LAMININS AS A POTENTIAL ENHANCER OF BETA CELLS: PROLIFERATION AND SUBSEQUENT GENE EXPRESSION FOR THERAPEUTIC TREATMENT OF DIABETES MELLITUS

by

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in association with SymbioCellTech, LLC

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ABSTRACT

Islet of Langerhans transplant is a proven treatment for autoimmune Type 1 Diabetes mellitus (T1DM), as has been demonstrated both pre-clinically in mice, rats and dogs, and clinically in humans. When administered as a treatment those affected with T1DM, these islets are potent enough to eliminate the patient's need for insulin for up to five years, albeit with the need for immunosuppression. One challenge faced by investigators, however, is the lack of abundance of islets for treatment due to donor scarcity. Islets must be harvested from the pancreata of deceased organ donors, and up to five donors are needed to cure a single patient. Thus, many researchers are currently focused on effectively proliferating the insulin-producing islet beta cells. Beta cells are difficult to culture and tend to proliferate slowly, even in very rich growth mediums. Passaging multiple times to exponentially expand these cells is one solution to the problem of low cell counts, but another issue arises: the potency of the beta cells decreases as the passage number increases.

Islet beta cells are understood to have reduced gene expression after each successive passage (i.e. using small volumes of one culture to seed multiple new cultures, thus increasing overall cell yield exponentially). This reduced gene expression is particularly observed in genes relating to T1DM, namely the genes for insulin, glucagon, and others. In past experiments, it has been determined that islet cells tend to lose both growth and treatment potency around the third or fourth passage—or in other words, around the fourth or fifth successive culture grown from harvested cells.

In this study, both technical challenges—low cell count yields and loss of potency in later passages—were addressed by studying the effect of human recombinant laminin-

511 on the proliferation and gene expression of dog islet beta cell cultures. SymbioCellTech, located in the University of Utah Research Park, has developed a therapeutic that, after a single treatment, has been shown in pre-clinical testing in both immune-competent, non-obese diabetic (NOD) and non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice to be a lifelong functional cure for diabetes. This treatment was developed using wild-type Islet of Langerhans cells harvested from both mouse and domestic dog pancreata. The study finds that there is no statistically significant difference in the growth rate, gene expression, or potency between laminin-enhanced and unenhanced dog islet cell cultures expanded from the same animal; human recombinant laminin-511 does not bear significant benefits or enhancing effects on the growth of SCT dog islet beta cell cultures under standard culture conditions.

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INTRODUCTION

In our modern world, diabetes is steadily reaching epidemic proportions: the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014, with the global prevalence among adults rising from 4.7% to 8.5% in the same period (World Health Organization, 2016). In 2012, an estimated 3.7 million deaths were directly caused by diabetes or attributed to hyperglycemia (elevated blood glucose levels); furthermore, it is projected that diabetes will become the 7th leading cause of death by 2030 (Mathers & Loncar, 2006). If left poorly or uncontrolled over time, diabetes leads to serious damage to numerous body systems—especially the nerves and blood vessels (World Health Organization, 1999)—and can cause blindness, heart attacks, stroke, kidney failure, and lower limb amputation (World Health Organization, 2016).

Diabetes is a chronic disease that comes in two forms: either the pancreas does not produce enough insulin for the body's needs (Type 1 Diabetes Mellitus, T1DM) or the body cannot effectively utilize the insulin that is produced by the pancreas (Type 2 Diabetes Mellitus, T2DM) (World Health Organization, 2016). The root cause of diabetes, therefore, can be traced back to insulin, which primarily functions to stimulate the uptake of glucose from the blood and into somatic cells—primarily myocytes (muscle cells) and adipocytes (fat cells) (Nguyen, Maalouf, Sakhaee, & Moe, 2011). As a signal molecule for energy storage and satiety after eating glucose-rich foods, insulin is the key that opens glucose channels which in turn allow glucose molecules to enter the cell and be used for various energy storage and metabolic processes (illustrated in Fig. 1).

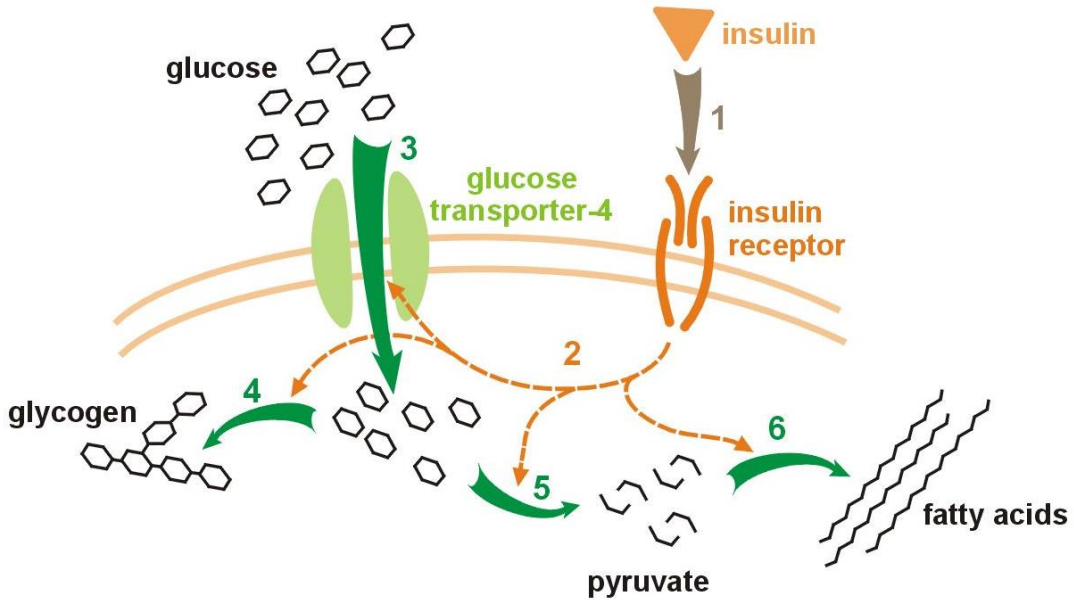

Figure 1. Insulin directly affects glucose uptake and metabolism.

Insulin binds to its receptor (1) which in turn starts many protein activation cascades (2). These include: translocation of Glut-4 transporter to the plasma membrane and influx of glucose (3), glycogen synthesis (4), glycolysis (5) and fatty acid synthesis (6).

The particular focus of this study is T1DM, previously known as insulin-dependent, juvenile, or childhood-onset diabetes. T1DM results from deficient insulin production caused by autoimmune attacks on insulin-producing pancreatic islet beta cells (Murphy & Weaver, 2017). Although the specific mechanism of this autoimmunity is not clear nor preventable with current knowledge (World Health Organization, 2016), past studies on the NOD (non-obese diabetic) mouse model of T1DM have demonstrated that pathogenic CD8+ T-cells target and destroy islet beta cells, confirming insulin as one of the principal autoantigens in T1DM NOD mice (Murphy & Weaver, 2017). The destruction of pancreatic islet beta cells, for which there is no proven method of prevention (Chiang, Kirkman, Laffel, & Peters, 2014), leads to nonproduction of insulin and the onset of T1DM. Despite insulin therapies to reduce the burden of diabetes, many patients still develop complications that compromise multiple organs and ultimately result in early death.

The complications of existing T1DM treatments are numerous: despite rigorous insulin therapy, diabetes can result in failure of the renal, nervous, or cardiovascular systems, as well as lower limb amputations, blindness, stroke, heart attack, and even early death. Other previously studied therapies, such as pancreas and islet cell transplants, turn out to be impractical because up to five suitable deceased donors are required to treat a single patient (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017). Furthermore, patients receiving these transplants find themselves bound to a lifetime of immunosuppressant drugs which elevate the risk of serious infections, cancers, and kidney disease (SymbioCellTech, 2017). The two largest technical obstacles to eliminating the need for exogenous insulin therapy in T1DM are thus defined: the donor

scarcity challenge, wherein several organ donors are needed to supply the necessary number of islet beta cells for just one patient; and the immune attack challenge, in which the treatment must prevent further immune system attack by the very same CD8+ T-cells that destroyed the original beta cells of the pancreas (SymbioCellTech, 2017).

The mission at SymbioCellTech (SCT) is to develop an effective treatment for T1DM. Located in the University of Utah Research Park, SCT has developed a therapeutic that, after a single treatment, has been shown in pre-clinical testing to durably eliminate the need for exogenous insulin without immunosuppressive agents in spontaneous or chemically induced T1DM (SymbioCellTech, 2017). In a great triumph over the existing treatment challenges, the proprietary therapeutic developed at SCT functionally cures T1DM without immunosuppressants and without the need for a large number of tissue donors. SCT laboratory studies demonstrate that tissue culture expanded bone marrow-derived or adipose-derived mesenchymal stem cells (MSCs), when grown together with culture expanded islet cells, create aggregates of allogeneic islet cells and MSCs, which SCT terms “Neo-Islets” (NIs). These NIs combine the anti-apoptotic and anti-inflammatory properties of MSCs with the insulin-producing properties of islet beta cells (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017), thereby directly addressing both the immune attack and donor scarcity challenge when administered as a therapeutic for curing T1DM. Not only does this simple out-patient procedure show great potential to permanently cure T1DM in humans, but it is slated for adaptation by SCT for T2DM in the near future (SymbioCellTech, 2017). Based on laboratory tests performed both in parallel and as a result of my research at SCT in 2015 and 2016, we have received FDA approval to conduct pilot studies in insulin-dependent, spontaneously diabetic pet dogs.

