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**The species origin of the cellular microenvironment influences markers of beta cell fate and function in EndoC-H1 cells.**

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**Abstract**

Interaction between islet cell subtypes and the extracellular matrix influences beta-cell function in mammals. The tissue architecture of rodent islets is very different to that of human islets; cell-to-cell communication and interaction with the extracellular matrix may vary between species. In this work, we have compared the responses of the human EndoC-H1 cell line to non-human and human-derived growth matrices in terms of growth morphology, gene expression and glucose-stimulated insulin secretion (GSIS). EndoC-H1 cells demonstrated a greater tendency to form cell clusters when cultured in a human microenvironment and exhibited reduced alpha cell markers at the mRNA level; mean expression difference -0.23 and -0.51; p= 0.009 and 0.002 for the Aristaless-related homeobox (*ARX*) and Glucagon (*GCG*) genes respectively. No differences were noted in the protein expression of mature beta cell markers such as Pdx1 and NeuroD1 were noted in EndoC-H1 cells grown in a human microenvironment but cells were however more sensitive to glucose (4.3-fold increase in insulin secretion following glucose challenge compared with a 1.9-fold increase in cells grown in a non-human microenvironment; p = 0.0003). Our data suggests that the tissue origin of the cellular microenvironment has effects on the function of EndoC-H1 cells in vitro, and the use of a more human-like culture microenvironment may bring benefits in terms of increased physiological relevance.

**Introduction**

Communication between pancreatic beta cells and other endocrine cell types is important for the maintenance of beta cell fate and function.(1-3) Within islets, beta cells interact intimately with the microenvironment, critical for glucose sensing and the regulation of islet cell function.(1-3) The cellular microenvironment of beta cells is also known to influence cell behaviour and survival (4-6) and can influence aspects of growth morphology, such as cell density, which is vital to beta-cell survival as well as influencing differentiation, cell-cell signalling and function (7, 8). Differences in pancreatic islet architecture, between humans and other species such as rodents, have also been shown to have implications for cell-to-cell crosstalk and beta-cell function (9-11).

Accordingly, several features of beta cell function and behaviour also differ between species. Glucose sensitivity and transport are different between human and rodent beta cells, with the major glucose transporter being GLUT2 (encoded by the solute carrier family 2 member 2 *(SLC2A2)* gene) in rodents and GLUT1 and GLUT3 (encoded by the *SLC2A1* and *SLC2A3* genes) in humans (10-13). Proliferation rate and expression of cyclin dependent kinases also vary between rodents and man, as do responses to cellular stress, pancreatic beta-cell injury and inflammation (10, 11, 14-18). There are also important transcriptomic distinctions between species, with insulin being coded for by one gene (*INS*) in humans but two genes (*Ins1* and *Ins2*) in rodents (10, 19-22). Gene expression differences, especially in relation to stress response, are also seen in human beta cells compared with rodent cells, most notably in neurogenin3 (*NGN3),* NANOG homeobox (*NANOG),* POU class 5 homeobox 1 *(POU5F1* *(Oct4)),* L-Myc-1 proto oncogene *(MYCL1),* paired box 4 *(PAX4)* and *MAF BZIP transcription factor B (MAFB)* expression (16).

Given the importance of cellular microenvironment to beta-cell fate and function, it follows that the nature of the microenvironment may influence these features in *in vitro* culture systems. Model systems to investigate the effect of a non-human cellular microenvironment have been difficult to source until recently since fully functional human beta cell lines have been few and far between. The creation of EndoC-H1, a human cell line derived from fetal pancreata, has provided a new and physiologically-relevant model system for the investigation of beta cell function in vitro (10, 14, 19, 22). These cells demonstrate stable insulin gene expression across multiple passages and are responsive to glucose (10, 14, 19, 22). Like many other differentiated cell lines, EndoC-H1 cells require an extracellular matrix (ECM) coat containing fibronectin and other components such as Fraction V for culture (22). This requirement allows investigation of the effects of different components of the cellular microenvironment on aspects of beta cell behaviour.

