Title: Human umbilical cord perivascular cells improve human pancreatic islet transplant function by increasing vascularization

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One Sentence Summary: Human umbilical cord perivascular mesenchymal stromal cells improve vascularization and glycemic control of human islets implanted in mice.

Abstract: Islet transplantation is an efficacious therapy for Type 1 diabetes, however islets from multiple donor pancreata are required and a gradual attrition in transplant function is seen. Here we manufactured human umbilical cord perivascular mesenchymal stromal cells (HUCPVCs) to Good Manufacturing Practice (GMP) standards. HUCPVCs showed a stable phenotype while undergoing rapid ex vivo expansion at passage 2-4 and produced pro-regenerative factors, strongly suppressing T cell responses in the resting state and in response to inflammation. Transplanting an islet equivalent (IEQ):HUCPVC ratio of 1:30 under the kidney capsule in diabetic humanized NSG mice demonstrated the fastest return to normoglycemia by 3 days post-transplant: Superior glycemic control was seen at both early (2.7 weeks) and later stages (7, 12, and 16 weeks) versus ratios of 1:0, 1:10, and 1:50, respectively. Syngeneic islet transplantation in immunocompetent mice using the clinically-relevant hepatic portal route with a marginal islet mass showed that mice transplanted with an IEQ:HUCPVC ratio of 1:150 had superior glycemic control versus ratios of 1:0, 1:90, and 1:210 up to 6 weeks post-transplant. Immunodeficient mice transplanted with human islets (IEQ:HUCPVC ratio of 1:150) exhibited better glycemic control for 7 weeks post-transplant versus islet transplant alone, and islets transplanted via the hepatic portal vein in an allogeneic mouse model using a curative islet mass demonstrated delayed rejection of islets when co-transplanted with HUCPVCs (IEQ:HUCPVC ratio of 1:150). The immunosuppressive and pro-regenerative properties of HUCPVCs demonstrated long-term positive effects on graft function in vivo, indicating they may improve long-term human islet allotransplantation outcomes.

INTRODUCTION

Islet transplantation is effective for stabilizing glycemic control and restoring awareness of hypoglycemia in people with Type 1 diabetes (1-9), and there is evidence of diminished

progression of microvascular and macrovascular complications (10). However, transplant is suboptimal and individuals typically require two to three islet transplants to improve their glycemic control (4). Inflammatory and immunological rejection mechanisms lead to the early loss of islets (11, 12) and contribute to the attrition in graft function seen over time (4). Repeat immune sensitization events may arise due to the necessity for multiple transplants. Furthermore, the number of people that may receive transplants is limited by the availability of suitable donor pancreata (13).Therapeutic strategies to improve graft function are urgently needed. Ideally, a therapeutic strategy would modulate the inflammatory and immune response and enhance engraftment of islets through a variety of mechanisms, reducing the need for multiple transplants and high doses of immunosuppression. This would allow more patients to be treated and would enable the use of pancreata with marginal numbers of isolated islets to be used.

Mesenchymal stromal cells (MSCs) are multipotent cells found in most tissues which are capable of self-renewal and have potential for tri-lineage differentiation: osteogenic, adipogenic, and chondrogenic (14). They have been shown to promote regeneration of tissues by supporting blood vessel formation through production of a broad spectrum of growth factors and extracellular matrix building molecules (15). MSCs suppress the host immune response, both innate and adaptive pathways, through activating anti-inflammatory factors, suppressing B- and T-cell proliferation, and increasing production of regulatory T-cells and polarized M2 macrophages. Such functions are highly relevant to the therapy of the chronic inflammatory autoimmune disease Type 1 diabetes (16).

Bone marrow-derived MSCs (bmMSCs) have been the gold standard in clinical practice, enhancing donor bone marrow cell engraftment and chimerism (17), and have been co-transplanted with islets in animal models of diabetes with favorable effects on graft function and longevity (18). However, bmMSCs are present at low frequency in the marrow

compartment, cell harvesting is painful and invasive, and bmMSCs also exhibit a reduced *ex vivo* expansion capacity as donor age increases (19). MSCs from other sites, including umbilical cord, have been used in animal models of diabetes, although not in animal models of islet transplantation (20). Cellular preparations derived from umbilical cord can be obtained in clinically-relevant quantities with procurement advantages: cell harvesting is non-invasive, and cells are highly expandable ex vivo. Preparations from umbilical cord, which include MSC-type cells and other cell types (epithelial), can undergo freeze thaw cycles for off-the-shelf use (21) and have been used in clinical trials (20, 21). However, before such MSCs can be used in clinical islet allotransplantation they must be fully characterized, demonstrate greater efficacy on co-transplantation with islets versus islet transplantation alone, and they must be compliant with GMP.

There have been no studies in islet transplantation in man using allogeneic MSCs. MSC studies in Type 1 diabetes are heterogeneous and difficult to compare because the numbers and types of MSCs used have differed, the time interval between diagnosis of diabetes and infusion of stem cells is highly variable, and some studies use autologous whereas other studies use allogeneic stem cells (22; 23). No studies in man have developed and/or characterized GMP compliant umbilical cord MSCs for co-transplant with islets or have systematically examined the islet:MSC ratio, which has the greatest impact on short, and long, term glycemic control.

Here, we have adapted existing methods for production of well characterized human HUCPVC (24-27) to a fully GMP compliant, xeno-free culture system. Our aims were to repeatedly derive and characterize GMP grade HUCPVCs with respect to immune regulatory and regenerative properties *in vitro,* their metabolic efficacy in co-transplantation experiments with islets *in vivo* [including infusion via the hepatic portal vein (HPV) route used in human transplantation], and to quantify islet graft vascularization using rodent models of islet transplantation.

RESULTS

Generation of GMP grade HUCPVCs with stable MSC phenotype

Six donations of umbilical cord were processed into HUCPVC lines, frozen after the initial seed culture was established, and then thawed and expanded up to passage 4 (p4) using 3 GMP compliant media: StemMACS (SM), SM + group AB donor plasma (SMAB), and SM + platelet lysate (SMPL). HUCPVCs exhibited the characteristic MSC phenotype [International Society for Cellular Therapy criteria (28)]: expressing the markers CD73, CD90, and CD105 but negative for expression of CD34, CD45, CD14, and CD11b (Fig. 1A). HUCPVCs also consistently expressed CD10, which is definitively associated with perivascular cells of the Wharton's Jelly (29, 30). SMPL gave the shortest population doubling times, however, differences were not significant and all 3 media gave satisfactory results (Fig. 1B). The HUCPVC phenotype was stable throughout serial passage of the cells and HUCPVCs maintained similar stemness potential in all media, assessed by Colony Forming Unit-Fibroblast (CFU-F) assay. Lipoaspirate-derived MSCs, derived from abdominal subcutaneous tissue (used as a comparator material, see characterization in fig. S1), thawed, and grown in identical medium from p2-p4, demonstrated significantly lower CFU-F activity in the same assay (Fig. 1C). HUCPVCs exhibited the ability to differentiate into adipocytes, osteocytes, and chondrocytes *in vitro* (Fig. 1D). One donation from an individual umbilical cord could be manufactured into a cell product containing a minimum of 1.04 $x10^8$ cells (DMEM+PL (DMPL) at initiation: $1.04x10^8$ to $2.97x10^8$; n=3) or min $4.35x10^8$ cells (SMPL at initiation: $4.25x10^8$ to $24.5x10^8$; n=3) by passage 2. HUCPVCs constitutively expressed the T cell inhibitory surface molecules PD-L1 (CD274) and PD-L2 (CD273) (Fig 1E). "Licensing" (exposure to inflammatory cytokines) of HUCPVCs with Interferon-gamma (IFN- γ) increased the relative fluorescence intensity of CD273 and CD274 (Fig 1E).

GMP HUCPVCs are potent T cell inhibitors that do not require *a priori* licensing by IFN-gamma

HUCPVCs exhibited robust inhibition of T cell proliferation. Addition of these MSCs to mitogen-stimulated peripheral blood mononuclear cells (PBMCs) in a ratio of 2:1 PBMCs:HUCPVC inhibited T cell division (Fig. 2A), with an effect still measurable at 8:1 (Fig 2A). T cell inhibition by HUCPVCs was achieved without licensing). Addition of IFN- γ as a single licensing cytokine did not significantly increase the ability of the HUCPVCs to suppress T cell proliferation in this assay (Fig. 2B). Licensing of HUCPVCs with multiple cytokines - IFN- γ, Interleukin-1-beta (IL-1β), and Tumour necrosis factor-alpha (TNF-α) did not increase the ability of HUCPVCs to suppress T cell proliferation compared to unlicensed cells (fig. S2). Unlicensed HUCPVCs grown in all 3 GMP compliant media types efficiently suppressed T cell proliferation irrespective of whether the complete SM medium was supplemented with AB plasma or PL.

GMP HUCPVCs express multiple immune-regulatory and pro-regenerative factors

HUCPVCs were assessed in the unlicensed and licensed state for the expression of a variety of anti-inflammatory and pro-regenerative factors, comparing to lipoaspirate-derived MSCs. Both MSC types expressed steady amounts of transcript for TGF-beta with and without licensing (raw CT range: 21-24, Fig. 3A). Licensing of MSCs strongly increased the expression of TSG-6 and IDO (raw CT range: unstimulated 20-23, stimulated 14-16) (Fig. 3, B and C). CD274 expression was also significantly increased after licensing in HUCPVCs compared to lipoaspirate-derived MSCs (Fig. 3D). HUCPVCs significantly upregulated production of CXCL8 and VEGF protein after licensing (Fig. 3, E and F). Lipoaspirate-derived MSCs also produced increased amounts of CXCL8 and VEGF in response to licensing, although this did

not reach significance (Fig. 3, E and F). Both factors are strongly associated with blood vessel formation; VEGF is also a strong T cell activation inhibitor. Measurement of MSC-derived TGF-beta was not possible in the SM-based medium, as this medium contains TGF-beta as a supplement.

Comparative expression of multiple chemokines in the resting and licensed state of HUCPVCs and adipose tissue MSCs

HUCPVCs and MSCs derived from adipose tissue were assessed in the unlicensed and licensed state with respect to changes in chemokine expression. Chemokine expression profiles associated with an inflammatory state, an anti-inflammatory state, a proangiogenic state (chemokines containing the ELR motif), and a potentially angiostatic state (chemokines lacking the ELR motif) were assessed using 4 separate analyses. The ELR motif consists of 3 amino acids, Glu, Leu, Arg, present in a family of chemokines which are potent inducers of angiogenic activity (31).

The heat map in Fig. 4 demonstrates major differences between the MSC types, showing anti-inflammatory and proangiogenic properties of HUCPVCs and pro-inflammatory and potentially angiostatic activity of the lipoaspirate-derived MSCs. Of note the HUCPVCs do not express inflammatory-associated chemokines in either the steady state or after cytokine challenge, whereas lipoaspirate-derived MSCs express a suite of potentially inflammatory chemokines at rest and then switch to a different inflammatory state after cytokine challenge.

Kidney capsule transplantation in immunodeficient mice

To begin to evaluate the therapeutic potential of GMP HUCPVCs, experiments were set up to firstly examine islet grafts: for this the kidney capsule route was used. All immunodeficient

mice were transplanted with a marginal mass of human islets (3000 IEQ/mouse) within four weeks of administration of streptozotocin (STZ). Prior to transplant, there were no differences in glucose concentrations or weights among groups [glucose: 30.1±1.0 vs. 32.6±1.0 mmol/L (mean ± SEM), weight: 26.4± 0.5 vs. 25.6 ± 0.6 g, islet transplant group vs. islet+HUCPVCs group, respectively ($P > 0.05$)]. In the pilot experiments transplanting human islets (4000 IEQ/mouse) within four weeks of administration of STZ, there were no differences in glucose concentrations or weights among groups [glucose: 22.7±1.6 vs. 20.8±0.9 vs. 24.1±1.4 mmol/L (mean ± SEM), weight: 33.7± 0.5 vs. 29.8± 1.5g vs. 31.9±0.8g, islet transplant vs. islet+HUCPVCs vs. islet+Lipo-MSCs (all $P > 0.05$)]. Tables S1 and S2 show the clinical information for the human islet preparations used in these kidney capsule transplant studies, including donor characteristics and *in vitro* static glucose stimulated insulin release (GSIS) and oxygen consumption rate (OCR) normalized to DNA.

1:30 IEQ:HUCPVCs in a marginal islet transplant model in immunodeficient mice produced optimal long-term metabolic results

In initial pilot experiments, 4000 IEQs were transplanted in NOD SCID gamma (NSG) mice with or without $1x10^5$ HUCPVCs or Lipoaspirate-MSCs. Lipoaspirate-MSCs did not enhance islet function (fig. S3A). In mice receiving islets alone or islets plus HUCPVCs, glycemic control was tighter at 6 weeks post-transplant (fig. S3B, tables S1 and S2 – islet preparation 1). Stimulated blood glucose concentrations were below counterpart control NSG mice that had not received STZ [with normal glucose tolerance (NGT), fig. S3B]. Therefore, a marginal islet mass approach was adopted and subsequent experiments were performed using 3000 IEQs per mouse via the kidney capsule route, and only HUCPVCs were tested further for their ability to optimize islet transplant (tables S1 and S2 – islet preparation 2).

Mice receiving 3000 IEQs plus 90,000 HUCPVCs (1:30 IEQ:HUCPVCs) reached "cured" blood glucose concentrations post-transplant (defined as non-fasted plasma glucose <11 mmol/L after STZ and transplant) faster compared to mice receiving grafts of islets alone or lower numbers of HUCPVCs (Fig. 5A, post-hoc tests $P < 0.001$). The average number of days to cure was 16.0±1.7, 12.0±2.6, 3.0±0.4, and 9.0±3.6 for mice receiving grafts of 1:0, 1:10, 1:30, and 1:50 IEQ: HUCPVCs, respectively.