The crucial element in the successful development of the SCT therapeutic has been to produce islet cells *in vitro* resulting in 1) a large quantity of cells, and 2) cells with high potency, i.e. the expression of relevant genes for curing T1DM (namely insulin, glucagon, and others). The development of Neo-Islets means that culturing a large number of cells *in vitro* allows just one tissue donor to potentially treat a large number of T1DM patients, which greatly increases the number of patients that can be treated even from a scarce pool of donors. Ensuring that our islet beta cells are potent enough in their insulin-producing capabilities allows the efficient usage and effective treatment of T1DM patients through the NI therapeutic. Thus, the contributions made by this study on islet beta cells have helped SCT reach this breakthrough.

Islet beta cells are known to be difficult to culture and retain *in vitro*; the mass of natural insulin-producing beta cells in the pancreas increases to meet the functional demand and maintain euglycemia (healthy blood glucose levels) and only occurs via two pathways: 1) the expansion by replication of pre-existing beta cells, and 2) neogenesis by proliferation and subsequent differentiation of pancreatic ductal epithelium (Bonner-Weir & Smith, 1994). Numerous growth factors are involved in these pathways (shown in Fig. 2 and Fig. 3), and special culture conditions are necessary to encourage the proliferation and retention of beta cells *in vitro*.

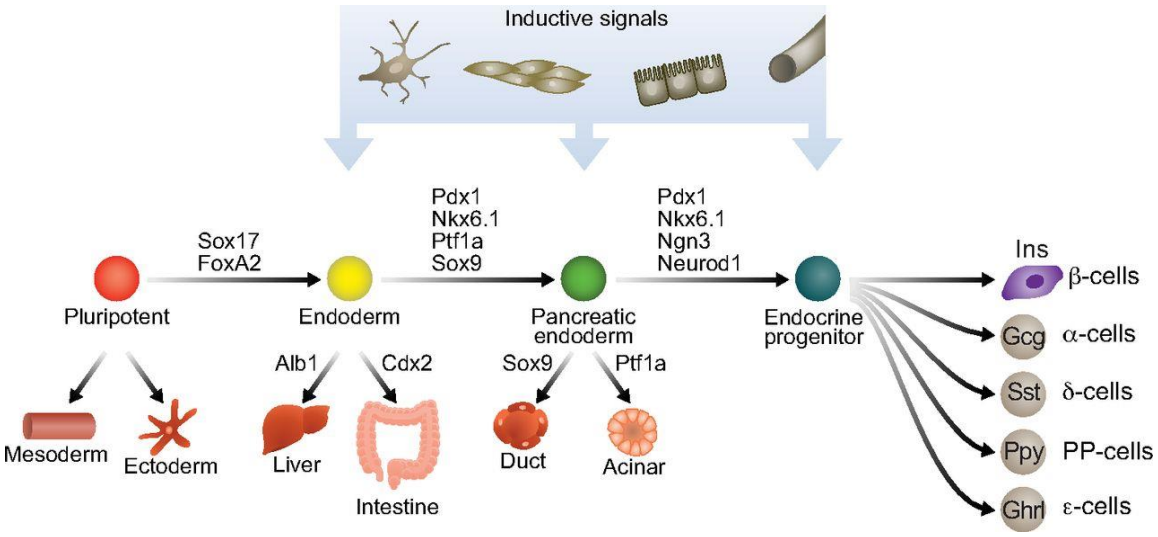

Figure 2. Development pathway of a pancreatic endocrine progenitor.

A pluripotent stem cell receives many signals and undergoes several stages before becoming a pancreatic endocrine progenitor. This progenitor can then differentiate into numerous pancreatic cells. Of interest is the pathway resulting in insulin-producing beta cells.

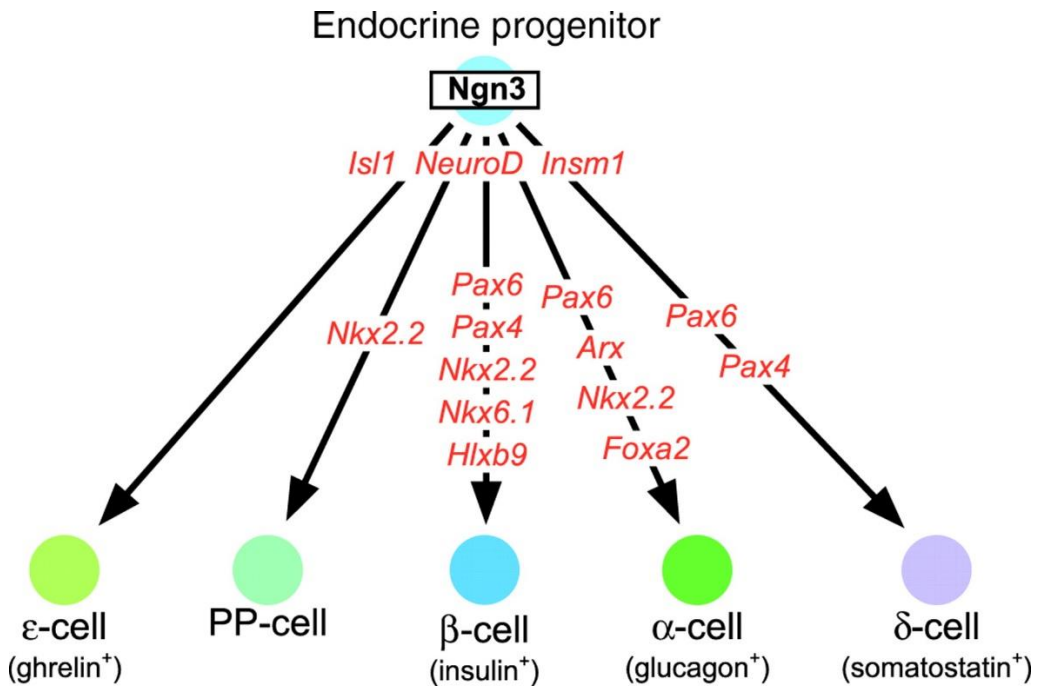

Figure 3. Differentiation possibilities of a pancreatic endocrine progenitor.

The endocrine progenitor can differentiate into a variety of pancreatic cell types depending on which signals are received. The signals resulting in the development of an insulin-producing beta cell (shown in light blue) include Pax6, Pax4, Nkx2.2, Nkx6.1, and Hlxb9.

Mindful of the difficulty in culturing islet cells *in vitro*, it was necessary to explore possible ways to improve the culturing process of our islet beta cells. A number of fairly recent studies have demonstrated the enhancing effects of laminins on the proliferation rates and potency of cell types that are usually difficult to culture: it was first discovered in the early 1980s that human laminins can be isolated in a reactive form and effectively used in laboratory conditions (Wewer, et al., 1983). Laminins (one form of which is illustrated in Fig. 4) are a naturally-occurring group of heterotrimeric proteins found in the extracellular matrix, and play a major role in forming the basal lamina protein network in the basement membranes of most cells in the body (shown in Fig. 5). They are biologically active, influencing the adhesion, migration, and cell differentiation processes of their surrounding cells (Timpl, et al., 1979).

In 2008, Japanese researchers further demonstrated that undifferentiated human embryonic stem cells successfully proliferated on recombinant human laminin isoforms (specifically rhLM-111, -332, and -511) for several passages while preserving their pluripotency (Miyazaki, et al., 2008). Further exploration of r-laminin-511 found that it adequately enhances growth for mouse embryonic stem cells to self-renew for 31 passages, providing a stable basis on which undifferentiated mammalian embryonic stem cells can grow *in vitro* (Domogatskaya, Rodin, Boutaud, & Tryggvason, 2008).

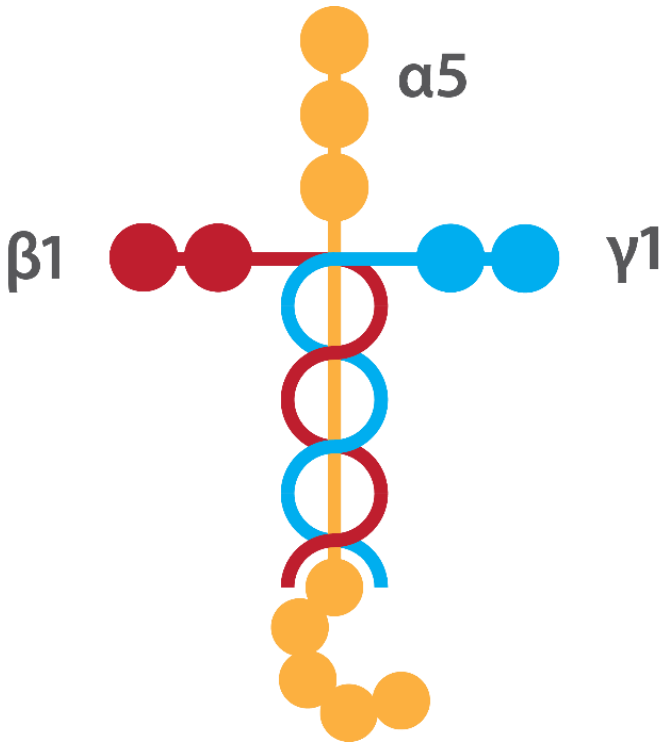

Figure 4. Human recombinant laminin-511

The diagram above shows r-laminin-511, which consists of alpha-5, beta-1, and gamma-1 domains. This form of laminin has been found to enhance growth in mouse embryonic stem cells and thus was hypothesized as an enhancer of islet beta cells in this study.

