

Review

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Directed differentiation of rhesus monkey ES cells into pancreatic cell phenotypes

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Abstract

Embryonic stem cells (ES) can self-replicate and differentiate into all cell types including insulin-producing, beta-like cells and could, therefore, be used to treat diabetes mellitus. To date, results of stem cell differentiation into beta cells have been debated, largely due to difficulties in defining the identity of a beta cell. We have recently differentiated non-human primate (rhesus) embryonic stem (rES) cell lines into insulin producing, beta-like cells with the beta cell growth factor, Exendin-4 and using C-peptide as a phenotype marker. Cell development was characterized at each stage by gene and protein expression. Insulin, NKX6.1 and glucagon mRNA were expressed in stage 4 cells but not in early undifferentiated cells. We concluded that rES cells could be differentiated *ex vivo* to insulin producing cells. These differentiated rES cells could be used to develop a non-human primate model for evaluating cell therapy to treat diabetes. To facilitate the identification of beta-like cells and to track the cells post-transplantation, we have developed a marker gene construct: fusing the human insulin promoter (HIP) to the green fluorescent protein (GFP) gene. This construct was transfected into stage 3 rES derived cells and subsequent GFP expression was identified in C-peptide positive cells, thereby substantiating endogenous insulin production by rES derived cells. Using this GFP detection system, we will enrich our population of insulin producing rES derived cells and track these cells post-transplantation in the non-human primate model.

Review

Diabetes Mellitus (DM) is a collection of heterogeneous disorders that result in glucose homeostasis abnormalities and produce metabolic complications that are frequently debilitating and life threatening. Currently, approximately 17 million Americans [1-6] are affected by DM; and this number is expected to increase by 165% in the USA in the next 30 years [7]. Identifying methods to treat

or cure DM, along with efforts to prevent its development, will be a key in stemming this pandemic.

Central to the development of DM is the relative loss of insulin production from the pancreatic beta cells. Replacing these cells has been a therapeutic goal for decades and could prevent the morbidity and mortality associated with DM. Recently, islet transplantations were successful in restoring normal glycemic control [8]. This success

provides proof that replacing functional β cell mass is an effective treatment for DM.

Although islet transplantation has shown significant promise, it remains an unlikely therapy for patients with DM primarily due to the lack of available human islet tissue [9,10]. Furthermore, individual patients will require repeat islet transplantations to offset the slow but progressive loss of transplanted islet function [11]. Since β cells are the only sources of insulin in the body, an unlimited and renewable supply of β cells or islets will be needed to successfully treat DM by transplantation [12-14].

An ideal tissue source for transplantation would be β cell lines with glucose-mediated insulin release, that are not immunogenic, tumorigenic or at risk of transmitting infectious disease, and are able to replicate *ex vivo* without losing their differentiation potential [15]. While such a cell line does not yet exist, islet progenitor (adult stem cells) or embryonic stem (ES) cells are prime candidates [13]. Both adult and embryonic stem cells have the potential to proliferate *ex vivo* and differentiate into islet-like cells [16,17]. If these techniques can be translated into the growth and isolation of islet cells, this would provide a source of replaceable islet tissue.

Embryonic stem cells

ES cells, present in the inner cell mass of the pre-implantation embryo, are immortal and pluripotent [18]. Clonal mouse ES cell lines differentiate into islet-like phenotypes, *ex vivo* [19] and *in vivo* [17]. This methodology has also been applied to human ES cells; however, the process produces a mixed population of cells containing only about 3% insulin positive cells [20]. Although ES cells have the potential to differentiate into islet like cells, early work was limited by the identification of the β cell phenotype using insulin immunocytochemistry. This identification method has recently been invalidated because insulin is a growth factor present in the conditioned media used to differentiate and grow the cells [21]. A recent publication demonstrates that insulin in the media is pinocytosed into apoptotic cells and thus, is indistinguishable to endogenous insulin when identified by immunocytochemical or radioimmuno assays. Therefore, the identification of insulin can falsely identify apoptotic cells as insulin producing cells [21]. Subsequent to this publication, mouse ES cells were differentiated into insulin producing cells in media containing no additional insulin demonstrating the capacity of ES cells to develop into insulin-producing, β -like cells [22]. These studies highlight the need for specific and irrefutable markers of the β cell phenotype.