In this work, we have compared the effects of a non-human culture microenvironment with those of a culture system containing only human components on aspects of cell morphology and culture dynamics, beta-cell function (insulin content, insulin processing and glucose-stimulated insulin secretion), and on the transcriptional output of genes involved in beta-cell differentiation status and beta-cell identity. Cells cultured according to these modified protocols grow reliably in pseudo islet-like structures, and can be propagated for long periods of time without compromise to beta-cell survival, function or phenotype. Cells also show evidence of a more consolidated beta-cell phenotype at the transcriptional level, with increased expression of mature beta cell markers and decreased expression of markers associated with other islet subtypes. These data highlight the potential advantages of propagating human beta cell lines in a microenvironment more reminiscent of human physiology, when considering aspects of islet biology in experimental systems.

**Methods**

*Modified culture conditions for EndoC-H1 cell microenvironment.*

Non-human microenvironment – EndoC βH1 cells (Endo Cells, INSERM, France) were cultured on an ECM coat consisting of DMEM 4.5 g/L (Thermo Fisher, Waltham, MA USA), matrigel (100 μg/mL) fibronectin from bovine plasma 2 μg/mL coat (both from Sigma-Aldrich, Steinheim, Germany), and cultured in DMEM 1 g/L glucose (Thermo Fisher, Waltham, MA, USA) 2% BSA fraction V (Merck Chemicals, Darmstadt, Germany), 10 mM nicotinamide (Sigma-Aldrich, Steinheim, Germany) 50μM β-2-Mercaptoethanol, 5.5 μg/ mL transferrin, 6.7 ng/mL sodium selenite, and 100 U/mL penicillin and 100 ug/ mL streptomycin (Sigma Aldrich, Steinheim, Germany)

Human microenvironment - EndoC-H1 cells were cultured on Maxgel ECM mixture liquid (Sigma Aldritch, Steinheim, Germany) containing fibronectin from human plasma (Sigma-Aldritch, Steinheim, Germany). Culture medium (DMEM 1 g/L glucose, Thermo Fisher Waltham, MA USA) contained 2% human serum albumin fraction V (Merck Chemicals, Darmstadt, Germany), 100 U/mL Penicillin and 100 μg/mL streptomycin, 50 μM β-2-Mercaptoethanol (Sigma- Aldrich, Steinheim, Germany), 10 mM nicotinamide (Sigma Aldritch Steinheim, Germany), 5.5 μg/mL human transferrin (Sigma Aldritch, Steinheim, Germany) and 6.7 ng/mL of sodium selenite (Sigma Aldritch, Steinheim, Germany). Cells were passaged using TRYPLE 1x (Thermo Fisher, Waltham, MA, USA) and neutralised with heat inactivated human serum from human male AB plasma (Sigma Aldritch, Steinheim, Germany).

*Immunofluorescence characterisation of EndoC-H1 cells cultured in human microenvironment*

Immunofluorescence staining was used to determine expression of mature beta cell markers in EndoC-H1cells grown in conditions to mimic a more human microenvironment, compared with cells grown using the standard non-human culture reagent protocol. EndoC-H1cells were cultured on coverslips for 72 hours before fixing with 4% paraformaldehyde for 15 minutes at 4oC. Primary antibodies for PDX1 (Abcam Ab47267, rabbit polyclonal) and NEUROD1 (Abcam Ab60704, mouse monoclonal) were diluted in phosphate buffered saline (PBS) with 0.1 M Lysine, 10% donor calf serum, 0.02% sodium azide and 0.02% Triton (ADST), to permeabilise the cell membranes, at concentrations of 1/500 and 1/400 respectively and incubated overnight. Primary antibodies were visualised using highly cross-absorbed secondary antibodies (Life Technologies) also diluted in ADST at 1/400: goat anti-rabbit for PDX1 at 1/400 (555nm) and goat anti-mouse for NEUROD1 at 1/400 (555nm) and incubated for 1 hour. Insulin antibody (80564, LN10088287 Dako) was diluted in ADST at a concentration of 1/700 and incubated for 1 hour. Secondary goat anti guinea-pig insulin along with DAPI (Sigma-Aldritch D9542), were diluted in ADST at respective concentrations of 1/400 and 1 μg/ml. Slides were visualised using a LEICA DM4000 B-LED fluorescence microscope using LAS X image software.

