cardiotoxin-induced injury is a reproducible model of porcine skeletal muscle damage

In order to determine a robust model to investigate allogeneic pPIC regenerative therapy *in vivo*, two different models of porcine skeletal muscle damage were first evaluated. Freeze/crush and cardiotoxin (CTX) injury were assessed as they have been extensively used to model skeletal muscle damage in small animals (*28*). The freeze/crush injury is a segmental model of trauma, selectively affecting muscle fibers and interstitial tissue without disrupting the main innervation of the tissue. The CTX injury, induced using a series of cardiotoxin injections, is a diffuse injury model. All animals recovered from the surgical procedure without complications and were fully ambulatory within a few hours after surgery with only minor swelling observed at the surgical site. Histological assessment of explanted muscle revealed that the freeze/crush injury, as expected, was restricted to the superficial layer of muscle adjacent to a deep uninjured region (Fig. S1). CTX-injured muscles showed a diffuse pattern of damage throughout the tissue (Fig. S1). Both injuries resulted in significantly ($p<0.05$) decreased muscle fiber CSA (CTX-injured 53% ± 7% per total area; freeze/crush-injured 66% ± 7% per total area), compared to uninjured contralateral control muscle (83% ± 4% per total area) (Fig. S1). Moreover, CTX-injured muscle exhibited a significant ($p<0.05$) reduction in muscle fiber CSA, compared to freeze/crush-injured muscle (Fig. S1), showing that CTX injury damaged a greater number of myofibers. There was also a significant ($p<0.05$) increase in the abundance of connective tissue in both injury models (Fig. S1). Both injuries resulted in an overall reduction in muscle fiber diameter with CTX injury showing the greatest reduction (37.9 ± 8.5µm), which was statistically significant ($p<0.05$), compared to uninjured-control (63.3 ± 15.7µm) (Fig. S1).

A decreased myofiber diameter implies the occurrence of small, newly formed, regenerating myofibers. Therefore, centralized nuclei, a marker of newly regenerated fibers, were evident in both injuries however they were more prevalent in the freeze/crush-injured muscle (27.27 ± 11.34 per 100 myofibers; $p<0.05$), compared to CTX-injured muscle (10.8 ± 6.0 per 100 myofibers) and uninjured control (Fig. S1), suggesting that freeze/crush-injured muscles were more advanced in the regeneration process at 14 days post-injury, compared to CTX-injured muscles. Immunohistochemical staining of injured muscle was performed to determine the extent of regeneration with respect to early myosin isoforms. Neonatal myosin heavy chain (nMHC) staining was observed in both larger fibers and small angular myofibers of CTX and freeze/crush-injured muscle at 14 days post-injury (Fig. S1) and in the freeze/crush injury nMHC expressing myofibers were restricted to the uppermost superficial layer of the muscle. The CTX-injured muscles showed a diffuse pattern of nMHC expression and $nMHC^{pos}$ myofibers were more prevalent in CTX-injured muscle (37.2% ± 5.7%) compared to freeze/crush injured muscle (25.6% ± 5.3%). $nMHC^{pos}$ fibers were also smaller in CTX-injured muscle (21.7µm ± 4.9µm) compared to those identified in the freeze/crush-injured muscle (24.8µm ± 5.3µm), supporting our observation that freeze/crush injury regeneration is more advanced at 14 days compared to CTX-injury, which induces widespread injury with incomplete regeneration at 14 days post-injury (Fig. S1).

These results confirmed that CTX injury is a reproducible and diffuse injury model for testing skeletal muscle regeneration in the pig. However in order to confirm whether 14 days was an appropriate time point for studying porcine skeletal muscle regeneration, a subset of animals were also sacrificed 21 days post-CTX injury. Immunohistochemical staining of muscle cross-sections revealed that at 21 days there was no significant ($p<0.05$) difference between the muscle

fiber or connective tissue CSA (75% ± 3% muscle fiber per total area), compared to uninjured control (82% ± 3% muscle fiber per total area) (Fig. S2). These findings suggest that 21 days after CTX injury the muscle has undergone almost complete regeneration. Therefore, to measure whether allogeneic pPIC transplantation could enhance the regeneration of skeletal muscle, CTX was selected as the injury model with sacrifice of the pigs at 14 days post-injury.

