

Generation of insulin-expressing cells from mouse embryonic stem cells

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Abstract

The therapeutic potential of transplantation of insulin-secreting pancreatic β -cells has stimulated interest in using pluripotent embryonic stem (ES) cells as a starting material from which to generate insulin secreting cells in vitro. Mature β -cells are endodermal in origin so most reported differentiation protocols rely on the identification of endoderm-specific markers. However, endoderm development is an early event in embryogenesis that produces cells destined for the gut and associated organs in the embryo, and for the development of extra-embryonic structures such as the yolk sac. We have demonstrated that mouse ES cells readily differentiate into extra-embryonic endoderm in vitro, and that these cell populations express the insulin gene and other functional elements associated with β -cells. We suggest that the insulin-expressing cells generated in this and other studies are not authentic pancreatic β -cells, but may be of extra-embryonic endodermal origin.

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Recent studies have demonstrated that type 1 diabetes can be successfully treated by transplantation of islets of Langerhans derived from heart beating donors [1], but there is insufficient transplant material to meet the clinical demand. Embryonic stem (ES) cells are a potentially useful starting material from which to generate insulin-containing cells since they renew themselves indefinitely in culture in vitro, and can therefore be readily expanded to the large numbers required for transplantation therapy without losing their pluripotent phenotype. However, progress in reproducibly differentiating ES cells into a functional pancreatic endocrine lineage has been slow. To date, differentiation strategies have focused mainly on driving differentiation of mouse ES cells by withdrawing the pluripotency maintaining cytokine, leukaemia inhibitory factor (LIF), from the

culture medium and growing the cells as non-adherent clusters known as embryoid bodies (EBs). Several similar differentiation protocols are reported to generate islet-like structures containing a significant number of insulin-expressing cells (e.g. [2–4]).

Most protocols rely on RT-PCR to detect markers associated with endodermal or pancreatic development, and it is often assumed that these markers indicate cells of embryonic origin. However, the extra-embryonic endoderm that constitutes part of the yolk sac of the developing conceptus shares many characteristics with those of embryonic endoderm including expression of the early transcription factor network. Primitive endoderm, the precursor to yolk sac endoderm, is the first cell type to differentiate from the epiblast cell layer [5], and it has been detected in the peripheral cell layer of ES cells grown as embryoid bodies in the presence of LIF, while cells forming the core of the EB remained undifferentiated [6]. In this paper, we present evidence that mouse

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ES cells readily differentiate into a population of cells which display endodermal and some β -cell characteristics, but which are likely to be extra-embryonic in origin.

Methods

Materials. The CCE mouse ES cell line was obtained from Stem Cell Technologies (Vancouver) and the D3 mouse ES cell line was a kind gift from Professor Sigurd Lenzen (Hannover). Tissue culture reagents were obtained from Sigma (Poole, UK), except for foetal bovine serum (ES cell tested) which was purchased from PAA laboratories (Austria) and LIF, obtained from Chemicon International (CA, USA). Other general-purpose laboratory reagents were purchased from Sigma. RNA extraction and PCR reagents were obtained as described in [7]. The anti-OCT-4 antibody was obtained from Santa Cruz (CA, USA) and fluorescein-conjugated IgG was from Vector Laboratories (CA, USA).

Cell culture. The CCE and D3 mouse ES cell lines were routinely propagated on tissue culture plastic coated with 0.1% (w/v) gelatin in DMEM (25 mM glucose) containing 15% foetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, non-essential amino acids, 0.1 mM β -mercaptoethanol, and 1000 U/ml LIF. Medium was changed daily and cultures were passaged every 2 days onto fresh gelatin-coated plastic at a split ratio of between 1:6 and 1:10. Cultures were washed with Mg^{2+}/Ca^{2+} free phosphate-buffered saline (PBS) to remove residual FBS and then detached from the growing surface with a trypsin (2.5 g/L)–EDTA (1 mM) solution. Prior to seeding into new flasks the ES cells were dissociated to single cell suspensions by repeated pipetting with a small bore pipette.

Differentiation of ES cells. Differentiation of ES cells in the presence or absence of LIF was induced by growing cultures to a higher density than would normally be employed in routine ES cell culture. ES cells were passaged as described above and standard ES cell medium with or without the addition of LIF (1000 U/ml) was replaced every 2 days. The differentiated ES cells (DES cells) were then harvested after between 4 and 12 days growth by trypsinisation and samples were prepared for analysis of gene expression by RT-PCR, or seeded onto coverslips for Ca^{2+} microfluorimetry or immunostaining. Unless stated otherwise results are representative of four separate cycles of the differentiation process from frozen stocks of the ES cell lines.

