Photosynthetic Algae/Insulinoma Cell Fusion Creating Self-Sustaining Insulin Producer

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Introduction

Insulin is a hormone that regulates blood sugar concentration. In the approximately 300 million diabetic patients worldwide, utilization and production of insulin is substantially, and in the case of Diabetes mellitus type I patients, completely, impaired [4]. These patients require the direct pumping of exogenous insulin into the blood. There are currently two methods of exogenous insulin production: extraction from other mammals, such as cattle, and genetic transfection of bacteria with the human genes necessary for insulin production. The former has become increasingly uncommon due to patients' allergic reactions to the insulin produced by genetically dissimilar animals. The second method is inefficient in that it requires the use of complex protocols in order to generate sufficient quantities of insulin [5].

The purpose of this study was to create a new method for the efficient *in vitro* production of exogenous insulin. The basis of this method is the creation of a low maintenance, plant-animal cell hybrid that produces insulin while remaining self-sustaining via photosynthesis. The unicellular, photosynthetic green-algae, *Chlorella kessleri*, was fused with rat insulinoma RIN-5F cells, or with primary cultured rat pancreatic islet cells to create cell hybrids, referred to as Modified Insulin Production (MIP) cells.

It was hypothesized that successful fusion of algae and insulinoma cells would lead to an efficient, inexpensive approach to *in vitro* insulin production via plant-animal cell hybrids containing the biochemical properties of each cell type. Prior to fusion, the rigid cell walls of the algae cells were removed by the cell wall-degrading enzymes cellulase and pectinase, creating algal protoplasts. Algae cells were imaged by transmission electron microscopy prior to fusion, and protoplast formation was observed via light microscopy. Protoplast isolation is essential, as the rigid cell walls prevent membrane interaction among algae cells with those in surrounding media. Fusion was then stimulated with polyethylene glycol (PEG, MW 4000). Ultra-Sensitive Rat Insulin ELISA was performed on the pre-fused insulinoma cells and on the MIP cell hybrids to quantify insulin secretion. Successful fusion was observed via both scanning electron microscopy and light microscopy. The algae/insulinoma and algae/rat pancreatic islet cell fusion process represents the potential for the inexpensive production of life-saving bio-products by means of cell hybrids.

Methods

Cell Culture

Rat pancreatic insulinoma cells (RIN-5F) were cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum, and incubated at 37°C and 5% CO_2. Cells were sub-cultured twice each week.

Primary rat pancreatic islet cells were cultured directly from extracted rat pancreas. Sterile scalpel was used to loosen the tissue, after which cells were cultured identically to RIN-5F cells.

Photosynthetic green-algae, *Chlorella kessleri* , was cultured in ATCC Medium 5 (without agar), incubated in an environmental chamber with 14 hour light/10 hour dark cycle. Algae cells were sub-cultured every other week.

Chemical and Experimental Design

Polyethylene glycol (MW 4000) was diluted to 0.125 mM in deionized water. Cellulase and pectinase were diluted to 4% and 0.4% solutions by volume, respectively, in deionized water.

Algae/Insulinoma, Algae/Islet cell Fusion

Fusion was stimulated by combining approximately 5 mL of *C. kessleri* protoplasts in ATCC Medium 5, with cultured RIN-5F or rat islet cells in DMEM/F-12 media. 1 mL of 0.125 mM PEG solution was added to the cell mixture. The flask was incubated overnight prior to light microscopy analysis.

Microscopy

Transmission electron microscopy was used to obtain high resolution cross-section images of algae cells. Cells were prepared by pelleting at 300 x g for 5 minutes, fixation in 5% glutaraldehyde/4% formaldehyde, post-fixation in 2% osmium tetroxide, dehydration in a graded series of acetone, embedding and curing in PolyBed 812 epoxy resin. 100 nm sections were cut and placed on 200 mesh copper TEM grids. Grids were stained with 2% uranyl acetate followed by 0.5% lead citrate and imaged with a 200 kV JEOL JEM-2100.

Light microscopy was used for qualitative analysis of RIN-5F cells, *C. kessleri* cells, and MIP cell hybrids. Cells were imaged with a *Nikon Eclipse TS100* light microscope.

ELISA

Rat insulin in media from both RIN-5F cells and MIP cell fusions was measured by Ultra Sensitive Rat Insulin Enzyme-Linked Immunosorbant Assay. Absorbances were measured at 450 nm and 620 nm and were compared to standard rat insulin (0 ng/mL, 1 ng/mL, 2 ng/mL, 4 ng/mL, 8 ng/mL, 16 ng/mL, 32 ng/mL, and 64 ng/mL). ELISA was performed on RIN-5F cells before fusion to establish a control, and on MIP cell hybrids after fusion was confirmed via light microscopy.