Transplantation of allogeneic GFP^{pos} pPICs improves skeletal muscle architecture and accelerates myofiber regeneration

pPICs, which have previously been phenotyped and propagated over long term culture maintaining phenotypic and genomic stability (*16*), were transduced with a lentiviral vector encoding a GFP reporter. 87.2% of pPICs were GFP-positive as determined by flow cytometry (Fig. S3). GFP^{pos} pPICs (GFPpPICs) were propagated over 9 passages (P3-P12) to generate the required number of cells for transplantation (20x10^6 per animal). GFPpPICs maintained a PIC phenotype after GFP transduction, which was stable up to P12, remaining positive for PW1, CD34 and negative for CD45 and Pax7 (Fig. S3). Furthermore, GFPpPICs at P12 were comparable in their gene expression profile and bi-potent differentiation potential to unlabelled pPICs (Fig. S3).

Initial histological observations indicated that pPIC-treated muscle had significantly improved muscle architecture and increased BrdU^{pos} centralized myonuclei, indicative of undergoing accelerated regeneration, compared to CTX-PBS treated muscle (Fig. 1a-b). There was a significant ($p<0.05$) increase in muscle fiber CSA in pPIC-treated muscle (78% ± 5% per total area), compared to CTX-PBS (62% ± 2% per total area) (Fig. 1c) and the ratio of muscle

fiber:connective tissue CSA of pPIC-treated muscle (78%:22% ± 5% per total area) at 14 days was closer to uninjured contralateral control muscle (87%:13% ± 3% per total area) (Fig. 1c).

Immunohistochemistry revealed a marked increase ($p<0.05$) in the number of centralized, BrdU^{pos} myofiber nuclei, which indicates newly regenerated fibers, in pPIC-treated muscles (15% ± 3% /total myofibers), compared to CTX-PBS treated muscles (2% ± 1% /total myofibers) (Fig. 1b & d). The uninjured contralateral control muscle had a very low number of BrdU^{pos} nuclei and centralized nuclei, indicating the low turnover of skeletal myofibers in uninjured, resting muscle (0.04% ± 0.08%) (Fig. 1b & d). The diameter of the BrdU^{pos} regenerating myofibers in pPIC-treated muscles were found to be significantly larger (41.4µm ± 10µm) than those of CTX- PBS treated muscles (23.8µm ± 7.3µm) 14 days post-injury (Fig. 1e). Taken together, these findings support an improved and accelerated regeneration of injured skeletal muscle, following transplantation of allogeneic pPICs.

Allogeneic pPICs stimulate neo-angiogenesis when transplanted into injured porcine skeletal muscle

Immunohistochemical staining revealed a significant ($p<0.05$) increase in capillary density with CTX-pPIC treatment, compared to CTX-PBS and uninjured muscle (Fig. 2a-b). Moreover, the presence of BrdU^{pos}/vWF^{pos} capillaries in the pPIC-treated muscle confirmed their formation post-injury and pPIC transplantation (Fig. 2c). Quantification revealed a significant ($p<0.05$) increase in the number of BrdU^{pos}/vWF^{pos} capillaries in pPIC-treated skeletal muscle (93.94 ± 2.86 per myofiber), compared to CTX-PBS-treated (55.84 ± 2.86 per myofiber) (Fig. 2d). These

findings show that allogeneic pPIC therapy stimulated angiogenesis in the injured skeletal muscle.

Allogeneic pPIC transplantation activates endogenous PICs

To determine whether GFPpPIC transplantation had stimulated the activation of endogenous progenitor cells, sections were stained for PW1, Pax7 and GFP. We found a significant ($p<0.05$) increase in the number of endogenous PW1^{pos}/Pax7^{neg}/GFP^{neg} PICs in skeletal muscle that had been treated with allogeneic GFPpPICs (7.9% ± 1.9%/total nuclei), compared to CTX-PBS-treated CTRL (4.6% ± 0.5%/total nuclei) (Fig. 3a-b). We also quantified the number of PW1^{pos}/Pax7^{pos}/GFP^{neg} satellite cells, and found that pPIC treatment did not significantly alter the number of satellite cells (2.6% ± 0.4%/total nuclei), compared to CTX-PBS treatment (2.4% ± 0.5%/total nuclei) (Fig. 3c-d). These data show that at 14 days post-injury allogeneic pPIC transplantation had a stimulatory effect on the endogenous pPICs population resulting in an increase in their number.