*Assessment of glucose-stimulated insulin secretion*

EndoC-H1cells were seeded onto 24 well plates at 4 x 105 per well and cultured for 72 hours in separate cultures. Cells were then transferred to a culture medium containing 2.8mM glucose as a starvation medium and cultured overnight, before being incubated for 1 hour in a starvation medium containing 0.5mM glucose in HEPES Krebs Ringer Buffer (KRB) at pH 7.4 containing: 116 mM/L NaCl, 5 mM/L KCl, 1 mM/L CaCl2, 1 mM/L MgCl2, 1.2 mM/L KH2PO4, 24 mM/L NaHCO3, 10 mM/L HEPEs, 0.2% Human Serum Albumin (HSA). After 1 hour the 0.5mM glucose starvation HEPES KRB starvation buffer was removed and replaced with HEPES KRB containing 20 mM glucose and cells incubated for 1 hour. Media was removed from each sample, spun at 1000 g for 5 minutes to remove protein debris and supernatant harvested for comparison with controls using an ELISA against fully processed human insulin. (Human Insulin ELISA, Crystal Chem, Downers Grove, IL, USA, assay range 0.9-220 mU/ L, analytical sensitivity 0.25 mU/ L, precision CV <10%, 1.2% cross reactivity with pro insulin).Absorbance was measured using a plate reader and measured A450 values, subtracting A630 values.

*Candidate genes for expression analysis*

Target genes were selected for their roles in beta cell fate, maturity and function. Selection included genes that are typically disallowed in beta cells due to their roles in inappropriate insulin release as a result of lactate and pyruvate metabolism in response to exercise, notably *SLC16A1* and *LDHA.* Alongside these, genes were also selected as indicators of cell stress and apoptosis, including *MYC, ARNT, HIF1a and DDIT3*. Transcription factors with roles in the regulation of beta cell fate were also selected, including: *PAX4, PDX1, ARX, MAFA, MAFB, FOXO1, NKX6.1, NGN3* and *NKX2.2. PAX4* and *ARX* act in a negative regulatory feedback axis. *PAX4,* favours the beta cell fate, while *ARX* is an alpha cell gene. It is also known to act in parallel with *NKX2.2* to regulate beta cell differentiation status and increase *PDX1* expression, which is known to be a master regulator of beta cell fate.(9, 23) *MAFA* regulates the insulin gene and is typically only expressed in mature beta cells and is also involved in increased expression of *PDX1*.(24) *MAFB*, although not typically expressed in mouse beta cells is known to be expressed in human cells.(25, 26) *FOXO1* is important in gluconeogenesis, beta cell differentiation and the maintenance of beta-cell function during cell stress via continued insulin secretion by acting on *MAFA* and *NEUROD1*.(27, 28) Reduced expression of *NKX6.1* has been reported in T2D, leading to a loss of beta-cell function.(24) It also negatively regulates *ARX* expression to maintain beta cell identity.(27) Reduced levels of NKX6-1 have also previously been shown to be associated with changes in beta cell fate, and in particular, with beta cell to delta cell transdifferentiation. (27) Although primarily required for induction of *PAX4*  expression in pancreatic endocrine development, *NGN3* persists in the mature pancreas maintaining islet cell identity and studies in mouse models suggest it plays a role in beta cell dedifferentiation in response to diabetogenic stimuli.(20) *NKX2.2* is restricted to beta cells and a subset of alpha and PP cells in mature islets and has roles in the maintenance of beta-cell function and fate.(27, 29)

*Quantitative real-time Polymerase Chain reaction (qRT PCR) assessment of gene expression*

RNA was extracted using the TRI®reagent (Sigma-Aldrich, Steinheim, Germany), chloroform (Thermo Fisher, Waltham, MA, USA) method. Cells were washed in Dulbecco’s phosphate buffered saline (D-PBS). TRI® reagent was used to harvest RNA, 10 μL of MgCl2(Thermo Fisher, Waltham, MA, USA) and 200 μLof Chloroform added. Samples were centrifuged at 14800 rpm, 4oC for 20 mins, the clear aqueous layer removed and 500 μL of isopropanol (Thermo Fisher, Waltham, MA, USA) added for overnight precipitation. Samples were centrifuged at 14800 rpm, 4oC for 45 minutes to form RNA pellet. The pellet was then repeatedly washed using 75% molecular grade ethanol (Thermo Fisher, Waltham, MA, USA), air dried and then re-suspended in 20 μL RNase-free water. cDNA synthesis was carried out using Superscript® VILO™ cDNA synthesis kit (Thermo Fisher, Waltham, MA USA). Samples were normalised to 100 ng/μL RNA prior to reverse transcription.

