

# A novel technique for the maintenance and propagation of adult human islets in long term cell culture

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## Significance

Pancreatic islet transplantation offers a potentially effective means of treating type I diabetes mellitus. The success of the Edmonton protocol in 2000, in which 7 consecutive type I diabetics were rendered insulin independent after intraportal injection of an average of 850,000 islets, represented a major breakthrough in islet transplantation and produced widespread public and scientific enthusiasm.(1) Despite this success, major obstacles remain before widespread application of islet cell transplantation is possible. Foremost, the replication and maintenance of differentiated human islets in vitro has proven extremely difficult, despite multiple approaches.(2-14)

Because of the difficulty in replicating and maintaining human islets in vitro, the current state of the art requires a single recipient to receive islets derived from multiple (generally 2 or 3) freshly processed cadaver pancreases. Given that only about 3500 suitable cadaver pancreases become available each year in the United States, islet cell transplantation remains inaccessible to the 35,000 newly-diagnosed type I diabetics.(9) The technology to replicate and maintain human islets in vitro would provide additional tissue mass for islet cell transplantation.

## Background

Long-term human adult islet culture has not been possible in the past because beta cells lose endocrine differentiation when placed in standard cell culture.(10) During this process, endocrine cells gradually transdifferentiate into ductal, acinar, and intermediary cells. These cell types can be characterized by multilabeling immunohistochemical and immunoelectron microscopic examination, insulin and other secretory hormone mRNA expression, and hormone secretion in response to secretagogues. With standard culture techniques, ductal cells develop within free-floating islets by 7 days.(10) Later, cultured cells express ductal and acinar cell markers with progressive loss of insulin expression and secretion over the next 8 weeks, such that by day 60, very few (if any) beta cells remain.(10) At this time, most cells appear undifferentiated and can only be maintained for 270 days before they become senescent.(11) This process of transdifferentiation occurs whether islets are cultured in clusters or allowed to form monolayers.(10)

Various attempts to expand and maintain differentiated beta cells in vitro using growth factors and extracellular matrix have generally not been successful.(2-15). Although short-term proliferation in response to mitogens can be achieved, generally loss of endocrine differentiation occurs rapidly thereafter.(6) Other approaches include co-localizing islets with pancreatic ductal epithelium or by adding secretory products of ductal cells.(12) Some have embedded islets in type I collagen gel or modified culture media with only limited success.(13-15) Even with tailored culture strategies, the longest published time that cultured adult islets may be maintained and successfully transplanted into a rodent model to achieve normoglycemia is 4 months.(16) At 4 months, only 3.6% of cells expressed insulin at time of transplant.(16)

## Preliminary Observations

A novel technique has been developed for long term maintenance and propagation of differentiated adult human islet cells in cell culture.(17) Unlike previous attempts, the islets maintain their differentiated state and secrete insulin in a dose-dependent manner in response to glucose even after 12 months in culture.

The technique involves obtaining whole islet extracts from cadaveric pancreases through commonly used collagenase digestion methods.(18) The islets then placed in a complex media specialized for beta cell growth, containing inorganic salts, amino acids, vitamins, bovine serum albumin, and various other components. Key components include zinc sulfate, myo-inositol, niacinamide, L-taurine, L-glutamine, free acid (reduced) glutathione, pyruvic acid, EDTA, L(+) lactic acid, and (+) a-tocopherol. The cells are incubated at 37 degrees Celsius, pH 7.40, 5% carbon dioxide, and 5-10% oxygen tension. After equilibration, the capsule surrounding the whole islet is then gently ruptured chemically or mechanically by microsurgery using sterile glass microneedles, which cause a tiny breach in the capsule surrounding the islet. This approximates the series of events that occur during pancreas development, where destabilization of a small area of the constricting basement lamina elicits subsequent islet cell proliferation and growth of the islet (19-20) The breach provides an opening in the basement lamina so that a few islet cells are gently extruded in an attached tiny nodule. This tiny nodule will remain attached to the large whole islet during the subsequent days or even weeks of culture. Cell proliferation occurs within and at the periphery of the tiny nodule so that it increases in size. Eventually the larger nodule will break off forming a “daughter-islet” containing one or more cells. Another series of islet cells will extrude through the hole in the basement lamina of the large whole islet to form another tiny nodule and the series of events will repeat. The independent daughter-islet will remain suspended in the culture medium or very loosely attached to any fibroblasts which might be tightly adhered to the bottom of the culture dish. These small daughter-islets may be transferred to new culture dishes giving sub-cultures of islet cells. The doubling time for these islet cells is about 10-14 days, resulting in a 20-fold increase in cell number by four months of culture. In general the media is changed about every 2-3 days and the cells are passed every 10-14 days. Functionally, the cells undergo a 90 to 120 day recovery period when glucose responsiveness is reduced, after which normal function resumes. Cells derived from daughter-islets in specialized media are currently continuing to divide and secrete insulin in response to glucose stimulation for over 17 months in culture.(17)

Preliminary characterization of cultured islets has been performed. Islets were maintained in continuous culture through 55 generations, at which time they had been in culture for 12 months. Glucose responsiveness was then assessed. The glucose concentration in the experimental medium alone was 1.1 mM. To this medium was added glucose in the concentrations shown in the table below, and the cells were incubated for various times. Samples were collected and assayed for insulin content by RIA.(17)

mM glucose added	mu IU insulin secreted / 1.5 million cells / mL (at various time points).					
	1 hour	2 hours	3 hours	5 hours	7 hours	24 hours
0mM	29	42	82	99	210	101
1mM	275	363	451	564	582	812
5.6 mM	3413	982	999	2383	2718	2760
11.0 mM	3376	1768	2220	1184	3292	1276
16.5 mM	3716	2415	2450	2589	4039	2099
22 mM	2538	1845	2966	1722	1329	989
33 mM	972	345	446	657	1109	1073

This in vitro response is comparable to that from freshly isolated adult human islets.(18) When further characterized by immunohistochemical staining, greater than 60% of cultured islets contain immunoreactive insulin and GABA, indicating the differentiated state of the cells.(17) Finally, cultured islets have been successfully transplanted into a small series of diabetic nude

mice resulting viable engraftment, human insulin secretion, and improved glycemic control.