At 2.8 weeks post-transplant, glucose control as determined in the intra-peritoneal glucose tolerance test (IPGTT) was superior in mice transplanted with islets plus HUCPVCs, with no statistical significance between the islet plus HUCPVCs groups (Fig. 5B; Table 1). At 7 weeks post-transplant, glucose control determined by IPGTT was superior with 3000 islets plus 90,000 HUCPVCs (ratio of 1:30 IEQ:HUCPVCs), with no significant difference between grafts with islets alone and other ratios of HUCPVCs (Fig. 5C; Table 1). At 12 weeks post-transplant, the tightest glycemic control was seen in mice receiving 1:30 and 1:50 IEQ:HUCPVCs, with no difference in those receiving islets alone and 1:10 IEQ:HUCPVCs (Fig. 5D). By 16 weeks, the best glycemic control was seen in mice transplanted with 1:30 IEQ:HUCPVCs (Fig. 5E). On 2-way ANOVA analysis there was an effect of time post-transplant on the integrated glucose response, with an interaction between time post-transplant and numbers of HUCPVCs received. However, post-hoc analyses examining the effect of time (from 2.8 to 16 weeks) on graft function found no deterioration with the 1:30 IEQ:HUCPVCs grafts.

The ratios of circulating C-peptide to glucose concentration at 60 minutes concurred with these observations and were elevated in mice transplanted with 1: 30 IEQ:HUCPVCs at 12 and 16 weeks (Fig. 5F and G). Control experiments were performed in STZ-treated mice receiving Matrigel alone versus HUCPVCs in Matrigel. Glucose concentrations did not fully

normalize in either group, however glucose concentrations were lower in mice receiving HUCPVCs in Matrigel versus Matrigel alone (fig. S4).

Confirmatory studies demonstrate hyperglycemia after removal of islet grafts

After 16 weeks, mice with transplants (n=5) underwent left nephrectomies to remove islet grafts; these mice reverted to diabetes and blood glucose concentrations increased from 5.0±0.1 mmol/L on the day of surgery to hyperglycemia (18.8±1 mmol/L) three days post-surgery. These results confirmed that the normoglycemic state was a consequence of islet transplant rather than the regeneration of mouse islets in the pancreas (fig. S5).

Vessel density in islet graft was increased with islets co-transplanted with HUCPVCs

To determine whether vascularization was increased in the grafts where islets were co-transplanted with HUCPVCs, vessel density was quantified within the islet grafts. Vessel density was increased in islet grafts co-transplanted with 3000 islets plus HUCPVCs, in a ratio of 1:10 and 1:50 (Fig. 6, A and B), as compared to grafts of islets alone. Results were confirmed using immunofluorescence analysis with a further endothelial marker to ERG (fig. S6). Grafts with 4000 islets and $1x10^5$ HUCPVCs were also examined and demonstrated non-significantly greater vessel density in these grafts with HUCPVCs ($p = 0.07$, fig. S7).

In vivo syngeneic, allogeneic, and human islet transplants in mice via HPV

To examine the metabolic function of islets with HUCPVCs, transplant experiments were done via the hepatic portal vein into the liver, emulating the clinical route used in man. Islet transplantation in man involves transplantation of islets from a donor with a different tissue and blood type to the recipient and islets may be rejected in part secondary to alloimmune

mechanisms. To model this allogeneic transplant in mice, immunocompetent C57Bl/6 mice may be transplanted with islets from Balb/C mice, eliciting a brisk rejection of transplanted Balb/C islets. A marginal Balb/C islet mass (500 IEQs/mouse) was used and IEQ:HUCPVCs ratios of 1:0, 1:90, 1:150, and 1:210 were explored. In a second experiment a curative mass was used (900 IEQ/mouse), comparing IEQ:HUCPVCs ratios of 1:0 versus 1:150. To investigate syngeneic transplant, immunocompetent C57Bl/6 mice were transplanted with islets from C57Bl/6 mice within 9 days of administration of STZ. Finally, immunodeficient NSG mice were transplanted with human islets from a single islet isolation (table S1 and S2; donor 3).

Prior to transplant, all groups of mice had established diabetes with blood glucose readings > 17 mmol/L and there were no differences in glucose concentrations or weights between treatment groups ($P > 0.05$, table S3). There was no evidence of cellular mediated rejection in the liver after transplantation of 150,000 HUCPVCs with mouse islets via the HPV of immunocompetent C57Bl/6 mice at day 1 and day 8 post-transplantation (fig. S8).

1:90 and 1:150 IEQs:HUCPVCs via the HPV produced optimal metabolic results in short-term allogeneic experiments

Using a marginal islet mass approach, transplant of 500 IEQs per mouse±HUCPVCs was tested to assess optimized islet function up to 10 days post-transplant. Islets from a BALb/C mouse were transplanted into a C57Bl/6 mouse via the HPV at IEQ:HUCPVCs ratios of 1:0, 1:30, 1:90, and 1:150. Blood glucose concentrations decreased most in the mice transplanted with 1:90 and 1:150 IEQ:HUCPVCs (Fig. 7A), although this decrease did not reach statistical significance. Blood glucose concentrations did not change in mice transplanted with islets alone. Blood glucose concentrations reached a nadir at day 2-3 post-transplant but glycemic control deteriorated in all groups by day 8 post-transplant, reflecting rejection of the islet grafts.

The experiments were repeated with 900 IEQs per mouse±HUCPVCs in a ratio of 1:150 versus 900 islets alone in an attempt to cure the mouse of diabetes and to ascertain whether HUCPVCs ameliorated or delayed graft rejection. Five of 6 mice receiving HUCPVCs were cured of diabetes at day 1 versus 3 out of 7 mice receiving islets alone. There was a slower increment in blood glucose concentrations in all mice receiving islets plus HUCPVCs, consistent with delayed graft rejection (Fig 7B). Frank hyperglycemia (plasma glucose ≥13 mmol/L) was evident by day 5 in all mice receiving islets alone whereas hyperglycemia in the mice receiving islets plus HUCPVCs was delayed until day 8 ($P = 0.04$).

1:150 IEQ:HUCPVC transplanted via the HPV in an immunocompetent syngeneic model produced optimal metabolic results in long-term experiments

Using a marginal islet mass approach, 500 IEQs were implanted per mouse with or without HUCPVCs to assess optimized islet function up to 6 weeks post-transplant. Islets from C57Bl/6 mice were transplanted into C57Bl/6 recipient mice via the HPV in the following IEQ:HUCPVCs ratios: 1:0, 1:90, 1:150, and 1:210.

Blood glucose concentrations decreased in the mice transplanted with 1:150 IEQ:HUCPVCs, and these mice maintained the tightest glycemic control over the 6 week duration of the experiment (Fig. 7C). The mice receiving an IEQ:HUCPVC ratio of 1:150 had the greatest insulin:glucose concentrations after IPGTT 6 weeks post-transplant (Fig. 7D). All mice (n=8) transplanted with 1:150 IEQ:HUCPVCs were cured from their diabetes versus n=3-4 mice in all other groups (χ^2=7.5; p=0.02). Liver function tests at cull were normal in both groups with no significant differences between the groups (fig. S9).

1:150 human IEQ:HUCPVC transplanted via the HPV in an immunodeficient mouse model cured diabetes and led to long-term cure rates

Using a marginal islet mass approach, 700 human IEQs per mouse with or without HUCPVCs in a ratio of 1:150 IEQ:HUCPVCs were transplanted to determine ability to cure diabetes and maintain cure for 7 weeks after islet transplantation. Blood glucose concentrations decreased significantly in the mice transplanted with 1:150 IEQ:HUCPVCs and diabetes in mice was cured, maintaining normoglycemia for the 7 week duration of the experiment (Fig. 8). At the end of the experiment, the average glucose concentration in mice receiving islet-only transplants vs. islet ±HUCPVCs was 34.7±7.2 vs. 11.2±2.6 mmol/L (mean±SEM, $P < 0.01$). Six out of 8 mice transplanted with 1:150 IEQ:HUCPVCs were cured from their diabetes versus n=0 mice in the group transplanted with islets without HUCPVCs ($\chi^2=6$; $p<0.01$).

DISCUSSION

Here we demonstrated that GMP compliant HUCPVCs produced in xeno-free conditions are capable of rapid post-thaw *in vitro* expansion at passage 2-4, with stability of phenotype throughout passages. HUCPVCs exhibit immunosuppressive properties in the non-licensed state. We also demonstrate that these specific MSCs are distinct from those derived from lipoaspirate, with anti-inflammatory and pro-regenerative properties in the licensed state. *In vivo,* using a kidney capsule route of administration, the ratio of HUCPVCs to human islets, which demonstrated optimal metabolic function (32), was 30 to 1. Transplanting grafts of this ratio led to rapid control of glucose by 3 days post-transplant and superior graft function at both early (2.7 weeks post-transplant) and later stages (7, 12, and 16 weeks post-transplant). After 100 days, defined as long-term in such models (33), there was no evidence of graft

dysfunction and there was increased vascular density in grafts containing HUCPVCs versus grafts of islets alone.

In immunocompetent mice, we demonstrated that these human umbilical cord MSCs did not elicit an immune response, consistent with other studies (34) and providing proof of concept of using this model in syngeneic islet transplant experiments in the absence of immunosuppression. Transplanting mouse islets with HUCPVCs (1:150 IEQ:HUCPVCs) by the HPV route in immunocompetent mouse models produced optimal glycemic results in the short and longer term (up to 6 weeks after transplant). Previous experiments had not systematically examined the dose of MSCs relative to islets for efficacy regarding glycemic control. We further confirmed that transplantation of human islets with HUCPVCs, transplanted via the HPV route, in immunodeficient mice led to a cure of diabetes in these mice which was maintained for the 7-week duration of the experiment without evidence of long-term adverse effects on the liver.

The ease of procurement of umbilical cord tissue over other tissue sources, ability to rapidly expand this MSC population, and stable phenotype makes this an ideal MSC source for off-the-shelf banking and use (35). MSCs used in these experiments were assayed after a cryopreservation step at p1, thawing cells in xeno-free GMP medium before use, with demonstrable *in vivo* function. The functional utility of thawed HUCPVCs used immediately following cryopreservation has also recently been demonstrated (36), contrasting with prior reports suggesting it is necessary to culture bone marrow mesenchymal cells after cryopreservation to restore their immune-regulatory properties (37; 38). In contrast to our findings, some groups have demonstrated that MSCs derived from adipose tissue have a beneficial effect on islet transplantation (39); the source of fat as well as other donor factors may be critical.

The ability to suppress T cell activation and express TGF-beta in the non-stimulated state has not previously been described for MSCs (40; 41) and is beneficial, along with the ability to upregulate pro-regenerative factors when induced by inflammation. TSG-6 expression has been recently shown to play a central role in MSC suppression of inflammation *in vivo* (42) and the HUCPVCs used here highly upregulated this factor in response to inflammation. At 24 to 48 hours post-islet transplant there is marked inflammation in the liver (11; 43; 44) which compromises islet survival and engraftment. MSCs may exert protective effects by promoting angiogenesis and immunomodulation (45; 46). With time, as inflammation becomes quiescent, vascularization of islets takes place and is largely complete within 4 to 6 weeks.

Autologous bmMSC trials are underway in different disease areas including patients with Type 1 diabetes (47). Furthermore in certain diseases including graft versus host disease (48-53), critical limb ischemia (54; 55), and acute myocardial infarction (56), allogeneic bmMSCs have been administered with considerable success, although no Phase III trials have been reported. No center has performed randomized controlled clinical trials using allogeneic MSCs in patients with Type 1 diabetes undergoing islet transplantation. One open label, randomized, pilot study in humans transplanting autologous bone marrow mononuclear cells (bmMNC) with a small proportion of Wharton's Jelly-derived cells that had not been phenotyped demonstrated evidence of preservation of graft function (23). No conclusions with respect to the effect of the mesenchymal cell population can be reached from these bmMNC studies, although such studies illustrate the clinical utility of MSCs for transplantation in Type 1 diabetes.

There are several small studies that have examined autologous bmMSCs in Type 1 diabetes and in animal models of islet transplantation. Many studies used an IEQ:MSC ratio of approximately 1:10 or lower (57-59), showing modest effects on graft function but pronounced

effects on graft vascularity. Although dosage experiments are difficult to perform and interpret in clinical practice, the optimum ratio that we demonstrate is in excess of previously reported ratios and in concord with MSC numbers used in primate studies (18). In our experiments, an IEQ:MSC dose of 1:10 did not yield sustained effects on graft function. It is of interest that the largest quantity of MSCs in our study did not demonstrate the greatest benefit in terms of graft function, indicating the possibility of a plateau effect with MSCs that may be tissue source dependent. This further emphasizes the importance of our dose response studies. The experimental route of human islet transplantation under the kidney capsule was selected for experiments in immunodeficient mice because islets remain at the site of delivery, enabling quantification of vasculogenesis; we confirmed vascular density using two different methodologies. Islet delivery via the hepatic portal route more closely reflects the clinical scenario, however the islets tend to disseminate throughout the liver as well as the systemic circulation and it is therefore not possible to quantify vasculogenesis. Our HPV studies in immunocompetent mouse models using mouse islets, as well as in immunodeficient mouse models using human islets, inform the optimal ratio of islets to HUCPVCs to use via the hepatic portal route of delivery in man and we further confirmed longevity of graft function to 7 weeks in these models using a separate isolation of islets.

The fate of MSCs post-transplant is largely unknown; tracking cells to determine whether they engraft or differentiate is necessary but remains a limitation of the present work. We presented extensive data regarding expression of inflammatory response modifying factors by the MSCs, which associated with improved transplant outcome. However, the effects of transplanted MSCs on recruitment and modification of other host cell populations in the transplant setting is not yet determined. Immune modulation and angiogenic factor expression is strongly upregulated in vitro after licensing with cytokines, and repeating our observations with licensed MSCs could aid interpretation of the therapeutic relevance of the many factors

expressed by the MSC. Here we examined both grafts formulated in Matrigel and transplanted into the kidney and grafts introduced to the liver via the HPV, the route used in humans. We cannot robustly compare the numbers of HUCPVCs used successfully in this study to numbers of bmMSCs used in other published studies due to differences in the experimental route of transplant. Other limitations include that the HUCPVC studies were not carried out with immunosuppression in immunocompetent recipients and that the experiments were confined to small animal models using only male animals. Dose escalation studies are required to fully evaluate the effect of HUCPVCs with immunosuppression in man.

