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**IMPACT OF DONOR AGE AND WEANING STATUS ON PANCREATIC EXOCRINE  
AND ENDOCRINE TISSUE MATURATION IN PIGS**

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Miranda Stiewig – Data analysis/interpretation, data collection

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Tom Spizzo – Concept/design, data collection, critical revision of article

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Edwin S Monuki – Data analysis/interpretation, critical revision of article

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## ABSTRACT

**Background:** During the process of islet isolation, pancreatic enzymes are activated and released, adversely affecting islet survival and function. We hypothesize that the exocrine component of pancreata harvested from pre-weaned juvenile pigs is immature and hence pancreatic tissue from these donors is protected from injury during isolation and prolonged tissue culture.

**Methods:** Biopsy specimens taken from pancreata harvested from neonatal (5-10 days), pre-weaned juvenile (18-22 days), weaned juvenile (45 -60 days) and young adult pigs (>90 days) were fixed and stained with Hematoxylin & Eosin. Sections were examined under a fluorescent microscope to evaluate exocrine zymogen fluorescence intensity (ZFI) and under an electron microscope to evaluate exocrine zymogen granule density (ZGD).

**Results:** Exocrine content estimation showed significantly lower ZFI and ZGD in juvenile pig pancreata ( $1.5 \pm 0.04 \text{U}/\mu\text{m}^2$ , ZFI;  $1.03 \pm 0.07 \times 10^3 / 100 \mu\text{m}^2$ , ZGD) compared to young adult pigs ( $2.4 \pm 0.05 \text{U}/\mu\text{m}^2$ , ZFI;  $1.53 \pm 0.08 \times 10^3 / 100 \mu\text{m}^2$  ZGD). Islets in juvenile pig pancreata were on average smaller ( $105.2 \pm 11.2 \mu\text{m}$ ) than islets in young adult pigs ( $192 \pm 7.7 \mu\text{m}$ ), but their insulin content was comparable ( $80.9 \pm 2.2\%$  juvenile;  $84.2 \pm 0.3\%$  young adult,  $p > 0.05$ ). All data expressed as Mean  $\pm$  SEM.

**Conclusion:** Porcine islet xenotransplantation continues to make strides towards utilization in clinical trials of Type 1 diabetes. Porcine donor age and weaning status influence the extent of exocrine maturation of the pancreas. Juvenile porcine pancreata may represent an alternative donor source for islet xenotransplantation as their exocrine component is relatively immature; this preserves islet viability during extended tissue culture following isolation.

**KEY WORDS:** Exocrine, Pancreas, Xenotransplantation, Histology, Zymogen

**Abbreviations**

T1D – Type 1 Diabetes

PAS – Periodic Acid Schiff

## **Introduction**

Type 1 Diabetes (T1D) is an autoimmune disorder that occurs due to a rapid destruction of insulin-producing beta cells in the pancreas, leading to profound insulin deficiency and a loss of the ability to regulate blood glucose levels (1). While T1D can be managed through administration of frequent exogenous insulin through injections or infusion pumps, there is no permanent cure. As many as three million people in the United States suffer from T1D, and the permanence of the diagnosis necessitates a lifetime of treatment, which accounts for a cost of almost \$15 billion dollars annually in the US alone (2). A feasible alternative to exogenous insulin administration is islet transplantation, with human allotransplantation being the obvious first choice. However, there is an acute scarcity of transplant-worthy islets from deceased human donors which creates the need for an alternative islet source. Porcine islet xenotransplantation is considered a viable alternative. Porcine insulin is physiologically similar to human insulin and the donor pool is virtually limitless. Although cross-species transplantation (i.e. xenotransplantation) was attempted as early as the 17<sup>th</sup> century (3), it still faces many challenges that affect the survival and viability of tissue transplanted from one animal species to another, not least of which the immunosuppressive therapy is required to prevent immediate rejection of islet xenografts after transplantation (4).

The Spring Point Project (Spring Point, MN, USA) is a nonprofit organization that aims to make porcine islets an unlimited resource for use in xenotransplantation. It is currently the only source of viable porcine tissue and organs that has been deemed clinically suitable by the Food and Drug Administration (FDA).

