A PRE-EXISTENT HYPOXIC GENE SIGNATURE PREDICTS IMPAIRED ISLET GRAFT FUNCTION AND GLUCOSE HOMEOSTASIS

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Abbreviations

DMOG: dimethyloxaloylglycine; GSIS, glucose-stimulated insulin secretion; HIF, hypoxia inducible factor; i.v.; intravenous;

DMOG: dimethyloxaloylglycine; GSIS, glucose-stimulated insulin secretion; HIF, hypoxia inducible factor; i.v.; intravenous; hypoxia-inducible factor 1α (HIF-1α)

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ABSTRACT – 200 WORD MAX

We examined whether hypoxic exposure prior to the event of transplantation would have a positive or negative effect upon later islet graft function.

Mouse islets, exposed to hypoxic culture (1% O_2 for 16 h) were transplanted into syngenic recipients. Islet graft function, beta cell physiology, as well as molecular changes were examined. Expression of hypoxia-response genes in human islets pre- and post-transplant was examined by microarray.

Hypoxia-pre-exposed murine islet grafts provided poor glycemic control in their syngeneic recipients, marked by persistent hyperglycemia and pronounced glucose intolerance with failed first and second phase glucose-stimulated insulin secretion in vivo. Mechanistically, hypoxic pre-exposure induced HIF-1a and a concomitant increase in hypoxic-response genes including LDHA; a molecular gene set which would favor glycolysis and lactate production and impair glucose sensing. Indeed, static incubation studies showed hypoxia-exposed islets exhibited dysregulated glucose responsiveness with elevated basal insulin secretion. Isolated human islets, prior to transplantation, express a characteristic hypoxia-response gene expression signature, including high levels of LDHA, which is maintained post transplant.

Hypoxic pre-exposure of an islet graft drives a HIF-dependent switch to glycolysis with subsequent poor glycemic control and loss of GSIS. Early intervention to reverse or prevent these hypoxia-induced metabolic gene changes may improve clinical islet transplantation.

A major obstacle to clinical islet transplantation is the poor function of islets after engraftment. Poor function is evidenced by the necessity to transplant multiple islet preparations to achieve successful islet transplant outcomes (1). Also, surviving human islet grafts exhibit poor metabolic function in vivo (2), and demonstrate a steady decline in function over time (3). A number of processes may reduce the metabolic performance of transplanted islets and in particular, hypoxia has been suggested to be a critical insult contributing to the poor performance of islet grafts (4-6). Islet grafts are known to have insufficient blood supply after transplantation (7) due to poor re-vascularization after engraftment (8) compared to the intimate spatial relationship pancreatic islets share with blood vessels in situ (9). Poor vascularization coupled with the implantation of islets into the relatively de-oxygenated blood of the portal vein would result in reduced oxygen tension of islet grafts (7; 10). Further, isolated islets cultured in standard conditions are exposed to dynamic changes in oxygen partial pressures ranging from normoxic, through hypoxic, to anoxic levels.

In the context of islet transplantation it remains to be established if hypoxic exposure prior to the event of transplantation will result in impaired graft function. Many cell types exhibit a regulated hypoxic response, a common feature of which is the co-ordinated expression of hypoxic-response genes mediated through the activity of the transcription factor hypoxia-inducible factor 1α (HIF-1α)(11). The induction of certain hypoxia-response genes may be considered to be beneficial for islet transplantation. Lack of nutrients and oxygenation as a result of inadequate vascularization is considered a major obstacle to early graft function (12). Murine islets respond to reduced oxygenation in culture and post-transplant by activating HIF1a and VEGFA to enhance local angiogenesis (13-15). Moreover, increasing islet vascularization, by forced expression of VEGF, improves transplant outcomes in the early post transplant period (16). In addition some reports show that HIF, together with its binding partner ARNT, is required for maintaining normal beta cell function (17; 18). Increasing HIF-1a stabilisation by administration of iron chelators (11) improves human islet function in rodent transplant model recipients (19). Thus increased HIF-1α activation,

secondary to hypoxia during islet isolation, could be expected to improve graft function by acting to promote local angiogenesis and glucose sensitivity. Indeed, hypoxic preconditioning has a protective effect for heart transplants (20) and this involves mechanisms including the up-regulation of HIF-1α (21), thus hypoxic pre-conditioning of isolated islets prior to transplantation may improve post transplant graft function.

In contrast, other studies would predict hypoxic pre-exposure to have a negative impact upon subsequent islet graft function. Hypoxic culture conditions are detrimental to isolated islet glucose-stimulated insulin release in vitro due to energy depletion and induction of cell death (6; 22), however as this effect is reversible, its longer-term impact on post graft function is not known. Following transplantation, islet grafts accumulate HIF-1α protein which then attenuates with time (15; 23). The period of high HIF-1α expression is associated with reduced insulin secretion and increased apoptosis, suggesting a link between HIF-1α accumulation and impaired graft function (15; 24). Further to this, hypoxia induces an NF-κB-dependent transcriptional program in islets characterized by increased expression of pro-apoptotic genes and increased cell death (25). Studies also show that specific induction of HIF-1α in beta cells diverts glucose metabolism to favor glycolysis and lactate production, resulting in impaired ATP production, reduced insulin secretion and systemic glucose intolerance (26-28). Thus, exposure of islets to hypoxia has been proposed as one explanation for impaired islet graft function (4-6).

Based on current literature it is difficult to predict what the impact of hypoxia will be for subsequent islet graft function and further definitive studies addressing this question are lacking. Therefore in this study we determined the impact of pre-transplant hypoxic exposure on post-transplant islet graft function and whole body glucose homeostasis in mice. Hypoxia response-gene expression was also analyzed in isolated human islets prepared for transplantation.