Allogeneic GFP^{pos} pPICs are cleared by the host's immune system

To determine whether allogeneic GFPpPICs had persisted in the skeletal muscle and directly contributed to the regeneration or, as expected, had been cleared by the host's immune system, skeletal muscle cross-sections were co-stained for GFP and laminin. Only a few GFPpPICs (1 GFP^{pos} nuclei/1560 counted) persisted within the skeletal muscle 14 days post-injury (Fig. 4a-b). Moreover, we found that the very few donor GFPpPICs that had remained, had taken up residence within the interstitial spaces and maintained expression of PW1. No GFP^{pos} nuclei were identified underneath the basal lamina in a satellite cell position (Fig. 4a, Figure S4). We

found only a rare occurrence of a single GFP^{pos} myofiber (per 1000 myofibers counted) (Fig. 4a-b). At 14 days post-injury no GFPpPICs were identified in other tissues (lung, spleen or liver) or in the uninjured contralateral control muscle (Fig. S4).

Muscle sections were stained for the infiltration of cytotoxic T cells, cytotoxic T cells/natural killer cells and macrophages expressing CD8a, Granzyme B and IBA-1, respectively (Figure S5). $CD8a^{pos}$ and Granzyme B^{pos} cells were significantly upregulated in CTX-pPIC treated muscle, compared to CTX-PBS and uninjured muscle (Fig 4c,d). In terms of the innate immunity, IBA-1-positive macrophages infiltration was significantly increased in both CTX-PBS and CTX-pPIC treated muscle relative to uninjured muscle (Fig. 4e).

To document that the immune cells (cytotoxic T cells/NK cells) were responsible for clearance of pPICs we performed cytotoxicity assays with porcine peripheral blood mononuclear cells (PBMCs) *in vitro*. Porcine PHA-activated and inactivated PBMCs were co-cultured with pPICs at several seeding densities and apoptosis (Annexin V/7-AAD) of pPICs measured by flow cytometry. Activated PBMCs induced apoptosis between 70-80% in pPICs (Fig. 4f). Apoptosis of pPICs did not occur when inactive PMBCs were co-cultured with pPICs (Fig. 4g).

Taken together these data suggest that allogeneic pPICs exert their action via a paracrine effect, activating endogenous progenitors before being cleared by the host immune system post-injury.

Allogeneic pPICs express an array of pro-regenerative paracrine factors

To identify the mechanism through which pPICs were able to promote regeneration in skeletal muscle, we looked to their secretome. Porcine PICs were found to express a range of cytoprotective/ pro-regenerative growth factors and cytokines (Fig. 5a) similar to that observed for stem/progenitor cells, such as mesenchymal stem cells (MSCs) (*29)* and satellite cells (*30*).

In particular we noted the greatest relative expression (>10,000) was Chemokine (C-C Motif) Ligand 2 (CCL2), monocyte chemotactic and activating factor, which is considered to be essential for stimulating muscle repair (*31, 32*) (Fig 5a). TIMP1 and TIMP2, involved in tissue remodeling were also highly expressed (>100 relative expression; Fig 5a). Factors which were 10-100 relative expression included those implicated in the activation, migration, proliferation and differentiation of muscle stem/progenitor cells, such as Periostin, Neuregulin 1/2, TGF-βs, FGFs, HGF, IGF-1, INHBA, LIF, IL-6 and SCF (*33-42*) (Fig.5a). Interestingly, GDF-11, which has been shown to ameliorate the age-related dysfunction of skeletal muscle by rescuing the function of aged muscle stem cells (*43*), was significantly expressed by pPICs (Fig.5a). Pro-angiogenic factors including VEGFa, PDGFs and IL-8, capable of stimulating recruitment of endothelial cells and initiating vascularization following injury (*44-46*), were also found to be highly expressed (>10) in pPICs (Fig. 5a).

To enrich for a highly paracrine secreting pPIC population, we identified whether pPICs expressed differential levels of factors depending on their differentiation status. This was necessary because following pPIC transplantation into injured skeletal muscle the microenvironment could facilitate pPICs to differentiate into a myogenic precursor-like cell type, which could then express differential levels of pro-regenerative factors, compared to its undifferentiated counterpart. Therefore, a comparative qRT-PCR analysis was performed between undifferentiated pPICs and those which had undergone 24 hours of myogenic differentiation *in vitro* (differentiated pPICs). Overall, there were distinct differences between the differentiated and undifferentiated pPICs (Fig. 5b-c; Table S1).