However, other hurdles that can potentially derail the application of xenotransplantation to the human setting still need attention. There has been considerable scientific interest in the



effect of islet isolation on the pancreatic tissue itself. It has been observed that during the islet isolation procedure, the exocrine acinar tissue shows elevated levels of necrosis and apoptosis (5). Autoactivation of pancreatic exocrine enzymes by the collagenase/protease enzyme mixture results in elevated levels of serine proteases (trypsin, chymotrypsin and elastase) during islet isolation and in vitro culture, which correlated negatively with islet survival (5-7).

The release of these enzymes has also been correlated with increased oxidative stress-induced islet injury, leading to decreased viability and survival in pancreatic islets (8, 9). It has been demonstrated that inhibition of some or all of these pancreatic enzymes is correlated with improved islet function and survival (10-12). In addition to the presence of exocrine enzymes in the culture media during isolation and prolonged in vitro culture, other donor parameters can also affect islet yield (13).

To date, there have been few studies evaluating the effect of porcine donors of different ages and weaning status on the exocrine component of the pancreas in these donors. Nagaraju S et al. reported that neonatal pigs might be a viable source of islets for xenotransplantation studies (14). However, in their report, no distinction was made regarding the weaning status of the porcine donors. This analysis is vital as neonatal pigs that have not yet been weaned on solid food would exhibit an inactive or immature exocrine component and hence would be more appropriate as islet donors since their islets would not be exposed to high levels of harmful enzymes during the isolation or in vitro culture period.

The purpose of this study was to evaluate the exocrine and endocrine components in porcine donors of various ages and weaning status (neonatal or 5-10 days old, **Group I**; pre-weaned juvenile or 18-22 days old, **Group II**; weaned juvenile or 45-60 days old, **Group III**; young adult >90 days old, **Group IV**). In so doing, we expected to be able to correlate the effects

of exocrine tissue maturity on islet function and survival. Through this, we hope to be able to determine the optimum porcine donor age and weaning status that would be most likely to provide the highest yield of viable islets using a scalable isolation protocol for the purpose of clinical xenotransplantation trials.

**We hypothesized that pancreata harvested from pre-weaned pigs would have limited exocrine enzyme reserves, and that their exocrine component will mature after weaning, resulting in a rapid increase in enzyme reserves. The aim of this study was to determine whether donor age and weaning status impact pancreatic exocrine maturity.**

To test this hypothesis, we characterized the exocrine content of pancreatic tissue harvested from pigs of different ages (neonatal, pre-weaned, recently weaned, or young adult pigs) to compare the exocrine enzyme content and acinar maturity by analyzing their structures using light and ultramicroscopic imaging techniques. We then evaluated and compared islet morphology and cell composition. Comparing changes among the different groups studied will allow us to analyze the effect of donor age and weaning status on exocrine and endocrine tissue maturity, and thus, allow us to predict the effect of donor age and weaning status on islet yield and function.

## **Research Design and Methods**

### **Experimental Groups:**

Pancreata were harvested from neonatal Yorkshire pigs (5-10 days old, **group I**); pre-weaned juvenile pigs (18-22 days old, **group II**); weaned juvenile pigs on solid feed for 4 weeks (45-60 days old, **group III**); and young adult pigs on solid feed (>90 days old, **group IV**) (Table 1).

### *Pancreas harvest*

Pancreases were obtained from male Yorkshire Pigs (n=8 per group, S&S Farms, Ramona, CA). The pigs were anesthetized by intramuscular injections of ketamine (20mg/kg) [Phoenix Pharmaceutical, St. Joseph, MO] and xylazine (2mg/kg) (Phoenix Pharmaceutical, St. Joseph, MO). They were maintained under isoflurane anesthesia (Phoenix Pharmaceutical, St. Joseph, MO) at 5 MAC at a flow rate of 5 L/min O<sub>2</sub> (Airgas, CA) (15-17).