RESEARCH DESIGN AND METHODS

Human islets and cadaver pancreas specimens.

Eight human pancreatic islet isolates were prepared at the Centre for Transplant and Renal Research, Westmead Hospital by a closed loop method as previously described (29). Immediately after extraction, ~5, 000 IEQ pure islets (fractions 3-8) were re-suspended in mRNA isolation buffer (RLT lysis buffer, Qiagen) and immediately stored at –80°C. Seven cadaver pancreas specimens were obtained from the Islet Cell Resource Center of the Joslin Diabetes Center. Hyperglycemic donor samples were excluded. Frozen pancreatic tissue was sectioned at 8 μm in a cryostat, mounted on uncoated glass slides at –20°C, and immediately stored at –80°C.

Laser Capture Microdissection (LCM). LCM was performed on frozen pancreatic sections using PixCell II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA): laser power, 35 mW; pulse duration, 3.0 msec; spot size, 7.5 μm. Each section typically had 3-10 islets, and on average, 6-7 sections provided 800 pulses required for sufficient RNA for arrays. Beta cell enrichment was determined by the ratios of expression (RTqPCR) of glucagon or somatostatin to insulin in β–cell enriched LCM samples; glucagon/insulin expression ratio for β-cell selected versus generalized non-selected islet tissue was 0.238±0.132; somatostatin/insulin expression ratio for β-cell selected tissue versus generalized non-selected islet tissue was 0.584±0.277.

RNA extraction, amplification and labeling. LCM samples: Total RNA (PicoPure RNA Isolation Kit, Arcturus) was subjected to two rounds of amplification by T7-based linear amplification using T7-oligo-dT-primers (RiboAmp HS RNA Amplification Kits, Arcturus) (2). Islet samples: Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and single stranded cDNA was produced using the Superscript III Reverse Transcriptase Kit (Invitrogen, Australia).

Microarray analysis

Briefly, expression data from isolated islets (Affymetrix HGU133+2 arrays) and LCM beta cells (Affymetrix U133 X3P) were independently normalised using RMA and collapsed from probe to gene-level by picking the probe with the highest average expression level. The two datasets were quantile normalised and those genes that were in common were selected for comparative analysis. Heatmaps were created in GENE-E.

RTqPCR analysis

Primers were designed with Primer3 based on Genbank sequences and synthesized by Sigma Aldrich. PCR reactions were performed on the RG300 Real Time PCR System (Rotorgene) using FastStart SYBR Green Master Mix (Roche Diagnostics). GAPDH was used as the housekeeping gene, and data was analyzed using the ΔΔCt method.

Animal studies

All animal studies were approved by the Garvan/St.Vincent's Animal Ethics Committee. Animals were housed no more than 5 mice per cage with free access to standard mouse chow and water, and were maintained on a 12hour light/12 hour dark cycle.

Mouse islets and hypoxic treatment.

Murine islets of Langerhans were isolated exactly as we have previously described (30; 31) and cultured in RPMI1640 media (Invitrogen) under normoxic (21% O_2, 5% CO_2, 74% N_2) or hypoxic (1% O_2, 5% CO_2, 94% N_2) conditions for 16-18 hours as previously described (26) and indicated in text. To activate HIF-1a islets were cultured with 0.5 mmol/l dimethyloxalylglycine (DMOG) for 16 hours (26). HIF-1a protein was assessed by Western blotting as per (26) using anti-mouse Hif-1α (Novus Inc.) and anti-β-Actin (BD biosciences).

In vitro insulin secretion

Insulin secretion was determined as described (32). In brief, islets were cultured for 18hours under normoxic or hypoxic conditions in RPMI1640 media, then moved to Krebs-ringer buffer containing 10mmol/l Hepes (KRBH) with 2mmol/l glucose for 30mins under normoxic conditions. Next, batches of 5 islets were handpicked, and cultured in 130ul of KRBH containing 2 or 20mmol/l glucose for 1 hour under normoxia. Insulin secreted into the KRBH was quantified by radioimmunoassay (Linco/Millipore).

Islet Transplantation.

Isolated islets from C57BL/6 mice were transplanted into C57BL/6 mice, at a ratio of 3 donor pancreata per 1 recipient, as previously described(30). Recipient mice were rendered diabetic by streptozotocin (Sigma) injection (180 mg/dL i.p.). Diabetes was determined as blood glucose levels ≥25 mM on two consecutive readings. Fed blood plasma was harvested by sampling ~20ul of blood from the tail using plastic capillary tubes (Starstedt), followed by centrifugation at 12,000g for 5mins to remove erythrocytes and collection of the supernatant. Insulin levels were determined by insulin ELISA (Crystalchem Inc.).

Intravenous glucose tolerance testing

Intravenous (iv.) glucose tolerance testing of mice was performed 14 days post-transplantation following an overnight (16hour) fast with ad libitum access to water. D-glucose (20% solution) was injected into the tail vein of conscious mice at a dose of 1g/kg. Blood glucose levels were determined using an Accu-chek performa glucometer (Roche), and 5ul blood samples were collected using glass capillary tubes (Sigma) at the indicated time points. Insulin levels were determined by insulin ELISA (Crystalchem Inc.).

Statistical analysis

Statistical significance was determined using Mann-Whitney U tests or two-tailed Student's t-tests as appropriate and as indicated in the figure legends. A *P*-value of less than 0.05 was regarded as statistically significant. Statistical analysis was performed in R (version 2.8.0; (33), 1996) and Graphpad Prism (version 5.0a; Graphpad Software).

RESULTS

Expression of hypoxic-response genes in isolated human islets and laser dissected beta cells.