Volcano plot analysis highlighted factors that were significantly (>2-fold) down- or upregulated in differentiated compared to undifferentiated pPICs (Fig. 5c; Table S1). Cytokines and growth factors involved in stem cell migration and proliferation, such as HGF (*9*), were upregulated in undifferentiated pPICs. Whereas, a significant upregulation of factors involved in stem/progenitor activation and differentiation, such as INHBA, SPP-1, IL-8, BMP4, IGF-1, NRG-1 and Periostin, were expressed in differentiated pPICs. (Fig. 5c). We confirmed changes in expression at the protein level of undifferentiated compared to differentiated pPICS for a selected panel of up- or down-regulated factors, which supported the transcript data (Fig. 5d). These data suggest that both undifferentiated and differentiated pPICs could be used to exert a pro-regenerative paracrine effect, but differentiated pPICs upregulate expression of a more vast array of pro-regenerative factors.

The pPIC secretome promotes a regenerative response in vitro

We next examined the effect of the pPIC secretome on human myoblast and pPIC proliferation and differentiation *in vitro*. pPIC conditioned media significantly ($p<0.05$) increased proliferation of pPICs (Fig. 6a) and myoblasts (Fig. 6b), compared to unconditioned media and heat-inactivated media where the secreted factors were denatured (Fig. 6a-b). Next we showed that factors that were expressed by pPICs such as, IGF-1, HGF, and NRG-1, when supplemented to pPICs *in vitro,* increased their proliferation (Fig. 6c) and for TGF-β1, increased pPIC differentiation (Fig. 6d-e) assessed by quantifying the fusion index of MHC^{pos} cells, compared to unsupplemented control. Moreover, *in vitro* angiogenesis assays confirmed that secreted factors in pPIC conditioned media promoted HUVECs to form endothelial networks, compared to unconditioned media and heat inactivated media (Fig. 6f, 6g and Fig. S5). Taken together these

data demonstrate that factors secreted by pPICs promote proliferation and differentiation of skeletal muscle progenitors, PICs and stimulate angiogenesis *in vitro*.

Administration of HGF and IGF-1 improves skeletal muscle regeneration, but not as great as pPIC transplantation

To determine whether cytokines produced by other cell types other than pPICs could contribute to the repair, we immunostained the muscle sections for 4 growth factors, HGF, IGF-1, TGFβ1 and NRG1. CTX:pPIC transplanted muscle showed increased expression of all factors, compared to CTX:PBS and uninjured muscle. Specifically, HGF expression was mainly confirmed to the myofibers, IGF-1 and NRG1 expression was confined to the myofibers and interstitial cells and TGFβ1 expression was confined to the interstitial cells, following CTX:pPIC transplantation (Fig. S7).

To determine if IGF-1 and HGF could improve regeneration to a similar degree as pPIC transplantation, we injected IGF-1 and HGF into CTX injured skeletal muscle, and we also administered the growth factors through a UPy hydrogel, as we have done previously in the porcine chronic MI model (*47*). We found that IGF-1/HGF administration improved muscle regeneration, increased the number of $BrdU^{pos}$ myofiber nuclei (8% ± 2%/total myofibers vs. 2% ± 1%/total myofibers CTX:PBS) and $BrdU^{pos}$ regenerating myofibers were larger (33.28µm ± 6.3µm vs. 23.8 µm ± 7.3 µm CTX:PBS) (Fig. 7). However, these effects were not enhanced if the growth factors were administered through the UPy hydrogel ($BrdU^{pos}$ myofiber 8 ± 3% /total myofibers and $BrdU^{pos}$ regenerating myofibers diameter 35.58µm ± 4.98µm), and more importantly the improvements were not as great as transplantation of pPICs (Fig. 1). Therefore, pPICs are superior to targeted growth factor administration as they release a plethora of growth

factors and cytokines which activate multiple endogenous repair mechanisms, contributing to accelerated and effective repair and regeneration *in vivo*.

Discussion

The present study documents that in a porcine model of skeletal muscle damage, which is applicable to humans, transplantation of allogeneic pPICs persist long enough to elicit a paracrine effect by secretion of regenerative cytokines and growth factors. These factors stimulate endogenous progenitor cell activation and differentiation, leading to accelerated and improved autologous myofiber regeneration and microvessel formation.