The animals were then placed in the supine position and the surgical area was disinfected with 70% ethanol (Fisher Scientific, Pittsburgh, PA) followed by povidone iodine (Phoenix Pharmaceutical, St. Joseph, MO). Under sterile conditions, a single midline incision was made from the xiphoid process to the pubic symphysis. The underlying muscle was identified and the abdominal cavity was entered using a pair of sharp scissors. Abdominal muscles and overlying skin were retracted to expose the abdominal organs and the stomach was retracted away from pancreas using surgical clamps. The tail of the pancreas was identified and quickly dissected away from the spleen and the left kidney. Care was taken not to damage the underlying splenic vessels.

Once the tail of the pancreas had been dissected away until the body was reached, the organ was bisected, removed, and placed in cold organ preservation solution (Mediatech, Manassas, VA, USA). The first, second, and third parts of the duodenum were identified and secured using blunt intestinal clamps. The head of pancreas was dissected away from the duodenum and the right half of the body was excised with the head *en bloc* and placed in University of Wisconsin (UW) organ preservation solution. The entire procedure was completed in less than five minutes to minimize tissue damage due to cold ischemia. Immediately after extirpation, the pancreas (including the head, body and tail) was weighed. All animal procedures including but not limited to monitoring, surgery, and euthanasia were performed with approval from the University of

California Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine. The body weights, pancreas weights and ages of the donors were recorded.

### *Tissue Preservation*

Three biopsy specimens (10x10 mm sections) of the tail of the pancreases from each animal in each group were procured and immediately transferred to 10% neutral buffer formalin (Sigma Aldrich, St. Louis, MO) where they were allowed to fix for 48 hours (18). The tissue was then prepared for paraffin (Sigma Aldrich, St. Louis, MO) fixation using a Leica TP1020 (Leica Microsystems, Buffalo Grove, IL) as previously described (19). After preparation (involving serial dehydration in alcohol solutions of increasing concentrations), the tissue was blocked in paraffin using a Leica EG 1150C (Leica Microsystems, Buffalo Grove, IL) and 5  $\mu$ m sections of the tissue were cut using a Leica RM 2255 microtome (Leica Microsystems, Buffalo Grove, IL). The sections were incubated at 65°C for an hour after which they were stained using techniques described below.

### *Histology & Histomorphometry*

Histological sections from the tail of the pancreas from each experimental group were stained with Hematoxylin & Eosin (Sigma Aldrich, St. Louis, MO) and Periodic Acid Schiff (PAS) (Sigma Aldrich, St. Louis, MO) staining without diastase digestion (Figure 2) using standard protocols and then evaluated (20). Sections were examined under a digital inverted light microscope (EVOS<sup>TM</sup> xl core, Fisher Scientific, Waltham, MA) to compare acinar size, structure, and morphology (Figure 1). Hematoxylin & Eosin sections were also examined under an inverted fluorescent microscope (Nikon Ti-E, Nikon, Tokyo, Japan) using laser-excitation (ex/em 488/540 nm) to quantify zymogen granule density (Figure 3) (21). This was calculated by

evaluating the fluorescent signal intensity using standard histomorphometric image analysis techniques (22).

Slides stained using Periodic Acid Schiff (without diastase digestion) were used to evaluate the maturity of the exocrine pancreas. Briefly, the slides were fixed in formaldehyde (Sigma Aldrich, St. Louis, MO), washed with PBS (Sigma Aldrich, St. Louis, MO) and distilled water and then stained with periodic acid. The slides were then washed again, stained with hematoxylin, and washed a final time. The slides were then ready for examination and imaging.

#### *Electron Microscopy*

1  $\mu\text{m}$  thick sections were cut and stained with toluidine blue (Sigma Aldrich, St. Louis, MO) to identify optimal sections for electron microscopy following which sections were imaged using a transmission electron microscope (Tecnai 12 biotwin/FP, FEI, Oregon, USA) (23). Acinar sections from porcine pancreas tissue samples taken from animals of different ages were analyzed under an electron microscope. Images of electron dense zymogen granules were obtained at a magnification of 5600x and a minimum of 10 sections (per sample) were analyzed using image analysis algorithms (ImageJ, National Institutes of Health, Bethesda, MA) to quantify mean granule size and density.