An important aspect of these studies is that human GMP grade HUCPVCs at passage 3 and human islets were used in immunodeficient mouse models; furthermore, a single large islet donation was used for the main transplant experiments via the kidney capsule and the HPV route in the mice, facilitating interpretation of the findings. It is well recognized that there is considerable donor heterogeneity in islet samples. Thus, such studies are difficult to perform in animal models with respect to timing.

The findings of this study have major clinical implications. At present, subjects with Type 1 diabetes need to receive islets from at least two donor pancreata to improve glycemic control; there is a shortage of suitable donor pancreata, and attrition in graft function is seen over time, likely secondary to loss of beta cells through autoimmune, alloimmune, and inflammatory mechanisms. Furthermore, over 60% of islets are lost in the first 24 to 48 hours post-transplant (11; 43; 44). Our study demonstrates the mechanism of action of GMP grade HUCPVCs *in vitro* and positive effects *in vivo* on graft function *in vivo.* These results lay the foundation for future safety studies in animal models, which should include the tracking of MSCs by state-of-the-art molecular techniques and an assessment of long-term safety and tumorigenesis. With such data it will be possible to proceed with randomized controlled clinical trials in man.

MATERIALS AND METHODS

Study design

The purpose of this study was to explore whether MSCs improved islet transplantation outcomes, and to elucidate the mechanism of action. We carried out islet transplantation experiments using the kidney capsule route to examine the islet graft in its entirety, as well as transplantation via the HPV to emulate the transplant route used clinically. MSCs manufactured using GMP compliant methods from human umbilical cord and adipose tissue were extensively characterized at a phenotypic and molecular level, including expression of inflammation and angiogenesis-modulating chemokines and cytokines.

Human islets were transplanted into immunodeficient mouse models and mouse islets were transplanted in immunocompetent mouse models. The optimal ratio of islets to HUCPVCs was determined. Sample sizes were determined on the basis of prior experience with similar studies and pilot experiments. All mice used were males to eliminate any potential confounding influences of differences in engraftment secondary to gender. All mice were randomly assigned to treatment groups and all analysis performed blinded to treatment condition. No surviving animals were excluded from analysis and no outliers were excluded. The number of biologic replicates is specified in the figure legends.

Materials

All human tissue (donated material) was fully consented for use in research, and ethical committee approval was obtained [Pancreatic islets ethics approval (SNBTS) REC reference: 13/NW/0105 IRAS project ID: 124650 and sample governance 14-29v2 and University of Alberta Research Ethics Board, Pro00013094]. The use of umbilical cord material was governed under ethics approval (Edinburgh Reproductive Tissue Biobank (ERTBB)-097 and

NHS Greater Glasgow & Clyde Project REC reference GN14NN406). All blood samples used in immune response assays were governed under the SNBTS sample governance committee approval process permission 14-02.

Generation of GMP grade HUCPVC

HUCPVC were generated for use in this study under a collaborative research agreement with Tissue Regeneration Therapeutics Inc. Methodology was based on published methods for establishment of HUCPVC from Wharton's Jelly (25) with modifications to use commercially available xeno-free medium with or without clinical grade AB plasma or platelet lysate supplements. Briefly, umbilical cords were aseptically dissected as described (26), and the Wharton's Jelly (WJ) was placed in culture. The cells are described as HUCPVCs as the WJ was harvested from the clearly distinguished tunica media of the 3 vessels of the cord [as discussed in Davies *et al.* (30)]. The cells are thus perivascular stromal cells, but not pericytes. Umbilical cord of 6-10 cm yielded approximately 2 ml of dissected material that was used to seed one 175 cm^2 flask. Explants were cultured in DMEM (Gibco), Heparin (Leo Labs) at a final concentration of 2 IU/mL, 1x Non-essential Amino Acids (Gibco) supplemented with 25% human platelet lysate (PL) in Corning Cell Bind flasks with no additional substrate. Once the cells had reached 80-90% confluence the adherent MSCs were recovered with a 10 min incubation at 37°C with 10 mL of 1 x TryplE (Gibco). To remove cell debris the material was passed through a 100 µm cell strainer (Falcon). The cells were counted using a haemocytometer and designated as passage 1 (for standardization purposes; the growth and time in culture for each cell line was recorded according to doubling rate). These MSCs were then cryopreserved at $1x10^6$ / vial in Cryostor (CS10, Sigma Ltd). Later in the study a further 3 cell isolates cells were grown to p1 in StemMACS medium supplemented with 25% PL instead of the DMEM-based medium. Thawed cells were washed once in StemMACS medium and re-cultured at

3000 cells/cm^2 in fresh flasks for up to 4 passages. Cells were cultivated from passage 2 to passage 4 in 3 GMP complaint xeno-free media – StemMACS MSC XF (SM) (Miltenyi Biotec), Stem MACS MSC XF supplemented with 25% human Platelet Lysate (SM PL), or Stem MACS MSC XF supplemented with 10% human AB plasma (SM AB).

Preparation of mouse models prior to islet transplantation experiments

All animal procedures complied with Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, and conformed to local ethical requirements of the University of Edinburgh. Male NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ /NOD SCID Gamma) mice were used to establish the human islet transplant models, male C57Bl/6 mice were used to establish syngeneic and allogeneic islet transplant models. Animals (n=8-10/group) were rendered hyperglycemic at 16-17 weeks old by administration of 160-180 mg/kg i.p. STZ (Sigma-Aldrich) in ice cold acetate buffer, pH 4.5, following a 4-hour fast. Daily tail blood glucose measurements were taken using a glucometer (One Touch Verio, LifeScan). Mice were classed as hyperglycemic if their non-fasted glucose concentrations were ≥20 mmol/L for two consecutive days. Once diabetes was confirmed, daily subcutaneous injections (0.5-2.5U) of insulin glargine (Lantus, Sanofi) were administered, until the day prior to islet transplantation. All islet transplantations took place within 4 weeks of administration of STZ.

Procurement and preparation of human islets and HUCPVCs

Three human islet preparations were isolated from donor pancreata by the Islet isolation laboratories using the Edmonton Protocol (2). In the clinical islet isolation laboratory (SNBTS) those islet preparations that did not meet clinical release criteria because of numbers (<200,000 IEQs) and that were consented for research purposes were utilized in the laboratory. Due to the inherent heterogeneity of donor islet preparations, one large islet preparation was used in the

longitudinal NSG mouse experiments with 3000 IEQs transplanted via the kidney capsule route as described (Table S1 and S2). Prior to this one preparation had been used in pilot experiments that utilized 4000 IEQs transplanted via the kidney capsule route. Similarly in the HPV transplants in NSG mice, one large islet preparation from a single islet isolation (donor preparation 3; Alberta Diabetes Institute, Islet Core Facility) was transplanted. Upon receipt of islets by the research lab, the islets were cultured in CMRL-1066 media (Mediatech), supplemented with 10% FCS (v/v, Scientific Laboratory Supplies), heparin (10 U/ml, Leo Pharma), penicillin/streptomycin (100 U/ml and 100 µg/ml respectively, Gibco), and 2mM L-Glutamine (Gibco), at 22°C/5% CO_2. All HUCPVCs for *in vivo* use were thawed from the p1 stock and grown to p3 for use. Cells were cultured in StemMACS (Miltenyi Biotech), and 5% Human PL.

Islet transplantation in immunodeficient NSG mouse model via kidney capsule route

Islet transplantation was carried out at 4000 IEQs initially ±1x10^5 Lipoaspirate MSC or HUCPVC with relevant control groups in the initial studies (fig. S1). Subsequently a marginal mass model using 3000 IEQs were transplanted with 3 ranges of HUCPVCs (ratios: 1:10, 1:30, 1:50) with parallel control groups including with islets alone, matrigel and HUCPVCs plus matrigel (6 groups).

In all co-transplantation experiments of MSC plus islets, both islets and HUCPVCs were placed into 0.5ml eppendorf tubes in co-culture for an hour at room temperature before transplant. Excess medium was drawn off to leave an islet pellet to which 10-20µl of ice cold matrigel (reduced-growth factor, Corning) was added. The islet-matrigel solution was drawn up into PE50 tubing just prior to transplant.

All mice were 20-21 weeks old at the time of transplant. Mice were anaesthetized using isoflurane (1.5-3%), the left kidney was exposed under aseptic conditions and the matrigel

(±islets±HUCPVCs) transplanted under the left kidney capsule. The wound was sutured and clipped before recovery. Control experiments were performed at the same time in the same cohorts of mice.

In vivo assessment of efficacy of HUCPVCs in islet engraftment and function in an immunodeficient NSG mouse model and immunocompetent C57Bl/6 mouse model

The function of the islet plus MSC grafts were assessed with an IPGTT. At 2.7, 7, 12 and 16 weeks post-transplantation under the kidney capsule. NSG mice were fasted overnight and, following a basal tail blood glucose reading, received 2g glucose/kg fasted body mass by *i.p* injection. Glucose measurements were then taken at 15, 30, 60, 90, and 120 min post-glucose injection. A blood sample was taken at the 60 min time point (into EDTA) for stimulated plasma C-peptide analysis. At the 16-week IPGTT further blood samples were also taken at 0 min and 120 min time points. IPGTTs were similarly carried out following transplantation of C57Bl/6 mouse islets via the HPV route into C57Bl/6 mice. At 6 weeks post-transplant, a blood sample was taken fasting and at the 60 min time point (into EDTA) for stimulated plasma insulin analysis.

Unilateral left nephrectomy as confirmation of insulin-secreting function of islets ± HUCPVCs engrafted kidney

The left kidneys of half the mice (receiving 1:30 or 1:50 IEQ:HUCPVCs at transplant) were removed via laparotomy at day 101, defined as the point of long-term engraftment (60). These mice were followed for a further five days to confirm the return of hyperglycemia.

All mice were culled following the IPGTT at 16 weeks and histology, including assessment of vascular density, performed.

Mouse islet isolation and culture

Pancreatic islets were isolated from C57BL/6, BALB/c mice (9 weeks old, 20-22 g) by a collagenase digestion method (61). The islets (250 islet/mL) were cultured free floating (37-C, 5% CO2) in RPMI medium 1640 (Bio-Whittaker, Walkersville, MD) supplemented with L-glutamine (Sigma), penicillin-streptomycin (1000 U/mLY10 mg/mL; Sigma) and 10% (vol/vol) fetal calf serum (HyClone, Celbio, Logan, UT) for up to 12 hours before the transplant. Islet purity was ≥90%.

Transplantation experiments via the HPV route in immunocompetent mice

All mice were 12-14 weeks old at the time of transplant. Animals were anaesthetized using isoflurane (1.5-3%), and HPV transplant was performed following a midline laparotomy exposing the HPV with subsequent injection of islets into the vein using a 27G needle followed by control of bleeding and resuturing of the skin wound.

Islets ± HUCPVC transplant experiments in mice via the HPV route

HUCPVCs (n=150,000) plus islets versus control experiments with islets alone (500IEQ/mouse) were injected into C57Bl/6 mice via the HPV route.

Mice were sacrificed at day 3 and day 8 (n=8/group). The livers were removed, step sectioned (5µm) and H and E stained for an immune infiltrate and quantified using the Nikon Eclipse TE2000-S microscope.

Allogeneic islet ± HUCPVCs transplants from BALB/c into C57Bl/6 mice

Allogeneic experiments used islets from BALB/c mice transplanted into C57Bl/6 mice via the HPV route with IEQ: HUCPVC ratio of 1:0, 1:30, 1:90 and 1:150 (n=6/group; n=500 IEQs per mouse). Mice were sacrificed at day 10 post-transplant.

In a second series of experiments, mice were transplanted with IEQ: HUCPVC ratio of 1:0 and 1:150 (n=8/group; 900 IEQs per mouse). Mice were sacrificed at day 21 post-transplant.

Syngeneic islet ± HUCPVCs transplants from C57Bl/6 donor and recipients

Syngeneic experiments used C57Bl/6 mice as donors and recipients with an IEQ: HUCPVC ratio of 1:0, 1:90 and 1:150 and 1:210 (n=7-9/group). Mice were sacrificed at 6 weeks post-transplant. At cull bloods were analyzed for liver function tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin].

Statistical analysis

Data was tested for normality and transformations were applied as appropriate. The integrated area under the curve (AUC) for glucose was calculated by the trapezoid rule (62). Glucose AUC measurements, time to cure diabetes with IEQ± HUCPVCs, 60-minute C-peptide:glucose ratios, and graft vessel densities were compared by ANOVA with post-hoc testing between groups. Gene expression and protein concentrations were examined by ANOVA, or in the case of two data sets, unpaired t-tests or Mann Whitney U test as specified. Statistical significance was taken as $p<0.05$. Group sizes of n=6 for the mouse studies (59) demonstrated 80% power to detect a 20% difference in response at the 95% level of significance.

Supplementary Materials

Materials and Methods

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cytokine analyses. AB, JN, PB, RC, and NM assisted and advised with metabolic assays. AB helped draft methods. All authors commented and reviewed manuscript. **Competing interests:** There were no conflicts of interest. **Data and materials availability:** All data associated with this study are present in the paper or the supplementary materials. Islets from the Alberta Diabetes Institute, Islet Core Facility were obtained under a material transfer agreement.