Skeletal muscle while capable of undergoing extensive regeneration exhibits diminished regenerative capacity in ageing and disease. The loss of regenerative capacity is largely thought to be due to intrinsic and extrinsic changes that ultimately abrogate resident progenitor cell competence (*48, 49*). Intrinsic mechanisms include epigenetic changes, telomere attrition, DNA damage or mitochondrial dysfunction. Extrinsic changes are associated with alterations of the muscle progenitor cell niche, which can be influenced by both systemic circulating factors and local changes associated with ageing, disease or metabolic pathways, which can negatively impact endogenous progenitor cells.

To date, most attempts to regenerate skeletal muscle have focused on delivery of culture-expanded myoblasts (*50*) or CD133^{pos} cells (*51*), which have been shown to engraft and generate functional satellite cells after xenotransplantation in rodent models, suggesting the potential for autologous regenerative applications. Despite advances, the results of clinical trials (*52*, *53*) collectively show low engraftment efficiency with prevailing issues associated with immune rejection. Over recent years, a large number of reports have shown that transplanted stem cells

mediate their beneficial effects via several indirect mechanisms, such as recruitment of endogenous progenitor cells, induction of angiogenesis, protection of existing survived cells and reduction in fibrosis and inflammation *(9, 54)*. These processes are regulated by a variety of small molecules, proteins, mRNAs/miRNAs and paracrine factors produced by the adoptively transferred stem cells in the damaged tissue milieu (*54-56*). This concept has been prevalent for many years, where it was shown the infusion of human umbilical cord blood cells can aid in stroke recovery due to enhanced angiogenesis, which may have induced neuroblast migration to the site of injury (*57*). Moreover, transplanted macrophages can promote liver repair by activating hepatic progenitor cells (*58*). We have previously documented the protective and regenerative effects of allogeneic cardiac stem/progenitor cell (CSC) transplantation or growth factor administration in porcine models of MI (*9, 12*). Cardiac stem/progenitor cells express high levels of numerous cytokines, including chemokines (TCA-3, SDF-1, 6Ckine), vascular growth factors (VEGF, EPO, bFGF, SCF), survival and activation factors (IGF-1, HGF, PDGF's), and cardiac differentiation factors (Activin A, Dkk-1, TGF-β), which are released at the site of injury, activating endogenous repair mechanisms and improving cardiac function (*9, 12, 59, 60*).

To our knowledge this is the first study to show allogeneic stem/progenitor cell transplantation elicits a paracrine effect by activating endogenous repair processes to improve and accelerate muscle regeneration in the pig. To date pre-clinical studies have not addressed the magnitude of the regeneration required to target whole skeletal muscle regeneration in the clinic. The best published results report regeneration in the region of milligrams of skeletal muscle in small mouse animal models, while humans would likely require at least a 3-fold increase in muscle mass in comparison (*61*). Here, we developed a large animal, pre-clinical skeletal muscle injury model, which is easily reproducible and is a useful, clinically applicable model to assess the

effects of proof-of-concept regenerative therapies designed to regenerate large volumes of muscle. The animals utilised were juvenile animals, ~2 month old pigs, which were selected due to their reduced size and practicability issues. They also had a functioning immune system required to test our theory that transplanted pPICs would be cleared by the host immune system. We showed that transplanted pPICs were cleared from the host by 14 days post-transplantation and activated PMBCs from the pig killed pPICs in a cytotoxicity assay *in vitro*. Analysis of the immune response in revealed that CTX:pPIC muscle had increased CD8 (adaptive immunity) and Granzyme B (innate immunity) cells and both CTX-PBS and CTX-pPIC –treated muscle had comparable infiltration of macrophages that were significantly greater than in uninjured muscle. Taken together we propose that the donor pPICs were removed in the early phases of regeneration and the increased regenerative response was mediated as a consequence of the activated endogenous progenitors.

Transplantation of allogeneic pPICs were found to stimulate the endogenous repair mechanisms of this tissue leading to improved and accelerated skeletal muscle regeneration, with increased muscle CSA, an increased number of BrdU^{pos} centralized nuclei and larger regenerating myofibers compared to CTX:PBS-treated muscle. In addition to myofiber regeneration following muscle trauma, damaged blood vessels lead to tissue hypoxia at the injury site (*63*) therefore new capillary formation after injury is also necessary for functional muscle recovery (*64*). Secretion of angiogenic factors, such as VEGF at the injury site is an important mediator of this process and several studies have shown that VEGF improves skeletal muscle repair by promoting angiogenesis (*65-66*). This study identified that following allogeneic pPIC transplantation there was a significant increase in new capillary formation, which would have contributed to the improvement in muscle regeneration observed.