We measured the expression levels of 34 target genes by quantitative real-time PCR. Endogenous control genes were *PPIA, UBC, HPRT1, GUSB, B2M* and *IDH3B* Reaction mix included 2.5 μL Taqman® Universal PCR mastermix II (no AmpErase® UNG) (Thermo Fisher, Waltham, MA, USA), 1.75 μL dH2O, 0.5 μL cDNA and 0.25 μL Taqman® gene assay (Thermo Fisher, Waltham, MA, USA) in a 5 μl reaction volume. The reaction mixes were spun, vortexed and re-spun at 3000 rpm to ensure reagents were well mixed before being added to a 384 well qRT-PCR plate. Cycling conditions were: 50 °C for 2 min, 95 °C for 10 min and 50 cycles of 15 seconds at 95°C for 30 s and 1 minute at 60°C. Reactions were carried out in 3 biological replicates and 3 technical replicates. The relative expression of each test transcript was calculated by thecomparative Ct technique which was used to calculate the expression of each test transcript. Expression was assessed relative to the global mean of expression across all transcripts which was empirically determined not to vary across test conditions. Expression levels were normalised to expression levels as seen in unstimulated EndoC-H1 cells cultured in a non-human microenvironment. The data were log transformed to ensure normal distribution and differences in gene expression levels were then investigated by student independent t-test carried out using SPSS version 23 (IBM, North Castle, NY, USA). Data were presented as means ± S.E.M.

**Results**

*EndoC-H1 cells show morphological changes when cultured in a more ‘human-like’ microenvironment*

EndoC-H1 cells cultured in a cellular microenvironment containing only human components showed no observational change in replication rate compared with cultures grown according to standard protocols, and this remained stable over eight passages. Population doubling (PD) showed no differences between culture methodologies (p= 0.975, mean difference 0.023). Culture in human reagents showed populations doubling values of (mean PD = 18.98hrs, n=3) while those cultured using non-human reagents had values of (mean PD = 19.0hrs, n=3). The cells showed an increased tendency to form three-dimensional structures resembling pseudo-islets under human culture conditions, which were visible under fluorescence microscopy, whereas cells cultured in a non-human microenvironment, grew as a flat confluent monolayer (figure 1, images C and D). EndoC-H1 cells grown in the ‘human’ microenvironment, analysed and quantified, appeared smaller and more rounded compared with cells grown in a non-human microenvironment; the median area in μM for cells cultured in non-human reagents was 81.2 μM compared with 77.6 μM for cells in human reagents although this was not quite statistically significant (p= 0.070). However, despite this difference in area, there was no increased evidence of nuclear blebbing when cells were visualised using a nuclear dye (figure 1).

*EndoC-H1 cells cultured in a more human microenvironment demonstrate no alteration in insulin secretion, insulin processing or protein expression of mature beta cell markers.*

We measured insulin secretion, insulin processing and expression of the mature beta cell proteins Pdx-1 and NeuroD1 in EndoC-H1cells cultured in a human-like microenvironment by immunofluorescence (IF). EndoC-H1 cells stained positively with antibodies against both insulin and pro-insulin, suggesting that they contain both pro-insulin and processed ‘free’ insulin, and there was no discernible difference in localisation or intensity of the insulin or pro-insulin staining between the two conditions (figure 2). EndoC-H1 cells cultured in both systems were also assessed for expression of the mature beta cell markers NeuroD1 (p= 0.335; mean difference -2.72, standard error 2.79; figure 3) and Pdx-1 (p= 0.164, mean difference 3.91, standard error 2.76; figure 3). No difference in the level of protein expression of either marker was identified in EndoC-H1 cells cultured using either methodology.