Figure legends

Fig. 1. HUCPVCs grown in different GMP compliant media. (**A**) Representative phenotypic analysis of HUCPVCs grown in GM-compliant medium (SMAB). HUCPVCs passage 3, baseline fluorescence set using matched isotype controls. Right: Percentage of HUCPVCs expressing a CD14-CD45-CD73+CD90+CD105+ phenotype across 3 serial passages (p2-p4). *n* = 3 individual donations. (**B**) HUCPVCs grown in different GMP media across 3 passages (p2-p4) from *n* = 5 donated samples of umbilical cord. Mean doubling time ± SEM (days) using different GMP compliant media. No significant differences between media used to expand cell lines 2-way Anova with Tukey's multiple comparison test. P ranges from 0.22 to 0.99. (**C**) Colony forming unit scores for HUCPVCs and lipoaspirate MSCs grown in different GMP compliant media from 3 different tissue donations across 3 passages (p2-p4) with numbers of colonies obtained from plating 10 cells/cm^2 in CFU-F medium quantified mean ± SEM shown, CFU-F significantly higher in HUCPVC than lipoaspirate cells ($p<0.01$) paired T-test in all media tested. (**D**) Differentiation of HUCPVCs to adipocytes, osteocytes, and chondrocytes. Samples were stained with Oil Red-O (adipo), Alazarin Red (osteo), or Safranin O (chondro) as described (63). Bright field microscopy magnification 40x. (**E**) Analysis of PD-L2 (CD273) and PD-L1 (CD274) expression on HUCPVCs with and without licensing. Light grey peak /green outline– isotype control; grey peak/blue outline – unlicensed; white peak licensed. SM – StemMACS, SMAB – SM + AB plasma, SMPL – SM + platelet lysate; CFU-F – Colony forming unit-fibroblasts.

Fig. 2. Inhibition of T cell proliferation and requirement for IFN-γ licensing for HUCPVCs to mediate this effect. (**A**) Representative dye dilution results measuring inhibition of T cell proliferation by HUCPVCs. HUCPVC were grown in SMAB, assayed at p4, and cultured with Ef670-stained PBMCs; ratios are PBMC:HUCPVC at outset of culture. PHA: phytohemagglutinin. (**B**) Inhibition of T cell proliferation with and without IFN-γ licensing. Comparative measurement of inhibitory effects was made by comparing the percentage of T cells remaining in the undivided peak after activation with or without titrated amounts of MSCs. Mean±SD of 3 different HUCPVC lines grown in SM PL, ratios are PBMC:HUCPVC. (No significant differences between licensed and unlicensed groups at each concentration, paired T-test).

Fig. 3. Gene expression and protein production by resting and licensed MSCs. (**A**) TGFβ, (**B**) CD274, (**C**) IDO, and (**D**) TSG6 gene expression in resting and licensed MSCs from lipoaspirate (blue) and HUCPVCs (red), measured by qRT PCR and expressed as $2^{(-\Delta CT)}$ against expression of RPLP0. Means ± SEM n=3 donors, MSC at passage 3. * $P<0.05$, ** $P<0.01$. Where plain bars absent, gene expression was lower limit of detection and are not included in measurement of significance. (**E**) CXCL8 and (**F**) VEGF production by resting (plain bars) and licensed (hatched bars) MSCs from liposaspirate (blue) and HUCPVCs (red) measured by Luminex 100, in pg/ml. Means±SEM n=3 donors, HUCPVCs and MSCs at passage 3. Protein measurements shown net of background measurement of analyte in culture medium alone.

Fig. 4. Chemokine expression by resting and licensed Lipo-MSCs and HUCPVC. The expression of inflammatory, ELR+ve and ELR-ve chemokines by resting (-) and licensed (+) MSCs from Lipo-MSC (adipose) and HUCPVCs (umbilical cord) were measured by RT^2

Profiler PCR Arrays. The expression of anti-inflammatory genes was measured using qRT PCR as indicated. All samples were run at the same time on the same plate. In each case, the average $2^{(-\Delta CT)}$ is plotted and heatmaps were generated using Heatmapper software (http://www2.heatmapper.ca/). Each group of genes was analyzed separately. Genes are clustered using the single linkage method. n>3<6 donors, HUCPVC /MSCs at passage 3. (Expression of CD274, TSG-6, IDO, and TGF-beta same samples as Fig 3.A)

Fig. 5. Time to cure diabetes with IPGTTs in NSG mice receiving 3000 IEQs and HUCPVCs. (**A**) All mice were transplanted with 3000 IEQs plus different ratios of IEQ:HUCPVCs (1:0, 1:10, 1:30, and 1:50). Time to normoglycemia (non-fasted glucose <11.0 mmol/L) was determined. Tail vein sampling for glucose concentrations (mmol/L) during IPGTT (2g/kg) was done at 0, 15, 30, 60, 90 and 120 minutes following IPGTT. Stimulated C-peptide concentrations (pmol/L) were sampled at 60 mins post IPGTT. Glucose concentrations at (**B**) 2.8 weeks, (**C**) 7 weeks, (**D**) 12 weeks, and (**E**) 16 weeks after islet transplant. Stimulated C-peptide concentration (pmol/L) divided by glucose concentration (mmol/L) at (**F**) 12 weeks and (**G**) 16 weeks. N=6-8 mice/group. Data presented as Mean±SEM. See Table 1 for additional information. Black bar: 3000 IEQs alone; red bar: 1:10 ratio of IEQ: HUCPVCs; red horizontal lined bar: 1:30 ratio of IEQ: HUCPVCs; red vertical lined bar: 1:50 ratio of IEQ: HUCPVCs. Post-hoc analyses for significant differences between ratios of IEQ: HUCPVCs are denoted: a -1:0 vs. 1:10; b – 1:0 vs. 1:30; c -1:0 vs. 1:50; d - 1:10 vs. 1:30; e - 1:10 vs. 1:50; f – 1:30 vs. 1:50.

Fig. 6. Vessel density in islet grafts implanted under kidney capsule at 16 weeks post-transplant. Diabetic NSG mice were transplanted with 3000 IEQs±HUCPVCs (IEQ:HUCPVCs of 1:0, 1:10, 1:30, and 1:50). Serial sections (5 μm) in n=5 mice per group were cut through the entire islet graft below the kidney capsule and vessel density associated with the islet graft was quantified. (**A**) Vessel density at 16 weeks post-transplant; Data presented as Mean±SEM. (**B**) Immunofluorescence image of a representative section showing vessel quantification in an islet graft. Endothelial cells (Isolectin B4) and mature vessels (anti-smooth muscle alpha-actin antibody) were identified by immunostaining. Black bar: 3000 IEQs alone; red bar: 1:10 ratio of IEQ: HUCPVCs; red horizontal lined bar: 1:30 ratio of IEQ: HUCPVCs; red vertical lined bar: 1:50 ratio of IEQ: HUCPVCs. Post-hoc analyses for significant differences between ratios of IEQ: HUCPVCs are denoted: a -1:0 vs. 1:10; b – 1:0 vs. 1:30; c -1:0 vs. 1:50; d - 1:10 vs. 1:30; e - 1:10 vs. 1:50; f – 1:30 vs. 1:50.

Fig. 7. Blood glucose concentrations after islet transplant ±HUCPVCs in mismatch (allogeneic) and syngeneic transplants. (**A**)**.** Immunocompetent C57Bl/6 mice (n=4-6/group) were transplanted with allogeneic Balb/C islets (n=500 IEQs) and HUCPVCs (ratios 1:0, 1:30, 1:90 and 1:150). (p>0.05) Mean values of glucose concentration are shown. (**B).** Glucose concentration over time, comparing islet-only grafts (n=900 IEQs) to IEQ:HUCPVCs (1:150) in immunocompetent C57Bl/6 mice transplanted with Balb/C islets (n=8 mice /group). (**C**). Immunocompetent C57Bl/6 mice (n=8/group) were transplanted with syngeneic C57Bl/6 mouse islets and HUCPVCs (ratios 1:0, 1:90, 1:150, and 1:210). Mean values of glucose concentration are shown. Tx: transplant. (**D**). Stimulated (60 min) insulin concentration (pmol/L) post IPGTT divided by glucose concentration (mmol/L) at 6 weeks in immunocompetent syngeneic islet transplants. N=8 mice/group. Data presented as Mean±SEM.

Fig. 8. Blood glucose measurement after human islet ±HUCPVCs transplant in immunodeficient mice. Immunodeficient NSG mice (n=8/group) were transplanted with 700 human islets ± HUCPVCs in a ratio of 1:150 IEQ:HUCPVCs. Mean±SEM values shown; difference between groups $p<0.01$.

Table 1. AUC of glucose concentrations after IPGTT. Data presented as mean±SEM. The integrated area under curve of glucose concentrations were calculated during 120 minute IPGTT (mmol/Lxmin^2) at 2.8, 7, 12 and 16 weeks post islet transplant; N=6-8 mice/group. One way ANOVA analyses comparing effect of integrated glucose response with ratio of islets: HUCPVCs transplanted under the kidney capsule were compared at separate time intervals. Tx: transplant. Post-hoc analyses for significant differences between ratios of islets to HUCPVCs are denoted: a -1:0 vs. 1:10; b – 1:0 vs. 1:30; c -1:0 vs. 1:50; d - 1:10 vs. 1:30; e - 1:10 vs. 1:50; f – 1:30 vs. 1:50.

Weeks post-Tx	**Islet:HUCPVC 1:0**	**Islet:HUCPVC 1:10**	**Islet:HUCPVC 1:30**	**Islet:HUCPVC 1:50**	**p ANOVA**
2.8 weeks	970±8	430±20	457±9	457±23	$<0.0001^{a,b,c}$
7 weeks	902±171	672±35	473±15	691±53	0.02^{b}
12 weeks	1111±123	1218±40	418±34	441±30	$<0.0001^{b,c,d,e}$
16 weeks	1089±60	1045±58	544±29	721±19	$<0.0001^{b,c,d,e,f}$

Supplementary Materials and Methods

Human platelet lysate

Platelet lysate supplement (PL) was produced by repeatedly freezing and thawing date-expired Human Platelet (SNBTS) packs to -80°C for 12 hours and thawing at room temperature. The freeze/thaw cycles were repeated 3 times. Upon final thaw 10 platelet donation packs were pooled and centrifuged at 350g, before decanting the supernatants as 50mL aliquots and storing at -40°C.

CFU-F assay

Cells were diluted to 1000 cells/mL in HBSS. 100µL of cell suspension was plated in triplicate into 6 well plates (10 cm^2, Corning, Cellbind) and CFU-F growth medium added [DMEM (Gibco), 10% FCS (PAA), 1xNEAA (Gibco)]. Final cell density was thus 10 cells/cm^2. Plates were incubated at 37°C in 5% CO_2 and assessed for outgrowth of cells by visual inspection every 2-3 days. After 16-21 days, the wells were washed with PBS three times and the cells incubated with 1% crystal violet solution (Sigma) for 15 mins. The wells were washed in excess PBS, allowed to air-dry, and colonies >2 mm in size were counted.

Lineage differentiation

MSCs were assessed for their differentiation capacity using the Human Mesenchymal Stem Cell Functional Identification Kit (R&D systems Cat# SC006). Briefly, MSCs were cultured for 7-21 days in adipogenic, osteogenic (HUCPVC only), or chondrogenic differentiation media as detailed in the kit. The mature phenotype of adipocytes, osteocytes and chondrocytes were defined by staining as detailed in (61) (HUCPVC) or the binding of antibodies against FABP-4 and aggrecan

supplied in the kit (adipocytes and chondrocytes respectively, Lipo-MSC) and were assessed by microscopy.

RT^2 Profiler PCR Arrays

RT^2 Profiler PCR Arrays Human Chemokines & receptors (Qiagen) were prepared and performed using a 7900HT (ABI) sequence detection system in accordance with manufacturer's guidelines. Briefly, 102 µl of cDNA synthesis reaction was added to 650 µl of RT^2 SYBR Green Mastermix and 548 µl of RNase-free water. Volumes were scaled up appropriately depending on sample size. 10 µl of the PCR mastermix was added to each well of the RT^2 PCR array. Once loaded, the plate was sealed using optical adhesive film. The plate was centrifuged at 1000g at RT to remove bubbles and run on an applied Biosystems 7900HT cycler. Cycling conditions were as follows: 1 cycle for 10 mins at 90ºC followed by 40 cycles of 15s at 95ºC/ 1 min at 60ºC. Upon completion of the cycles a manual cycle threshold value of above background and within the linear phase was chosen. Data analysis was performed using QIAGEN's GeneGlobe data analysis center. Any gene with a CT value of >35 was determined as undetectable. Data is presented as $2^{(-\Delta CT)}$ and normalized to the reference gene *Beta-2 Microglobulin (B2M)*.

Gene expression

MSCs from the different tissues were plated at a density of $1x10^5/cm^2$ and grown for 24 hours in DMEM PL. Medium was removed and replaced with either complete medium or complete medium supplemented with 10 ng/mL IFN-gamma, IL-1b, TNF-a (R&D systems). Cells were incubated for a further 24 hours, and cells were harvested as above. Supernatants were frozen at -80C for Luminex analysis (see below).

Expression of chemokine, cytokine, chemokine receptor and cytokine receptor genes were assessed using quantitative PCR. Briefly, total RNA was isolated from cells using the RNeasy mini Kit protocol (QIAGEN) according to the manufacturer's instructions - cells were lysed using RLT buffer, then homogenized using a QIAshredder spin column. Genomic DNA was digested as described in the protocol and RNA was eluted from the RNeasy spin column by adding 30-50µl of nuclease free water (QIAGEN). This was then quantified by using a nanodrop (Nanodrop 1000 Thermo Scientific) and stored at -80ºC. cDNA was synthesized using the high capacity RNA to cDNA kit (Life Technologies) to a final concentration of 168ng/µl and frozen at in -20 ºC until required for gene expression studies.

Prior to use, cDNA was diluted 1 in 5 and then added to a mastermix consisting of PerfeCTa SYBR Green FastMix with ROX reference dye (VWR-International), primers and nuclease free water. Master mix (9µl) was dispensed into each well of the 384 well plate. Using a repeat dispenser pipette, 1µl of sample cDNA or –RT cDNA was dispensed into each well with the exception of no-template control wells, where cDNA was substituted with nuclease free water. Samples were run on an Applied Biosystem 7900HT thermal cycler. Cycling conditions were as follows: 95ºC for 10 minutes (1x cycle) followed by 95ºC for 3 seconds then 60ºC for 30 seconds (x40 cycles). All primers were designed using Primer3 Input software version 4.0.0 (http://primer3.ut.ee/). Primer details in supplementary methods table 1.

