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Review Article

A review of piscine islet xenotransplantation using wild-type tilapia donors and the production of transgenic tilapia expressing a "humanized" tilapia insulin

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Abstract: Most islet xenotransplantation laboratories have focused on porcine islets, which are both costly and difficult to isolate. Teleost (bony) fish, such as tilapia, possess macroscopically visible distinct islet organs called Brockmann bodies which can be inexpensively harvested. When transplanted into diabetic nude mice, tilapia islets maintain long-term normoglycemia and provide human-like glucose tolerance profiles. Like porcine islets, when transplanted into euthymic mice, they are rejected in a CD4 T-cell-dependent manner. However, unlike pigs, tilapia are so phylogenetically primitive that their cells do not express α (1,3)Gal and, because tilapia are highly evolved to live in warm stagnant waters nearly devoid of dissolved oxygen, their islet cells are exceedingly resistant to hypoxia, making them ideal for transplantation within encapsulation devices. Encapsulation, especially when combined with co-stimulatory blockade, markedly prolongs tilapia islet xenograft survival in small animal recipients, and a collaborator has shown function in diabetic cynomolgus monkeys. In anticipation of preclinical xenotransplantation studies, we have extensively characterized tilapia islets (morphology, embryologic development, cell biology, peptides, etc.) and their regulation of glucose homeostasis. Because tilapia insulin differs structurally from human insulin by 17 amino acids, we have produced transgenic tilapia whose islets stably express physiological levels of humanized insulin and have now bred these to homozygosity. These transgenic fish can serve as a platform for further development into a cell therapy product for diabetes.

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Key words: bio-artificial pancreas – Brockmann body – cell transplantation – diabetes – encapsulation – insulin – pancreatic islet – teleost fish – transgenic fish – xenotransplantation

Abbreviations: BB, Brockmann body; BBs, Brockmann bodies; GLP, glucagon-like peptide; HAR, hyperacute rejection; IVC, inferior vena cava; NPIs, neonatal porcine islets; DPF, designated pathogen-free; SST, somatostatin; STZ, streptozotocin.

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Mammalian species have very large numbers of very small islets scattered among the exocrine pancreas, comprising about 1% of its total volume; this makes mammalian islets expensive and tedious to isolate. In stark contrast, bony fish (or teleosts) have much smaller numbers of very large islets existing as distinct islet organs called Brockmann bodies (BBs; Fig. 1). Because BBs are macroscopically visible and simple to harvest, they played an important role in the discovery of insulin and have even been used sporadically to extract insulin for treatment of patients with diabetes [1,2].

Since 1991, our laboratory has used tilapia (*Oreochromis niloticus*), a large, commercially important warm freshwater teleost species, as a source of islets for xenotransplantation research [3,4]. We and others have shown that tilapia islets transplanted under the kidney capsules of