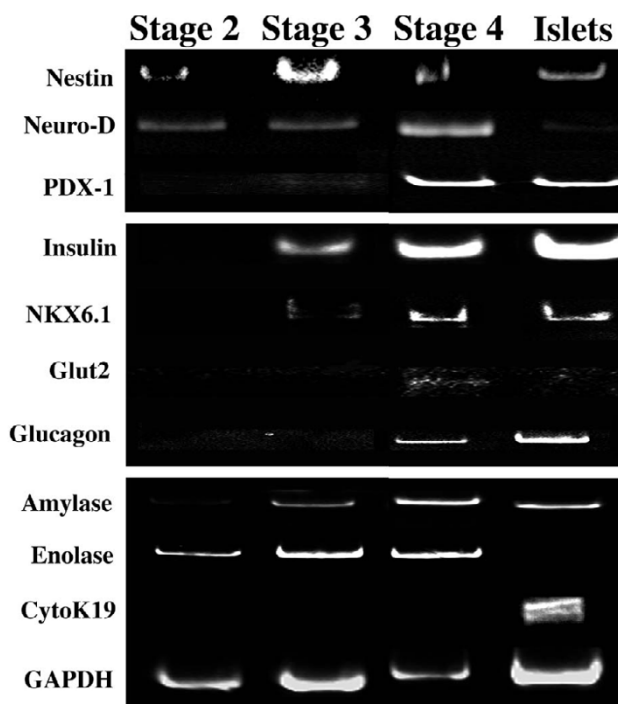


Figure 1
Pancreatic Gene Expression RT-PCR was performed on cells from each stage of differentiation, from 3 independent differentiation experiments. Genes from the pancreatic cell lineage were identified and compared to control samples from rhesus adult islets. Reverse transcriptase negative samples confirmed the absence of genomic DNA (data not shown).

Identifying beta like cells

Gene expression can be used to identify cell lineage and is not adversely affected by compounds in the media. In addition to insulin production and release, lineage restricted gene expression can also be used to identify developing β cells [23,24]. We utilized this information to identify β cell like phenotype development from differentiating rhesus ES cells and adult monkey islet cells. Total RNA was isolated from ES cells used in three separate differentiation experiments and RT-PCR performed using oligonucleotides based on the human gene sequence for genes of the pancreatic lineage. Genes associated with early β cell development such as NeuroD [25,26] and Nestin were identified in stage two cells while PDX-1 and insulin gene expression was detectable only after stage two, consistent with the presence of cells with a differentiated phenotype (Figure 1). Gene markers of other cell lineages including amylase and enolase were detected in stage four cells. This suggested that cells have undergone some degree of differentiation into β -like cells as indicated by the expression of insulin and PDX-1.

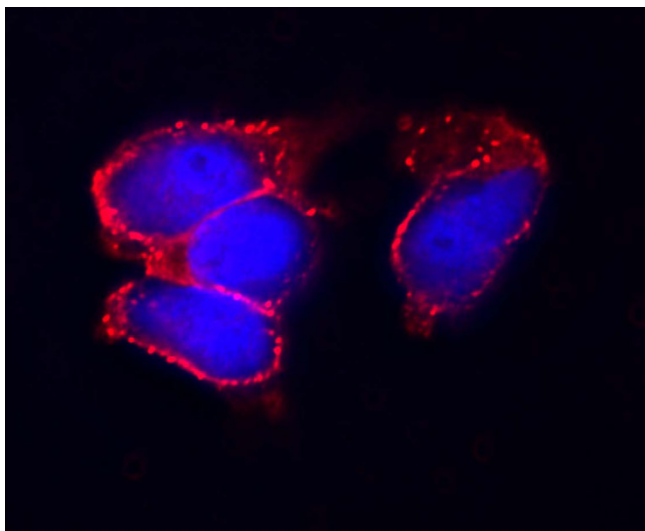


Figure 2
C-peptide Expression Rhesus ES cells were grown in media containing 1 nM Exendin-4 to promote differentiation to β like cells. The cells were fixed, permeabilized and probed with anti-C-peptide antibody (1/500 dilution) from Linco. C-peptide staining is visualized with Texas-Red secondary anti-body and the nuclei are stained with DAPI.

In addition to identifying β -cell lineage restricted genes, C-peptide, cleaved from proinsulin during insulin processing, can be used to identify an insulin producing, β -like cell. This methodology circumvents the problems associated with measuring mature insulin. Since C-peptide antibodies do not cross react with mature insulin or insulin in the growth media C-peptide is a direct measure of endogenous insulin production [27]. We were able to identify the production of insulin by rES cells grown in the presence of insulin using C-peptide immunocytochemistry (Figure 2). This data, along with the gene expression data, suggests that the rES cells are differentiating into insulin producing cells. At this time, however, further modifications to the culture conditions are needed to enhance cell differentiation and develop islet-like structures. Development of additional methods to identify the β cell phenotype that are sensitive and specific would greatly enhance these differentiation protocols.