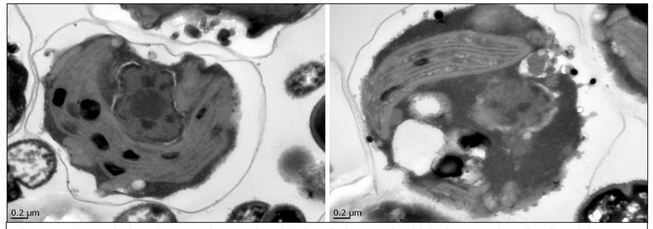

Figure 1: Transmission electron micrographs of *C. kessleri* . Note the thick glycoprotein cell wall and the stacked thylakoid membranes.

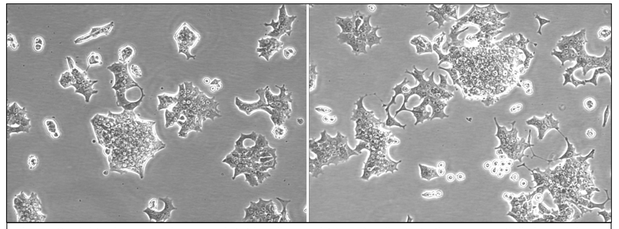

Figure 2: Light microscopy images of Rat insulinoma (RIN-5F) cells. Note the exponentially larger size of insulinoma cells in comparison to the size of the unicellular algae (Figure 3).

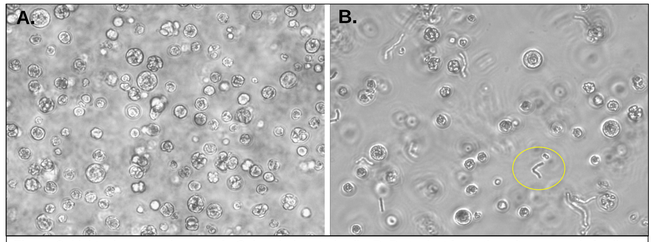

Figure 3: Light microscopy images. **A**: *C. kessleri* control cells. **B**: Protoplast formation after removal of cell walls. Note separated cell walls surrounding *C. kessleri* protoplasts.

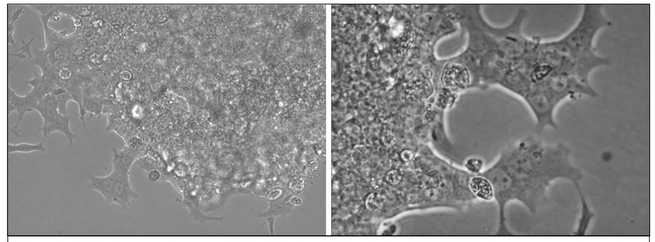

Figure 4: Light microscopy images of MIP cell fusions (*C. kessleri* + RIN-5F). Note the numerous algae cells fused with the much larger insulinoma cells.

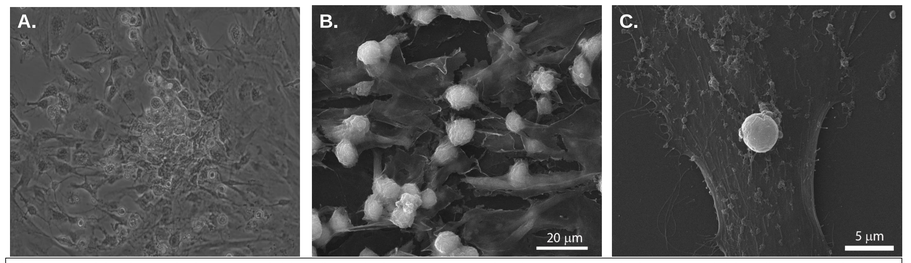

Figure 5: A: Light micrograph of rat pancreatic islet cells acquired at 200X. **B:** Scanning electron micrograph of rat pancreatic islet cells . **C:** Scanning electron micrograph of *C. keslerri* protoplast (spherical cell) fusion with islet cells isolated from rat pancreas (background cell).

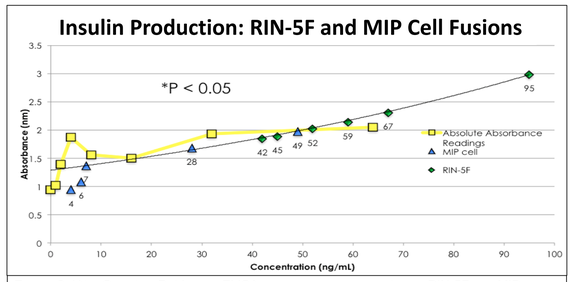

Figure 6: Ultra Sensitive Rat Insulin ELISA showing insulin production in RIN-5F and MIP cell fusions. Data based on rat insulin standards. *Denotes p<0.05 for Two-tailed T-test between each cell type and standard values.