*EndoC-H1 cells cultured in a more human microenvironment exhibit enhanced glucose stimulated insulin secretion (GSIS) compared with those cultured in a non-human microenvironment*

We assessed basal insulin release and glucose-stimulated insulin secretion (GSIS) in cells cultured in non-human or human-like microenvironments by ELISA. Cells cultured in a non-human microenvironment stimulated with 20mM glucose showed a doubling in insulin secretion compared to unstimulated controls which reproduces results found by Anderson et al (0.13 – 0.24 n = 10, p= 0.047; SD = 0.015; figure 4).(19) However, we used a Bonferonni correction to take into account multiple testing and under these conditions (p = <0.016) the cells cultured in a non-human environment did not show a statistically significant increase in insulin secretion following glucose stimulation. In contrast, the cells cultured in a human-like microenvironment showed a much stronger response, demonstrating a 4-fold increase in insulin secretion compared to unstimulated cells following glucose stimulation (0.11 – 0.47 p = 0.006; SD = 0.03; figure 4). A significant difference in insulin release in glucose-stimulated cells cultured in a human microenvironment (0.471 mU/ L) was detected compared with those cultured in a non-human microenvironment (0.239 mU/ L; p = 0.0003).

*EndoC-H1 cells cultured in human reagents have an increase in key genes involved in beta-cell fate*

We compared the expression of a panel of candidate genes involved in beta-cell function or maintenance of beta cell differentiation status in EndoC-H1cells cultured in a more human microenvironment compared with those cultured in a non-human microenvironment. The genes assessed are described in table 1. Although overall there was little difference in the gene expression profile between the two different culture methodologies, we did identify some changes in genes relating to alpha cell-like phenotypes. We identified a decrease in the alpha cell marker aristaless related homeobox (*ARX)* in a human-like microenvironment compared with cells cultured in a non-human microenvironment; (mean difference -0.623, standard error 0.072, p= 0.003; figure 4). Expression of transcripts encoding glucagon (*GCG*) showed lower expression in EndoC-H1 cells cultured in a more human microenvironment compared with cells cultured in a non-human microenvironment (RQ mean difference -0.577, standard error 0.064, p=0.001; Figure 4). There was a concomitant increase in *PAX4* expression in a human-like microenvironment compared with in cells cultured in a non-human microenvironment (mean difference 0.349, standard error 0.092, p= 0.032; Figure 4)*.* Finally, we noted an increase in the expression of transcripts encoding the pancreatic glucose sensor glucokinase (*GCK*); mean expression in cells cultured in a human microenvironment when compared with cells cultured in a non-human microenvironment; (mean difference, 0.244, standard error 0.070 p= 0.028; Figure 4).

**Discussion**

Beta cells in the human native islet are subject to intimate interaction with the extracellular microenvironment, and communication between pancreatic endocrine cells is important for regulation of beta-cell function and cell identity.(1-3) There are major differences between rodent islets and human islets in terms of cellular signalling, glucose transport and transcriptomic output, which have consequences for beta cell fate and beta-cell function.(9, 12, 13, 21) It follows therefore, that the composition of the cellular microenvironment may influence studies of beta cell biology in human cells in vitro. Here, we assess differences in beta-cell growth, glucose sensitivity and the expression of genes associated with beta-cell function or differentiation in the human beta cell line EndoC-H1 cultured in human and non-human cellular microenvironments. We demonstrate that EndoC-H1 cells demonstrate altered cellular morphology and enhanced response to glucose when cultured in a more human microenvironment. These features are accompanied by changes in the expression of genes associated with beta cell differentiation or beta-cell function consistent with a more consolidated beta-cell phenotype.

