Glucagon-like peptide-1 enhances production of insulin in insulin-producing cells derived from mouse embryonic stem cells

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Abstract

Embryonic stem cells (ESCs) can be differentiated into insulin-producing cells by a five-stage procedure involving altering culture conditions and addition of nicotinamide. The amounts of insulin in these cells are lower than those found in pancreatic β cells. Glucagon-like peptide-1 (GLP-1) induces the differentiation of β cells from ductal progenitor cells. We examined the possibility of GLP-1, and its long-acting agonist exendin-4, enhancing the differentiation of insulin-producing cells from mouse ESCs (mESCs). A five-stage culturing strategy starting with embryoid bodies (EBs) was used in this study. mRNA for pancreatic duodenal homeobox gene 1 (PDX-1) and neurogenic differentiation (NeuroD) was detected from stage 1, hepatocyte nuclear factor 3 beta (HNF3 β) and insulin 2 from stage 2, Ngn3 and glucose transporter 2 (GLUT2) from stage 3, and insulin 1 and other β -cell markers, at stages 4–5. Cells at stage 5 secreted C-peptide,

being 0.68 ± 0.01 pmol/ 10^6 cells per 2 days, and had an immunoreactive insulin content of 13.5 ± 0.7 pmol/ 10^6 cells. Addition of GLP-1 (100 nM) and nicotinamide (10 mM) at stage 5 resulted in a 50% and 48% increase in insulin content and C-peptide secretion respectively compared with nicotinamide alone. Glucose-induced insulin secretion was enhanced 4-fold by addition of both growth factors. The GLP-1 receptor was present at all five stages of the culture. Addition of exendin-4 to cells at stage 2 resulted in a 4.9-fold increase in expression of the gene for insulin 1 and a 2-fold increase in insulin content compared with the effect of nicotinamide alone at stage 5. It is concluded that both GLP-1 and exendin-4 enhance the level of expression of insulin in glucose-responsive insulin-producing cells derived from the R1 mESC line.

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Introduction

Glucagon-like peptide-1 (GLP-1) is a peptide secreted from the gut in response to food. It acts directly on β cells, enhancing the effect of glucose in stimulating insulin secretion from these cells. When administered to diabetic mice, GLP-1 lowers blood glucose levels and stimulates insulin secretion (Gutniak *et al.* 1992). In addition, GLP-1 increases the β -cell mass by inducing the differentiation and neogenesis of ductal progenitor cells into islet endocrine cells (Hui *et al.* 2001, Abraham *et al.* 2002). We have previously demonstrated that GLP-1 is capable of enhancing fetal pig β -cell differentiation from progenitor epithelial cells as well as initiating their functional maturation in islet-like cell clusters (ICCs) (Hardikar *et al.* 2002). Exendin-4, a long-acting GLP-1 receptor (GLP-1R) agonist, binds to and activates the GLP-1R with the same potency as GLP-1 (Xu *et al.* 1999). Exendin-4 is resistant to the enzyme dipeptidyl peptidase 4 (DPP-IV), which is present in serum. It has been reported previously that exendin-4 is capable of stimulating both the differentiation of β cells from ductal progenitor cells and proliferation of

β cells when given to rats and human (Xu *et al.* 1999, Zhou *et al.* 1999, Stoffers *et al.* 2000, Habener 2001).

Embryonic stem cells (ESCs) are pluripotent and can be directed to differentiate along ectodermal, mesodermal and endodermal lineages to become different cell types. ESCs have been manipulated by using stringent culture conditions, or by the introduction of chimeric gene construct, into cells that synthesize and secrete insulin (Soria *et al.* 2000, Lumelsky *et al.* 2001, Hori *et al.* 2002, Blyszczuk *et al.* 2003). Manipulation of culture conditions in mESCs was employed in two of the studies (Lumelsky *et al.* 2001, Hori *et al.* 2002), which resulted in the production of a mixed population of endocrine cells including those that produced insulin, but at levels lower than those in pancreatic islets. However, another group (Rajagopal *et al.* 2003) has indicated that this may be due to an artifact caused by the presence of insulin in unconditioned culture medium.