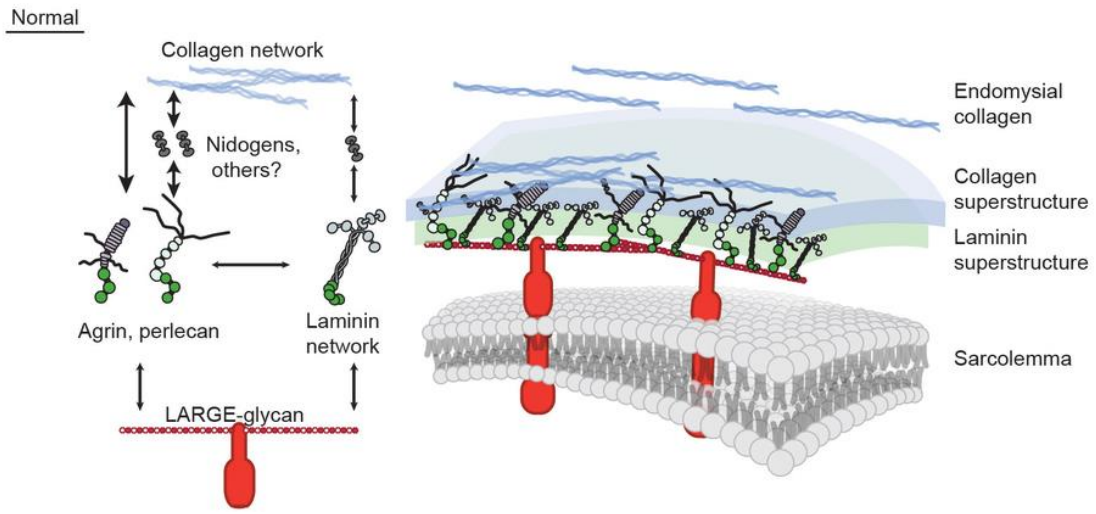

Figure 5. Laminin superstructure assists in the formation of basement membranes.

In skeletal muscle, laminin is one of several ligands (alongside agrin and perlecan) that bind to the LARGE-glycan on α-DG. Perlecan and agrin function to directly or indirectly (via accessory proteins such as nidogen) to laminin and collagen networks, forming strong linkages which likely enables compaction of the basement membrane which contributes cell health.

The research described in this study follows from the hypothesis that 1) growing beta islet cell cultures in culture flasks enhanced with a laminin coating will improve the proliferation rates of the cells for multiple passages, leading to greater abundance of cells cultured, and 2) laminin-enhanced cultures will have improved gene expression in later passages of islet cells, leading to greater potency of later passages for T1DM therapy despite having lower gene expressions than previous passages for relevant genes.

In aggregate, the present data demonstrate that there is no statistically significant difference in the growth rate, gene expression, or potency between cultures expanded from the same dog. Disproving our hypothesis about laminins, we conclude that r-laminin-511 does not bear significant benefits or enhancing effects on the growth of SCT dog islet beta cell cultures under our growth conditions.

MATERIALS AND METHODS

Reagents

Cell culture reagents: BioLamina LN511 Human Recombinant Laminin-511, Lonza DMEM:F12 Mixture, HyClone Fetal Bovine Serum (US Origin), Gibco Antibiotic-Antimycotic, Gibco Penicillin-Streptomycin-Glutamine (GPS), Gibco Trypsin EDTA, and BioLifeSolutions Cryostor CS10 Freeze Media.

RT-PCR primers and reagents: QIAGEN RNeasy Mini Kit (Buffer RLT, RW1, RPE, RNase-free water), Applied Biosystems TaqMan Universal Master Mix II with UNG, Invitrogen SuperScript II RNase H- Reverse Transcriptase, Invitrogen 5X First Strand Buffer, Invitrogen Random Primer, Thermo Scientific DTT (dithiothreitol), Thermo Scientific dNTP Mix (10 mM), and Canine gene primers (16): ActB, Pdx1, B2M, NKX6-1, INS, PAX4, GCG, PAX6, SST, VEGFα, GLP1R, CXCL12, abcc8, TGFB1, KCNJ11, and IGF1.

Overview of Methods

Islet beta cells from two unique dogs (Dog 18 and Dog 20 in the SCT experimental group) that demonstrated the greatest proliferation rate in previous SCT experiments were selected for the treatment. These two lines of islets were cultured in parallel in identical culture flasks, half with a r-laminin-511 (human recombinant laminin-511) coating that was manually applied to the flasks for 24 hours prior to seeding. After allowing all P0 cultures ample time to achieve the necessary confluence for passaging, which was approximately one week with one medium exchange on the fourth day of growth, each culture was passaged into a new flask at P1. These cultures were passaged continuously until the cells took an extended amount of time to reach ideal

confluence, or failed to reach confluence at all. This usually occurred around P3, when the islet cells did not reach desired confluence after up to ten days. A sample of cells from each culture and passage was stored for a Real-Time Polymerase Chain Reaction (RT-PCR) analysis throughout the culturing and passaging process. Cell counts were also documented at each passage for each culture. RT-PCR experiments were subsequently prepared and analyzed for each islet cell culture, passage number, and gene primer combination, with sixteen dog gene primers used in total for the gene expression analysis. This culture protocol allowed us to directly test both parts of our hypothesis with a parallel comparison between the control (no laminin) and treatment (r-laminin) groups for two distinct cell lines: the first part is tested based on cell counts and culture expansion rates, and the second part is tested based on gene expression results from the RT-PCR analysis.

Islet Isolation from Dogs

(protocol adapted from SCT SOPs (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017))

Fresh pancreata were obtained from euthanized dogs through an NIH sharing agreement and inflated via the common bile duct using 1 mg/ml Collagenase P solution, and islets were isolated from inflated pancreases as referenced in SCT publications (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017). In brief, the distended dog pancreas was cut into 15 to 20 pieces and placed in a 50 ml tube containing 20 ml of 1 mg/ml Collagenase P solution. The tube was placed into a 37°C water bath with the shaker set at 120 rpm. Islet content in the solution was monitored by microscopic examination of dithizone (DTZ) stained samples obtained from aliquots taken at 5 min intervals. Digestion was continued until approximately 50% of islets were free of acinar

tissue and was stopped with 20 ml of medium A solution (HBSS supplemented with 10mM HEPES + 1% BSA). The tissue suspension was then gently sieved through a 400 μm screen and centrifuged for 10 seconds at 100 x g at 4°C. The pellets were washed once and centrifuged for 10 seconds at 200 x g at 4°C. Three layer-density gradients were created by resuspending the pellets in 10 ml Histopaque-1.119, slowly layering on top 10 ml of Histopaque-1.077 followed by another layer of 10 ml of serum free medium. The tubes were centrifuged at 750 x g for 20 min at 4°C without brake. Islets were collected from the top interface and transferred to a 50 ml tube that contained medium A solution. The purified islet suspensions were washed with serum free medium and centrifuged for 10 seconds at 200 x g (4°C) twice and passed through a 40 μm cell strainer. Five 50 μl aliquots from each preparation were collected and used to assess the islet yield. Finally, hand-picked (to remove acinar cell content) islets were cultured in 20% FBS supplemented RPMI 1640 medium at 37°C, in a 5% CO2 incubator at pH 7.4.

Canine Islet Cell Culture

(protocol adapted from SCT SOPs (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017))

Initial Culture: Islets were further purified by capturing them in the top of a 40 μm filter strainer. Islet cells were cultured by placing whole islets on Laminin-511 coated wells, and allowing the islet cells to outgrow from the islets at 37°C until 90% confluent in DMEM-F12 + 20% FBS + GPS, which results in their dedifferentiation. Culturing in this manner further purifies islet cells and removes remaining exocrine cells.

Passaging: Dog islet cells were grown to approximately 90% confluence. They were then trypsinized (1x Trypsin-EDTA for 5-10 min), pelleted by centrifugation at 600x g for 5 min, washed with DMEM-F12 + 20% FBS + GPS, and seeded into T75

flasks. Passaged islet cells were cultured in DMEM-F12 + 20% FBS + GPS. Frozen cells were stored in 10% DMSO.

RT-PCR

(protocol adapted from SCT SOPs (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017))

RNA was extracted from 1x10e6 cells (QIAGEN RNeasy Mini Kit). Reverse transcription was performed using SuperScript II Reverse Transcriptase for 60 min. at 42°C. All reactions were carried out in a total volume of 20 µL with TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA) and the ABI 7500 Real Time PCR System. Utilized PCR primers are listed under Reagents. Reaction conditions were 50°C for 2 min, followed by a 95°C for 10 min start, and 40 cycles of melting at 95°C for 15 seconds and annealing at 60°C for 1 min. All samples were run in duplicate, and the average threshold cycle (Ct) value was used for calculations. RQ was calculated through normalization to internal controls (beta actin and beta 2 microglobulin (B2m), and the ABI 7500 software. Results are presented as log10(RQ) ± log10(RQmin and RQmax). Differences greater or less than log10(RQ) 2 or -2 were considered significant.