The cellular microenvironment is known to influence cell fate and differentiation status for many types of cells. (30-33). Fibronectin, in particular, is known to mediate mesodermal cell fate decisions (34). The extracellular matrix has particular importance in determining the functionality and survival of pancreatic beta cells (1-3). Species differences in characteristics of extracellular matrix have previously been reported in relation to extracellular matrix components (35). Given these observations, it is perhaps unsurprising that cells may behave differently when cultured in cellular microenvironments derived from different species. The increased tendency of the cells to form beta cell only pseudo islet structures when cultured in human derived reagents may improve cell-cell communication and previous studies have shown its importance for insulin secretion in islets.(36, 37) Whilst basal insulin secretion was similar in EndoC-H1 cells cultured in human and non-human cellular microenvironments, cells maintained in a more human-like microenvironment demonstrated an enhanced response to glucose in terms of insulin secretion. Human beta cell lines typically exhibit 2-3 fold increases in insulin secretion in response to a glucose stimulus, representing a release of approximately 10% of their insulin content (19, 38, 39) and our results are consistent with this. Since the cells were seeded at the same density and have equivalent PD times, it is not thought that the differences in insulin secretion were caused by differences in cell numbers in culture. Our data suggest that the glucose sensing and secretion machinery remains intact in EndoC-H1 cell cultured according to our protocols, and that these responses may perhaps be more physiologically representative of primary human beta cells when these cells are cultured in a more human microenvironment.

We also assessed the effects of human and non-human cellular microenvironments on the expression of genes involved in beta-cell function or beta cell identity in EndoC-H1 cells. Although we saw little change in expression levels of the majority of genes expressed, changes in markers of alpha cell function (*GCG*, *ARX*, which were downregulated) and some genes involved in maintenance of beta cell state (*PAX4*, which was upregulated) were noted. This finding is consistent with models in the literature which describe a regulatory feedback axis between *ARX* and *PAX4*, where the latter downregulates *ARX* expression to maintain beta cell fate. (27, 40-42) However, a recent study comparing rodent and human beta cell transcriptomes noted that in the mice, while Pax4 is required for *Arx* repression, it was undetectable in mature mouse beta cells.(43) Reduced *NKX6.1* expression may also lead to a consolidation of beta cell fate and improved beta cell function in cells grown in a human microenvironment, by virtue of its role in the regulation of somatostatin. (27)  Total somatostain RNA levels were lower in cells cultured in human reagents, but this did not reach statistical significance (table 1). We also identified an increase in the expression of glucokinase (*GCK*) transcripts in EndoC-H1 cells cultured in a more human-like microenvironment*.* Glucokinase is a metabolic sensor which is involved in the regulated secretion of insulin (44), and is also a MODY gene (MODY subtype 2) (45). The changes in gene expression that we note in our work are probably not confined to the genes we have tested, and emphasise the importance for transcriptomic studies to be carried out using the most physiologically-relevant model systems (21, 24, 25, 46, 47).

The existence of a human cell model such as EndoC-H1 is a great advantage for characterising disease in humans, but there are inevitably some limitations. EndoC-H1 cells have been reported to express some disallowed genes (19). These are genes which are typically not expressed in human beta cells due their roles in pyruvate and lactate metabolism, as this is imperative to protect against exercise induced insulin secretion. In primary beta cells the lactate dehydrogenase A (*LDHA)* and solute carrier family 15 member 1 (*SLC16A1)* genes are typically expressed at minimal levels (48). We identified that regardless of the culture microenvironment, EndoC-H1 cells express the *LDHA* and *SLC16A1* genes at relatively high levels. *LDHA* and *SLC16A1* code for proteins involved with pyruvate metabolism. They are disallowed in beta cells to prevent both inefficient processing of glycolytic products negatively impacting insulin secretion as well as exercise induced insulin release due to inappropriate pyruvate metabolism (48).

The strengths of this study are the creation of a fully human *in* vitro model system for the study of beta cell biology in the context of human disease. Although islets remain the gold standard for analysis of human disease, EndoC-βH1 represent a good step forward and offer an acceptable, and tractable, substitute, comparable to newly developed Def-PANC IPSC cell lines. Although the findings of this study show that the use of human derived reagents provides a culture environment which is equivalent to the standard culture methodology for EndoC-βH1 cells, there have been some challenges in developing the new protocol. This was particularly relevant for replacing the non-human ECM with a human equivalent and took some time to optimise. Further there are greater cost implications for sourcing human derived reagents. Despite such limitations it is clear that the novel culture methodology described here can improve the physiological relevance of the cellular micro-environment and provides a more human-like *in vitro* model system. We have shown that the species origin of the cellular microenvironment has affects and needs to be considered. Furthermore, our findings suggest that for EndoC-βH1 cells, this improves GSIS response and potentially consolidates mature beta cell status in this cell line. This study also represents the most comprehensive gene expression profile to date for genes involved with beta cell differentiation status and function, demonstrating the usefulness of this model for studies of islet function in man.