#### *Immunohistochemical evaluation of the Islets of Langerhans*

After formalin fixation and paraffin embedding (FFPE), the blocks obtained were sectioned to obtain 5  $\mu\text{m}$  slices which were then mounted on slides and stained using standard protocols (19). Briefly, the slides were deparaffinized and rehydrated using xylene and a serial dilution of alcohols. They were then heated in a 10mM solution of sodium citrate buffer (Sigma Aldrich, St. Louis, MO), rinsed in a mixture of Tris-Buffered Saline and Tween 20 (TBST) (Sigma Aldrich,

St. Louis, MO) and blocked with 10% goat serum (Sigma Aldrich, St. Louis, MO). Following this, primary antibodies against porcine insulin (Mouse anti-insulin, Abcam, Cambridge, MA) and glucagon (Rabbit anti glucagon, Abcam, Cambridge, MA) were added. Appropriate secondary antibodies against insulin (Anti Ms IgG-Alexa 488(green), Abcam, Cambridge, MA), and glucagon (Anti Rb IgG-Alexa 555(red), Abcam, Cambridge, MA) were added. Afterwards, the tissue was mounted with mounting media containing DAPI (4', 6-diamidino-2-phenylindole) (Vectashield, Vector Laboratories, Burlingame, CA) which binds to A-T rich regions in the DNA resulting in blue fluorescence under a fluorescence microscope. A minimum of 100 islets were imaged and analyzed using image analysis algorithms (ImageJ) to determine the percentage of insulin positive and glucagon positive cells within islets in a given sample.

### **Statistical Analysis:**

All data is presented as mean plus or minus the standard error of the mean (mean±SEM). A one-way ANOVA followed by a post-hoc Tukey's HSD test, was used to determine significance of differences between experimental groups with the level of significance set at  $p < 0.05$ .

### **Results**

#### *Characteristics of the pancreas in pigs by age and weaning status*

The gross appearance of the pancreas showed definite variations between neonatal, juvenile and young adult pigs. Of the groups evaluated in this study pancreases from young adult pigs (25-32kg) were the largest in size, very firm in consistency, with a well formed head (~25% of total pancreas weight) and a well-developed capsule. Pancreases from weaned juvenile pigs (45-60 days old) were nearly four-fold smaller in size compared to those from young adult pigs. They demonstrated a well formed head constituting ~25% of the weight of the pancreas, The were firm

and demonstrated a complete capsule reminiscent of young adult pancreases. Pre-weaned juvenile pancreases, (Group II) were significantly smaller than weaned juvenile (Table 1,  $p < 0.01$ , ANOVA) and young adult pig pancreases (Table 1,  $p < 0.05$ , ANOVA), and showed a well-developed head that constituted ~15% of the total weight of the pancreas. A capsule was noted around the tail of the pancreas but was incomplete. In neonatal pigs, the pancreas was small, soft in consistency, friable, with an underdeveloped head that formed  $< 5\%$  of the total weight of the pancreas. There was minimal connective tissue capsule around the neonatal pancreas at the time of harvest.

#### *Analysis of the Exocrine & Endocrine Pancreas – Histology*

For the purpose of comparing the structural composition of pancreatic tissue samples, tissue samples stained with H&E were compared between those of young adult pigs (Group IV) pancreases, neonatal pigs (Group I) and juvenile pigs (Groups II and III). Histological analysis revealed that neonatal pancreases demonstrated diffusely scattered insulin-positive cells without well-demarcated islet structures (Figure 5), in contrast to the well-defined islets found in juvenile and young adult pig pancreas (Figures 1, 5).