Some studies indicate that isolated human islets express high levels of hypoxia-regulated genes (34), and it has been suggested that induction of this hypoxia-response contributes to the poor performance of islet grafts (4; 24). Indeed, from genome transcript profiling analysis comparing the gene expression profile of eight independent human islet isolates prepared within our clinical islet transplant centre, with seven preparations of laser dissected human beta cells from human pancreata, we could identify increased expression of characteristic (11) hypoxia-response genes in isolated islets (Fig. 1). Increased genes included CITED2 and DDIT4 as well as PDK1, MAFF and SLC7A1. Therefore, isolated human islets exhibit a gene expression profile characteristic for that of cells responding to hypoxia stress. This gene signature is present prior to the event of transplantation.

Effect of pre-transplant hypoxic stress on islet graft function.

To test how hypoxia-pre-exposure would affect islet transplant outcomes, we established a model whereby primary C57BL/6 mouse islets otherwise isolated under optimal conditions (anaesthetized living donor), were then exposed to hypoxia by controlled culture in 1% O_2 for 16 h and subsequently transplanted under the renal capsule of diabetic and syngeneic recipients. Because islet grafts achieve near normal levels of oxygenation under the kidney capsule (10), this approach would reveal how pre-exposure to hypoxia would impact later graft function. The results from these transplant studies are presented as both individual blood glucose plots (Fig. 2*A*) and pooled median values (Fig. 2*B*). Islet-grafts prepared under normoxic (21% O_2) conditions were able to rapidly restore metabolic control to normal levels, reliably keeping blood glucose in a tight range around 5 mM. The function of normoxic cultured islet grafts was no different to that of freshly isolated and immediately transplanted islet grafts (data not depicted). In marked contrast, mice receiving hypoxia-pre-exposed islet grafts exhibited poor glucose homeostasis after transplantation; indeed

the mean blood glucose reading for mice receiving hypoxia-pre-exposed grafts was >15 mM for the majority of the post operative observation period ($P < 0.05$).

Hypoxic exposure impairs glucose-stimulated insulin release in vivo.

Consistent with the significantly elevated fed and fasting glucose levels of mice receiving hypoxia-pre-exposed islet grafts, these mice also exhibited impaired insulin release in the fed state (Fig. 2*C*). Whereas fed insulin levels for mice receiving control normoxia grafts where similar to that of non-transplanted control mice ($P > 0.05$; $n > 6$). This suggested that mice receiving hypoxia-pre-exposed islet grafts harboured a beta cell defect. When challenged with an exogenous intravenous injection of glucose, mice receiving hypoxia pre-treated grafts were found to be severely glucose intolerant, due to a highly-significant defect in GSIS (Fig. 3*A*). The impaired GSIS related to a complete loss of both first and second phase insulin response to a glucose challenge (Fig. 3*B*). After a 16 hour fast, mice receiving hypoxia-pre-exposed grafts had normal insulin levels and a slight elevation in fasting blood glucose (Fig. 3C and D). Thus pre-transplant exposure of islets to hypoxia is sufficient to severely impair graft function, resulting in the inability of a normally adequate mass of transplanted islets (3 donor: 1 recipient) to control glucose homeostasis.

Hypoxic pre-exposed islet grafts exhibit disrupted architecture.

Histological analysis of hypoxia-pre-exposed islet grafts 10 days post transplantation revealed poor islet architecture and patchy to non-existent insulin staining relative to normoxic islet grafts (Fig. 4). These data show that hypoxic pre-exposure has a deleterious effect upon later graft function, with depleted insulin content contributing to poor post-transplant glycemic control.

Hypoxia induced molecular changes in isolated rodent islets.

We next considered possible molecular mechanisms for the hypoxia-induced impairment in islet

graft function. We noted that a common feature of the hypoxic response genes expressed by isolated human islets presented in Figure 1 was their co-ordinate regulation by the transcription factor HIF-1α (11). Some studies (26-28) show that stabilisation of the HIF-1α protein in rodent beta cells impairs ATP production and insulin secretion by increasing expression of genes that oppose glucose oxidation including LDH, PDK1 and MCT4, whereas GLUT2 and GCK are negatively regulated. To determine whether hypoxic-pre-exposure had triggered such a HIF-1α-dependent molecular switch to anaerobic glycolysis, primary mouse islets were exposed to hypoxia (1% O_2, 16 h) and subsequently examined for HIF-1a accumulation and glycolytic gene expression. Western blot analysis demonstrated stabilisation of HIF-1α protein levels in islets cultured under hypoxic conditions as compared to control normoxic conditions (Fig. 5*A*). Further to this, and in association with HIF-1α stabilisation, hypoxic islets exhibited increased mRNA levels for LDHA, PDK1, GLUT1 and MCT4 (Fig. 5*B*). In addition, GLUT2 and GCK expression was down regulated by hypoxia. Thus, exposure of murine islets to hypoxia stabilised HIF-1α protein levels and altered the expression pattern of metabolic genes to favour anaerobic glycolysis and lactate production. This gene expression pattern associated with poor post transplant performance.

HIF-1α stabilisation is sufficient to alter glycolytic gene expression in rodent islets.

Hypoxia can activate multiple stress pathways including the NF-κB and HIF-pathways (35). To test whether hypoxia-induced activation of the HIF-1α pathway alone was sufficient to induce changes in glycolytic gene expression, rodent islets were treated with the pharmacologic HIF-1α activator DMOG and HIF-1α stabilisation was determined. Isolated islets treated with DMOG showed increased HIF-1a stabilisation (Fig.5A). The accumulation of HIF-1α occurred with a concomitant increase in mRNA levels for LDHA, GLUT1 and MCT4 (Fig. 5*C*). Also GLUT2 and GCK were down regulated, albeit non-significantly. This same pattern of HIF-1a stabilisation and gene expression was similar to that seen for islets cultured under hypoxic conditions (Fig. 5*B*). Thus stabilisation of HIF-1α by hypoxic exposure altered the expression pattern to glycolytic genes that

would favour anaerobic glycolysis.