**Author contributions**

LH conceived and managed this study. NJ carried out all the experiments and wrote the manuscript. SR oversaw the immunofluorescence work. CB oversaw the work on glucose response. RS and colleagues were the originators of the EndoC-H1 cell line.

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**Table 1.** Expression of genes involved in beta cell differentiation, fate or function in EndoC-βH1 cells cultured in human versus non-human microenvironments. SE = Standard Error, 95% CI = 95% Confidence intervals. Genes showing expression differences in cells grown in ‘human’ or ‘non-human’ microenvironments are indicated in bold italic type.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | t-test for Equality of Means | | | | |
| Gene | p value (p = 0.050) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference | |
|  | Lower | Upper |
| HPRT1 | .092 | .200 | .086 | -.057 | .458 |
| IDH3B | .143 | .146 | .077 | -.085 | .378 |
| PPIA | .159 | .132 | .074 | -.085 | .348 |
| ARNT | .406 | .098 | .104 | -.203 | .399 |
| **ARX** | **.003** | **.624** | **.072** | **.400** | **.847** |
| DDIT3 | .795 | .031 | .107 | -.358 | .419 |
| FOXO1 | .540 | -.145 | .213 | -.783 | .493 |
| **GCG** | **.001** | **.578** | **.065** | **.398** | **.757** |
| **GCK** | **.028** | **-.244** | **.071** | **-.445** | **-.043** |
| HES1 | .192 | .166 | .096 | -.159 | .491 |
| HIF1A | .241 | .124 | .088 | -.136 | .384 |
| INS | .107 | .193 | .093 | -.067 | .453 |
| LDHA | .173 | .134 | .080 | -.093 | .362 |
| MAFA | .960 | -.011 | .205 | -.580 | .558 |
| MAFB | .462 | -.124 | .152 | -.554 | .306 |
| MYC | .930 | .019 | .193 | -.790 | .828 |
| NANOG | .238 | -.149 | .107 | -.448 | .149 |
| NEUROD1 | .221 | -.190 | .131 | -.555 | .174 |
| NKX2-2 | .575 | -.083 | .135 | -.464 | .298 |
| NKX6-1 | .870 | -.031 | .180 | -.532 | .469 |
| PAX6 | .056 | -.250 | .093 | -.510 | .010 |
| **PAX4** | **.032** | **-.349** | **.093** | **-.643** | **-.055** |
| PDX1 | .605 | -.089 | .158 | -.533 | .356 |
| POU5F1 | .079 | -.127 | .052 | -.279 | .025 |
| PTPN1 | .244 | .104 | .071 | -.130 | .337 |
| SLC16A1 | .285 | .139 | .109 | -.190 | .468 |
| SLC2A2 | .936 | .010 | .113 | -.338 | .358 |
| SLC2A4 | .385 | .132 | .124 | -.343 | .608 |
| SOX9 | .090 | .346 | .129 | -.111 | .802 |
| SST | .275 | -.426 | .337 | -1.362 | .510 |
| STK11 | .176 | -.060 | .036 | -.163 | .043 |
| SYP | .339 | .111 | .091 | -.253 | .475 |

**Figure legends**

**Figure 1. Culture morphology and pseudo islet structure of EndoC-H1 cells grown in different species microenvironments**. Morphological differences between EndoC-H1 cells grown in a human microenvironment compared with the same cells grown in a non-human microenvironment. **A**. Cells cultured in human derived culture reagents. **B**. Cells cultured in non-human derived culture reagents. Cells cultured in human reagents showed increased tendency to form three dimensional structures resembling beta cell only pseudo islets. Those cultured in non-human reagents form a confluent monolayer. **C.** DAPI nuclear stain in blue cells cultured in human derived reagents. No increase in nuclear blebbing. **D.** DAPI nuclear stain. Cells cultured in non-human derived reagents. No increase in nuclear blebbing.