A reference gene Ribosomal Lateral Stalk Subunit P0 (RPLP0) which was most stably expressed throughout all samples was chosen for normalization. Any gene with a CT value of >35 was determined as undetectable. Data was expressed as expressed as $2^{(-\Delta CT)}$ against expression of RPLP0.

Protein expression

Conditioned media (24h) from the identical samples used for transcript analysis were collected and analyzed using a Luminex 100 analyzer (Bio rad) and premixed magnetic multi-analyte kits (R&D systems cat No. LXS-AHM-2) in accordance to the manufacturer's instructions. Samples were diluted 2-fold with calibrator diluent. The pre-coated microparticle cocktail was added to each well of the 96 well microplates, followed by either 50µl sample or standard, sealed and placed on an orbital shaker for 2 hours at RT. The plates were washed twice with 100 µl/well wash buffer and then incubated with 50µl/well anti-biotin detector antibody for 1 hour at RT on the shaker then washed as before and 50µl/well of streptavidin-phycoerythrin was added and incubated for 30 mins at RT. Microparticles were resuspended in 100µl/well of wash buffer and immediately read on the Bio-Rad analyser. Each microparticle bead region was designated and doublets excluded as stated on the certificate of analysis.

Supplementary Methods Table 1

Primer pairs

Gene Name	Left Primer	Right Primer
IDO	gcaagaacgggacactttgc	tgcctttccagccagacaaa
CD274	gcctccactcaatgcctcaa	ctgtcccgttccaacactga
TGF-beta	aagtggacatcaacgggttca	gggtggccatgagaagca
TSG-6	agcacggtctggcaaataca	gcagcacagacatgaaatccaat
HGF	ttgcctgaaagatatcccgacaa	cgggtgtgagggtcaagag
CFH	ggaagggagaatgggttgct	gatgtccacagggccttttct

Fig.1

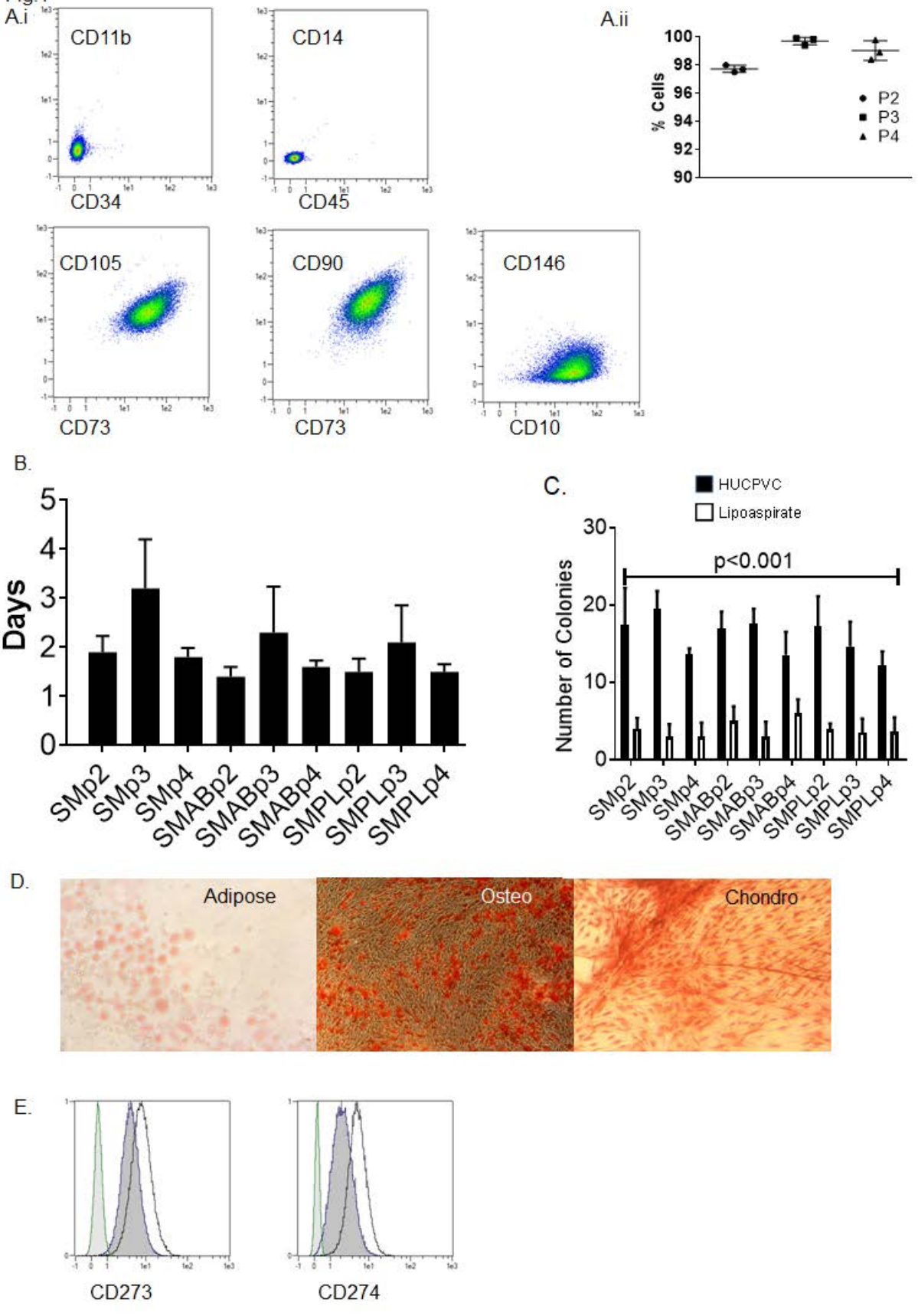
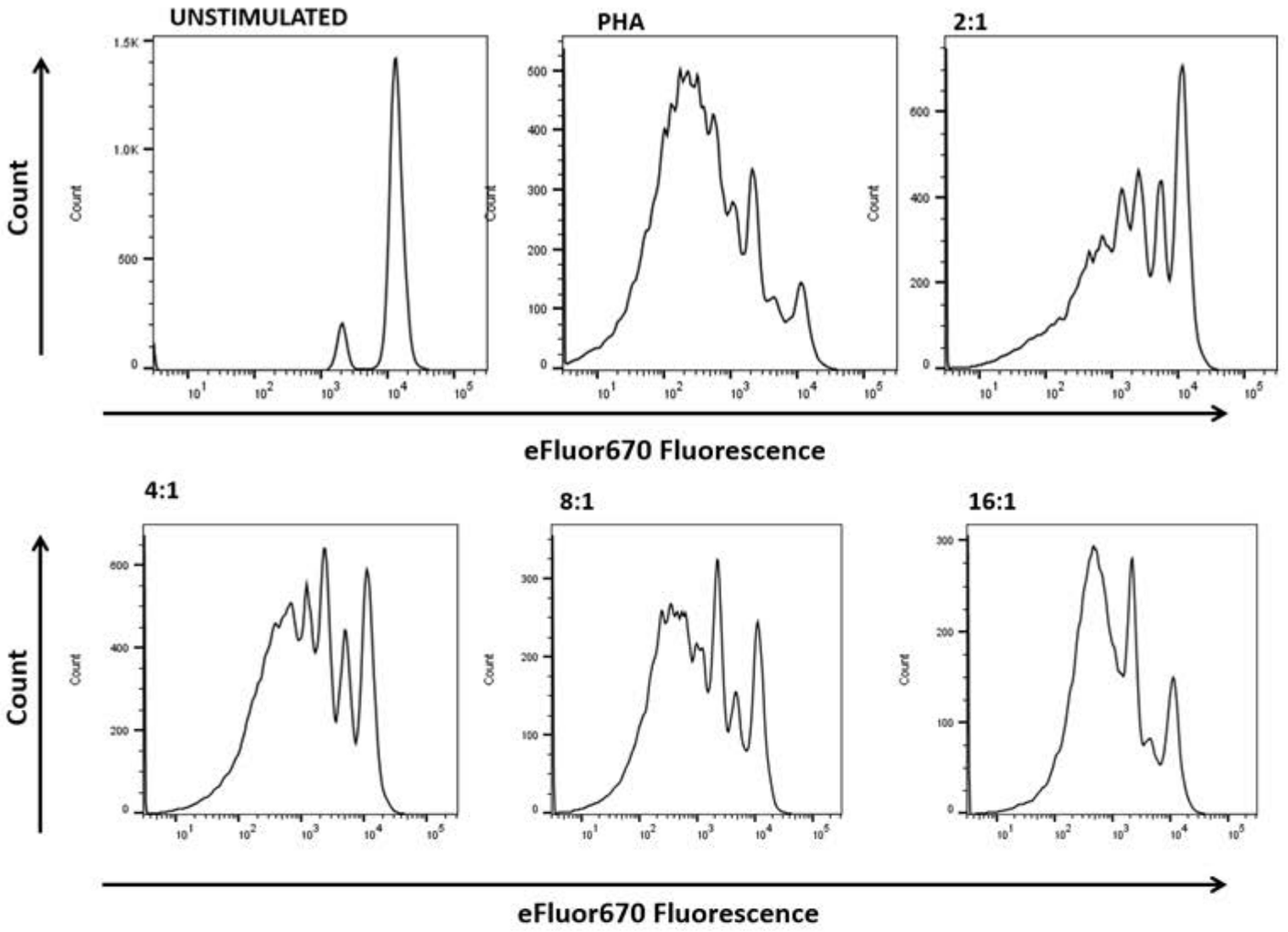

A.i
CD11b
CD14
CD34
CD45
CD105
CD90
CD146
CD73
CD73
CD10
A.ii
% Cells
100
98
96
94
92
90
P2
P3
P4
B.
Days
5
4
3
2
1
0
SMp2
SMp3
SMp4
SMABp2
SMABp3
SMABp4
SMPLp2
SMPLp3
SMPLp4
C.
HUCPVC
Lipoaspirate
p<0.001
Number of Colonies
30
20
10
0
SMp2
SMp3
SMp4
SMABp2
SMABp3
SMABp4
SMPLp2
SMPLp3
SMPLp4
D.
Adipose
Osteo
Chondro
E.
CD273
CD274

Fig.2

A.


UNSTIMULATED
PHA
2:1
4:1
8:1
16:1
Count
eFluor670 Fluorescence

B.