Allogeneic pPIC- treatment was shown to activate and increase the number of endogenous PW1^{pos}/Pax7^{neg} PICs, yet a similar increase in number was not found for PW1^{pos}/Pax7^{pos} satellite cells. Therefore, pPICs may either have a greater stimulatory effect on the endogenous PICs or, more likely, the time point of 14 days is insufficient to accurately measure the effect of pPIC transplantation on satellite cell activation. As satellite cells are the main contributor to muscle regeneration (*62*) it is likely that the satellite cells became activated early (days 1-3) and had returned to normal, quiescent levels once the regeneration process was almost complete at 14 days. Moreover, the assessment of satellite cell activation is hampered by lack of markers to identify activated satellite cells in the interstitial space (*30*). Also, the endogenous PICs might have maintained an activated state for longer due to their role in vascular repair. However, these data suggest the existence of a feedback loop triggered by the transplanted pPICs that activates the production of growth and survival factors by the endogenous PICs, which could explain their persistence and longer duration of activation over 14 days. The relationship between satellite cells and endogenous PICs during the repair and regeneration of skeletal muscle thus warrants further investigation.

We determined if the paracrine effect of pPICs depended on their differentiation status and predicted that upon transplantation, the *in vivo* microenvironment encountered by the pPICs during the first 24 hours would promote pPIC differentiation. The secretome of 24hr differentiated pPICs showing increased expression of factors such as IGF-1, NRG-1 and TGF-β1 and periostin, which facilitated proliferation and differentiation of endogenous muscle stem/progenitor cells. *In vitro* assays confirmed that conditioned media containing these secreted factors stimulated the proliferation of both pPICs and human myoblasts, and stimulated endothelial network formation of HUVECs. Moreover, IGF-1, HGF and NRG-1 when

supplemented individually to the culture media stimulated the proliferation of pPICs, while TGF-β1 was shown to play a role in promoting pPIC myogenic differentiation. Therefore, allogeneic pPIC transplantation elicits a paracrine effect on endogenous progenitor cells, increasing and accelerating skeletal muscle regeneration.

Many different stem/progenitor cells (*67-73*) have been investigated for cellular therapy each with their own set of unique advantages and shortcomings. Given the striated muscle similarities between skeletal and cardiac muscle, skeletal-muscle derived myoblasts were the first cell type to enter the clinical arena of heart repair (*74*). Skeletal muscle-derived progenitors showed promise in that they were pro-angiogenic and relatively resistant to hypoxic conditions therefore improved functional outcomes upon transplantation (*75-76*). However, due to issues associated with limited expansion *in vitro* and arrhythmogenic complications *in vivo* (*77*) they are widely considered unsuitable for cardiac regeneration. Utilizing PICs as a source of stem/progenitor cells for allogeneic cell therapy overcomes several of the limitations encountered with other adult muscle progenitor therapies and mesenchymal allogeneic therapies. Indeed, harvesting of PICs from an easily accessible source such as skeletal muscle means a simple muscle biopsy would be performed, and with an unlimited proliferative capacity *in vitro* a large number can be propagated, maintaining a stable phenotype and genotype over passage number (*16*). Together with a rich secretome of regenerative factors, these properties make PICs an ideal candidate cell source for the repair of the heart as well as other tissues.

One of the main limitations associated with the clinical application of stem cell based therapies is the time and costs associated with generating the large number of clinical grade cells required for treatment. Therefore, it may be beneficial to assess the effect of different doses of pPICs to

ascertain whether fewer cells could stimulate the same effect. Nevertheless, we envision the main advancement of these findings to be in identifying specific factors responsible for eliciting accelerated and improved muscle and microvessel regeneration. This would enable us to develop cell-free therapies that match or exceed the level of regeneration reported here, which would greatly facilitate clinical application. However, the delivery of HGF and IGF-1 to the injured skeletal muscle did not result in equivalent levels of muscle regeneration documented for the pPIC transplantation. Therefore, delivery of a multitude of factors/molecules is necessary, at different time points after injury, to elicit optimal repair and regeneration of the tissue.