Microscopy

(protocol adapted from SCT SOPs (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017))

Bright field images were obtained using a Zeiss Axio Observer D1 microscope with EC Plan-Neofluar 10, 20 or 40x objectives and appropriate filters. Images were taken with the AxioCam MR3 camera or Axiocam ICc1 camera with a 0.63x camera adapter and ZEN Pro 2012 Blue Edition software v. 1.1.1.0 (Carl Zeiss Microscopy, GmbH, Germany).

RESULTS

Part One: Proliferation Rate

To test the first part of our hypothesis, we first examined whether laminin enhancement affected proliferation rate by documenting cell counts for each culture during each passage process.

Expansion times for each culture and passage are documented, along with any times when growth media was exchanged (data shown in Table 1 and Table 2). All cultures were prepared and incubated using the same reagents and methods as described in the previous section; laminin-enhanced and normal cultures were grown in parallel under identical conditions for the same length of time.

*Table 1. Dog 18 Culture Duration**

Culture →	**D18 P0**	**D18 P1**	**D18 P2**	**D18 P3**
Start	0 days	0 days	0 days	0 days
Exch. Media	7 days	4 days	4 days	4 days
Passage	10 days	7 days	7 days	7 days

*Each culture for Dog 18 was allowed to expand for 7 days, with the exception of the first culture grown from fresh islet cells; this required three additional days (10 days total) in order to allow for adhesion and growth into culture.

*Table 2. Dog 20 Culture Duration**

Culture →	**D20 P0**	**D20 P1**	**D20 P2**	**D20 P3**
Start	0 days	0 days	0 days	0 days
Exch. Media	6 days	4 days	4 days	4 days
Passage	9 days	7 days	7 days	7 days

*Each culture for Dog 20 was allowed to expand for 7 days, with the exception of the first culture grown from fresh islet cells; this required two additional days (9 days total) in order to allow for adhesion and growth into culture.

Next, before and after cell counts for each culture were taken for each passage (data shown in Table 3 and Table 4). All cell counts are approximate and are accurate to within ± 5%, based on cell density in suspension as counted under an optical microscope using a hemocytometer.

*Table 3. Dog 18 Cell Expansion – Counts**

Culture →	**D18 P0 Lam**	**D18 P1 Lam**	**D18 P2 Lam**	**D18 P3 Lam**
# Seeded	~1000 fresh†	0.15×10^6	0.15×10^6	0.15×10^6
# @ Harvest	2.04×10^6	1.22×10^6	1.39×10^6	1.22×10^6
# Expanded	2.52×10^6	1.07×10^6	1.24×10^6	1.07×10^6
Culture →	**D18 P0**	**D18 P1**	**D18 P2**	**D18 P3**
# Seeded	~1000 fresh†	0.15×10^6	0.15×10^6	0.15×10^6
# @ Harvest	2.52×10^6	1.56×10^6	2.16×10^6	0.98×10^6
# Expanded	2.52×10^6	1.41×10^6	2.01×10^6	0.83×10^6

*Number of cells expanded for Dog 18 did not differ significantly between cultures grown with and cultures grown without laminin enhancement.
Cultures were harvested at about 90% confluence at each passage, and re-seeded at 2000 cells/cm^2 equating to 150,000 cells per T75 (75 cm^2) culture flask.

† Fresh islet cells were seeded into culture directly from previously prepared cells.

*Table 4. Dog 20 Cell Expansion – Counts**

Culture →	**D20 P0 Lam**	**D20 P1 Lam**	**D20 P2 Lam**	**D20 P3 Lam**
# Seeded	~600 fresh†	0.15×10^6	0.15×10^6	0.15×10^6
# @ Harvest	1.08×10^6	2.36×10^6	2.30×10^6	0.81×10^6
# Expanded	1.08×10^6	2.21×10^6	2.15×10^6	0.66×10^6
Culture →	**D20 P0**	**D20 P1**	**D20 P2**	**D20 P3**
# Seeded	~600 fresh†	0.15×10^6	0.15×10^6	0.15×10^6
# @ Harvest	1.80×10^6	2.00×10^6	2.78×10^6	0.88×10^6
# Expanded	1.80×10^6	1.85×10^6	2.63×10^6	0.73×10^6

*Number of cells expanded for Dog 20 did not differ significantly between cultures grown with and cultures grown without laminin enhancement.
Cultures were harvested at about 90% confluence at each passage, and re-seeded at 2000 cells/cm^2 equating to 150,000 cells per T75 (75 cm^2) culture flask.

† Fresh islet cells were seeded into culture directly from previously prepared cells.

Finally, proliferation rates were calculated from cell counts and expansion time (data shown in Table 5 and Table 6). Out of the eight pairs of cultures where a direct comparison can be made between the laminin-enhanced culture and its unenhanced counterpart, only two pairs exhibited greater proliferation rate with laminin enhancement. In all other cultures, the unenhanced culture had a proliferation rate greater than or equal to the proliferation rate of the culture enhanced with laminin.

*Table 5. Dog 18 Cell Expansion – Rates**

Culture →	**D18 P0 Lam**	**D18 P1 Lam**	**D18 P2 Lam**	**D18 P3 Lam**
# Expanded	2.52×10^6	1.07×10^6	1.24×10^6	1.07×10^6
# of Days	10 days	7 days	7 days	7 days
Growth Rate (cells/day)	2.52×10^5	1.53×10^5	1.77×10^5	1.53×10^5
Culture →	**D18 P0**	**D18 P1**	**D18 P2**	**D18 P3**
# Expanded	2.52×10^6	1.41×10^6	2.01×10^6	0.83×10^6
# of Days	10 days	7 days	7 days	7 days
Growth Rate (cells/day)	2.52×10^5	2.01×10^5	2.87×10^5	1.19×10^5

*****Growth rates for Dog 18 did not differ significantly between cultures grown with and cultures grown without laminin enhancement.
Proliferation rate (cells/day) calculated from the number of cells expanded divided by the number of days in culture.

*Table 6. Dog 20 Cell Expansion – Rates**

Culture →	**D20 P0 Lam**	**D20 P1 Lam**	**D20 P2 Lam**	**D20 P3 Lam**
# Expanded	1.08×10^6	2.21×10^6	2.15×10^6	0.66×10^6
# of Days	9 days	7 days	7 days	7 days
Growth Rate (cells/day)	1.20×10^5	3.16×10^5	3.07×10^5	0.94×10^5
Culture →	**D20 P0**	**D20 P1**	**D20 P2**	**D20 P3**
# Expanded	1.80×10^6	1.85×10^6	2.63×10^6	0.73×10^6
# of Days	9 days	7 days	7 days	7 days
Growth Rate (cells/day)	2.00×10^5	2.64×10^5	3.76×10^5	1.04×10^5

*Growth rates for Dog 20 did not differ significantly between cultures grown with and cultures grown without laminin enhancement.
Proliferation rate (cells/day) calculated from the number of cells expanded divided by the number of days in culture.

Part Two: Gene Expression

To test the second part of our hypothesis regarding gene expression, RT-PCR experiments were performed on samples collected from each passage of each culture. Relative Quantitation (RQ) data collected from the experiment was plotted in RQ vs Sample bar charts using Microsoft Excel. RQ describes the fold-increase (or decrease) of an experimental sample compared to a reference sample. Each experiment included a positive control to provide the reference (fresh islet cells from Dog 28 which had never been expanded in culture) and a negative control (no islet cells added to the PCR reagents). Plotting the base-10 logarithmic values of RQ values (log 10 RQ) on the vertical axis of these charts allows us to directly compare the reduction of expression across passages, thereby tracking the decay of gene expression across passages; a difference of ± 2 log RQ is considered to be of statistical significance.

Sixteen genes were analyzed in each RT-PCR experiment, and complete data is provided in Supplemental Figures S1-S8 found in the Appendix. For simplicity in presentation, the data for the insulin (INS) and glucagon (GCG) genes from each experiment have been compiled in a composite chart (Fig.6) that clearly illustrates the differences in expression between laminin-enhanced cultures and their unenhanced counterparts. Insulin (INS) expression is highlighted in **red**, while glucagon (GCG) expression is highlighted in **blue**. Error bars are also shown in **green**, plotted using log RQ Min and log RQ Max values as reported in the RT-PCR data.