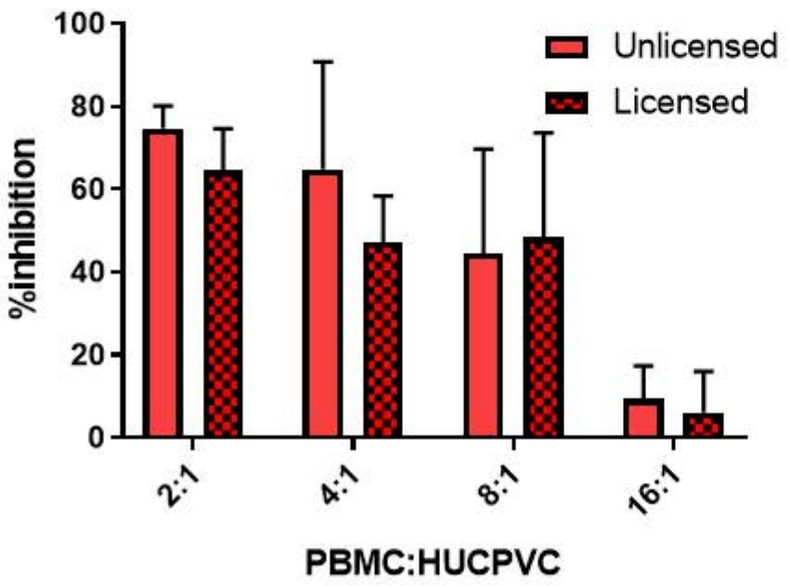

Unlicensed
Licensed
%inhibition
100
80
60
40
20
0
2:1
4:1
8:1
16:1
PBMC:HUCPVC

Fig.3

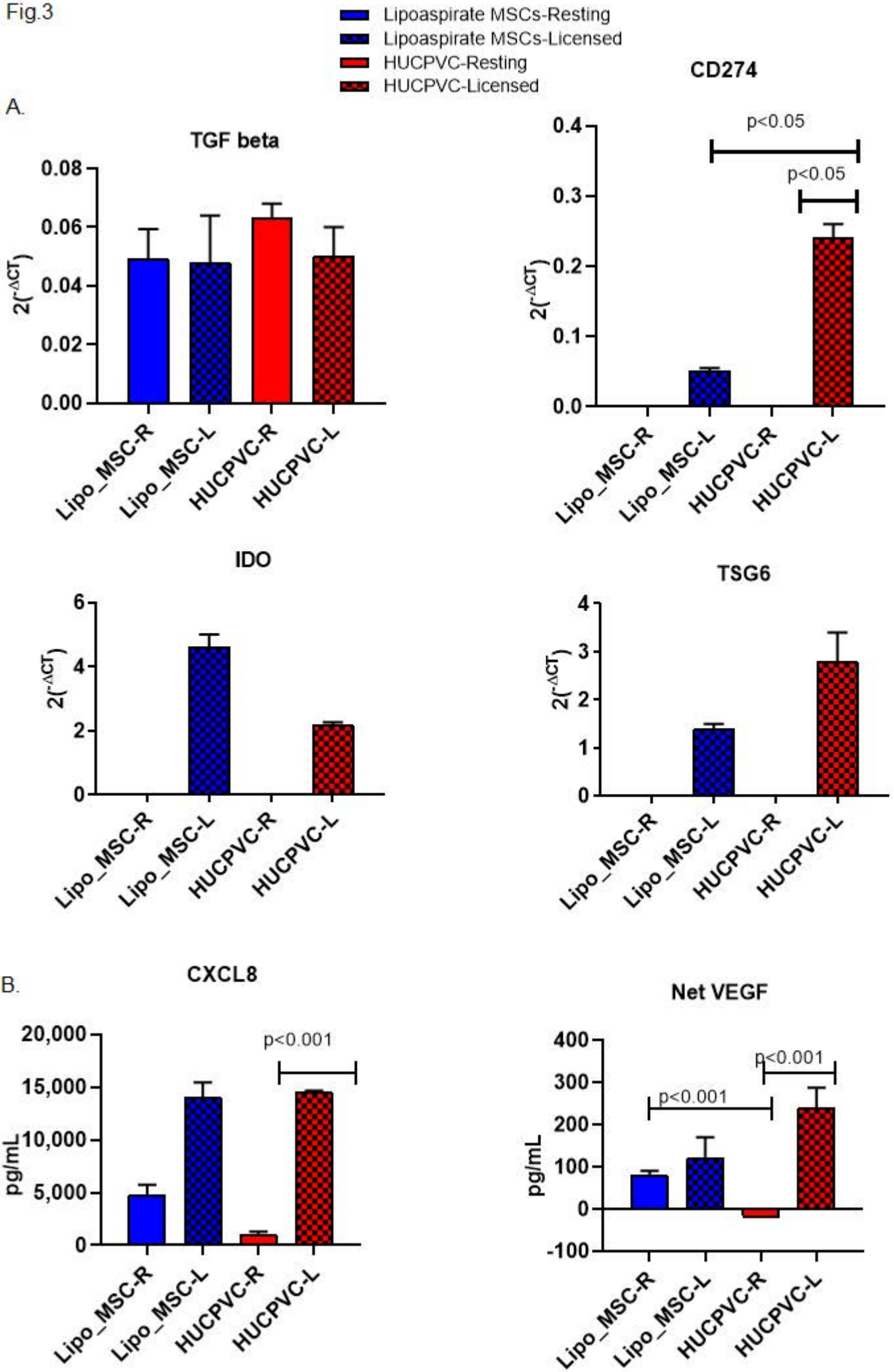
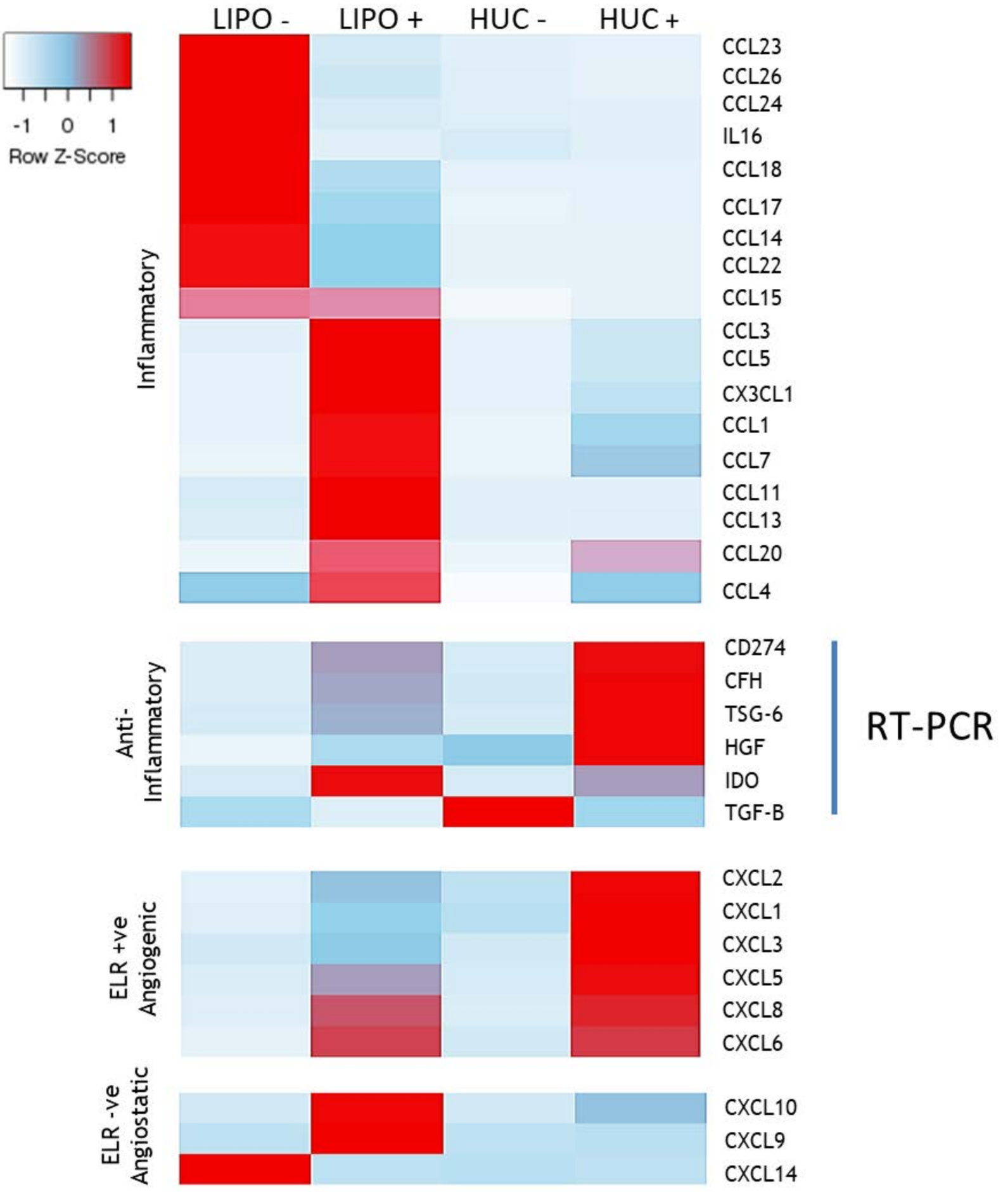

Lipoaspirate MSCs-Resting
Lipoaspirate MSCs-Licensed
HUCPVC-Resting
HUCPVC-Licensed
A.
TGF beta
CD274
p<0.05
p<0.05
IDO
TSG6
B.
CXCL8
p<0.001
Net VEGF
p<0.001
p<0.001
pg/mL
Lipo_MSC-R
Lipo_MSC-L
HUCPVC-R
HUCPVC-L

Fig. 4


LIPO -
LIPO +
HUC -
HUC +
-1
0
1
Row Z-Score
Inflammatory
CCL23
CCL26
CCL24
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CCL17
CCL14
CCL22
CCL15
CCL3
CCL5
CX3CL1
CCL1
CCL7
CCL11
CCL13
CCL20
CCL4
Anti-Inflammatory
CD274
CFH
TSG-6
HGF
IDO
TGF-B
RT-PCR
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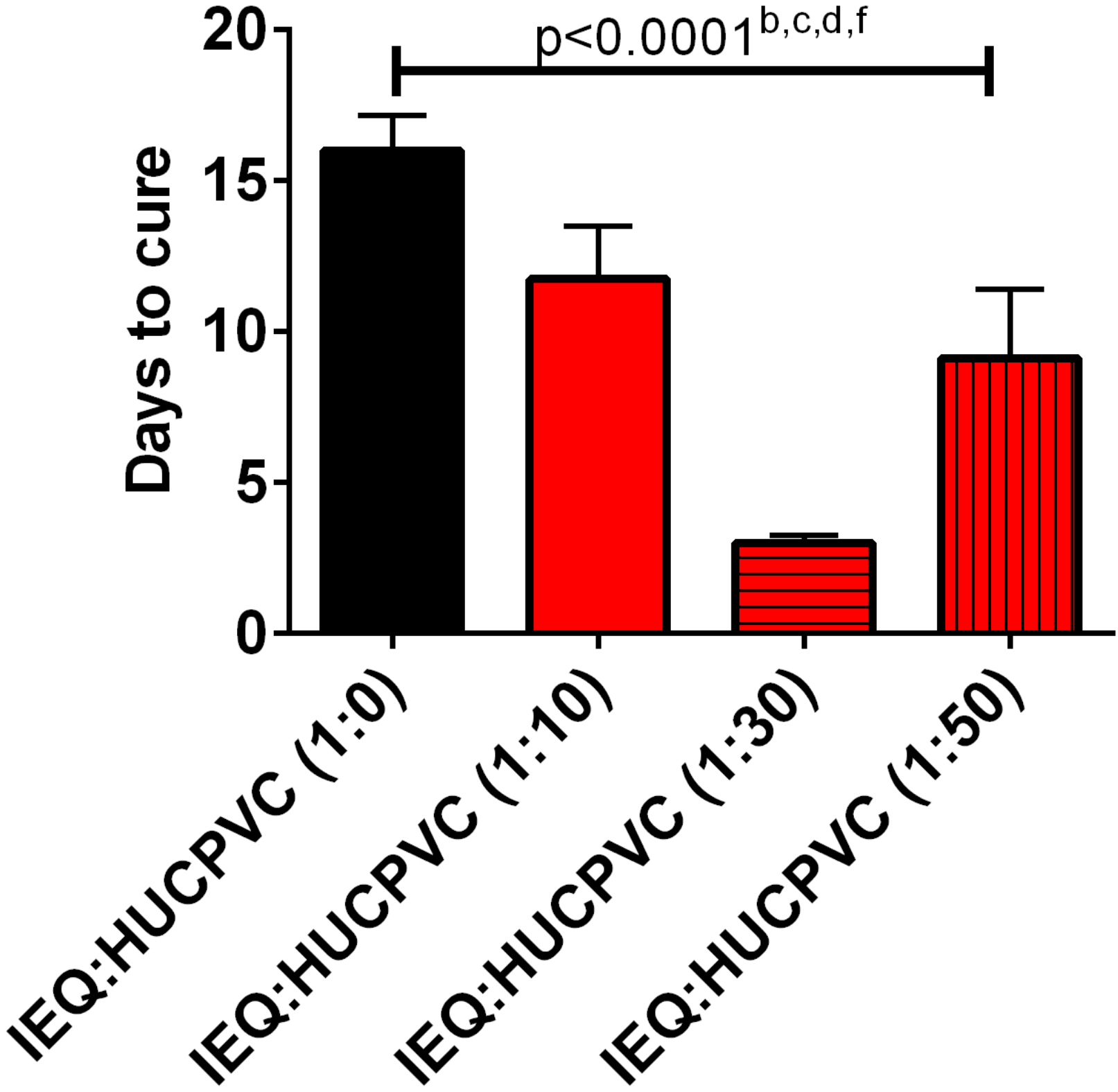
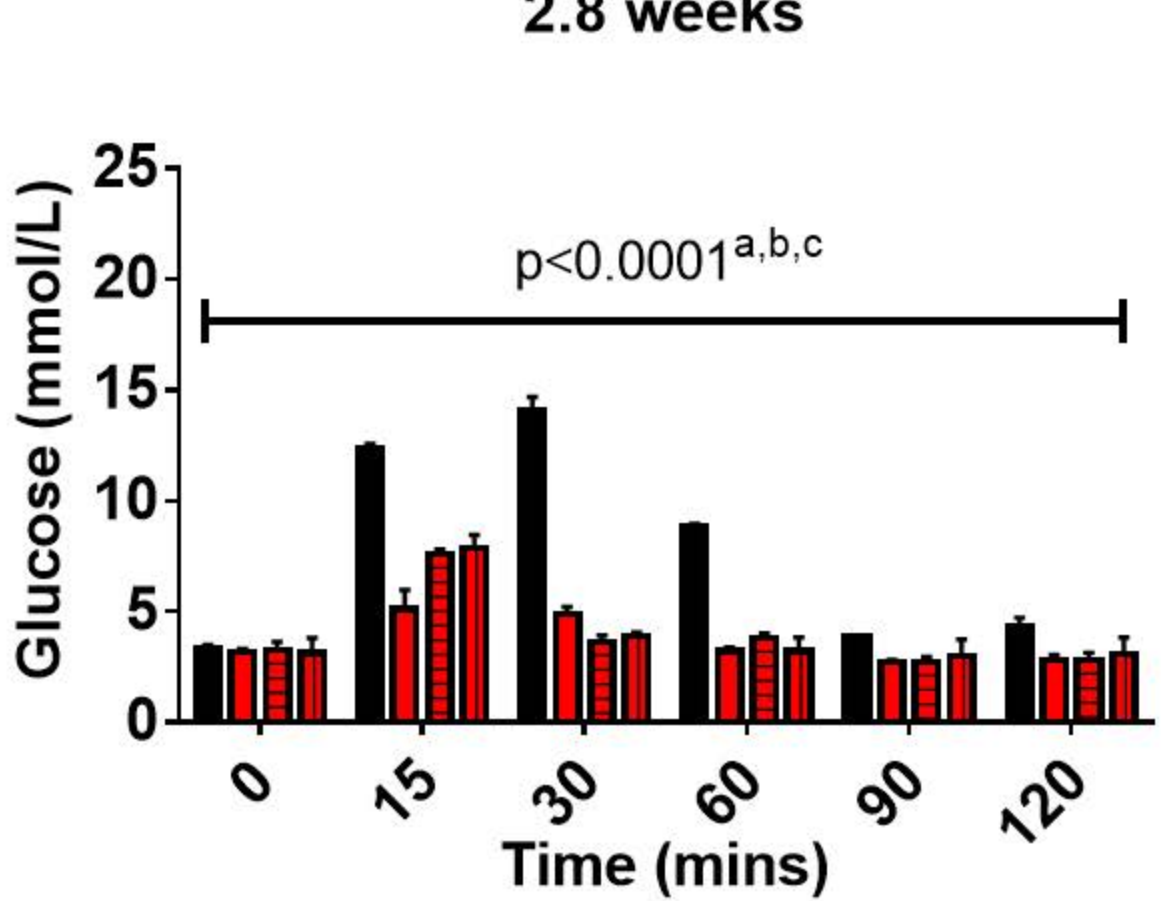
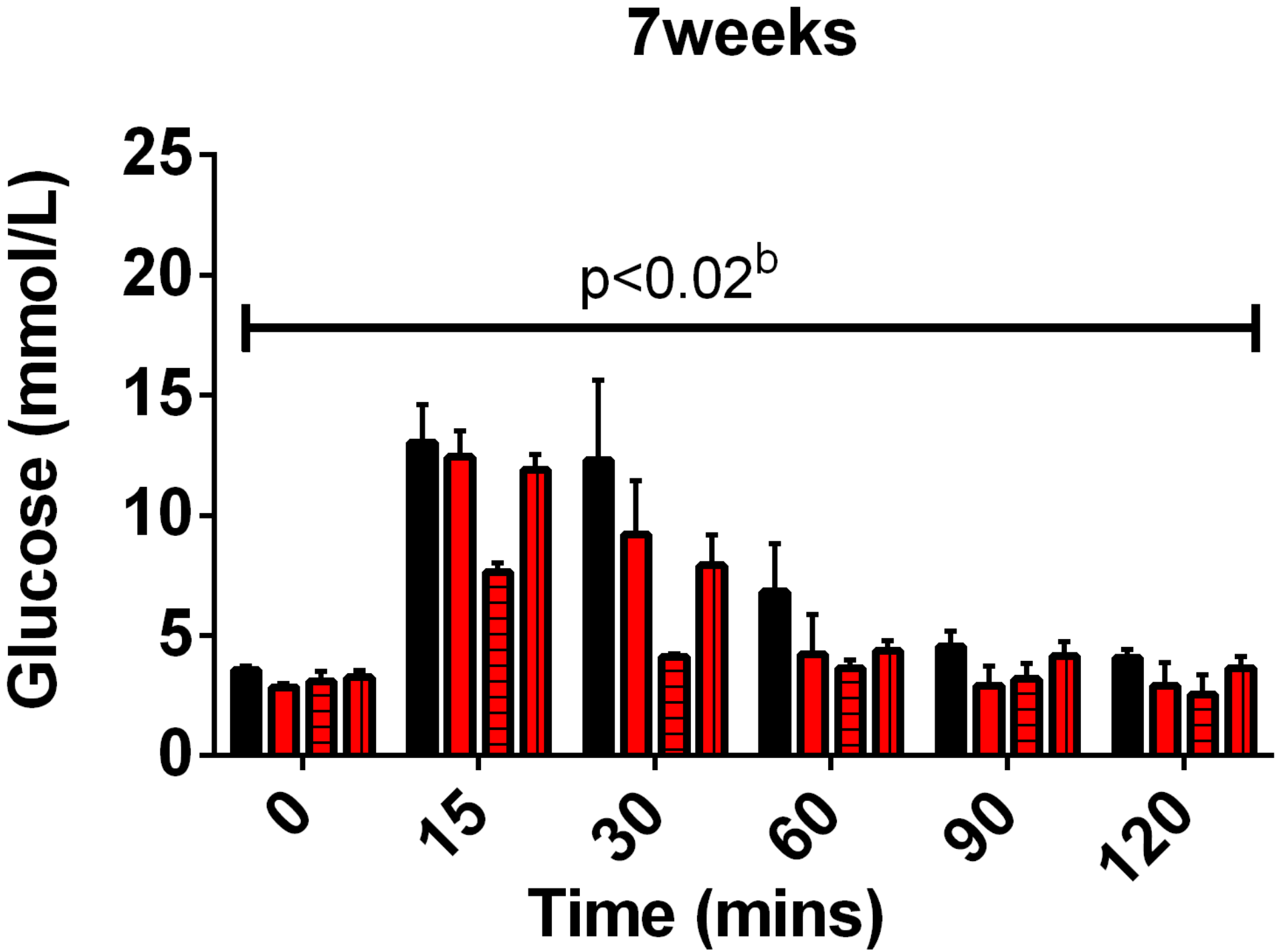
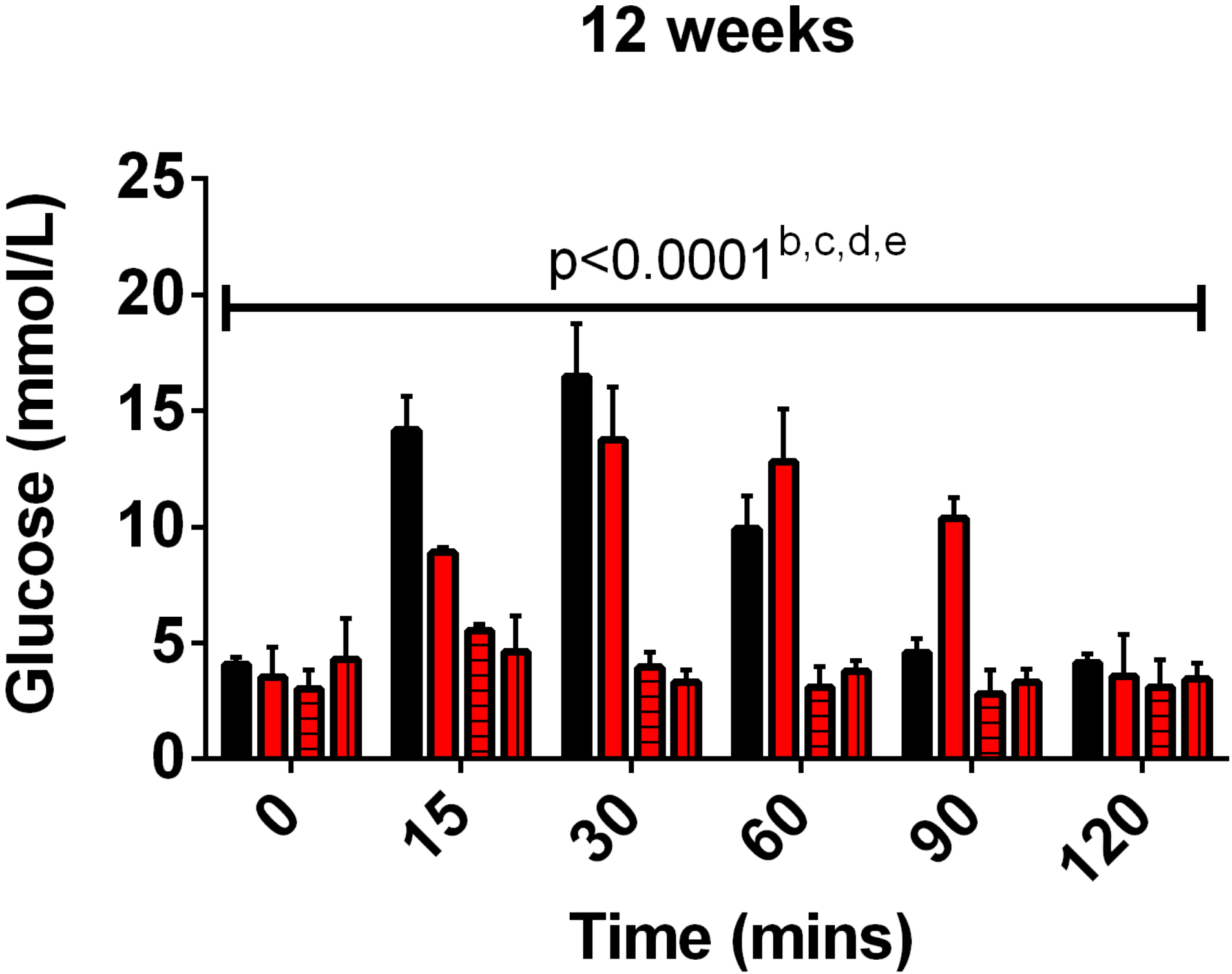
CXCL6
ELR -ve Angiostatic
CXCL10
CXCL9
CXCL14