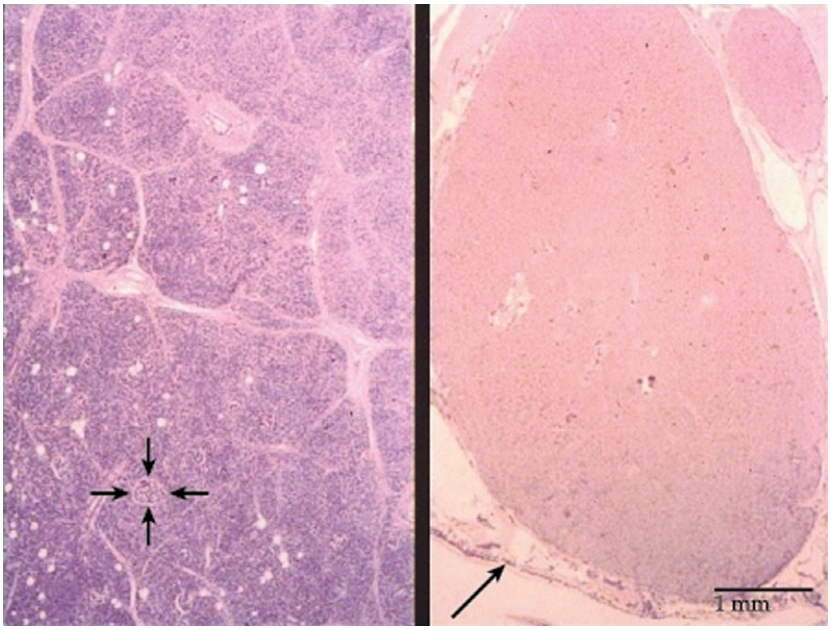

Fig. 1. Left. Histologic section of pig pancreas showing scattered islets (arrows indicate the largest islet). Histologic sections showing a large (center) and small (right upper corner) tilapia Brockmann body at the same magnification as the section of pig pancreas. Note the minimal exocrine contamination (arrow) admixed with the adipose tissue surrounding the BBs which digests away during the harvesting procedure (hematoxylin and eosin). Reprinted with permission from Wright JR Jr, Yang H. Tilapia Brockmann bodies: an inexpensive simple model for discordant islet xenotransplantation. Ann Transplant 1997; 2(3): 72–76.

streptozotocin (STZ)-diabetic athymic nude mice provide long-term normoglycemia and mammalian-like glucose tolerance profiles [3,5,6]. When transplanted into euthymic mice, tilapia islet grafts reject functionally in about a week [7], and rejection, characterized histologically by massive infiltration of macrophages, eosinophils, and T-cells [8], is temporally and mechanistically similar to rejection of pig or human islets [9]. Because of the low cost of isolating tilapia islets, it has for many years proven to be an excellent model to study islet xenograft rejection between discordant species [10–12].

Tilapia islets are more versatile than mammalian islets. Non-encapsulated tilapia islets can be transplanted under the kidney capsule [3], via the portal vein [13], and into the cryptorchid or non-cryptorchid testes [14]. When islets are transplanted into any of these sites, the grafts undergo neovascularization, one of the features that makes islet transplantation fundamentally different from directly vascularized whole organ grafts. Because BBs exist as multiple tiny discrete islet organs interconnected via an arterial and venous vascular pedicle and vascular "tree", it is actually possible to transplant them in athymic nude mice as immediately vascularized cluster grafts by microvascular surgical techniques [15], creating the unique ability to compare islet graft rejection as either neovascularized cell transplants or directly vascularized organ grafts. This is fascinating in the context that tilapia cells do not express $\alpha(1,3)$ gal, an antigen expressed on cells of most mammalian species that precipitates hyperacute rejection (HAR) [6]. Interestingly, like with our fish-to-rat heterotopic cardiac transplant model created to prove that fish-to-rodent is a discordant combination [16], directly vascularized BB cluster grafts hyperacutely reject in a matter of hours (see below). BBs are also unique in that they function after transplantation into the non-cryptorchid (i.e., intrascrotal) testis; in contrast, mammalian islets only function after intratesticular transplantation if the testis is raised into the abdominal cavity, thus raising the graft's ambient temperature to normal body temperature. Presumably, this difference is because fish are poikilotherms and, thus, their islets are fully functional at a wider range of body temperatures [14]. Another versatile feature is that tilapia islets function immediately after transplantation. Many xenotransplantation laboratories now use neonatal porcine islets (NPIs) [17,18] because adult porcine islets are difficult to isolate reproducibly [19]; while this solves the islet isolation problem, it creates a new one, as NPIs must mature for weeks or months after transplantation; therefore, function cannot be monitored to determine rejection. Tilapia islets are also better suited for encapsulation because they are exceedingly hypoxia resistant [20]. Finally, tilapia islets are essentially impervious to the β-cell toxins alloxan and STZ [21–23]. While all of these features support the use of tilapia islets, the primary advantage is the ease of harvesting BBs.

Harvesting tilapia islets

Harvesting tilapia islets is exceedingly simple and reproducible. Unlike isolating mammalian islets, there is no need to "inflate" pancreatic ducts with