Tagging ES cells for purification and identification post-transplantation

One approach to identifying the β cell phenotype is to tag insulin producing cells with a fluorescent marker. Marker genes such as the green fluorescent protein (GFP) can be used to purify cells populations by FACS and to track cells following transplantation [28]. We have generated a human insulin promoter (HIP)-GFP construct to be used

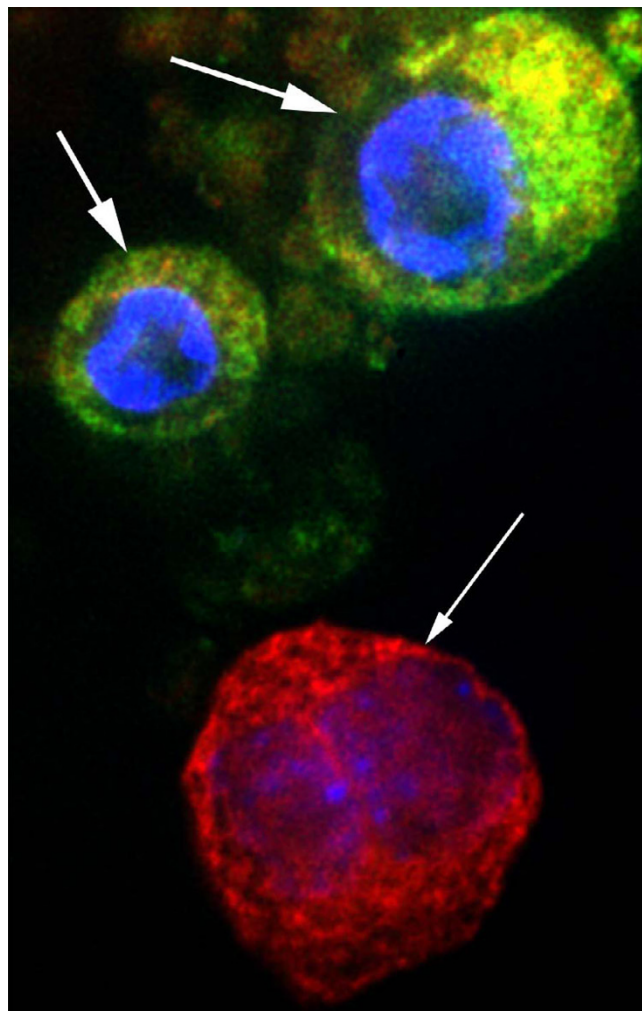


Figure 3
GFP and C-peptide expression in HIP:GFP transfected rES cells Rhesus ES cells were grown in differentiation media through stage 3 (bFGF). These cells, growing predominately as a monolayer on a glass coverslip, were transfected with the HIP:GFP construct using ExGen 500. Cells were allowed to differentiate in stage 3 media (bFGF) for 3 weeks, followed by 2 weeks in stage 4 media containing 1 nM Exendin 4. The cells were fixed in 3.4% formaldehyde and permeabilized with acetone. Cells were co-stained with DAPI and c-peptide. GFP expression was identified using a specific GFP filter and C-peptide detected by Tx-Red secondary antibody. In this field, two cells show both GFP and C-peptide expression (large arrows) and one cell expressing only C-peptide (small arrow).

specifically for these purposes. This construct will allow GFP expression to tag insulin producing cells and provide a means to purify insulin producing cells from the mixed cell population using fluorescent cell sorting (FACS).

We transfected rhesus ES derived cells at stage 3, prior to the addition of specific β cell growth factors. GFP expression was identified by fluorescent microscopy following 1 week of culture with the β cell growth factor, Exendin 4 [29-33]. Cells were co-stained for C-peptide to identify endogenous insulin production (Figure 3). We identified cells that co-expressed C-peptide and GFP supporting use of the HIP-GFP construct as a method to identify insulin producing ES derived cells. In addition to providing a means to identify and purify insulin producing cells, tagging the ES cells could allow their tracking post-transplantation.

Transplantation of ES cells into diabetic animal models

Development of animal transplantation models will be necessary to study the safety and efficacy of ES derived cell transplants. For DM therapies, ES derived β cells transplanted into the streptozotocin induced diabetic SCID mouse should provide elementary insights. In the past, use of rhesus monkeys and other non-human primate models provided critical information resulting in changes to the islet transplant protocol [34,35]. As with islet transplantation studies, the rhesus monkey has proved to be an invaluable model for other diabetes-related studies [36-38]. Therefore, an ideal primate model to study transplantation of ES cells is the chemically induced diabetic rhesus monkey. Tagging the ES derived cells prior to transplantation will provide an important tool in the investigation of ES cell transplantation.

Conclusion

The high cost and rising number of affected patients makes diabetes a major health crisis for the entire world. Current therapeutic options, with the exception of islet transplantation, have, at best, reduced the effects of diabetes. Stem cells (adult or embryonic) are currently the most promising candidates for islet cell replacement therapies. Recent advances differentiating embryonic stem cells into insulin producing cells have pointed out this potential, as well as, the pitfalls to the current approach. We have demonstrated insulin production from rhesus ES cells using C-peptide as a marker, thus avoiding the pitfall of insulin detection. In addition, we have transfected ES cells with a HIP-GFP construct to identify insulin producing, ES derived cells. This methodology will allow us to develop a pre-clinical model of cell transplantation in rhesus monkeys. Such a model is critical in the evaluation of the ES cell-based transplantation safety and efficacy.

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