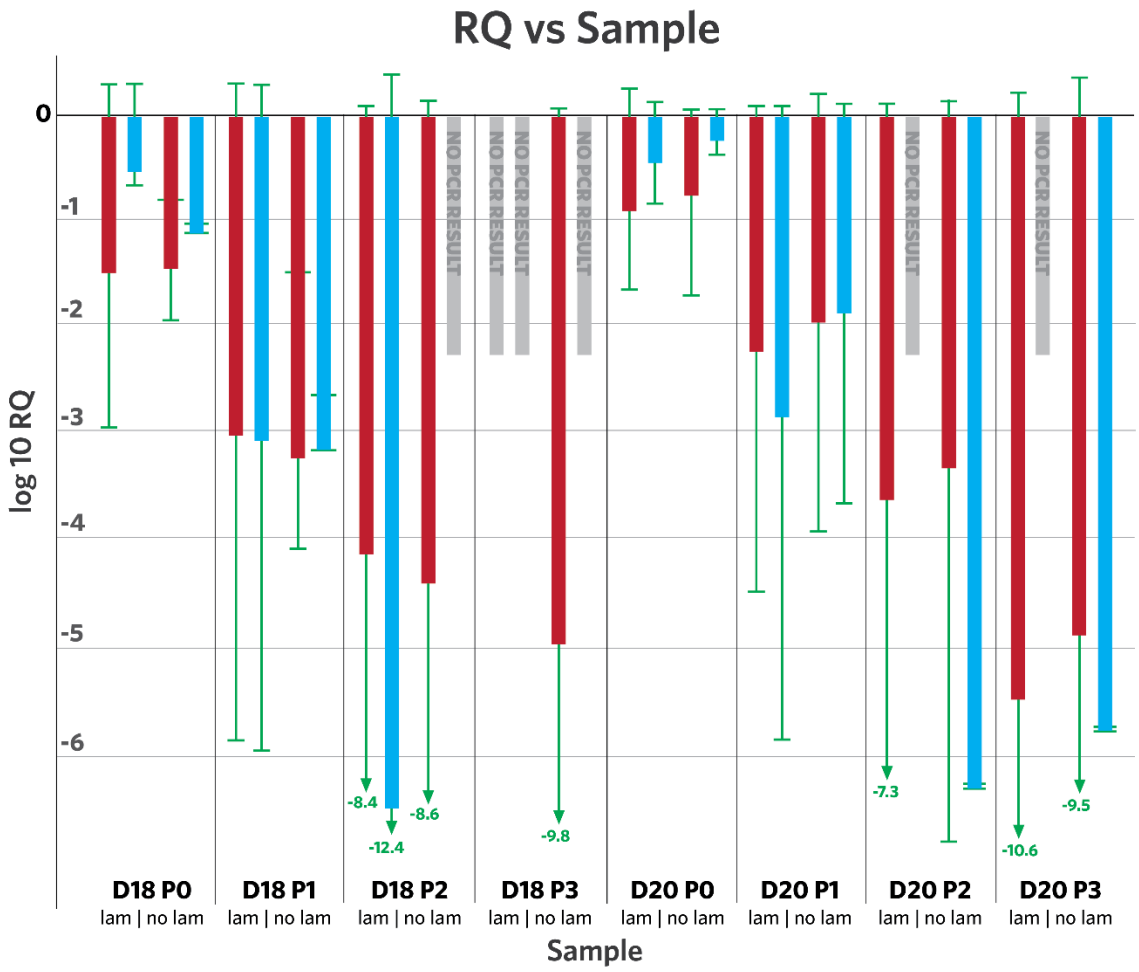

Figure 6. For every culture and passage, there was no significant difference between cultures grown with and cultures grown without laminin enhancement.

Insulin (INS) expression is shown in **red**, while glucagon (GCG) expression is shown in **blue**. Error bars are also shown in **green**, plotted using log RQ Min and log RQ Max values.

These data were taken directly from Supplemental Figures S1-S8 (see Appendix) and compiled into a single plot for ease of comparison across passages.

The composite chart clearly shows the comparison between the laminin-enhanced and unenhanced cultures for each passage within each vertical column, where laminin-enhanced expression are the two bars on the left within each section (one for insulin and one for glucagon) and unenhanced expression are the two bars on the right.

The data suggests that for every passage for which RQ data were generated, there is no major difference in gene expression between cultures that were enhanced with laminin and those that were not. In Dog 18 cultures, the unenhanced cultures, labelled "no lam", generally appear to have roughly equal or slightly lower expression of insulin than the laminin-enhanced cultures, labelled "lam". In Dog 20 cultures, the laminin-enhanced cultures consistently had lower expression than their unenhanced counterparts for both insulin and glucagon.

DISCUSSION

The present study was designed as an attempt to replicate successful experiments which demonstrated the enhancing effects of laminins on cell types which were difficult to culture. Specifically, our investigations addressed the hypothesis that laminins will enhance proliferation rates—measurable by cell counts—and gene expression—measurable by RT-PCR data—of dog islet beta cells. It was reasoned that due to the biological activity of laminins, which facilitate numerous cell processes in the body including adhesion to the basal lamina, laminins would naturally have an enhancing effect on islet cell cultures as well. Our hypothesis about islet cells was thus formulated based on this evidence suggesting that laminins improved cultures of other cell types that were similarly difficult to expand *in vitro*.

In addition to making a simple logical extension to the binding functions of laminins *in vivo* (Goddeeris, et al., 2013), there is a substantial amount of observations made on laminin from both human (Miyazaki, et al., 2008) and mouse (Domogatskaya, Rodin, Boutaud, & Tryggvason, 2008) embryonic stem cell cultures that would support our hypothesis. Therefore, it was reasonably thought that cultures of islet beta cells might benefit from laminin treatment prior to cell expansion. If laminins should significantly improve the proliferation rate and/or gene expression of islet beta cultures grown for therapeutic treatment, there could be great implications for the entire cell expansion process used throughout scientific research and for the T1DM therapeutic challenges of low donor cell numbers and reduced potency in later passages.

Despite these observations, however, it appears that our results do not support either part of the hypothesis. The use of laminin-enhanced culture flasks did not have any

significant effect on cell counts of cultures grown in parallel with unenhanced flasks, with only two out of eight pairs of cultures resulting in slightly higher cell counts with laminin enhancement. Additionally, laminin did not appear to have any significant effect on the gene expression of these islets as evaluated by RT-PCR; for every pair of laminin-enhanced and unenhanced cultures, there is an almost equal amount of expression decrease through each passage. With both parts of our hypothesis disproved, we find that there was no statistically significant benefit to using r-laminin-511 in our dog islet beta cell cultures.

It is important to note that it is entirely possible these experiments failed due to the need for species-specific laminins—a major limitation of this study. In these experiments, human laminins were used with canine cells simply because of commercial availability. Human laminins could possibly work only for human cells, and canine laminins could have a significant effect on canine cells were there a commercially available supply or were we able to purify a sufficient quantity. In support of this notion, SCT has found that canine cells—either ASCs (adipose-derived stem cells) or Islet cells—do not grow at all when cultured in human Platelet Lysate or serum, which routinely yields excellent growth for human cells. Canine cells have, however, been found to grow well when the serum source is bovine, and robustly in canine serum.

Since islet cells are a key component of an effective therapy for T1DM, it was important to explore any possible way to enhance the growth or potency of donor islet cells. As noted before, current treatment therapies require up to five suitable donors to successfully cure a single patient (Westenfelder, Gooch, Hu, Ahlstrom, & Zhang, 2017). Successfully culturing and expanding a great deal of donor cells *in vitro* would

significantly increase the impact that a single donor could have on islet cell recipients—and therefore revolutionize the way islet cultures are expanded in all scientific research. Due to its potential significance to the industry, the exploration of laminins as a potential enhancer of islet beta cells in their proliferation and subsequent gene expression for therapeutic treatment of T1DM is an approach that certainly merits further attention. Although canine laminins are not available commercially nor easily purified at SCT, additional study of the interaction between canine laminins and canine islet beta cells could yield different results than the ones presented in this study. Furthermore, pairing commercially-available human laminins with human islets may also result in enhancement; the availability of human islets for research, however, is understandably extremely low. There may also be a number of other enhancers for islet beta cells that have not yet been studied.

CONCLUSIONS

In conclusion, the present data demonstrate that there is no significant difference in the growth rate, gene expression, or potency between cultures expanded from the same dog islets, disproving our hypothesis. R-Laminin-511 does not bear significant benefits or enhancing effects on the growth of SCT dog islet cultures when grown using the conditions and methods presented in this study.

It is possible that performing these studies differently might yield important findings in biological and bioengineering applications: laminin could be used as an additive to the culture medium rather than as a coating on culture flasks, or, as mentioned, canine laminins instead of human laminins could be used with canine islets. Perhaps the culture conditions could also be varied through the use of different cell enhancers, growth mediums, flask sizes, medium additives, growth durations, passage confluence, or any combination of these. Although the unique culture conditions in this study did not show differences in islet growth or potency in the assays performed, any number of future studies may yield different results from ours.

The next phase of research that was performed subsequently during my time at SCT included working on confirming the dedifferentiation of islet cells, in which islets are redifferentiated alongside adipose-derived stem cells (ASCs) to confirm the possibility of restoring differentiation—and thus normal islet cell function. Following this, we then studied islet proliferation, where fresh dog islets from numerous canine donors were passaged for the first time to determine and preserve cell viability after long-term cryostorage.

After receiving our FDA approval for pilot studies in insulin-dependent diabetic pet dogs and clinical trials in humans, future directions for 2017 include studying the effectiveness of *in vivo* human cells in SCID mice. These studies will hopefully continue the progress that has been made at SCT towards a clinically-approved therapeutic for the permanent treatment of T1DM and eventually T2DM, with the ultimate goal of one day making diabetes mellitus a disease of the past.