Another interesting observation was the dramatic increase in eosinophilic cytoplasmic granules (Figure 1) in weaned juvenile (Groups III) and young adult pigs (Group IV). It is believed that this is strong proof of the effect of weaning on the exocrine moiety of the pancreas. In order to corroborate whether the abundant eosinophilic granules noted in juvenile weaned and young adult porcine pancreata were indeed due to the presence of greater exocrine enzyme reserves in the form of inactive ‘zymogens’ in the acinar tissue cytoplasm, tissue sections were stained using Periodic Acid Schiff (PAS) as it has been reported that exocrine enzyme vesicles in acinar tissue

stain positive for PAS, while endocrine hormone vesicles do not (24). The results demonstrated abundant PAS positive granules in the cytoplasm of exocrine acinar cells in juvenile weaned and young adult pigs while the cytoplasm of neonatal and juvenile porcine pancreases showed scant PAS positive granules (Figure 2).

#### *Zymogen Fluorimetry on Hematoxylin & Eosin Sections:*

Quantitative evaluation of zymogen granule fluorescence (Figure 3) measured after histological staining with H&E demonstrated that neonatal ( $1.2 \pm 0.01 \text{U}/\mu\text{m}^2$ ) and pre-weaned juvenile pigs ( $1.5 \pm 0.04 \text{U}/\mu\text{m}^2$ ) demonstrated significantly lower fluorescent signal intensity compared to weaned juvenile pigs ( $2.2 \pm 0.09 \text{U}/\mu\text{m}^2$ ) and young adult pigs ( $2.4 \pm 0.05 \text{U}/\mu\text{m}^2$ ). Results of statistical analysis are tabulated below (Table 2).

#### *Ultrastructural Analysis of the Exocrine Pancreas*

Evaluation of the ultrastructure of pancreatic exocrine acinar cells (Figure. 4) demonstrated a significantly lower density of zymogen granules in neonatal ( $0.82 \pm 0.03 \times 10^3/100 \mu\text{m}^2$ ) and pre-weaned juvenile pigs ( $1.03 \pm 0.07 \times 10^3/100 \mu\text{m}^2$ ) compared to weaned juvenile ( $1.44 \pm 0.07 \times 10^3/100 \mu\text{m}^2$ ) and young adult pigs ( $1.53 \pm 0.08 \times 10^3/100 \mu\text{m}^2$ ). Results of statistical analysis are tabulated below (Table 2).

#### *Immunohistochemical Analysis of the Endocrine Pancreas*

Islet cellular composition as evaluated by immunofluorescence microscopy demonstrated that neonatal porcine islets were smaller in size (Table 1), and had a significantly lower percentage of insulin positive cells than all other groups (Figure 5, Table 1). Juvenile porcine islets (pre-weaned and weaned) were significantly smaller than islets in young adult pigs ( $p=0.01$ ,



ANOVA), but their insulin content was comparable (Table 1). Results of statistical analysis are tabulated below (Table 3).

## **Discussion:**

Islet transplantation continues to be a promising curative therapy in the treatment of type 1 diabetes (T1D), but is currently relegated to last-line therapy due to a severe scarcity of human islets, inconsistency in islet yields, the need for life-long immunosuppression and unsatisfactory long-term success rates (25). Porcine islet xenotransplantation is a viable alternative as it addresses issues of tissue scarcity and **inconsistencies in the isolation procedure and islet yields**.

We have recently validated a novel, scalable method of isolating islets from juvenile pigs using gentle enzymatic digestion followed by in vitro culture over a 7-10 day period that avoids the need for purification using disruptive gradients while consistently preserving islet viability and providing excellent yields (16). On a per gram basis, using our isolation protocol, we are able to achieve yields upwards of 4000IE/g of pancreas, much higher than those reported from young adult pigs (26). Although several juvenile pigs would be required to attain the same yields as a young adult pig, the economics of producing suitable mature adult, retired breeder donor pigs suitable for pancreas harvest easily outweighs these benefits (14). Although young adult porcine islets release higher levels of insulin in response to glucose, they are friable and fragment easily.