In the present study, we examined the possibility of GLP-1 and its long-acting agonist, exendin-4, enhancing the differentiation of insulin-producing cells from mESCs.

Materials and Methods

Reagents

Culture medium knockout Dulbecco's modified Eagle's medium (KO DMEM), L-glutamine, β -mercaptoethanol, penicillin-streptomycin, fetal bovine serum (FBS), non-essential amino acids (NEAAs), B27 serum-free supplement, N₂ serum-free supplement, mouse basic fibroblast growth factor (bFGF), insulin-transferrin-selenium (ITS) solution were obtained from Gibco. Gelatin, fibronectin, transferrin, nicotinamide and chloroform were from Sigma while ESGRO leukaemia inhibitory factor (LIF) and exendin-4 were from the American Peptide Company (Sunnyvale, CA, USA). TRIZOL reagent, ethidium bromide, agarose, DNase 1 Amplification Grade Kit, SuperScript III First Strand Synthesis System Kit and Platinum Taq DNA Polymerase Kit were from Invitrogen. The Red Hot DNA Polymerase Kit was from ABgene (Epsom, Surrey, UK). The AmpliTaq Gold Kit was from Roche. Isopropyl alcohol was obtained from Merck and absolute ethanol was from Yarraville Distillery (Yarraville, Victoria, Australia). The labelled streptavidin-biotin (LSAB) 2 Horse Radish Peroxidase (HRP) Kit, amino-ethyl-carbazole (AEC) substrate chromogen and haematoxylin, polyclonal guinea pig anti-human insulin and rabbit anti-guinea pig immunoglobulin G (IgG) were obtained from DAKO (Carpinteria, CA, USA). The polyclonal rabbit anti-human GLP-1R antibody was received as a gift from Dr Daniel Drucker of The Banting and Best Diabetes Centre, University of Toronto, Canada.

Cell culture

Undifferentiated mESCs, from the -R1 cell line (Nagy *et al.* 1993) kindly supplied by Dr Patrick Tam from the Children's Medical Research Institute at Westmead, Sydney, were cultured on a feeder layer of irradiated mouse embryonic fibroblasts with medium containing KO DMEM, penicillin-streptomycin, 100 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 nM NEAA, 10% FBS and 1000 U/ml LIF. Medium was changed daily, and cells were split every 2–3 days.

To convert mESCs into insulin-producing cells, a five-stage culture strategy was used in the absence of a feeder layer. The strategy is a modification of that previously described (Lumelsky *et al.* 2001). Stage 1: mESCs were cultured on a gelatin-coated culture surface without a feeder layer but in the ES medium as described before for 2–3 days to expand the cells in an undifferentiated state. Stage 2: mESCs were harvested and then cultured in suspension for 3 days in the ES medium without LIF to generate embryoid bodies (EBs). Stage 3: EBs were transferred to culture dishes and allow to adhere overnight, then cultured for 6 days in serum-free medium supplemented with ITS solution, with final concentrations of

10 mg/l insulin, 56 mg/l transferrin, 6.7 mg/l selenium and 5 mg/l fibronectin, to select a specific cell population, thought to be nestin-positive cells. Stage 4: cells were transferred to gelatin-coated plates and cultured for 6 days in the presence of the mitogen bFGF 10 ng/ml, in N₂ serum-free medium containing B27 medium supplement, to expand the pancreatic progenitor cells. Stage 5: cells were cultured for 6 days in N₂ medium plus B27 supplement. In order to induce the differentiation of cells into insulin-producing cells, 10 mM nicotinamide and 100 nM GLP-1/10 nM exendin-4 were added. These concentrations of agents were selected based on maximal effects observed with them in differentiation of fetal pancreatic tissue (Hardikar *et al.* 2002, authors' unpublished observations).