Hypoxic exposure impairs glucose-stimulated insulin release in vitro.

Stabilisation of the HIF-1α protein via gene mutation in rodent beta cells impairs ATP production and impairs insulin secretion by increasing expression of metabolic genes favouring glycolysis and lactate production (26-28). We questioned whether hypoxic exposure of islets prior to transplantation also resulted in impaired beta cell function through a similar mechanism. Islets were cultured under normoxic, or hypoxic conditions for 18 hours before static incubations were performed for 1 hour under normoxic conditions to measure insulin release (Fig. 6). These experiments revealed that hypoxic islets exhibited higher than normal insulin secretion at 2 mM glucose as compared to islets cultured under normoxic conditions. Furthermore, islets exposed to hypoxia exhibited a reduced secretory response from ~6-fold of basal following normoxic culture to ~2.5-fold following hypoxic culture. This indicates that hypoxia pre-treated islets have an impaired ability to sense and response to changes in glucose concentration, consistent with our in vivo data demonstrating impaired GSIS in diabetic mice possessing hypoxia-pretreated grafts. Thus, stabilisation of HIF-1α by hypoxic exposure altered the expression pattern of glycolytic genes to favour anaerobic glycolysis, resulting in impaired islet graft function.

Expression of HIF-target genes in isolated human islets

Given that human islets expressed characteristic HIF-1α regulated genes (see Fig. 1), we next investigated whether isolated human islets exhibited a HIF-1α-dependent molecular switch to anaerobic glycolysis relative to beta cells in situ (Fig. 7*A*). This analysis revealed that relative to laser dissected beta cells, isolated human islet preparations showed an ~18 fold increase in lactate dehydrogenase exprssion, - ~6-fold increase in hexokinase 1 and 2, 3 fold increase in Glut1 and 70% decrease in glucokinase levels. This pattern resembles mouse islets exposed to hypoxia (see Fig. 5*B*) as well as those expressing high levels of HIF (26-28).

We next determined whether the molecular profile of isolated human islets would correct after transplantation. We compared glycolytic gene expression levels in human islet preparations prior to, and 4-weeks after, transplantation under the renal capsule of euglycemic immunodeficient RAG-/- mice by RTqPCR (Fig. 7 *B* and *C*). The RTqPCR data showing baseline mRNA expression levels for the glycolytic genes in isolated islets are presented in Fig 7*B*, whereas comparisons between pre- and post transplanted human islets are represented in Figure 7*C* as -ΔΔCt values. Note LDHA mRNA levels in isolated human islets were consistently in the range of expression levels for IAPP (Fig. 7*A*). Following transplantation, expression levels for LDHA and hexokinase were maintained at levels equivalent to that of newly isolated islets (Fig. 6A). In contrast GLUT1 levels decreased ~2-fold. Thus, despite providing similar levels of oxygenation to that of the native pancreas (10) sub-capsular transplantation could not reverse hypoxia-regulated metabolic gene expression to that of pancreatic islets in situ. Thus, isolated human islets express a molecular signature consistent with that of islets exposed to a hypoxic insult, which would, based on our animal modelling, predict impaired graft function and poor glycemic control. This profile is not corrected after transplantation into a relatively benign graft site – the kidney capsule – and would be potentially further exacerbated by transplantation into the de-oxygenated environment (10) of the portal vein.

DISCUSSION

Many clinical studies indicate that following isolation human islets typically show poor function in vivo. The clearest evidence for this is seen in the need to transplant multiple islet grafts before good metabolic control can be achieved (1-3). Therefore understanding the mechanisms responsible for poor islet graft function will allow the development of targeted strategies to improve the success of clinical islet transplantation.

Consistent with a previous report (34), we show that at the point of isolation human islets express a molecular profile characteristic of cells exposed to a hypoxic environment. Given the clinical practise to transplant human islets immediately after isolation, without culture (1), and the speculation that hypoxia is detrimental to islet function (4-6), it would be important to determine the effect of hypoxic pre-exposure upon latter islet graft function. Therefore we examined graft outcomes for islets prepared under optimal conditions versus those exposed to a hypoxic environment. Strikingly, hypoxic islet grafts failed to provide metabolic control to syngenic recipients demonstrating that hypoxic exposure prior to transplantation results in impaired islet graft function and whole body glucose intolerance. Mechanistically, the failure of hypoxic grafts to regulate glucose homeostasis related to a major defect in the ability of beta cells to secrete insulin in response to a glucose challenge.

Different to many other cell types, beta cells express relatively low levels of lactate dehydrogenase, monocarboxylate transporters and low-K_m hexokinases (36; 37). We have identified up-regulation of these enzymes in hypoxic islets, along with increased PDK1, which act to reduce glucose oxidation: PDK1 inhibits PDH activity preventing pyruvate entry to the mitochondrial TCA cycle, LDH converts pyruvate to lactate and MCT4 enables export from the cell. In addition, we identified a reduction of the high-K_m glucokinase and increase in low-K_m hexokinases 1 and 2, potentially further disrupting glucose sensing in hypoxic beta cells. Normoxic beta cells rely on mitochondrial glucose oxidation to generate sufficient quantities of ATP per molecule of glucose metabolized to trigger insulin secretion within the normal physiological range of glucose concentrations (38) and

thereby maintain whole body glucose homeostasis. Indeed, forced expression of LDHA (39) in beta cell lines impairs glucose sensing and insulin release, demonstrating that low expression of LDH is essential for normal beta cell function. Stabilization of HIF-1α protein, the principal hypoxia-induced transcription factor (11), in murine beta cells via genetic mutation alters beta cell metabolism to favour anaerobic glycolysis and leads to impaired glucose stimulated insulin release and glucose intolerance in vivo (26-28). This functional impairment relates to a loss of beta cell specialization, due to specific molecular changes including increased expression of the normally suppressed genes LDHA, PDK1 and MCT4, whereas GLUT2 and GCK are reduced (26-28). This HIF-1α-induced molecular re-configuration severely dampens mitochondrial metabolism and increases lactate generation; resulting in impaired glucose stimulated insulin release. These molecular and metabolic changes support the concept that islets exposed to low oxygen tension loose the specialisation of gene expression responsible for normal beta cell physiology (36) resulting in graft dysfunction and poor transplant outcome.