While porcine islet xenotransplantation is still in its experimental stages, centers all over the world have reported successful reversal of hyperglycemia in non-human primate recipients (27) and some have even commenced trials in human patients (28). Valdes-Gonzalez R et al. reported that type 1 diabetic patient that received porcine islet xenografts (specifically neonatal porcine islets) showed minimal post-operative complications (29). Research on porcine embryonic

pancreatic tissue transplantation, suggests that the gestational age of the sow from which the embryos are harvested might play a role in the outcome of the procedure (30). However, several factors still impede clinical translation of this promising therapeutic modality, one of which is the concern that zoonotic diseases could spread from pig donors to human recipients after islet transplantation. Elliott RB et al. reported no evidence of pig virus transmission into human recipients from a trial of 14 T1D patients that received porcine islet xenografts between 2009 and 2014 in New Zealand (28). Other concerns include the need for complicated immunosuppressive regimens with severe and often unpredictable adverse effects is one of the primary factors that stands between xenotransplantation and trials in human patients (31), if islet yields and function can be improved further, the impetus to clinical translation will be greater.

Several researchers have evaluated strategies to improve islet yields in porcine donors. In a study of 68 miniature pigs, Kim H et al reported that male gender and a positive history of pregnancy in females improved islet isolation outcomes (A). Bottino et al reported that islets from retired breeder pigs ( $\geq 2$  years) offered higher islet yields and intact islet morphology (B). However, the economics of housing donors for over 2 years makes them less suitable, and significantly impacts scalability of the entire process. Kim JH et al reported that Chicago-Medical School miniature pigs demonstrated consistently high islet yields ( $9589 \pm 2823$  IEq/g) compared to controls (C). Loganathan G et al reported that weaned pigs fed on a soybean oil enriched high-fat diet demonstrated higher islet yields ( $1578 \pm 994$  IEq/g) compared to pigs weaned on a normal diet (D). While these studies provide valuable insights into the various factors that trigger endocrine maturation and improve islet yields, few studies have been performed to evaluate the effect of weaning on exocrine maturity and its impact on islet yields.

One of the main factors that significantly reduces post-isolation islet yield and function is the activation of endogenous exocrine pancreatic enzymes during the islet isolation procedure leading to detrimental effects on the islets themselves (32). While selected enzyme inhibitors have been able to address this issue partially, several studies conducted over the last decade have attempted to discover an association between pig weaning status, feed composition and exocrine enzyme activity (33-36), as well as an association between donor age and enzyme activity (37, 38). Research has even delved into understanding the complex interactions between weaning, age, and enzyme activity (39-41). However, many of these studies were not interested in the applications of these results to islet isolation and xenotransplantation but rather towards understanding the effect of various dietary regimens on the content and volume of pancreatic enzyme secretions. Unlike previously published research, this study is the first to evaluate the role of weaning and porcine age on exocrine and endocrine maturity for the purpose of identifying the optimal age and weaning status of prospective islet donors for successful islet isolation and xenotransplantation

We have demonstrated the utility of zymogen fluorometry and granule density analysis in evaluating exocrine maturity and have demonstrated that after weaning, juvenile pigs show a rapid increase in pancreatic mass which is associated with a significant increase in pancreatic exocrine enzyme content. Thus, our hypothesis that pre-weaned juvenile porcine pancreata have depleted or inactive enzyme reserves, was supported by the results of this study.

Using immunofluorescence microscopy, we were able to evaluate the endocrine content of porcine islets from pancreas sections obtained from pigs of different age groups. We were able to evaluate islet morphology and insulin content and have reported that while islets in juvenile pigs

(weaned or pre-weaned) were significantly smaller than young adult porcine islets, the difference in their insulin content was not statistically significant [ $p=0.4$ , NS, ANOVA].

Recently, we have reported that when compared with neonatal and young adult pigs; pre-weaned juvenile pigs provided the highest, consistent yields of highly pure, viable islets that were able to respond satisfactorily to an in vitro glucose challenge (16). We then reported that islets from juvenile pigs isolated using our simple, scalable isolation method were able to reverse streptozotocin-induced hyperglycemia in kidney capsule of diabetic athymic nude mice for a period of 60 days (42). By selecting donor pigs of the appropriate age and weaning status, we are able to achieve consistently high yields of viable, functional islets for the purpose of islet xenotransplantation. Our method of isolation avoids disruptive purification gradients and allows for maturation of the islets during extended tissue culture during which the acinar tissue is gradually sloughed off to reveal intact, viable and functional islets. Islet isolation from juvenile pigs allows for scalability due to the consistency of yields, simplicity of the isolation procedure, and the availability of large litters of piglets with every farrowing. At our research facility, we have process as many as 25 pancreases from juvenile pigs at hour period, yielding more than 1million viable islets after 7 days of in vitro maturation in tissue culture (unpublished data).