Insulin content

Insulin was extracted from cells of each stage by incubating overnight with acid-ethanol at 4 °C. Immunoreactive insulin content was measured by RIA using a rat insulin standard; the lower limit of sensitivity of the assay was 0.25 ng/ml. Cross-reactivity with proinsulin was 73%.

C-peptide

To measure C-peptide release, the culture medium from each stage was collected and stored at –80 °C. The medium was concentrated by freeze drying and the amount of C-peptide in the culture medium was then measured by RIA using a rat C-peptide RIA kit (Linco, MO, USA); the lower limit of sensitivity of the assay was 25 pM. Rat insulin is not detected in the assay; cross-reactivity with human proinsulin is <0.01%, but cross-reactivity with mouse proinsulin is unknown.

Insulin secretion

For static incubation, cells from stage 5 were plated in 12-well culture plates at a density of 500 000 cells per well, allowed to attach to the bottom of the well and proliferate overnight. Cells were then washed thoroughly with basal medium (PBS containing 1 mM CaCl₂, 20 mM Hepes, 2 mg/ml BSA and 2.8 mM glucose) to remove culture medium. Monolayers were incubated in the basal medium for two consecutive 1-h periods to stabilize the basal secretion of insulin. Monolayers were then exposed to 20 mM glucose for 1 h. The medium was then collected, and its immunoreactive insulin content measured by RIA using a rat insulin standard.

RT-PCR

Total RNA was isolated from undifferentiated ES cells and the cells from stages 1 to 5 using TRIzol reagent and followed by DNase treatment; 1 μ g total RNA from each

Table 1 Primer sequences and PCR product size

Gene	Forward primer	Reverse primer	Product (bp)
GLP-1R	tcagagacggtgcagaaatg	cagctgacattcacgaagga	158
Oct-4	ggcgttctctttggaaaggtgttc	ctcgaaccacatccttctct	312
AFP	acaggaggctatgcatcacc	tggacatcttcacatgtgg	470
BMP-4	tgtgaggagtttccatcacg	ttattctcttctcctggaccg	566
Nestin	caagaaccactggggtctgt	tcccacctctgttgacttcc	248
PDX-1	ttagggcagtacgggtcctc	ccacccagtttacaagctc	325
NeuroD	cttgccaagaactacatctgg	cgtgttgaaagagaagttgcc	430
Nkx6-1	tcagtcaaggtctggttcc	cgatttgcctttttcagca	211
Ngn3	aagagcgagttggcactgagc	aagctgtggtccgctatgcg	223
GK	ggaacaacatcgtgggactt	tgaaggtgatttcgcacttg	783
Glut2	ggataaattcgctggatga	ttccttgggttctggaact	298
PC1	ttggctgaaaggaaagaga	gcttcatgtctctggttga	202
PC2	ctgtgacggctatgcttcaa	agctgcagatgtcccagagt	205
Insulin 1	atggccctgttggtgcacttcc	ttagttgcagtagttctccagctgg	320
Insulin 2	ccctgctggccctgctctt	aggcttgaaggtcacctgct	212
PP	ctgcctctccctgtttctc	ggctgaagacaagagaggc	337
SS	gtttctgcagaagtctctgg	agttcttgacgccagcttg	187
CK19	gtgccaccattgacaactcc	aatccacctccacactgacc	268
Amylase	cattgttgacacttgacacc	ttctgctgtttccctcatt	300
Carboxypeptidase A	gcaaatgtgtgttgatgcc	atgacaaaactcttgaccg	521
GAPDH	aatcccatcaccatctcca	ggcagtgatggcatggactg	336