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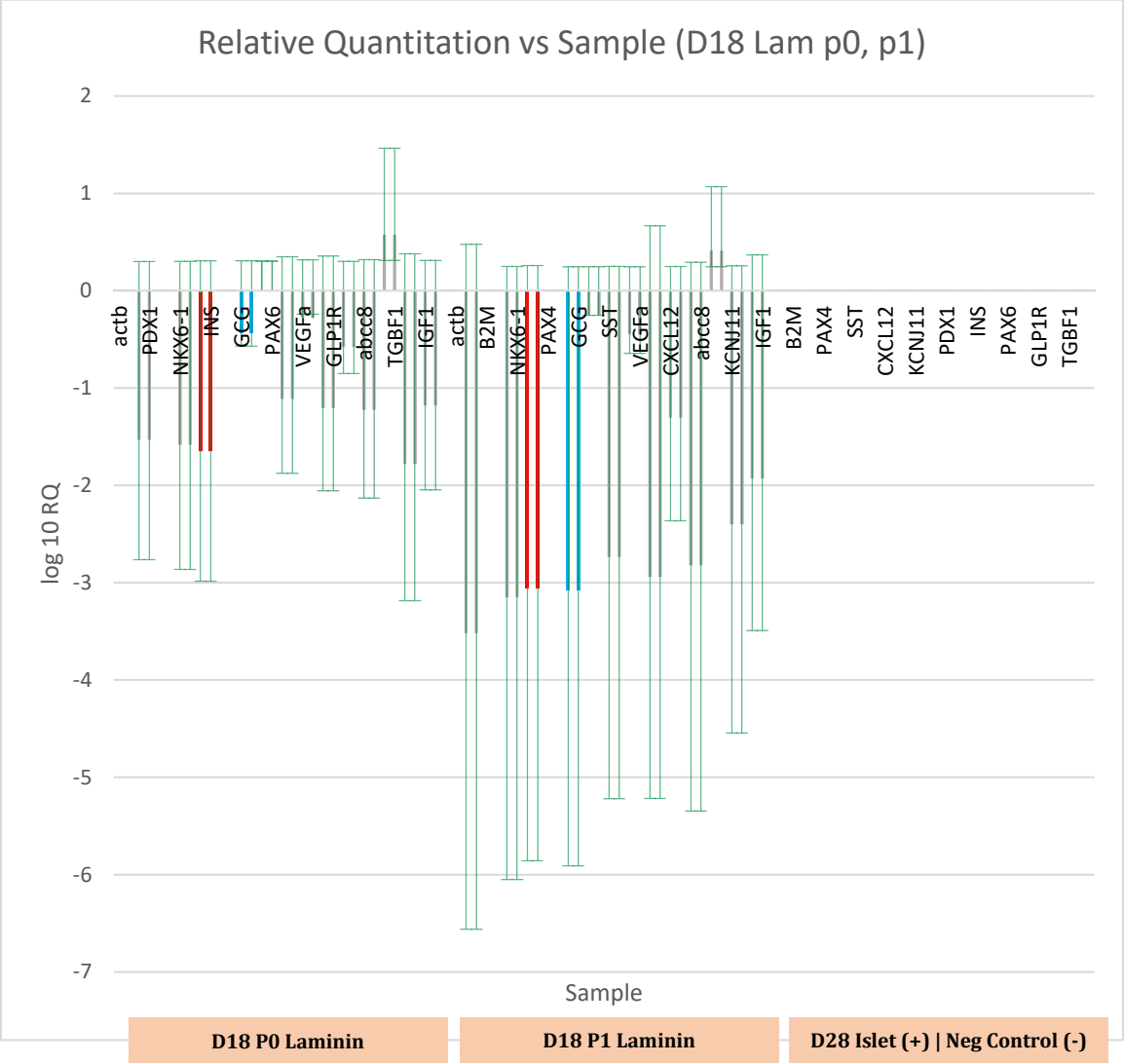
APPENDIX: SUPPLEMENTAL FIGURES

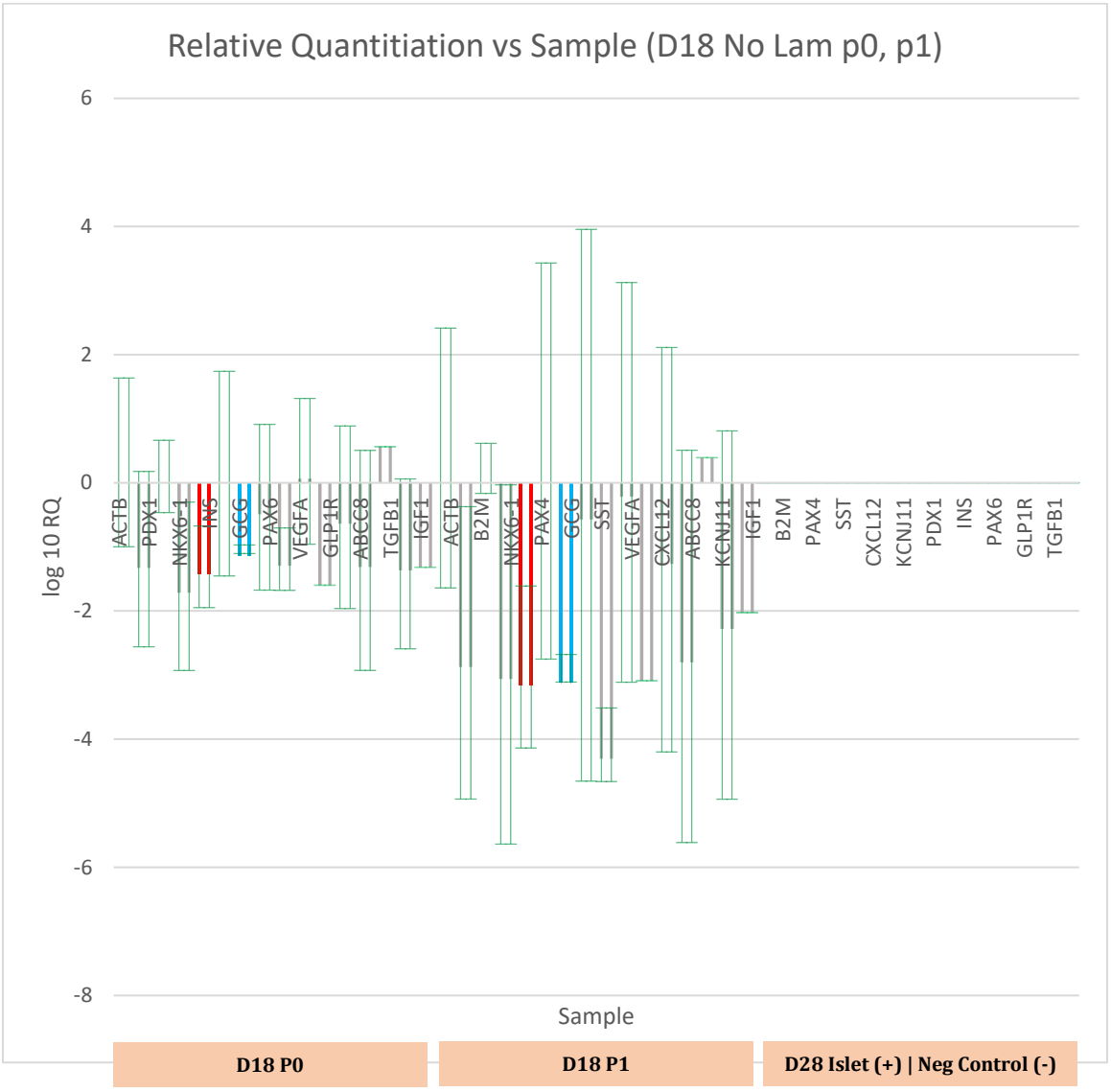
Sixteen genes were analyzed in each RT-PCR experiment, and complete data is provided in Supplemental Figures S1-S8 found on the following pages. In each of the Relative Quantitation vs Sample figures, insulin (INS) expression is highlighted in **red**, while glucagon (GCG) expression is highlighted in **blue**.

Since each culture in each experiment was run in duplicate, there are two bars for each expressed gene. Error bars are also shown in **green**, plotted using log RQ Min and log RQ Max values as reported in the RT-PCR data.



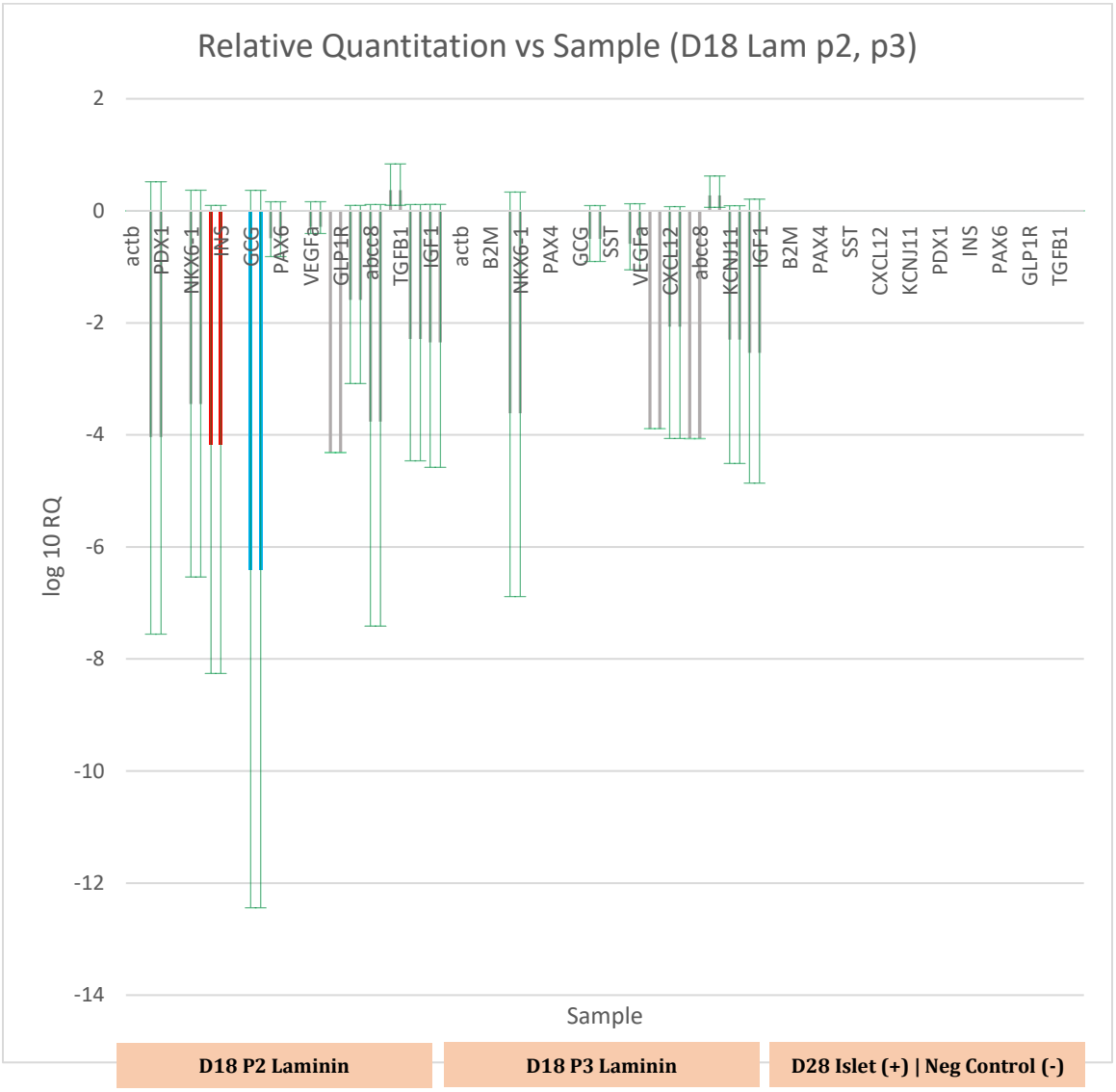
Supplemental Figure S1. RQ vs Sample of Dog 18 Laminin, P0 and P1.