Another limitation of this study is the unknown effect of transplanting PICs into diseased muscle, such as diabetes or muscular dystrophy models, where the harsh environment and metabolic elements affect stromal stem cell activity and potency (*78*). Moving towards clinical application this warrants investigation as we may require a combinatorial approach in order to overcome the harsh environments that the transplanted PICs will likely encounter. Taken together, these results provide the proof-of-concept needed to justify further experimental development and refinement of this allogeneic cell therapy approach, which ultimately could lead to an effective, simple, clinically applicable, and widely available protocol of tissue repair and regeneration.

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Figure Legends

Fig. 1. ***Transplantation of GFP^{pos} allogeneic, pPICs accelerates skeletal muscle regeneration***. (**a**) HVG staining of uninjured CTRL, CTX:PBS and CTX:pPIC-treated muscle showing improved skeletal muscle architecture in pPIC-treated muscle, scale bar 100µm. Inset shows 40x magnification, scale bar 50µm. (**b**) Immunohistochemical staining indicates an increase in BrdU^{pos} centralized nuclei in CTX:pPIC-treated muscle compared to uninjured:CTRL and CTX:PBS-treated muscle, scale bar 50µm. (**c**) Quantification of the ratio of skeletal muscle to connective tissue CSA in uninjured CTRL, CTX:PBS and CTX:pPIC-treated muscle determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. uninjured CTRL, **P=0.05 vs. CTX:pPICs. (**d**) BrdU^{pos} centralized nuclei per total myofibers, determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. uninjured CTRL, **P=0.05 vs. CTX:PBS. (**e**) Average myofiber diameter of regenerating (BrdU^{pos} centralized nuclei) myofibers determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. CTX:PBS.

Fig. 2. ***Allogeneic GFP^{pos} pPICs stimulate neo-angiogenesis following transplantation into injured skeletal muscle***. (**a**) Skeletal muscle cross-sections were stained for vWF to evaluate capillary density, scale bar 50µm. (**b**) Number of capillaries per myofiber, determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. uninjured, **P=0.05 vs. CTX:PBS. (**c**) Newly formed BrdU^{pos}/vWF^{pos} capillaries were identified 14 days post-injury, scale bar 20µm. (**d**) Percentage of BrdU^{pos} capillaries per total capillaries, determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. vs. CTX:PBS.

Fig. 3. ***Allogeneic GFP^{pos} pPICs stimulate endogenous pPIC activation*** (**a**) Representative image of GFP^{neg}/PW1^{pos}/Pax7^{neg} pPICs resident in CTX:pPIC-treated skeletal muscle 14 days post-injury, scale bar 50µm. (**b**) Number of GFP^{neg}/PW1^{pos}/Pax7^{neg} pPICs per total nuclei, determined from three fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. CTX:PBS. (**c**) GFP^{neg}/PW1^{pos}/Pax7^{pos} satellite cells per total nuclei, determined from three fields of view per muscle, n=5 animals per group. Data are mean ± SD. (**d**) Representative image of PW1^{pos}/Pax7^{pos} satellite cells resident in both CTX:PBS and CTX:pPIC-treated skeletal muscle 14 days post-injury, scale bar 50µm.

Fig. 4. ***Allogeneic GFP^{pos} pPICs are cleared by the host immune system*** (**a**) Immunohistochemistry identified a small number of donor GFP^{pos} pPICs at 14 days post-transplantation, scale bar 100µm. GFP^{pos} cells were found as nuclei within the interstitial spaces between the muscle fibers (bottom inset) and a rare GFP^{pos} muscle fiber was also identified (top inset). (**b**) Quantification of GFP^{pos} cells to total number of nuclei or myofibers in paraffin embedded skeletal muscle cross-sections, n=5. Data are mean ± SD, *P=0.05 vs. CTX:PBS. (**c**) Quantification of CD8^{pos} cells per total nuclei (%) determined from five fields of view per group. Data are mean ± SD, *P=0.05 vs. uninjured muscle, **P=0.05 vs. CTX:PBS. (**d**) Quantification of Granzyme B^{pos} cells per total nuclei (%) determined from five fields of view per group. Data

are mean ± SD, *P=0.05 vs. uninjured muscle, **P=0.05 vs. CTX:PBS. (**e**) Quantification of IBA-1^{pos} cells per total nuclei (%) determined from five fields of view per group. Data are mean ± SD. Porcine PICs were co-cultured with either (**f**) PHA-activated porcine PBMCs or (**g**) inactivated porcine PBMCs at different ratios and analysed for apoptosis using AnnexinV/7-AAD staining and flow cytometry. Early apoptosis of CD45^{neg} PICs is represented by AnnexinV expression, whereas late apoptosis is represented by double positive AnnexinV/7-AAD staining, determined from duplicate wells per group. Data are mean ± SD, *P=0.05 vs. pPICs only.