GLP-1R, glucagon-like peptide-1 receptor; Oct-4, Octamer-binding transcription factor 4; AFP, alpha fetoprotein; BMP-4, bone morphogenic protein-4; PDX-1, pancreatic duodenal homeobox gene 1; NeuroD, neurogenic differentiation; NKx6-1, NK6 transcription factor related, locus 1; Ngn3, neurogenin 3; GK, glucokinase; GLUT2, glucose transporter 2; PC1 & 2, prohormone convertase 1 & 2; PP, pancreatic polypeptide; SS, somatostatin; CK19, cytokeratin 19; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

sample was then used to prepare single-stranded cDNA with the SuperScript First-Strand System for RT-PCR. A standard 25 µl PCR reaction was performed in a thermal cycle using the following profile: an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 52–62 °C for 45 s (depending on the primer) and elongation at 72 °C for 60 s. The samples were finally incubated at 72 °C for 5 min. PCR products were separated on a 1% TAE agarose gel, photographed and analysed using the Gel Doc 1000 system (BioRad). Quantification of the blots and comparison with the housekeeping gene was performed with software ImageJ, with experiments being repeated 3–4 times for each gene. The primer sequences used for PCR are shown in Table 1.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections of cultured mESCs. After dewaxing and serial alcohol rehydration, the sections were exposed to 3% H₂O₂ to remove endogenous peroxidase, and 1% BSA to block non-specific binding. The sections were then incubated overnight with primary antibodies, anti-human GLP-1R polyclonal antibody at 1:1000. The next morning, the slides were washed with PBS, exposed to biotinylated anti-rabbit/mouse for 15 min and then incubated with

streptavidin-peroxidase conjugate for a further 15 min. Finally, the sections were treated with substrate-chromogen AEC. A standard concentration of haematoxylin (0.1%) was added as a counterstain. The primary antibody was omitted for the negative control.

Statistics

Data were analysed by Student's *t*-test when there were two groups to be compared, and by analysis of variance and Duncan's test when there were more than two groups. The statistical package used was Number Cruncher Statistical Systems (Kaysville, UT, USA).

Results

Insulin and C-peptide production

Intracellular immunoreactive insulin content was detected at stage 3 with a significant increase at stage 5 (Fig. 1A). Addition of GLP-1 at stage 5 resulted in a 50% increase in immunoreactive insulin content compared with the addition of nicotinamide alone (20.1 ± 3 pmol/10⁶ cells vs 13.5 ± 0.7 pmol/10⁶ cells).

Immunoreactive C-peptide was also produced from the cells at stage 5, which probably indicates their ability to

Figure 1 Immunoreactive insulin content (A) and C-peptide secretion (B) for mESCs at different stages of culture. The number of samples was 12 (4 replicates in 3 separate experiments). **Stage 5N & GLP > *stage 5N > stages 3 and 4; $P < 0.05$. Abbreviations: 5N, stage 5 cells with nicotinamide; 5N & GLP, stage 5 cells with nicotinamide and GLP-1.

cleave proinsulin in the same manner as a pancreatic β cell. An alternative explanation is that the cells were producing proinsulin (but not insulin) since the cross-reactivity of mouse proinsulin in the assay is unknown. This explanation seems unlikely since the RIA used detects neither insulin (rodent or human) nor human proinsulin. The addition of GLP-1 to stage 5 cells resulted in a 48% increase in C-peptide release (1.01 ± 0.01 pmol/10⁶ cells vs 0.68 ± 0.01 pmol/10⁶ cells) (Fig. 1B). Insulin secretion expressed as a percentage of content was 5%.

Finally, stage 5 cells, or mES-derived insulin-producing cells, secreted immunoreactive insulin in response to 20 mM glucose. There was a 2.2- and 4.4-fold increase in insulin secretion in the presence of nicotinamide alone and nicotinamide + GLP-1 respectively. Levels of immunoreactive insulin increased from 2.25 ± 0.95 to 4.99 ± 1.89 ng/10⁶ cells per hour for the nicotinamide group ($n=6$) and from 1.74 ± 0.34 to 7.68 ± 1.4 ng/10⁶ cells per hour for the nicotinamide + GLP-1 group ($n=6$).