Positive Control/Reference: Dog 28 Fresh Islet.



Supplemental Figure S2. RQ vs Sample of Dog 18 without Laminin, P0 and P1.

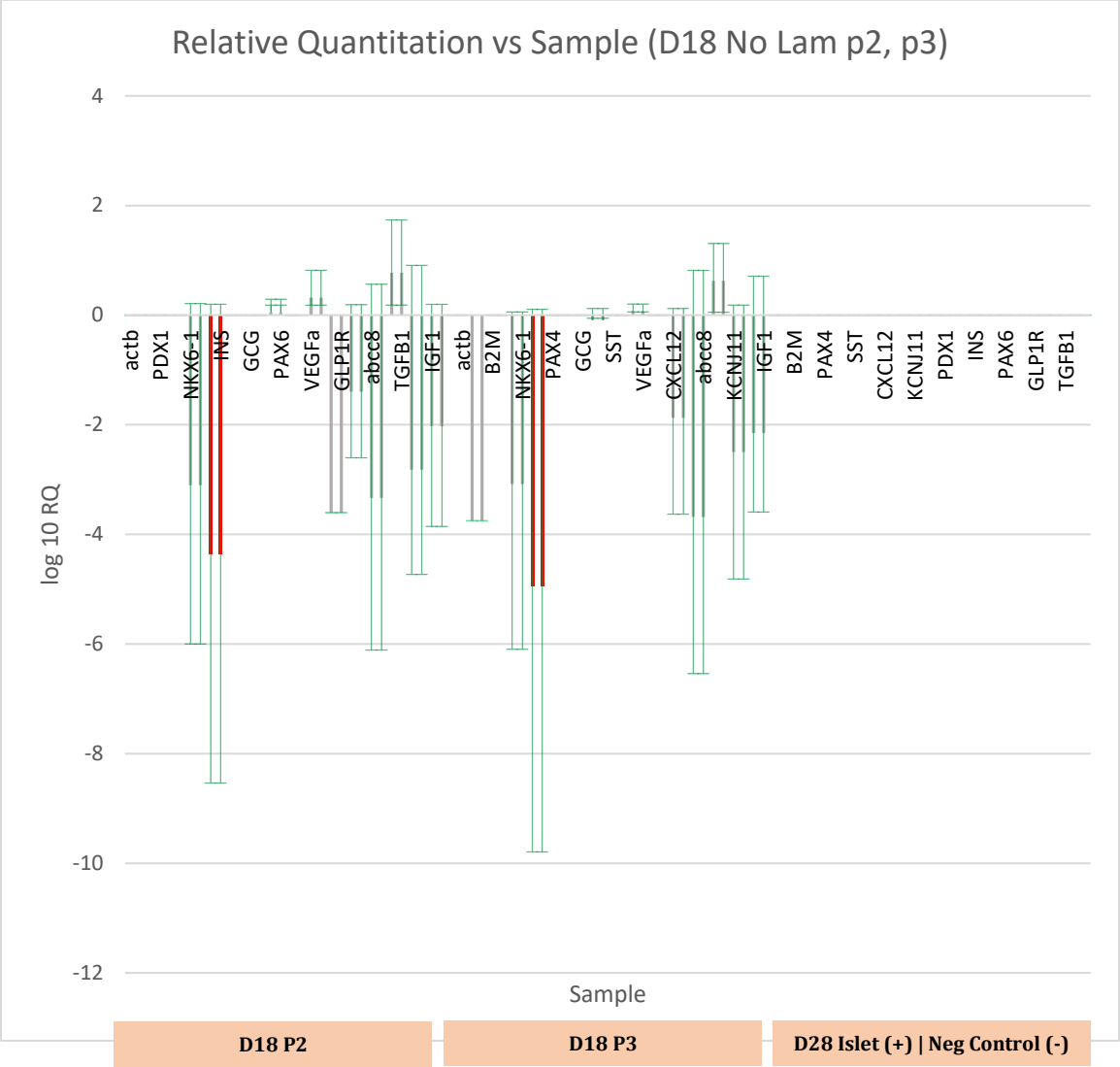
Positive Control/Reference: Dog 28 Fresh Islet.



Supplemental Figure S3. RQ vs Sample of Dog 18 Laminin, P2 and P3.

Positive Control/Reference: Dog 28 Fresh Islet.

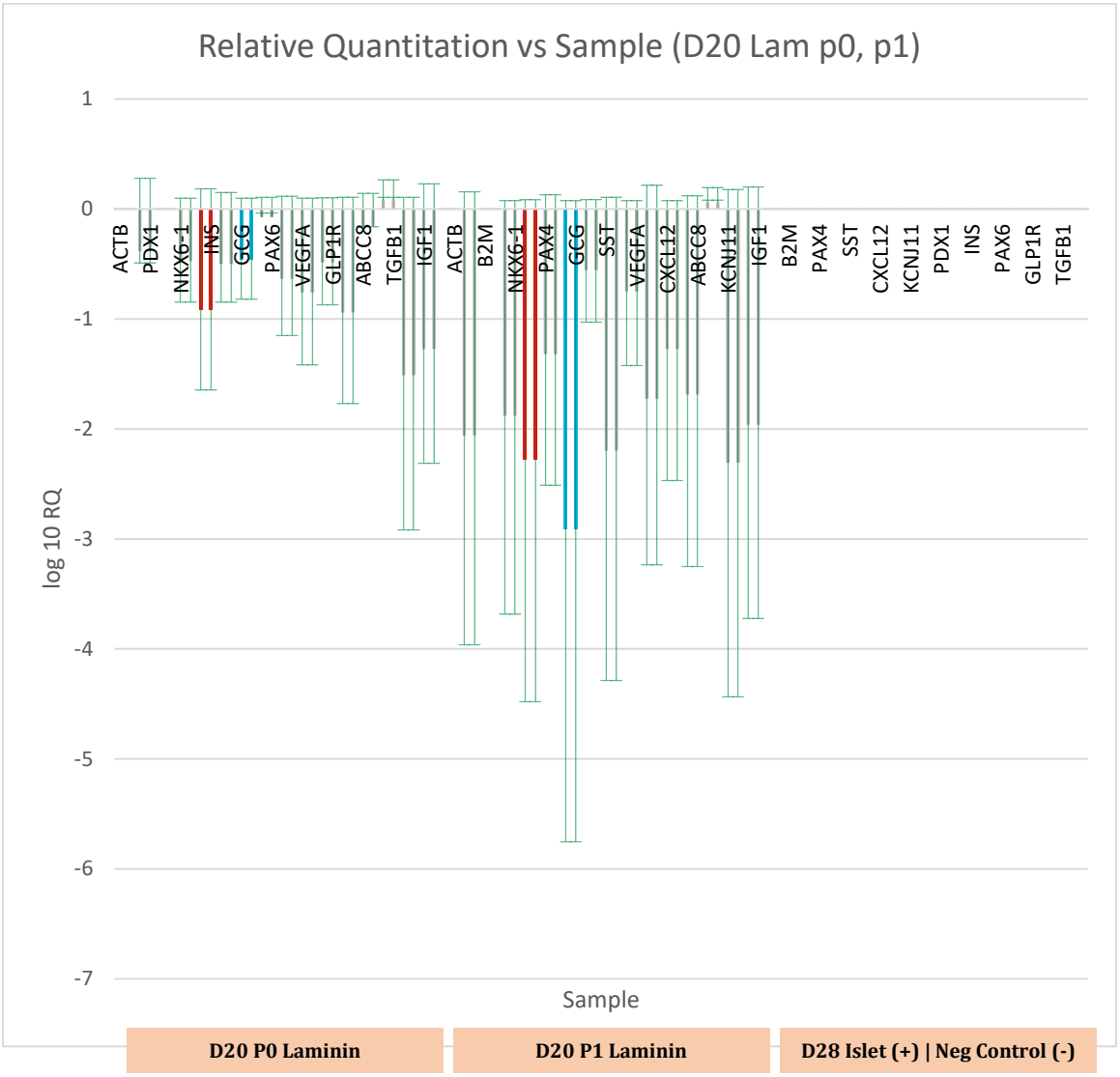
No RT-PCR data was generated for either INS or GCG in D18 P3.