This study demonstrates that hypoxic exposure of islets prior to transplantation has an overall negative impact on graft function and glucose homeostasis. The induction of a hypoxia-like genetic program results in dysregulated expression of genes that control metabolic glucose sensing, and suggests wider beta cell de-differentiation. It is possible that the negative impact of the hypoxic-response program in islets is masking potential positive effects; therefore further studies to specifically target positive consequences of HIF-1a activation, such as pro-angiogenic responses, may prove beneficial. Given isolated human islets, like hypoxic exposed rodent islets, show a loss of specialization with altered expression of genes regulating glucose stimulated insulin release, this may explain by extrapolation, why large numbers of islets and multiple transplants are required to reverse diabetes in clinical trials. Our findings also suggest that early intervention in reversing the hypoxic changes may be critical to improve graft function and transplant outcome.

AUTHOR CONTRIBUTIONS

J.C., S.N.W., M.H., A.W., M.J.C., W.K. and S.T.G researched data. M.H. and G.W. conducted microarray analysis on laser dissected human beta cells. W.J.H. and P.O.C. from the Australian Islet Transplant Consortium provided access to isolated human islets. J.C., S.N.W and S.T.G wrote the manuscript. The guarantor is S.T.G.

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DISCLOSURES

All authors concur that there are no conflicts of interest to disclose, financial or otherwise, as defined by the *Journal*.

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FIGURE LEGENDS

Gene expression analysis of hypoxia response genes in laser dissected human beta cells and isolated human islets. *A:* Heatmap of the global-normalized-log2 gene expression level for HIF-target genes; islet factors; islet hormones; and, non-islet factors as indicated by category. Each column represents a different sample of either laser dissected human beta cells designated with 'cadaver'; or isolated human islets designated with 'H'. Gene symbols are from the OMIM data-base.

Figure 2. Maintenance of blood glucose homeostasis by hypoxia-pre-exposed islet grafts. *A*: Blood sugar levels of individual mice receiving either control normoxic islet grafts (hatched line) or hypoxia-pre-exposed grafts (solid line). $n = 6$ per group. *B*: Median blood sugar levels derived from (*A*) for mice receiving either control normoxic islet grafts (hatched line) or hypoxia-pre-exposed grafts (black line). Error bars indicate median ± SEM; $n = 6$ per group per time. *C*: Fed plasma insulin levels for mice receiving either control normoxic islet grafts (open symbols) or hypoxia-pre-exposed grafts (closed symbols except diamonds) at post operative day (POD) 1, 2 and 3; $n = 6$ per group. 'Baseline' indicates random feed blood insulin levels from a cohort ($n = 14$) of age and sex-matched C57BL/6 mice provided for comparison (closed diamond symbols). Each point represents an individual mouse and bars indicate median value; $*P < 0.05$; $**P < 0.01$.

Figure 3. In vivo glucose-stimulated insulin secretion from hypoxia-pre-exposed islet grafts. *A*: Blood glucose; and, *B*: blood insulin levels; over time for mice receiving control normoxic islet grafts (hatched line) or hypoxia-pre-exposed grafts (solid line) following a 16 hour fast and intravenous glucose challenge (1g/kg). *C*: 16 hour fasting blood glucose; and, *D:* fasting blood insulin levels; from mice that received normoxic grafts (solid triangles) or hypoxia-pre-exposed grafts (open triangles). Data represents mean values ± SEM, $n \geq 6$ per time point; $*P < 0.05$; $**P < 0.01$.

Figure 4. Histological assessment of hypoxia-pre-exposed islet grafts. *A* and *B*; Examples of Haemotoxylin and Eosin staining for two representative control normoxic islet grafts; *C* and *D*; Examples of insulin labeling for corresponding hypoxia-pre-exposed grafts in *A* and *B*. *E* and *F*; Examples of Haemotoxylin and Eosin staining for two hypoxia-pre-exposed islet grafts; *G* and *H*; Examples of insulin labeling for corresponding hypoxia-pre-exposed grafts in *E* and *F*. Islet grafts were harvested at post-operative day 10.