Measurement of gene expression by RT-PCR. RNA extraction [7] and standard RT-PCR techniques, performed as described previously [8], were used to detect mRNAs for the genes listed in Table 1. PCRs were continued for 40 cycles in all cases.

Reaction products were separated on 1.8% agarose gels and stained with ethidium bromide (0.5 μ g/ml) to visualise the DNA bands. Primers were designed using mRNA sequence information and the nucleotide search engine BLAST, except for the following for which primer sequences and conditions have been published as indicated: HNF4 α and AFP [9], COUP TF1 [10], and Kir6.2 [11].

Quantification of preproinsulin, OCT-4, and β -actin. Quantitative measurement of preproinsulin (PPI) and β -actin mRNAs was performed using the Lightcycler rapid thermal cyclers system, and compared with expression levels in the MIN6 mouse insulinoma cell line. Generation of primers and standards, and measurements of mRNA levels were carried out as described [12]. Quantitative measurements of mouse OCT-4 mRNAs in CCE and D3 ES cells, and DES cells of both lines were also carried out essentially as described [12] under the following conditions: PCRs consisted of a 10 s denaturation step followed by 40 cycles comprising denaturation at 95 °C for <1 s, annealing at 56 °C for 10 s and an extension phase of 13 s. Three millimolar Mg^{2+} was included in the reaction mixture. Primer sequences for OCT-4 were: forward: GCGTTCCTTTGGAAAGGTGTC, reverse: CTCGAACCACATCCTTCCT. Reaction products were visualised by separation on 1.8% agarose gels and stained with ethidium bromide (0.5 μ g/ml) to verify the product size. The product bands were excised from their gels and purified using a spin column method. Samples were eluted in RNase free water and sequenced using fluorescence chain terminator methods.

Calcium microfluorimetry and immunocytochemistry. Undifferentiated ES cells and DES cells derived from CCE and D3 ES cells were seeded onto polyornithine (10 μ g/ml) and laminin (1 μ g/ml) coated glass coverslips at a density of approximately 5×10^4 cells/coverslip and allowed to adhere overnight in standard ES cell culture medium. Changes in intracellular calcium levels were measured by single cell microfluorimetry in Fura-2-loaded DES cells, as described previously for insulin-secreting cells [13]. Immunocytochemical detection of protein expression was performed after cell fixation using 4% paraformaldehyde. Cell samples were incubated overnight at 4 °C with a monoclonal anti-OCT-4 mouse primary antibody (1:500 dilution in a 1% milk solution in Tris-buffered saline with 0.1% Tween) followed by a fluorescein-conjugated secondary IgG at 1:200 dilution in 10 mM Hepes/0.15 M NaCl.

Results

Endodermal gene expression in differentiated cells

The expression of transcription factors associated with endodermal cell differentiation was assessed by RT-PCR in DES cells generated from CCE ES cells and maintained as monolayers in the presence of LIF. HNF-4 α and 3 β (FoxA2) were readily detectable in the DES cells, as was COUP TF1, a primitive endoderm and liver-specific transcription factor. PDX1, an endodermal and pancreatic transcription factor, was also detectable in the DES cells, whereas α -fetoprotein

Table 1
The sequences of primer pairs used for RT-PCR amplification of cDNAs of interest

Gene recognised	Forward primer sequence	Reverse primer sequence	T_{ann} (°C)	Product size (bp)
Preproinsulin	AAC CCA CCA GGC TTT TGT C	TGC AGT AGT TCT CCA GCT GG	57	267
Preproglucagon	TGC AGT GGT TGA TGA ACA CC	GGT GGC AAG ATT GTC CAG AA	55	269
HNF4 α	ACACGTCCTCCATCTGAAGGTG	CTTCCTTCTTCATGCCAGCCC	58	269
FoxA2 (HNF3)	GTG AGA AGC AAC TGG CAC TG	GGT GGT TGA AGG CGT AAT GG	58	337
PDX1	AAT CCA CCA AAG CTC ACG CGT GG	CCC GCT ACT ACG TTT CTT ATC TTC C	60	190
AFP	CCTGTGAACTCTGGTATCAG	GCTCACACCAAAGCGTCAAC	55	406
COUP TF1	AGCCATCGTGCTATTACAG	TTCTCACCAGACACGAGGTC	55	569
SUR 1	GCC GAG AGC GAG GAA GAT	GGA GCA GTT CCT GGT GGC	61	224
Kir6.2	GCC ATG CTG TCC CGA AAG	GGC CAG GGG ACA TTC CTC TGT	61	437