Fig. 5. ***Allogeneic pPICs express an array of pro-regenerative paracrine factors.*** (**a**) Expression profile of pPICs arrayed by qRT-PCR, relative expression to average expression of five housekeeping genes ACTB, B2M, GAPDH, HPRT1, RPL13A. Data are mean ± SD, n=3. (**b**) Hierarchical clustering of differentially regulated pPIC transcripts. The colors in ascending order from green to red represent the magnitude of gene expression for the measured average difference values. The tree on the left of the clustergram indicates the pairwise similarity relationships between the clustered expression patterns, n=3 for both undifferentiated and differentiated pPICs (24 hours in differentiation media). (**c**) Genes that were differentially regulated by at least two fold were selected by a t-test, p<0.01 and are illustrated in a volcano plot. Red indicates those genes that were significantly upregulated, black represents genes whose expression levels did not significantly change, while green indicates genes that were significantly downregulated in differentiated pPICs compared to undifferentiated pPICs, n=3. (**d**) Western blots confirmed that pPIC exposure to a myogenic permissive environment led to an increase in IGF-1, TGF-β1, NRG-1 and a decrease in HGF protein expression, n=3.

Fig. 6. *The pPIC secretome promotes a regenerative response in vitro* (**a**) Quantification of pPIC BrdU 24h proliferation assay in standard growth media (GM), 24h pPIC conditioned media (CM), unconditioned media (UM), or heat inactivated conditioned media (ICM). Total BrdU^{pos} cells per total nuclei, determined from five fields of view per group. Data are mean ± SD, *P=0.05 vs. all conditions. (**b**) Quantification of human myoblasts BrdU 24h proliferation assay in GM, CM, UM or ICM. Total BrdU^{pos} cells per total nuclei, determined from five fields of view per group. Data are mean ± SD, *P=0.05 vs. UM and ICM. (**c**) Quantification of BrdU 24h proliferation assay in media supplemented with IGF-1, HGF, TGF-β1 or NRG-1 compared to control. Total BrdU^{pos} cells per total nuclei, determined from five fields of view per group. Data are mean ± SD, *P=0.05 vs. control and TGF-β1. (**d**) Representative micrographs of pPIC immunocytochemical staining for MHC after 5 days of myogenic differentiation in the presence of IGF-1, HGF, NRG-1 or TGF-β1 compared to control. (**e**) Quantification of pPIC fusion index after 5 days of myogenic differentiation in the presence of IGF-1, HGF, TGF-β1 or NRG-1 compared to control. Data are mean ± SD, *P=0.05 vs. control for >3 nuclei. (**f-g**) Endothelial network formation quantified as capillary area (**f**) and total length of capillaries (**g**), after HUVECs were exposed to endothelial growth medium (GM), pPIC conditioned media (CM), unconditioned medium (UM) and heat-inactivated conditioned media (ICM). Data are mean ± SD, *P=0.05 vs. ICM and GM.

Fig. 7. ***Administration of IGF-1/HGF and UPy IGF-1/HGF stimulates moderate skeletal muscle regeneration***. (**a**) HVG staining of uninjured, CTX:IGF-1/HGF and CTX:UPy IGF-

1/HGF-treated muscle, scale bar 100µm. (**b**) Immunohistochemical staining shows a number of $BrdU^{pos}$ centralized nuclei in both CTX:IGF-1/HGF and CTX:UPy IGF-1/HGF-treated muscle compared to uninjured muscle, scale bar 50µm. (**c**) Quantification of the ratio of skeletal muscle to connective tissue CSA in uninjured, CTX:IGF-1/HGF and CTX:UPy IGF-1/HGF-treated muscle determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. uninjured CTRL. (**d**) $BrdU^{pos}$ centralized nuclei per total myofibers, determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. uninjured CTRL. (**e**) Average myofiber diameter of regenerating ($BrdU^{pos}$ centralized nuclei) myofibers determined from five fields of view per muscle, n=5 animals per group. Data are mean ± SD, *P=0.05 vs. uninjured CTRL.