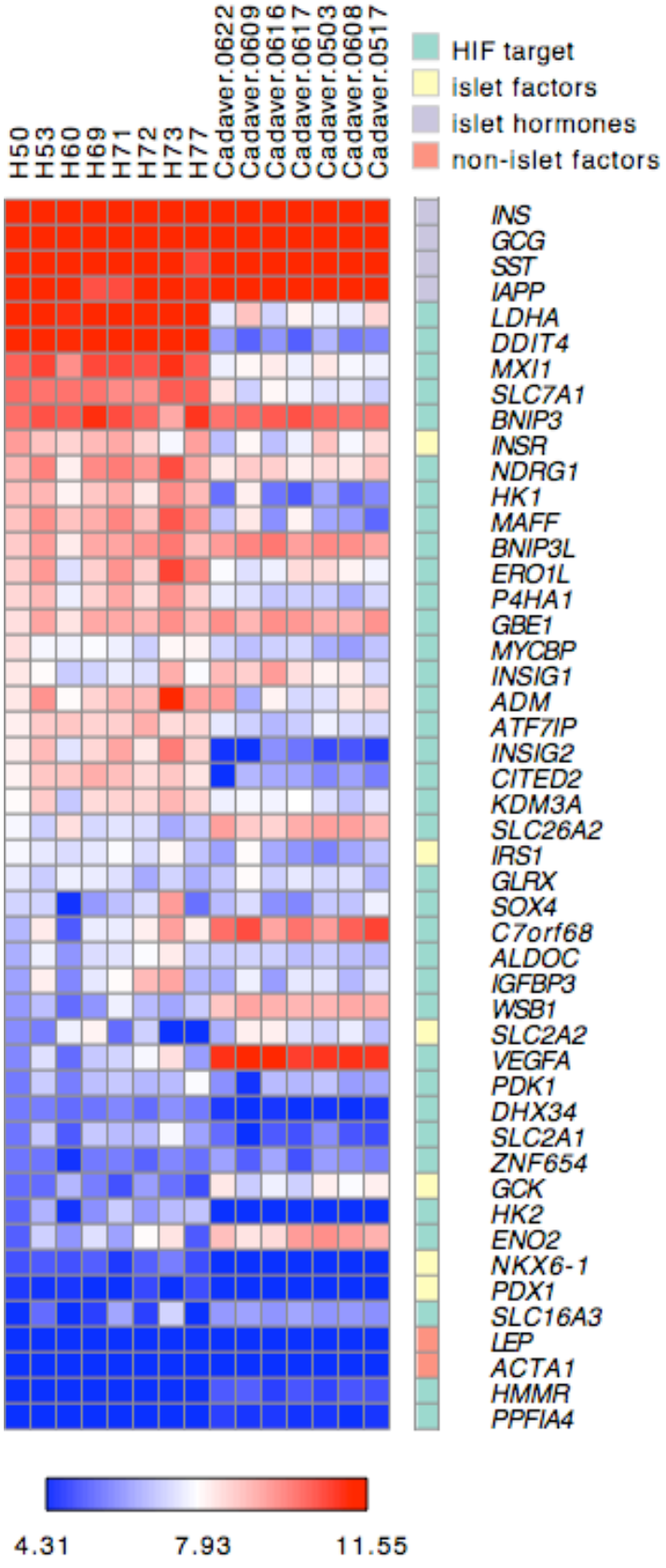
Figure 5. Molecular changes induced in primary mouse islets by hypoxia. *A*: Western Blot analysis of HIF-1α protein in freshly isolated mouse islets cultured for 16 hours under either normoxic (21% O_2), or hypoxic culture conditions (1% O_2), or in normoxic conditions supplemented with dimethyloxaloylglycine (DMOG; 0.5 mmol/l). Data from three independent islet batches per treatment. *B*: Gene expression levels for islets cultured as in (*A*) expressed as fold change comparing hypoxic versus normoxic culture conditions, $n = 3$ per group. *C*: Gene expression levels for islets cultured as in (*A*) expressed as fold change comparing DMOG-treated versus normoxic culture conditions, $n = 3$ per group; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Figure 6. Effect of hypoxic-exposure on in vitro GSIS. GSIS response of mouse islets cultured for 18 hours under either normoxic (21% O_2; open bars), or hypoxic culture conditions (1% O_2; closed bars) prior to static incubation for 1 hour at 2 mM or 20 mM glucose. Data represent mean ± SEM of eight replicates, from one of four independent experiments conducted; $*P < 0.05$

Figure 7. Altered expression of hypoxia-regulated metabolic genes is associated with the isolation of human islets. *A*: Using microarrays, gene expression levels of key metabolic hypoxia-response genes were measured, and used to calculate fold changes in gene expression associated with human islet isolation (n = 8), when compared to human islets in situ recovered by laser micro-

dissection (n = 7). Data represent median gene expression changes ± SEM. *B*: Expression levels for key hypoxia-response genes in isolated human islets as assessed by RTqPCR (-ΔCt, n = 8). For reference, mRNA levels for INS, IAPP, PDX1 and IRS1 are plotted. Horizontal bars show median values. *C*: Hypoxia-response gene expression stability in human islet grafts 4 weeks after transplant into RAG-/- mice, assessed using RTqPCR and normalized to pre-transplant levels. Individual grafts are plotted; black lines indicate the mean; $n = 6$ per group; $*P < 0.05$, $**P < 0.01$.

FIGURE 1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H60
H69
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Cadaver.0503
Cadaver.0608
Cadaver.0517
HIF target
islet factors
islet hormones
non-islet factors
INS
GCG
SST
IAPP
LDHA
DDIT4
MXI1
SLC7A1
BNIP3
INSR
NDRG1
HK1
MAFF
BNIP3L
ERO1L
P4HA1
GBE1
MYCBP
INSIG1
ADM
ATF7IP
INSIG2
CITED2
KDM3A
SLC26A2
IRS1
GLRX
SOX4
C7orf68
ALDOC
IGFBP3
WSB1
SLC2A2
VEGFA
PDK1
DHX34
SLC2A1
ZNF654
GCK
HK2
ENO2
NKX6-1
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ACTA1
HMMR
PPFIA4
4.31
7.93
11.55