## **Conclusion**

Pre-weaned juvenile pigs (15-20 days old) demonstrate tissue characteristics ideal to achieve good yields of viable, functional islets using our simple, scalable islet isolation method; a relatively immature, quiescent exocrine component with an endocrine component that is able to mature rapidly during in vitro culture. Comparing the exocrine and endocrine components of the

pancreas in pigs of different ages elucidates the developmental changes triggered in the exocrine pancreas and in *islets of Langerhans* with age and weaning.

The pancreas in juvenile pigs demonstrates compact islets with insulin content comparable to that in young adult pigs; islets sourced from juvenile pigs may thus be a promising source for transplantation into non-human primates and humans in clinical trials as treatment for type I diabetes. We believe that by identifying donor parameters that can help maximize porcine islet yield, function and viability, we will be able to optimize transplantation protocols for future xenotransplantation trials. In addition to evaluating pancreases from retired breeder pigs, specific studies examining the exocrine tissue from porcine pancreata and measuring enzyme levels and cytokines in culture media will also help elucidate the mechanisms behind our observations. We will also attempt to expand our work based on these initial observations to further improve islet yields with the goal of achieving consistent numbers of viable, functional porcine islets to support translation of our efforts to trials in non-human primates and human patients.

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## Figure Legends

**Figure 1. A Comparative analysis of Hematoxylin & Eosin (H&E) sections of pancreas from the four animal groups studied under a brightfield microscope.** Group I (A). Group II (B). Group III (C). Group IV (D). White arrows denote the islets. Scale = 200 $\mu$ m.

**Figure 2. A Comparative analysis of Periodic Acid Schiff (PAS) sections of pancreas from the four animal groups studied under a brightfield microscope.** Group I (A). Group II (B). Group III (C). Group IV (D). Note how islets in neonatal and pre-weaned juvenile pigs are hard to distinguish from surrounding exocrine tissue; islets in weaned juvenile and adult pigs are easily distinguished by a lack of intense eosinophilic cytoplasm (mature zymogen granules) seen in the surrounding exocrine tissue. White arrows denote the islets.

**Figure 3. A Comparative analysis of Hematoxylin & Eosin sections of pancreas from the four animal groups studied under a fluorescent microscope after excitation using a 488 nm HeNe Laser.** Group I (A). Group II (B). Group III (C). Group IV (D). Note the gradual increase in the intensity of the fluorescent signal with increasing donor age and weaning status. Scale = 200 $\mu$ m.

**Figure 4. A Comparative analysis of ultramicroscopic sections of pre-weaned and weaned porcine pancreas.** A representative section of the exocrine acinar tissue in 18-20 day old pre-weaned juvenile pigs (Group II) (A). A similar section from an adult porcine pancreas (Group IV) (B). Electron dense zymogen granules are seen in the apical cytoplasm of the acinar cells (Red arrows). Note the increase in zymogen granule size and number with an increase in donor age and change in weaning status. Magnification 5600x.

**Fig 5. Characterization of Porcine Islet Endocrine Composition.** Characterization of porcine pancreatic islet cellular composition using fluorescence immunohistochemistry. Neonatal porcine islets demonstrate a lower incidence of insulin positive cells (green) compared to juvenile and adult pigs and are significantly smaller in size ( $p=0.001$ ) (A). Juvenile porcine islets (weaned & pre-weaned) are significantly smaller ( $p=0.03$ , pre-weaned;  $p=0.04$ , weaned) but do not demonstrate significant differences in insulin content ( $p=0.4$ , pre-weaned;  $p=0.1$ , weaned). Glucagon positive cells stain red. A blue counterstain (DAPI) is used to stain the nuclei.