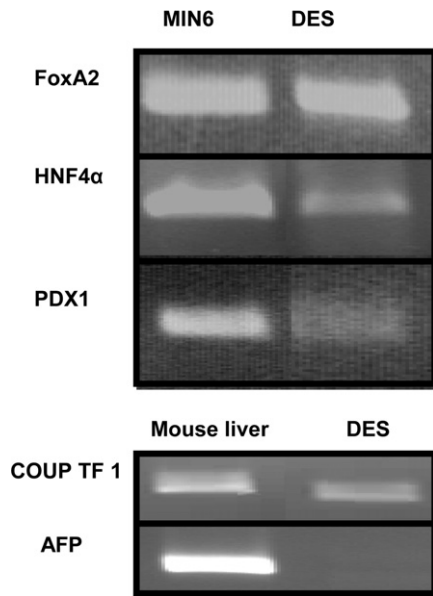


Fig. 1. Expression of endodermal-specific genes in differentiated (DES) cells, CCE cultures, and MIN6 cells. Markers associated with pancreas and liver development (HNF4α, FoxA2, and PDX1) were detected in DES cells by RT-PCR, as was COUP TF1, a transcription factor present in liver and extra-embryonic endoderm. AFP expression was absent in DES cells.

(AFP), which is abundantly expressed in the developing liver and the visceral endoderm cells of the yolk sac, was not detected (Fig. 1). Similar results were obtained using DES cells grown in the absence of LIF, with detectable levels of mRNAs for FoxA2, PDX1, and COUP TF1, but no detectable expression of AFP (results not shown).

OCT-4 expression in ES and differentiated cells

OCT-4 is expressed in pluripotent cells of the embryo [14], and expression is down-regulated following differentiation in most tissues. However, in the primitive endoderm OCT-4 expression is increased above that in undifferentiated cells [15,16]. We used quantitative RT-PCR and immunocytochemistry to assess the expression of OCT-4 mRNA and protein, respectively, in ES cells and in DES cells. The immunostaining in Fig. 2A shows widespread patterns of nuclear staining (>95% of cells stained) both in CCE ES cells and in DES cells derived from CCE cells. A similar pattern of OCT-4 immunostaining was detected in DES cells generated from D3 ES cells (results not shown). Quantitative measurements of OCT-4 gene expression in ES and DES cell