Effect of GLP-1 on mESC differentiation

The effect of GLP-1 on the differentiation of cells into insulin-producing cells was determined by analysis of gene

expression. The transcription factor Oct-4, a marker of ESC pluripotency, was down-regulated at the late stages of cell development and absent at stage 5 (Fig. 2). Three germ line markers, α -fetoprotein (AFP) (endodermal marker), nestin (ectodermal marker) and bone morphogenic protein-4 (BMP-4) (mesodermal marker) were also analysed within this modified differentiation protocol. All three lineage markers were present from stage 1 (Fig. 2). The expression of the endodermal marker AFP and ectodermal marker nestin were significantly up-regulated in cells of stage 2 (Fig. 2B); with no difference in the presence of nicotinamide alone and both nicotinamide and GLP-1. The expression of the mesodermal marker BMP-4 was down-regulated in cells of stage 5, particularly in the presence of GLP-1.

Expression of genes of β -cell development during the differentiation process

The transcription factors PDX-1, NKx6.1, NeuroD and Ngn3 (known to be involved in pancreatic β -cell development *in vivo*), and HNF3 β (a transcription factor involved in endodermal development) were analysed by RT-PCR.

Figure 2 RT-PCR analysis of gene markers for ectoderm, mesoderm and endoderm in differentiating mESCs. (A) Abbreviations: s1, stage 1 cells; s2, stage 2 cells; s3, stage 3 cells; s4, stage 4 cells; s5N, stage 5 cells with nicotinamide; s5N+G, stage 5 cells with nicotinamide and GLP-1. In order to show results clearly, the figure has been put together with gels carried out at different times. (B) Quantification of gene expression relative to the housekeeping gene GAPDH; $n=3-4$ for each gene; $*P<0.0001$ for stage 2 vs stage 1. AFP, α -fetoprotein; BMP-4, bone morphogenic protein-4.

PDX-1 was present in all five stages of development with slightly elevated levels at stage 5 (Fig. 3). HNF3 β was present from stage 2, Nkx6.1 and NeuroD from stage 3, and Ngn3 from stage 3 (Fig. 3).

Expression of mature pancreatic cell markers during the differentiation process

Several genes expressed in mature pancreatic β cells were analysed during the differentiation process (Fig. 4). Insulin 1 was present only at stage 5 while insulin 2 was present from stage 2 at a time when culture medium was not supplemented with insulin, and increased significantly thereafter (Fig. 4B). Glucagon was present only at stage 5, somatostatin from stage 3, but pancreatic polypeptide was undetectable at all five stages. Glucokinase, a β -cell marker, was present at stage 5 and glucose transporter 2 (GLUT2) from stage 3. PC1 and PC2, key enzymes involved in the cleavage of

proinsulin to insulin and C-peptide, were present from stage 4 with higher levels at stage 5.

Identification of the GLP-1R

RT-PCR and immunohistochemistry were used to determine the stage-specific expression of the GLP-1R during the differentiation of mESCs. GLP-1R gene was expressed in all stages of the differentiation protocol (Fig. 5A). The level of expression was similar in all stages throughout the differentiation process. A positive control was achieved by using cDNA from an insulinoma cell line NIT-1, and a sample negative control achieved by using adult mouse skeletal muscle cDNA. A water negative control was used to ensure there was no contamination of the PCR components.

Immunohistochemical staining confirmed the result. Cells of all stages were stained positively for the

Figure 3 Expression of pancreatic transcription factors in differentiating ESCs. (A) Abbreviations as in Fig. 2 caption. In order to show results clearly, the figure has been put together with gels carried out at different times. (B) Quantification of gene expression relative to the housekeeping gene GAPDH; $n=3-4$ for each gene.

GLP-1R (Fig. 5B). The staining was localized within the cytosol and the plasma membrane of positively staining cells. Semi-quantitatively, the percentage of positive staining cells of all five stages was similar (80%). Adult mouse pancreas was used as a positive control, while adult mouse testis was used as a sample negative control.