p<0.0001b,c,d,f
Days to cure
20
15
10
5
0
IEQ:HUCPVC (1:0)
IEQ:HUCPVC (1:10)
IEQ:HUCPVC (1:30)
IEQ:HUCPVC (1:50)


2.8 weeks
Glucose (mmol/L)
25
20
15
10
5
0
p<0.0001a,b,c
0
15
30
60
90
120
Time (mins)


7weeks
25
20
15
10
5
0
p<0.02b
Glucose (mmol/L)
0
15
30
60
90
120
Time (mins)


12 weeks
p<0.0001b,c,d,e
Glucose (mmol/L)
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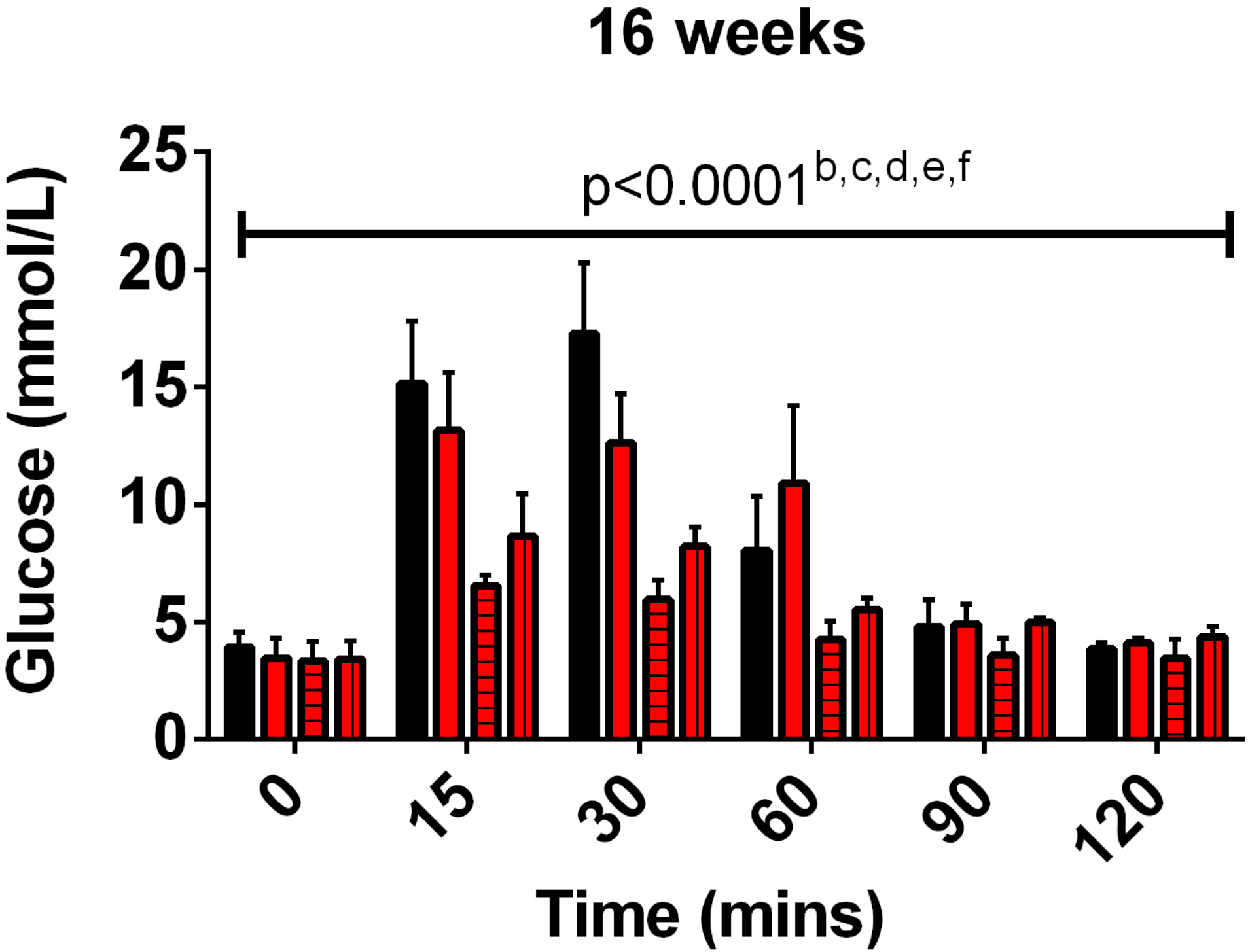
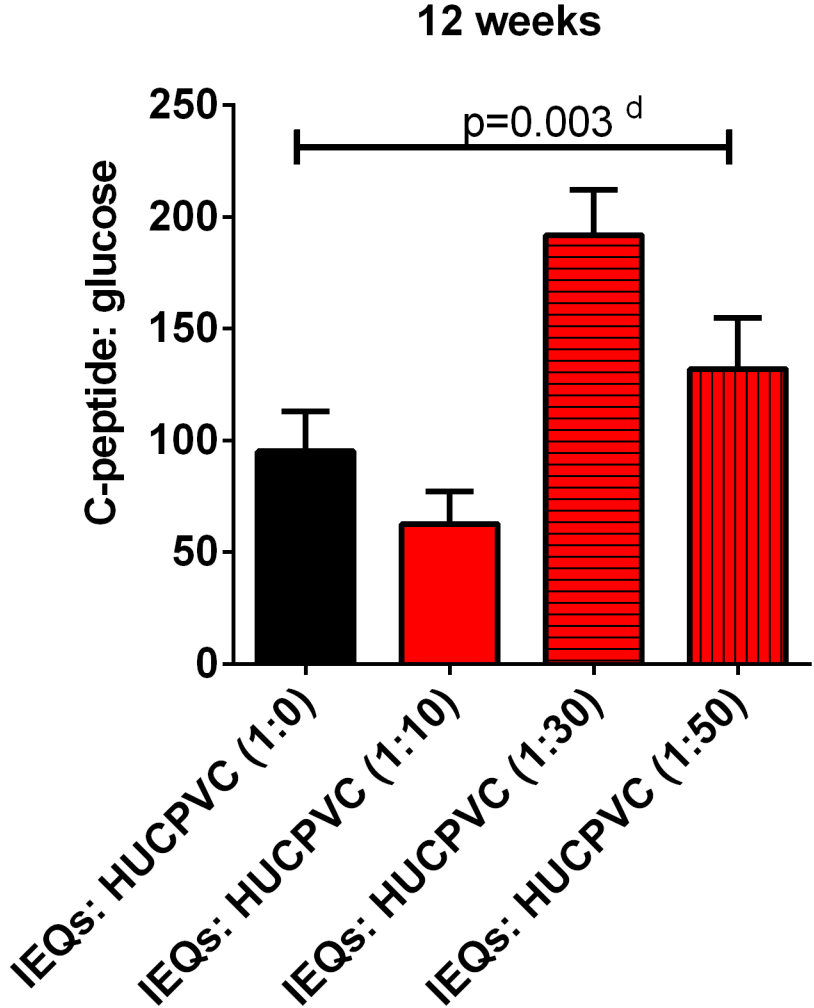
20
15
10
5
0
0
15
30
60
90
120
Time (mins)