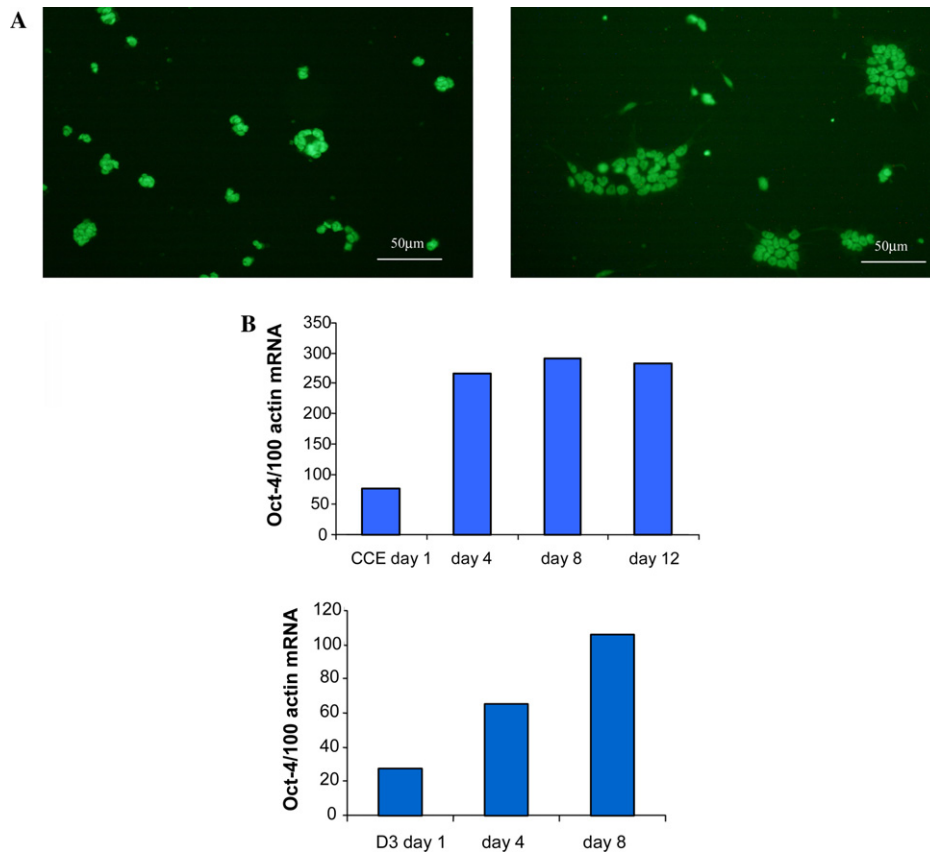


Fig. 2. (A) Fluorescence immunocytochemistry staining for OCT-4 revealed a widespread distribution of nuclear staining in both CCE ES cells (A, left panel) and CCE-derived differentiated (DES) cells (A, right panel). (B) Quantitative PCR in DES cell samples derived from CCE (upper panel) and D3 (lower panel) mouse ES cells showed a time dependent increase in levels of OCT-4 mRNA over that determined for the undifferentiated cells (day 1).

populations showed an approximately threefold increase in expression in the differentiated cells after 4 days, increasing to a fivefold increase in OCT-4 expression in DES cells derived from the D3 ES cells after eight days growth (Fig. 2B).

Expression of markers of β -cell function

Islet hormone expression was undetectable in undifferentiated ES cells, but the mRNAs for the islet hormones PPI and preproglucagon were expressed in the DES cell populations, as shown in Fig. 3A. Quantification of PPI mRNA levels in two separate DES cell preparations gave values of 60 fg PPI/ 10^4 fg β -actin and 0.7 fg PPI/ 10^4 fg β -actin, compared to an expression level in MIN6 mouse insulinoma cells of 150 pg PPI/ 10^4 fg β -actin. Differentiation also induced expression of functional elements normally associated with stimulus-response coupling in mature β -cells. Fig. 3A shows that mRNAs for both subunits of the ATP-sensitive K^+ channel (K_{ATP}), the inwardly rectifying K^+ channel Kir6.2 and the sulphonylurea receptor SUR1, were detected in DES cells but only Kir6.2 was detected in undifferentiated CCE cells. Microfluorimetric measurements demonstrated that the K_{ATP} channel expressed by DES cells was functionally active as assessed by tolbutamide-induced changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Thus, as shown in Fig. 3B a sub-popu-

lation of the cells showed reversible and re-producible elevations in $[Ca^{2+}]_i$ in response to the sulphonylurea tolbutamide (100 μ M, 8% of cells tested).

Discussion

The present results demonstrate that mouse ES cells will spontaneously differentiate towards an endodermal phenotype if maintained at high cell density in vitro, and suggest that the endodermal cells may be extra-embryonic in origin. Thus, DES cells derived from two different ES cell lines expressed a number of endodermal markers. Expression of the HNF family of transcription factors is an early event in liver and pancreas formation [17], and these transcription factors are also expressed in the endoderm of the yolk sac, which shares functional similarities to liver including nutrient uptake and delivery [18–20]. AFP and COUP TF 1 are involved in both liver and yolk sac endoderm development, although COUP TF 1 is expressed in the primitive endoderm, a precursor to the visceral and parietal cells of the yolk sac endoderm [10], while AFP is expressed in some cells of the visceral endoderm [21,22]. The DES cell populations also expressed the mRNA for PDX1, a transcription factor that is important in early pancreas specification [23,24] and for the maintenance of a mature β -cell phenotype [25]. PDX1 expression is often used as evidence for differentiation towards a pancreatic endocrine phenotype [3,2,26] but PDX1 mRNA has also been localised to visceral endoderm [21]. Thus, our measurements of gene expression suggest the presence of endodermal cells but these commonly used markers do not discriminate between endoderm of embryonic or extra-embryonic origin.