Effect of exendin-4 on mESC differentiation

Due to the expression of the GLP-1R at all stages, exendin-4, a long-acting agonist of the GLP-1R, was added to the culture media at stages 2 to 5. Addition of exendin-4 at the early stage resulted in a significant 4.9-fold increase in expression of insulin 1 compared with the addition of nicotinamide alone at stage 5 ($P=0.026$). However, there was no significant difference in expression compared with that observed when both nicotinamide and exendin-4 were added at stage 5 (Fig. 6A and B). Expression of the gene for insulin 2 in all three groups was not significantly different.

As might be expected, the immunoreactive insulin content of stage 5 ESCs was enhanced, 2-fold, when exendin-4 was added from stage 2 as compared with addition of nicotinamide alone at stage 5 (Fig. 6C).

Discussion

Recent studies have shown that mESCs can differentiate into insulin-producing cells and form structures that resemble those of pancreatic islets by varying the culture conditions (Lumelsky *et al.* 2001, Hori *et al.* 2002). The insulin-producing capacity of these cells has been questioned due to findings of Rajagopal *et al.* (2003), which indicated that insulin immunoreactivity was an artifact caused by insulin uptake from the culture medium. In addition, recent findings have suggested that insulin-producing cells derived from Lumelsky's protocol are not β cells but cells that have neuronal characteristics (Sipione *et al.* 2004). Our studies showed that using a modification

Figure 4 RT-PCR analysis of mature pancreatic cell markers in differentiating ESCs. (A) Abbreviations as in Fig. 2 caption. In order to show results clearly, the figure has been put together with gels carried out at different times. (B) Quantification of gene expression relative to the housekeeping gene GAPDH; $n=3-4$ for each gene; $*P=0.003$ for stage 2 vs stages 3–5.

of a five-stage method described by Lumelsky, mESCs were converted into insulin-producing cells. These cells secreted immunoreactive insulin in response to glucose, along with C-peptide, indicating that the immunoreactive insulin was being produced by the cells rather than being taken up from the culture media. Also, we found both insulin 1 and 2 mRNA expressed in the cells, with insulin 2 being expressed in cells at stage 2, when the medium is not supplemented with insulin. This is in contrast to the data from others, who detected no insulin transcripts (Rajagopal *et al.* 2003) or only insulin 2 (Sipione *et al.* 2004). While the expression of genes for the transcription

factors PDX-1 and Ngn3 that we found in the developing cells is not exclusive to pancreatic precursors and is present during neuronal development (Sommer *et al.* 1996, Perez-Villamil *et al.* 1999), at least some of the cells produced at the later stages of culture were of pancreatic origin. The cells expressed genes found in pancreatic exocrine and duct cells, all four pancreatic endocrine cells (α , β , δ and PP), as well as a number of genes found in pancreatic β cells (namely glucokinase, GLUT2, PC1 and PC2). The difference between our results and those of Sipione *et al.* (2004), whose insulin-producing cells were glucose unresponsive, and did not express insulin 1 and rarely glucagon,

Figure 5 Expression of receptor for GLP-1 in differentiating ESCs. (A) RT-PCR analysis of GLP-1R gene expression in all stages of differentiating ESCs. Abbreviations as in Fig. 2 caption. (B) ESCs at stage 1 stained with GLP-1R antibody. The receptor is identified as a dark stain on the surface of cells (arrows). Scale bar, 50 μ m.

may be due to the different mESC lines examined. Although the cells were able to produce immunoreactive insulin, the production of this hormone from the cells, 0.5 pmol/ 10^6 cells per day, was much lower than that from pancreatic β cells, 0.14 pmol/islet per day (Tuch *et al.* 1989), or 140 pmol/ 10^6 cells per day if it is assumed that there are 1000 cells in an islet. Also, the cell population was not homogenous. When transplanted into NOD/SCID mice, all mice developed teratomas (data not shown), which display features of tissues arising from three germ layers.