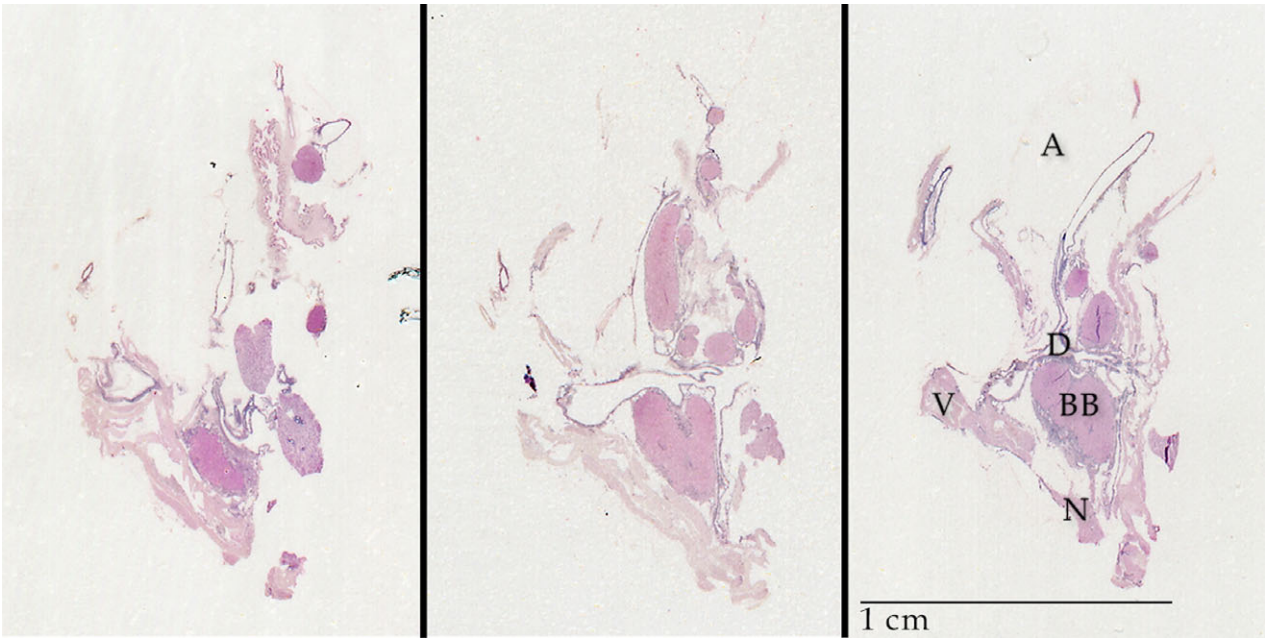

Fig. 2. Whole mount produced by processing an entire "BB region" for histology. Sections were cut at three different levels through the block to provide a three-dimensional view. Sections were stained with hematoxylin and eosin. The regions are composed of adipose tissue (A), bile and pancreatic ducts (D), blood vessels (V), nerve (N), and Brockmann bodies (BB). Twelve BBs can be identified in the center frame. Reprinted with permission from Yang H, Wright JR Jr. A method for mass-harvesting islets (Brockmann bodies) from teleost fish. Cell Transplant 1995; 4: 621–628.

expensive blends of special collagenases and there are no complicated, time-consuming islet isolation procedures with variable, unpredictable outcomes. Tilapia BBs are scattered within fat tissue surrounding the common bile duct (Fig. 2) in a triangular region bounded anteriorly by the edge of the liver, superiorly by the stomach, and inferiorly by the spleen and gall bladder (Fig. 3A,B). A layperson with no scientific training can be taught to identify and excise this "BB region" with <10 min of training.

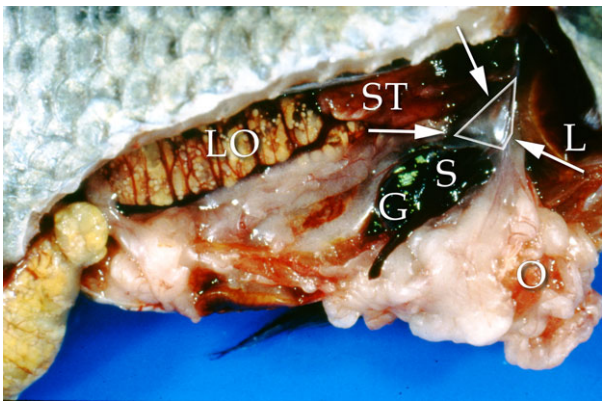

Fig. 3. Dissection of female tilapia with the right ovary and omentum (O) reflected downward reveals the roughly triangular "Brockmann body (BB) region" (outlined by arrows and lines) surrounded by the liver (L), stomach (ST), and spleen (S) and gall bladder (G). LO = left ovary. Reprinted with permission from Yang H, Wright JR Jr. A method for mass-harvesting islets (BBs) from teleost fish. Cell Transplant 1995; 4: 621–628.