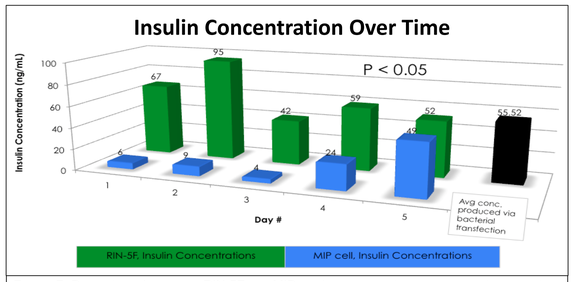

Figure 7: Production of insulin in RIN-5F and MIP cell fusions over time, compared to production of insulin via current bacterial transfection techniques. *Denotes p<0.05 for Two-tailed T-test between daily insulin concentrations for each cell type.

Results & Discussion

The inter-kingdom fusion process for exogenous insulin production was hypothesized to work effectively only if algae cell walls were successfully degenerated; and if PEG acted as a strong fusion agent, inducing RIN-5F cells to engulf *C. kessleri* protoplasts. The fusion process was used to show that the combination of two significantly different cell types can yield a resultant cell fusion that retains the biochemical characteristics of both cell types. The immortal, insulin-producing cell line, RIN-5F, derived from rat pancreatic tumor, was used as the foundation of the MIP cell fusions, as it is one of few known cell lines to readily produce insulin *in vitro*. The unicellular green-algae species, *C. kessleri*, was selected as the fusion partner due to its photosynthetic nature and low maintenance requirements for culture. Thus, fusion of these cell types was performed in an attempt to create a hybrid cell that produces useable quantities of exogenous insulin and remains self-sufficient and viable via photosynthesis.

The rigid, glycoprotein cell wall surrounding *C. kessleri* prevented fusion as it inhibited the flexibility of the algae cells to be fused inside the membrane of the RIN-5F cells (Figure 1). Cellulase and pectinase were used to remove the *C. kessleri* cell walls, forming protoplasts, approximately 72 hours before fusion. Light microscopy showed successful protoplast formation (Figure 3B), at which point centrifugation at 300 x g and removal of supernatant isolated the *C. keslerri* protoplasts.

Fusion between RIN-5F cells and algal protoplasts was then induced. 24 hours after PEG was introduced to the algae/insulinoma flask, light microscopy showed membrane interaction between *C. kessleri* and RIN-5F cells (Figure 4). Media samples were taken from the MIP cell fusions and from the RIN-5F control flask each day for one week following fusion, and samples were analyzed by ELISA.

It was determined that the MIP hybrids produced insulin in quantities consistent with insulin produced by RIN-5F cells alone (Figure 6). Furthermore, the amount of insulin produced by the MIP fusions was comparable to that produced by a similarly sized sample of bacteria by current bacterial transfection methods of exogenous insulin production according to the NIH (Figure 7). It is important to note that, without any additional culturing or exchange of media, the inter-kingdom fusions continued to produce consistent quantities of insulin, approximately 45 ng/mL, for 14 days after fusion was induced. This suggests that during this time, the cells remained viable and that the photosynthetic products of the algae in the MIP fusion was sustaining the hybrid cells.

The initial RIN-5F/*C. keslerri* fusion process was expanded by hybridization between the same algae species and primary islet cells cultured directly from an extracted rat pancreas. Fusion in this case was determined via scanning electron microscopy, and suggested a more superficial interaction between the membranes of the algae protoplast and the pancreatic cells (Figure 5B). ELISA analysis to test for insulin secretion in media by this cell hybrid is yet to be performed.

The inter-kingdom cell fusion process represents the potential for the efficient, inexpensive production of essential cellular bio-products. In this study, insulin was the bio-product of interest due to the properties of the components of the hybrid cells. However, altering the cell types used in the plant-animal cell fusion could lead to the production of numerous other hormones and biochemical cofactors for clinical use. The plant-animal cell fusion process also gives insight into inter-kingdom, multi-nuclei cell hybrid formation, an emerging technique that has the potential to yield remarkable results in mass production of useable bio-products.

References

[1] Y. Lee, et al., *J. General Microbiology* (1987) 635-641.
[2] L. Yang, et al., *Cellulose* (2008) 789-796.
[3] G. Yu, et al., *Internet Archives - Patent Application* (2008).
[4] A. Thayer, *Chemistry and Engineering News* (2011).
[5] A. Riggs, *Diabetes Care,* Vol. 4, No.1, (1981).

Acknowledgements

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