FIGURE 2

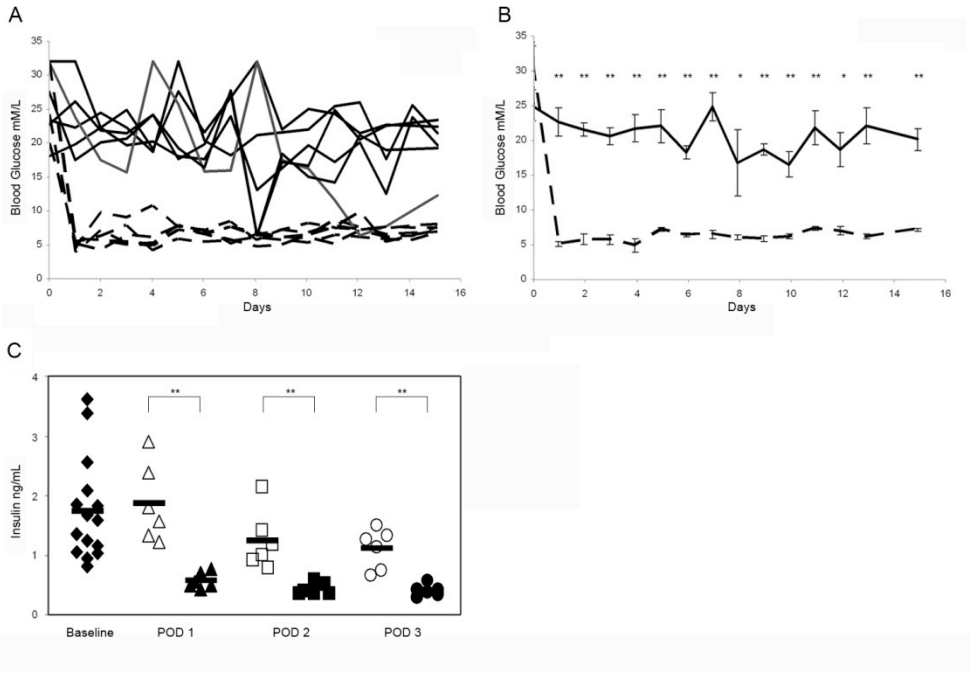
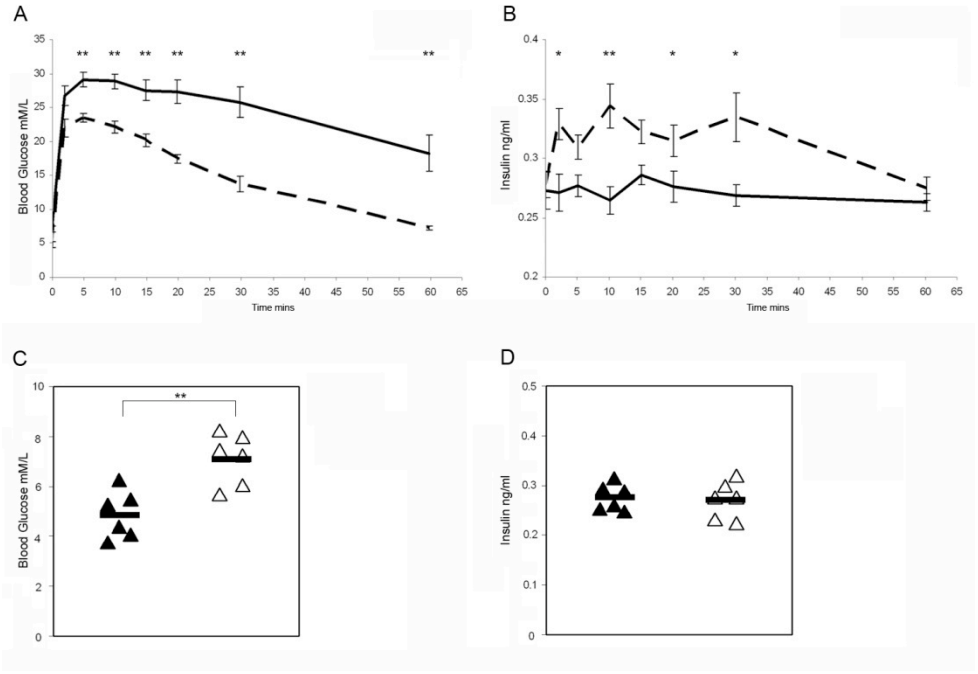

A
Blood Glucose mM/L
Days
B
Blood Glucose mM/L
Days
C
Insulin ng/mL
Baseline
POD 1
POD 2
POD 3

FIGURE 3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Blood Glucose mM/L
Time mins
B
Insulin ng/ml
Time mins
C
Blood Glucose mM/L
D
Insulin ng/ml