16 weeks
p<0.0001 b,c,d,e,f
Glucose (mmol/L)
25
20
15
10
5
0
0
15
30
60
90
120
Time (mins)


12 weeks
p=0.003 d
C-peptide: glucose
250
200
150
100
50
0
IEQs: HUCPVC (1:0)
IEQs: HUCPVC (1:10)
IEQs: HUCPVC (1:30)
IEQs: HUCPVC (1:50)

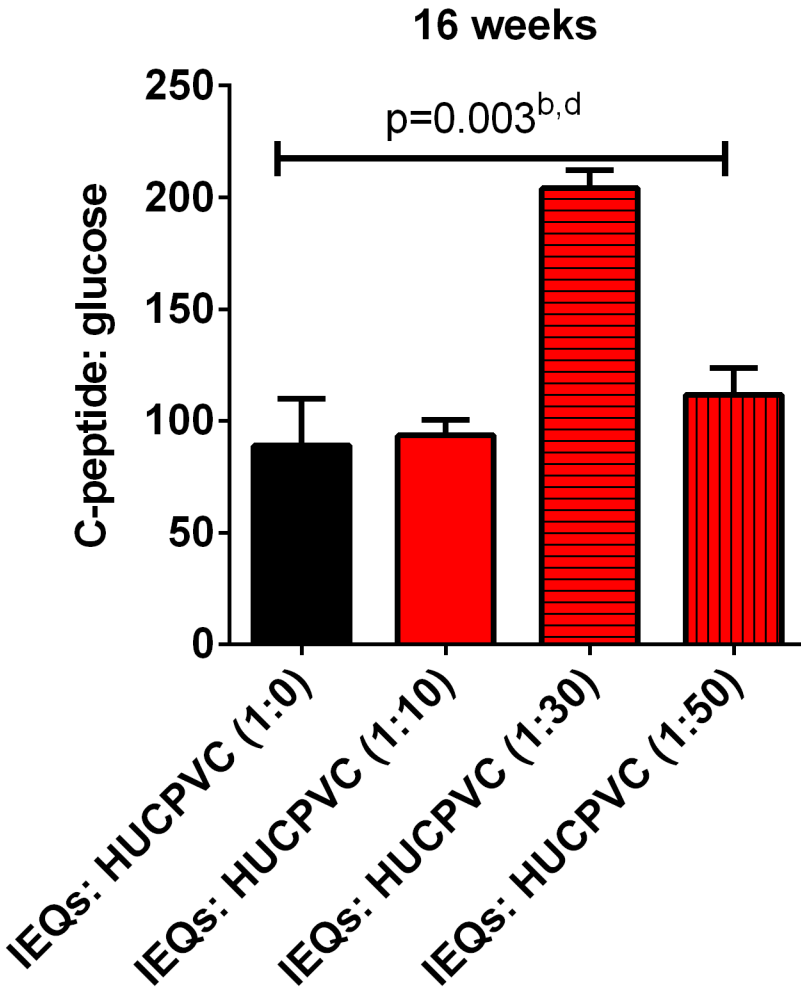
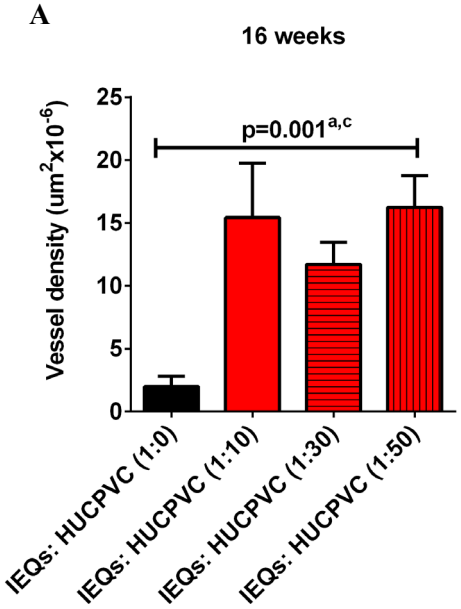
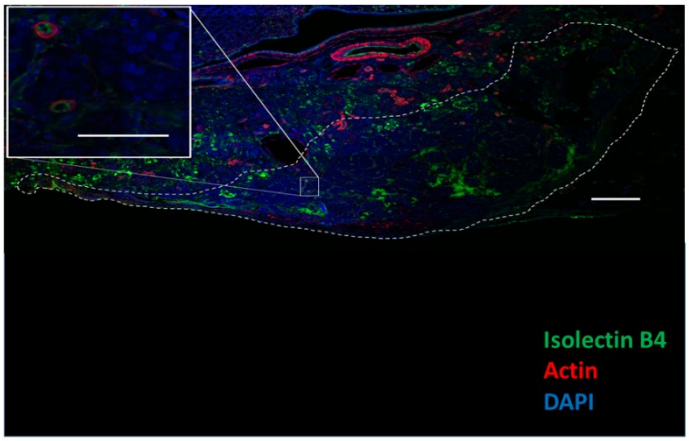

16 weeks
p=0.003b,d
C-peptide: glucose
250
200
150
100
50
0
IEQs: HUCPVC (1:0)
IEQs: HUCPVC (1:10)
IEQs: HUCPVC (1:30)
IEQs: HUCPVC (1:50)

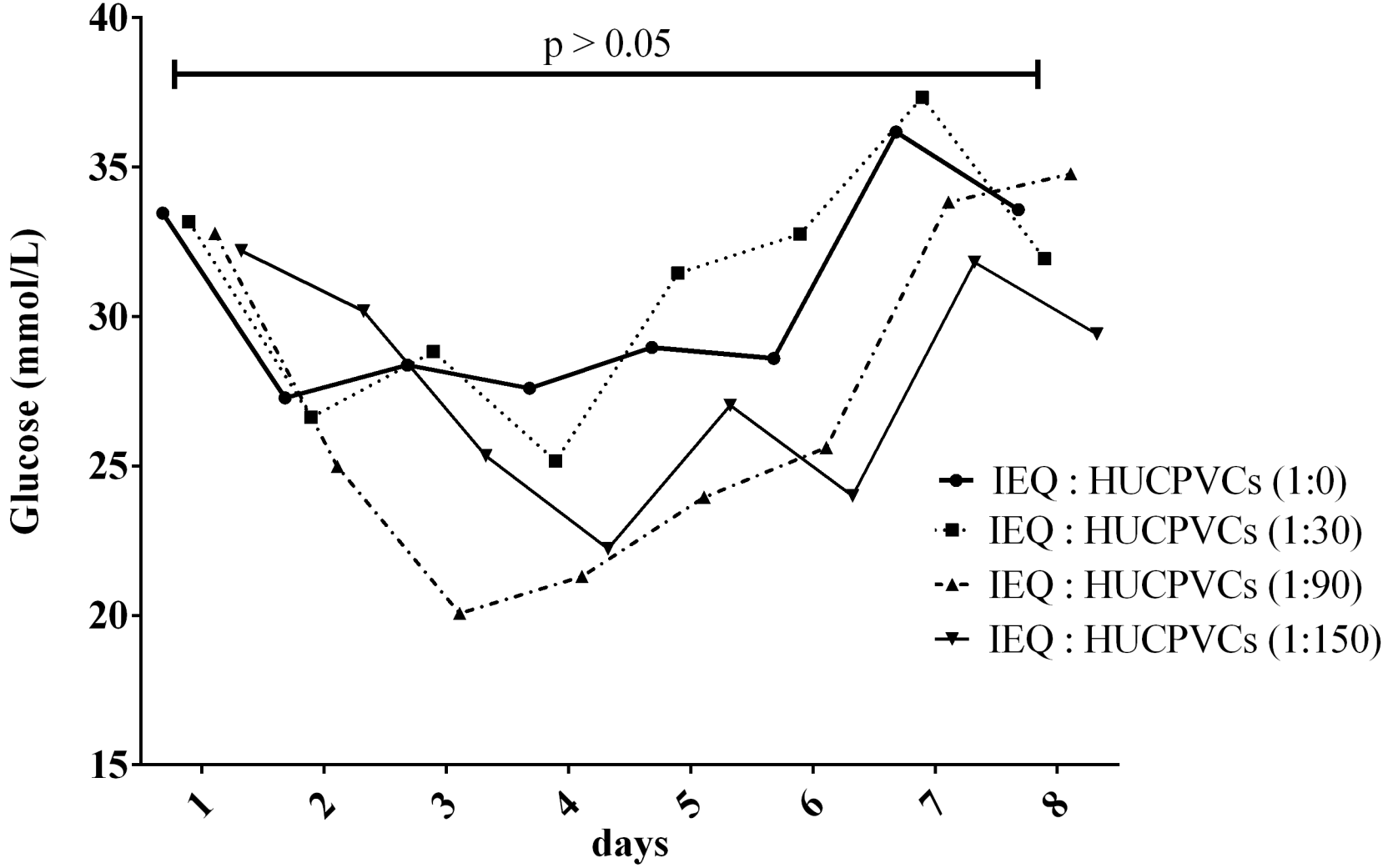
Fig. 6

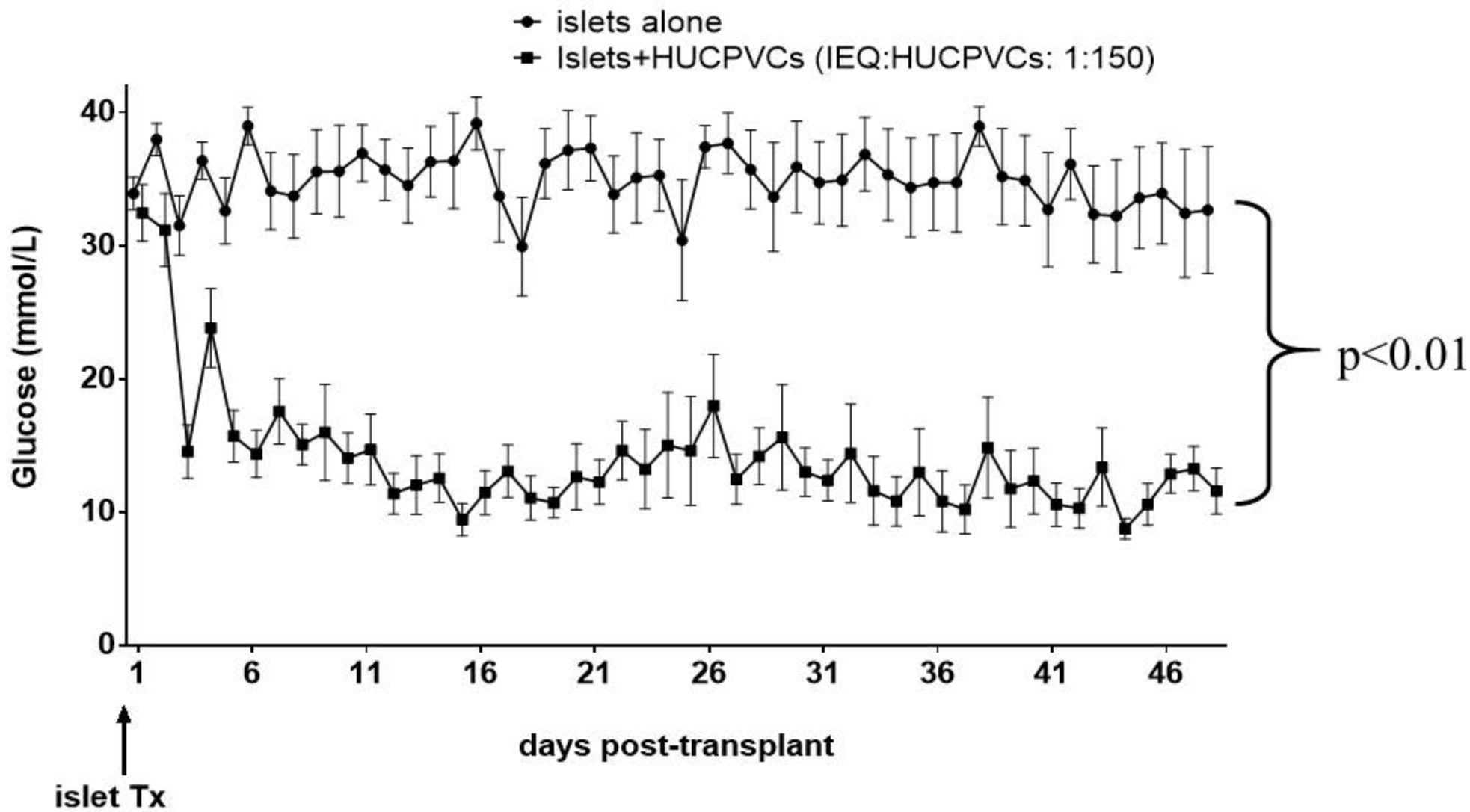
A


16 weeks
p=0.001a,c
Vessel density (um2x10-6)
25
20
15
10
5
0
IEQs: HUCPVC (1:0)
IEQs: HUCPVC (1:10)
IEQs: HUCPVC (1:30)
IEQs: HUCPVC (1:50)

B


Isolectin B4
Actin
DAPI


p > 0.05
Glucose (mmol/L)
40
35
30
25
20
15
1
2
3
4
5
6
7
8
days
IEQ : HUCPVCs (1:0)
IEQ : HUCPVCs (1:30)
IEQ : HUCPVCs (1:90)
IEQ : HUCPVCs (1:150)


islets alone
Islets+HUCPVCs (IEQ:HUCPVCs: 1:150)
Glucose (mmol/L)
40
30
20
10
0
1
6
11
16
21
26
31
36
41
46
days post-transplant
islet Tx
p<0.01