**Figure 2. Proinsulin and insulin expression in EndoC-H1 cells grown in human vs non-human microenvironments.** **1A-1D**: EndoC-H1 cells cultured in human reagents (n = 6). **1A**. DAPI stain. 1B. Proinsulin stain. 1C. Insulin Stain. 1D. Overlay image of 1A-C showing co-localisation of proinsulin with insulin as well as ‘free’ processed insulin indicating proper insulin processing in the cells cultured in human derived reagents. **2A-D**. EndoC-H1 cells cultured in non-human reagents (n=6) 2A. DAPI stain. 2B. Pro-insulin stain. 2C. Insulin stain. 2D Overlay image of 2A-C showing co-localisation of proinsulin with insulin as well as ‘free’ processed insulin indicating proper insulin processing in the cells cultured in non-human reagents. This experiment was carried out in 6 replicates.

**Figure 3. NEUROD1 and PDX1 expression in** **EndoC-H1 cells grown in different species microenvironments. 1 A-D**: EndoC-H1 cells cultured in human reagents (n = 6). 1A. DAPI stain. 1B NEUROD1 stain. 1C. Insulin Stain. 1D. Overlay image of 1A-C showing co-localisation of NEUROD1 with DAPI. **1 E-H**: EndoC-H1 cells cultured in human reagents (n = 6). 1E. DAPI stain. 1F PDX1 stain. 1G. Insulin Stain. 1H. Overlay image of 1E-G showing co-localisation of PDX1 with DAPI. **2 A-D**. EndoC-H1 cells cultured in non-human reagents (n=6). 2A. DAPI stain. 2B. NEUROD1 stain. 2C. Insulin stain. 2D Overlay image of 2A-C showing co-localisation of NEUROD1 with DAPI. **2 E-H**. EndoC-H1 cells cultured in non-human reagents (n=6). 2E. DAPI stain. 2F. PDX1 stain. 2G. Insulin stain. 2H Overlay image of 2E-G showing co-localisation of PDX1 with DAPI.

**Figure 4 A. Assessment of glucose stimulated insulin secretion and insulin content in EndoC-H1 cells grown in different species microenvironments.** Absorbance was measured at both 450 and 630nm. The 630 values were subtracted from the 450nm values according to the manufacturer’s guidelines. Human microenvironment not stimulated (HNS): EndoC-H1 cells cultured in human microenvironment and not stimulated with 20mM glucose. (OD 0.118 n=10) Human microenvironment stimulated (HS): EndoC-H1 cells cultured in human microenvironment and stimulated with a 20mM glucose for 1 hour. (OD 0.471 n =10) Non-human microenvironment not stimulated (ANS): EndoC-H1 cells cultured in non-human microenvironment and not stimulated with 20mM glucose. (OD 0.131 n =10) Non-human microenvironment stimulated AS: EndoC-H1 cells cultured in non-human microenvironment and stimulated with a 20mM glucose for 1 hour. (OD 0.239 n =10)

**Figure 4 B. Change in gene expression in EndoC-H1 cells in EndoC-H1 cells grown in different species microenvironments.** Expression levels of the *CGC, ARX* and *PAX4* genes in response to different species microenvironment are given. Independent student t-tests were used to determine changes in gene expression. Graph showing change in mature alpha cell marker *ARX* expression (p=0.003) between cells cultured in human reagents compared to those cultured in a non-human microenvironment. Decreased expression of *ARX* is consistent with the observed increase in *PAX4* expression, known to be a negative regulator of the mature alpha cell marker *ARX*. Changes in mature alpha cell marker, *GCG*, expression (p= 0.001) between culture methodologies. Changes in *PAX4* expression between culture methodologies. Increased expression in *PAX4* (p= 0.032) Changes in *GCK* expression between culture methodologies. Increased expression of *GCK* (p=0.028) Decreased *ARX* and *GCG* expression may suggest consolidated mature beta cell status.