Supplemental Figure S4. RQ vs Sample of Dog 18 without Laminin, P2 and P3.

Positive Control/Reference: Dog 28 Fresh Islet.

No RT-PCR data was generated for GCG in either D18 P2 or D18 P3.

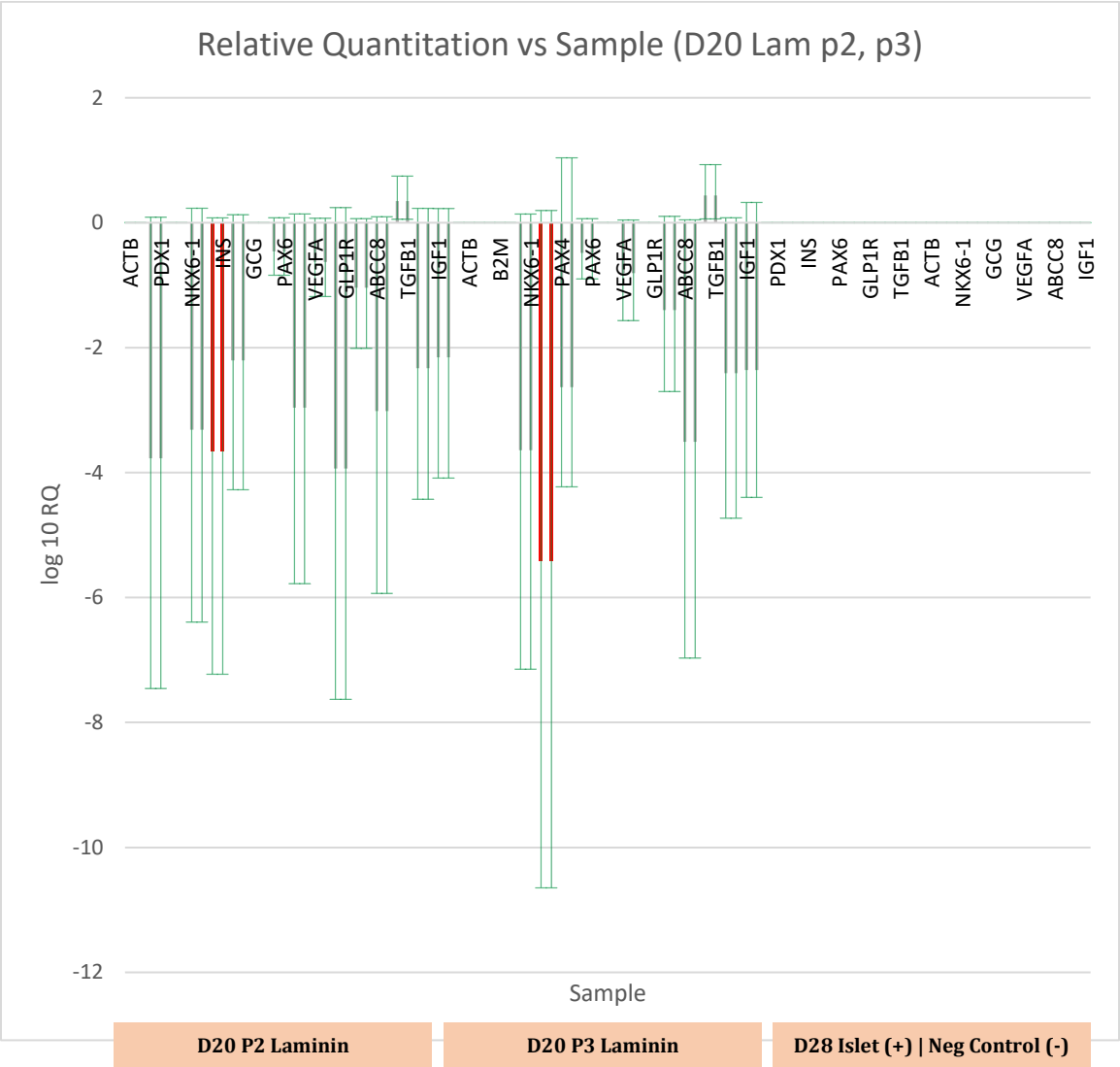


Supplemental Figure S5. RQ vs Sample of Dog 20 Laminin, P0 and P1.

Positive Control/Reference: Dog 28 Fresh Islet.

Supplemental Figure S6. RQ vs Sample of Dog 20 without Laminin, P0 and P1.

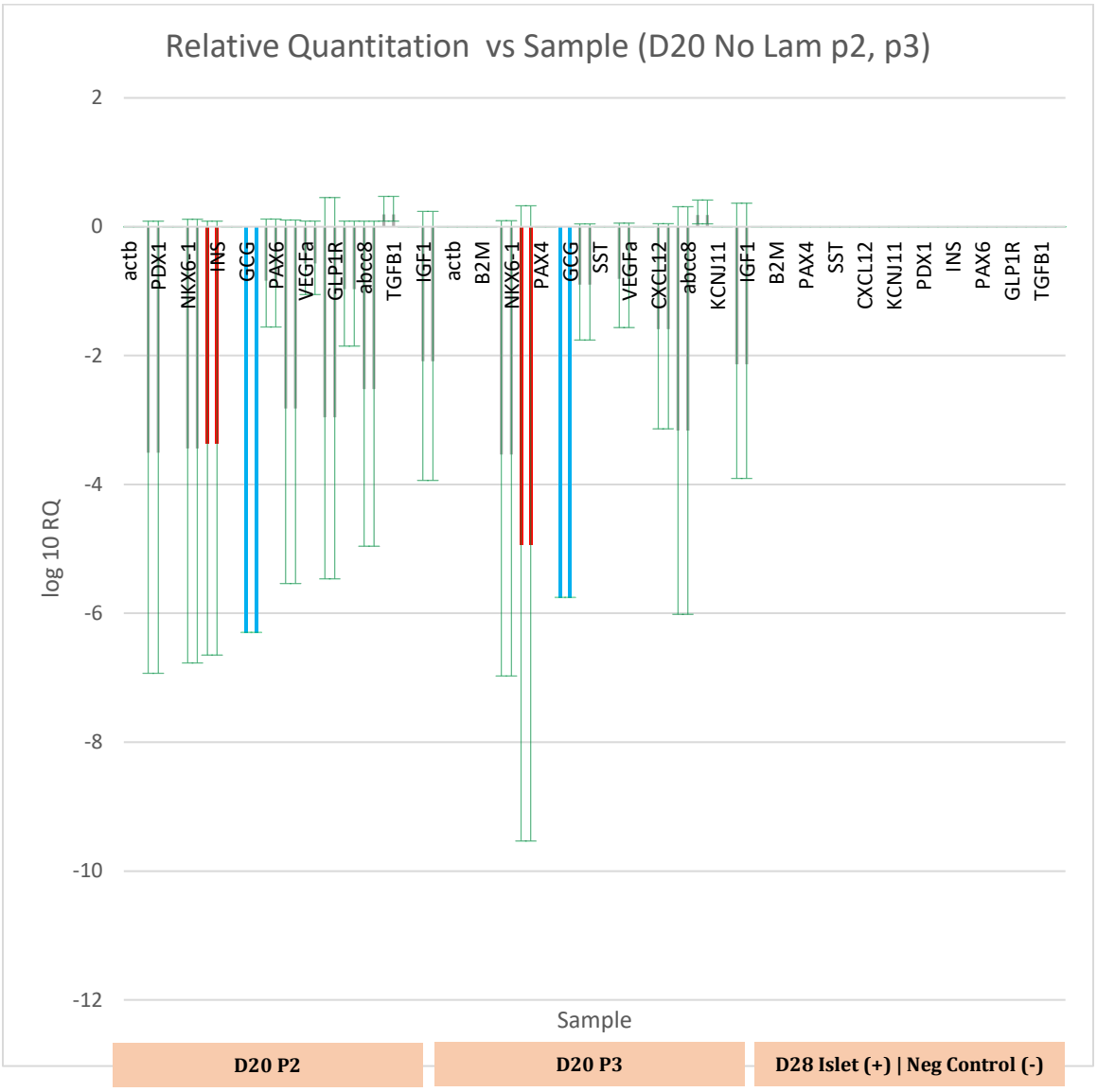
Positive Control/Reference: Dog 28 Fresh Islet.



Supplemental Figure S7. RQ vs Sample of Dog 20 Laminin, P2 and P3.

Positive Control/Reference: Dog 28 Fresh Islet.

No RT-PCR data was generated for GCG in either D20 P2 or D20 P3.



Supplemental Figure S8. RQ vs Sample of Dog 20 without Laminin, P2 and P3.

Positive Control/Reference: Dog 28 Fresh Islet.

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