FIGURE 4

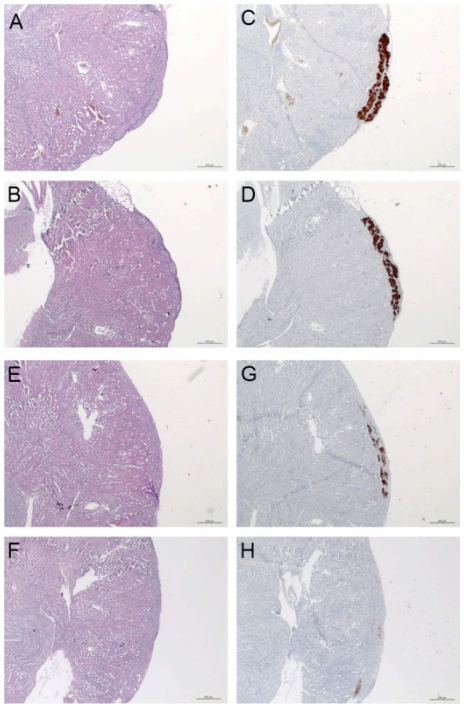


FIGURE 5

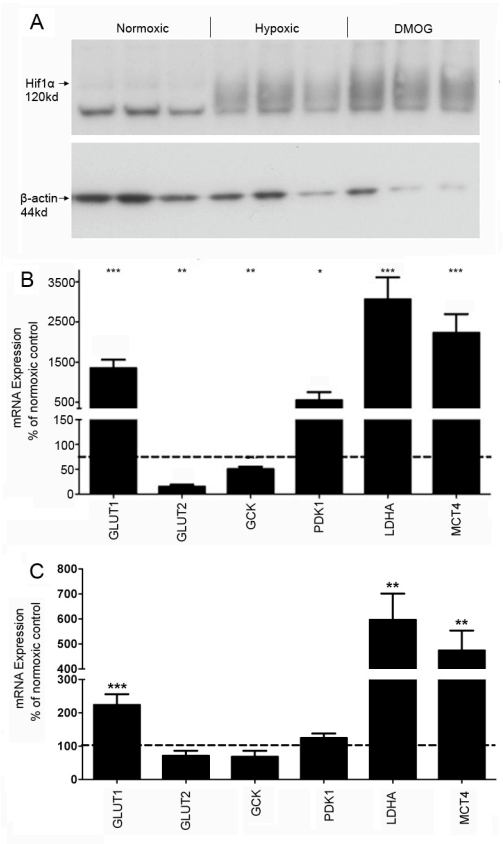

A
Normoxic
Hypoxic
DMOG
Hif1α → 120kd
β-actin → 44kd
B
mRNA Expression % of normoxic control
3500
2500
1500
500
150
100
50
0
***
**
**
*
***
***
GLUT1
GLUT2
GCK
PDK1
LDHA
MCT4
C
mRNA Expression % of normoxic control
800
700
600
500
400
300
200
100
0
***
**
**
GLUT1
GLUT2
GCK
PDK1
LDHA
MCT4

FIGURE 6

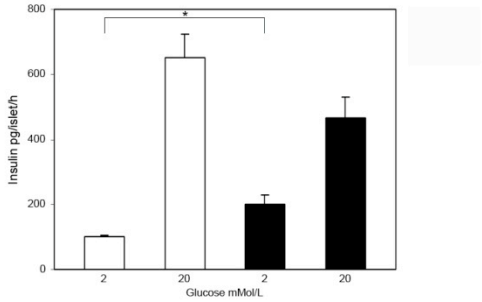
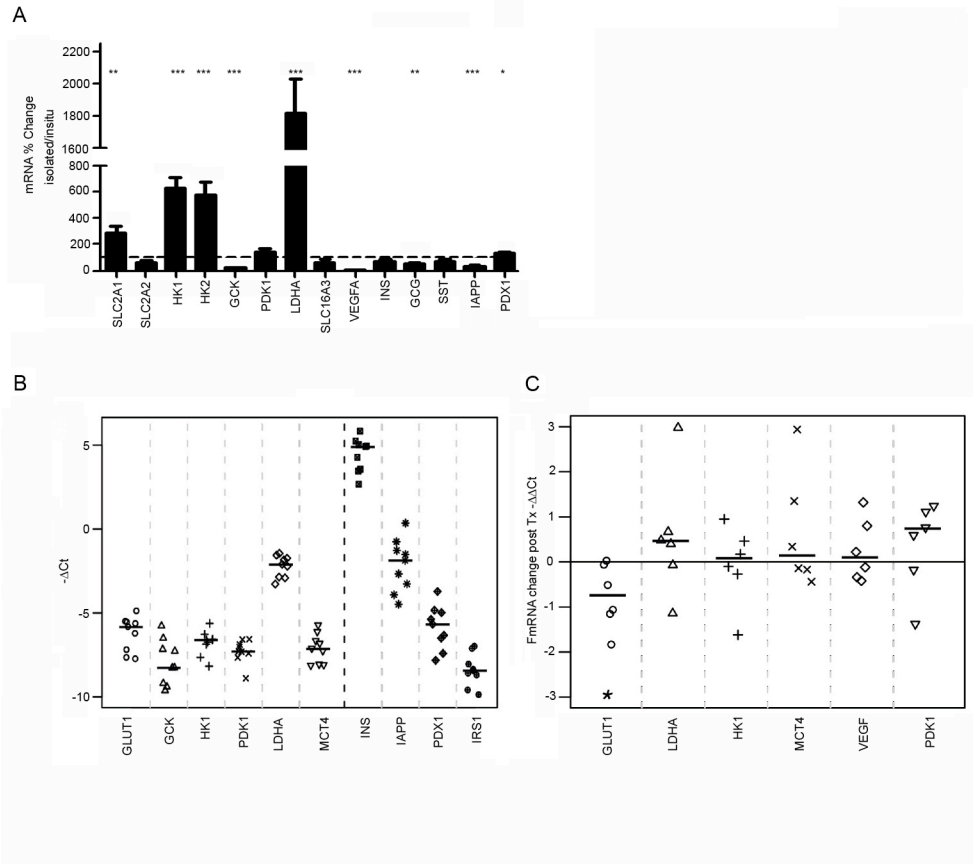


FIGURE 7


A
mRNA % Change isolated/insitu
2200
2000
1800
1600
800
600
400
200
0
**
***
***
***
***
***
**
***
*
SLC2A1
SLC2A2
HK1
HK2
GCK
PDK1
LDHA
SLC16A3
VEGFA
INS
GCG
SST
IAPP
PDX1
B
-ΔCt
5
0
-5
-10
GLUT1
GCK
HK1
PDK1
LDHA
MCT4
INS
IAPP
PDX1
IRS1
C
FmRNA change post Tx -ΔΔCt
3
2
1
0
-1
-2
-3
GLUT1
LDHA
HK1
MCT4
VEGF
PDK1