Differentiation towards embryonic endoderm is usually associated with reduced expression of the ES cell transcription factor OCT-4 [2,27]. In contrast, our measurements of OCT-4 mRNA and protein levels showed that differentiation was associated with increased OCT-4 expression, suggesting that the endodermal component of the DES cells was primitive endoderm, the precursor to yolk sac endoderm, rather than endoderm of embryonic origin. This conclusion is supported by the concomitant expression of the primitive endoderm-specific COUP TF 1, and the frequency of OCT-4 immunopositive cells in the DES cell populations suggests that extra-embryonic endodermal cells represented a large proportion of the differentiated cell population.

The DES cells also expressed functional pancreatic markers, including mRNAs and the subunits of the K_{ATP} channel, a key element in glucose sensing by mature β -cells [28]. The tolbutamide-induced increases in $[Ca^{2+}]_i$ in a sub-population of DES cells are consistent with functional K_{ATP} channels being closed by the

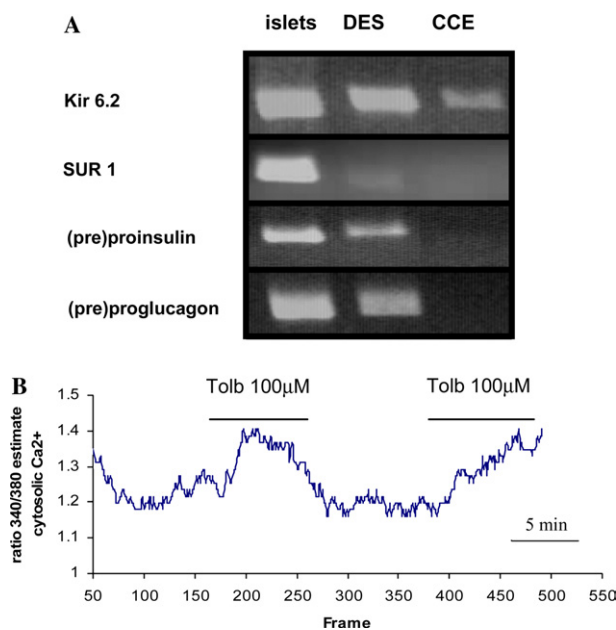


Fig. 3. (A) Markers of islet cell function were detectable by RT-PCR in DES cell cultures derived from CCE mouse ES cells. Only Kir6.2 was found to be expressed in the undifferentiated starting material as well as the DES. mRNAs for SUR 1, PPI, and preproglucagon were only detectable in the DES cell samples. (B) A small number of DES cells (8% of cells tested) responded to the K_{ATP} channel agonist tolbutamide by showing reversible elevations in intracellular calcium.

binding of tolbutamide to the SUR1 subunit, causing a depolarisation of the cells and the influx of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) through voltage-operated calcium channels. The reversibility of the response further suggests that the cells possessed mechanisms for regulating their internal calcium stores after the influx of $[\text{Ca}^{2+}]_o$. However, although important for insulin secretion, K_{ATP} expression is not an exclusive marker for pancreatic β -cells and functional K_{ATP} channels are not confined to endodermal endocrine cells, but have also been reported in tissues of neuroectodermal and mesodermal origin. Insulin gene expression is generally considered to be specific for pancreatic β -cells in adults but, in common with PDX1, insulin is expressed in other tissues during development. Thus, PPI mRNA and immunoreactive insulin are expressed in the extra-embryonic endoderm of the rodent yolk sac during development [21,29,30], with expression being localised to the visceral endoderm component [21].

These observations are consistent with our measurements of insulin gene expression in a cell population expressing markers of extra-embryonic endoderm, and suggest that the PPI mRNA expression in our DES cell populations may be in insulin-expressing yolk sac endoderm rather than pancreatic β -cells. This conclusion is further supported by previous studies showing that primitive endoderm is formed in the outer cell layer of EBs [6,31] and that insulin-positive cells were located exclusively on the periphery of mouse EBs, suggestive of extra-embryonic endoderm rather than pancreatic β -cells [26]. The low levels of insulin gene expression in our DES cell populations are consistent with other studies using EB-based protocols, in which insulin-expressing cells are estimated to represent no more than approximately 0.001% of the cell population [32].

In conclusion, our results raise the possibility that spontaneous differentiation to primitive endoderm may represent a default pathway for mouse ES cells maintained in some in vitro environments, and that these cells express low levels of the insulin gene. We therefore suggest that many of the studies reporting the differentiation of insulin-expressing cells from mouse ES cells are generating insulin-expressing extra-embryonic endoderm rather than the assumed authentic embryonic endodermal endocrine cells.

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