## **Methods and Experimental Design**

Our objectives are:

1. To characterize the cultured islet phenotype by expression of endocrine, ductal, acinar, and intermediary cell markers as well as insulin gene expression.
2. To further define glucose responsiveness of cultured cells, which particular attention to how glucose responsiveness changes over time during long term culture.
3. To show that normoglycemia can be achieved when cultured cells, with and without cryopreservation, are transplanted into a diabetic nude mouse model.

### **Objective 1: To characterize the cells' phenotype by expression of endocrine, ductal, acinar, and intermediary cell markers; insulin gene expression; proliferation; and genomic stability.**

Adult human whole islets will be obtained from the UCSF human islet isolation facility using standardized described liberase digestion and Ficoll gradient centrifuge purification techniques.(18) Using the capsular breach technique described above, whole islets will be cultured in specialized media with media change every 2-3 days and cell passage every 10-14 days. Rates of growth and viability will be assessed during cell passages with ethidium bromide/acridine orange staining and counting. Proliferation (DNA synthesis) will be assessed by nuclear incorporation of methyl H3 thymidine or bromodeoxyuridine coupled with insulin staining by indirect immunofluorescence. Karyotyping and STR analysis will be performed to assess genomic stability..

Cultured adult human islets maintained for at least 6 months will be fixed in 10% buffered formalin, embedded in paraffin, and processed for immunohistochemistry using the avidin-biotin method.(21) Expression of various ductal, acinar, and endocrine cell markers including insulin (endocrine), glucagon (endocrine), somatostatin (endocrine), pancreatic polypeptide (endocrine), CAII (ductal),  $\alpha$ -amylase (acinar), CK 7 (ductal), CK 19 (ductal), NSE (endocrine), Ki-67 (replicating cell), and CA19-9 (ductal) will be assessed to characterize the phenotype of cells maintained in long term culture. A portion of the cells will be reserved for a preproinsulin and PDX1 (a replication transcription factor) mRNA expression assay. The messenger RNA within will be converted to cDNA, then amplified using primers designed from the published preproinsulin and PDX1 sequence, with human actin performed in parallel to serve as an internal control.

### **Objective 2: To further define glucose responsiveness of cultured cells.**

Cultured adult human islets maintained for 3, 6, and 12 months will be centrifuged and then resuspended in a base medium consisting of 3 parts support media and 1 part DMSS. The cells will be incubated for 1.5 or 3 hours in D(+)glucose at concentrations ranging from 1 to 33 mM. ELISA analysis will be performed to show insulin response at 1,2,3,5, and 24 hours. A portion of cells will first be cryopreserved and then thawed before insulin responsiveness is assessed.

Also at 3,6, and 12 months, a sample of the cell culture will assayed for (1) gene expression through preproinsulin and PDX1 mRNA expression by reverse transcriptase PCR, (2) DNA synthesis by nuclear incorporation of methyl (3) H thymidine or bromodeoxyuridine, and (3) insulin staining by indirect immunofluorescence.

**Objective 3: To show that normoglycemia can be achieved when cultured cells, with and without cryopreservation, are transplanted into a diabetic nude mouse model.**

Male nude mice (20-25g) will be purchased as recipients of adult human islets maintained in cell culture for at least 6 months. Mice will be maintained at the UCSF Laboratory Animal Research Center in accordance with accepted ethical guidelines for care and under approval from the Committee on Animal Research. Diabetes will be induced by single dose injection of streptozotocin (250mg/kg i.p) and animals with blood glucose levels >300mg/dL for 1 week selected. The diabetic mice will then be randomized into treatment (transplant) and sham control groups. A portion of the treatment mice will receive islets which have first been cryopreserved. Under anesthesia, flank incision will be performed and the kidney exposed. Approximately 2000 islet equivalents in a volume of ~50 uL will be transplanted using a Hamilton syringe under the kidney capsule of the diabetic nude mice. Control mice will receive saline injection.

Serum glucose will be measured daily for 60 days. At 30 days, a glucose tolerance test will be performed and serial measurements of glucose, insulin, and c-peptide obtained. At 60 days, the animals will undergo nephrectomy for immunoperoxidase staining and assessment for a return-to-diabetes state. Finally, at 67 days mice will be euthanized and histological evaluation of the pancreas performed.

**Summary:**

From the above series of experiments, our objective is to show that adult human islets may be maintained in cell culture for at least 12 months and can be induced to replicate without undergoing neoplastic transformation. Such cells may continue to express phenotypic markers of differentiated beta cells without undergoing transdifferentiation. Cultured islets may also secrete insulin in response to glucose and amino acids in a physiologic manner and may be transplanted into a diabetic mouse model to achieve normoglycemia. Ultimately, these cells may be useful as a source of tissue for human islet transplantation trials.

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