The novel strategy of our approach was the addition of a gut hormone, GLP-1, and its receptor agonist exendin-4, to enhance mESC differentiation. Previously, we had demonstrated that GLP-1 promoted the differentiation of β cells from fetal pancreatic precursor cells (Hardikar *et al.* 2002). In the current experiments we have demonstrated that addition of GLP-1 as well as nicotinamide at stage 5 of the differentiation protocol resulted in a 50% increase in immunoreactive insulin content compared with nicotinamide alone. The increase in intracellular immunoreactive insulin was associated with an increase in secretion of C-peptide from the cell. The down-regulation of the mesodermal marker BMP-4 indicates that GLP-1 was exerting an effect on cell differentiation; however it is possible that the hormone was increasing the number of

insulin-producing cells by enhancing their proliferation and/or increasing the insulin production per cell. It is not possible to distinguish which of these three explanations was predominant, since immunoreactive insulin producing cells in stage 5 could not readily be identified histologically. The cells did not stain for C-peptide (DAKO anti-rodent antibody). It is also possible that the effect of GLP-1 was being exerted on only a fraction of the stage 5 cells. Glucose-induced insulin secretion, following addition of both growth factors, was enhanced 4-fold compared with a 2-fold increase when treated with nicotinamide alone. This effect might be due to a direct effect of GLP-1, an effect observed on pancreatic β cells.

Due to the favourable effects of GLP-1 on differentiating mESCs, we sought the GLP-1R at different stages of culture in order to determine the earliest time it would be logical to introduce this hormone or its long-acting agonist, exendin-4. Surprisingly GLP-1R mRNA was detected at all stages of culture and even in mESCs cultured on feeder layer (results not shown), indicating that the removal of the feeder layer was not a contributing factor determining its presence. This finding was confirmed using immunohistochemistry. The presence of the GLP-1R in undifferentiated mESCs may be a result of several factors. Firstly, pluripotent cells *in vivo* may express the GLP-1R. To date, no study has identified the

Figure 6 Effect of exendin-4 on differentiation of mESCs. Exendin-4 was added from stage 2. (A) Insulin 1 and 2 expression in differentiating mESCs. (B) Quantification of gene expression relative to the housekeeping gene GAPDH; $n=3-4$ for each gene. (C) Insulin content of differentiating mESCs. Abbreviations: Ex, adding exendin-4 from stage 2 of differentiation of ESCs; s5N+E, adding exendin-4 and nicotinamide at stage 5; s5N, adding nicotinamide at stage 5. *, $P<0.05$ for s5N+E and Ex vs s5N.

GLP-1R within the ICM of the developing blastocyst. Even if present, studies conducted by Scrocchi *et al.* (1998) have indicated that GLP-1R null mice are viable and reproduce normally, indicating a lack of function in the development of the embryo. Secondly, ESCs have been shown to express many otherwise tissue-specific genes with no apparent role (Friedrich & Soriano 1991). This *in vitro* characteristic of the differentiating ESCs may result in 'background' expression of genes not specific to the functional ESC. Thirdly, the presence of the GLP-1R may be a result of mESCs that have spontaneously differentiated into cells that characteristically express the GLP-1R gene. Due to the high percentage of stage 1 cells staining positively for the GLP-1R it is probable that its presence is characteristic of *in vitro* mESCs and not from cells that have spontaneously differentiated. Also of interest, stage 2 GLP-1R-positive cells were found predominantly in the single columnar layer lining the EBs. This layer of cells is of an endodermal lineage, expressing a

number of endodermal markers including AFP and HNF-3 β (Abe *et al.* 1996, Karbanova & Mokry 2002). Since the GLP-1R was present at all stages of cell development, we added a GLP-1 agonist (exendin-4) to cells as they started to differentiate (stage 2). As expected, this had a beneficial effect, with enhanced expression of the insulin 1 gene and immunoreactive insulin content.

In conclusion, we have demonstrated that mESCs can be converted into immunoreactive insulin-producing cells by varying the culture conditions. GLP-1 and exendin-4 have beneficial effects on the production of immunoreactive insulin from mESC. However, the levels produced are lower than those produced by pancreatic β cells, and the cell population is not homogenous, so that it is not suitable for transplantation as a therapy for diabetes.

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