There are two methods to harvest tilapia BBs. The "manual method", which we used when we began two decades ago, focuses only on procuring the larger BBs, which in large tilapia can measure up to 5 mm in maximum dimension. Larger BBs can be removed simply by excising the entire "region", placing it in a plastic Petri dish with Hank's balanced salt solution (HBSS), and microdissecting them from the fat surrounding the common bile duct using a dissecting microscope [24,25]. This method is fine for a few transplants in mice but is relatively slow and inefficient (i.e., misses the smaller islets). Although much of the islet biomass in each tilapia resides in the larger islets, it should be noted that tilapia produce new islets and their older islets grow throughout their life span and so there is a tremendous range in islet size [26].

The second method (i.e., enzymatic mass harvesting) allows one to collect large and small BBs. BB regions are removed from multiple fish simultaneously and sequentially placed in a tube of HBSS on ice. Sufficient BB regions (see below) are then placed in a tube with a warm type II (normally used to harvest adipocytes) or type VII collagenase solution and placed in a 37 °C shaker water bath for 10 min with moderate shaking. The digestion is

stopped by adding cold HBSS, causing the fat cells to float to the top of the tube and BBs to remain as a pellet [27].

Regardless of which method is used, large BBs are chopped with scissors into smaller fragments prior to transplantation [28]; the amount of chopping is determined by the transplantation route (i.e., "mammalian islet"-sized fragments are required for intraportal vein transplants, whereas larger fragments can be transplanted under the kidney capsule or into the testis). After overnight culture (37 °C/5% CO_2), these fragmented BBs "round up" and then, viewed with an inverted tissue culture scope, take on the appearance of slightly oversized mammalian islets. Fragmented islets can be transplanted immediately, cultured under various conditions [29], or cryopreserved in liquid nitrogen [30]. Remarkably, fragmentation does not affect the cellular composition or function of the islets because the large islets are comprised of repetitive units of the four major cell types [31]. There is a linear relationship between fish body weight and the number of islet endocrine cells [32]; therefore, the sum of the body weights of multiple donor fish can be used to predict the total islet cell mass as well as the number of transplants that can be performed [12]. Unlike isolating mammalian islets, it is not possible to calculate yield in islet equivalents per gram of donor pancreas, as there is no pancreas to weigh and the BB region is not a discrete organ that can be weighed. In experienced hands, the yield is not variable and approaches 100% of the islet biomass, at least until it is chopped. After isolation of mammalian islets, one typically needs to test viability and functionality (e.g., stimulation index). Although fish islet viability can be tested with standard methods such as ethidium bromide–fluorescein diacetate staining [20], we do not do this as, in our hands, the harvesting method is so simple and non-traumatic that viability is not a variable. The best measure of functionality is to transplant a "unit" (see below) of BB tissue under the kidney capsule of a diabetic nude mouse. This is important when learning to harvest BBs but is not necessary once one is experienced with the model.

Because there is a linear relationship between donor body weight and islet endocrine cell number [32], one can harvest sufficient islet tissue by simply weighing donor fish. A transplantable "unit" of BBs is the equivalent of all of the chopped BB tissue from an 800 g donor tilapia (~1.5 million islet endocrine cells) and is sufficient to assure long-term normoglycemia after transplantation under the kidney capsule of a 25–30 g STZ-diabetic nude mouse. The "unit" can be derived from one 800 g tilapia, two 400 g tilapia, half of the islet tissue from a 1600 g tilapia, or one-tenth of the total islet tissue harvested from multiple donors with an aggregate body weight of 8000 g. Although we tend to use donors weighing between 500 and 1000 g, this linear relationship holds for donors between 100 and 2000 g [32].

Xenograft rejection

At the time we began this work 23 yr ago, the vast majority of studies on islet xenograft rejection had been performed using the concordant rat-to-mouse model and many of these studies implied that islet xenograft rejection could be prevented by simple culture methods, depleting passenger leukocytes, or short-term immunosuppression. However, when these methods were applied to discordant species combinations (or even mouse-to-rat), most conferred little real protection (n.b., world literature prior to 1992 is comprehensively reviewed in [33]). Initially, we used tilapia BBs as an inexpensive tool to screen previously published methods which had been reported to prevent concordant islet xenograft rejection.

Intuitively, it seemed hard to imagine any species combination that would be more discordant than fish-to-mammal by virtue of the several 100 million yrs separating these orders phylogenetically, but we did eventually have to prove it using a tilapia-to-rodent heterotopic heart transplant model [16]. The "gold standard" for determining whether xenotransplantation between species is discordant is demonstration of HAR after directly vascularized whole organ transplantation. The literature was not helpful as we could not identify anyone who had performed a fish-to-rodent organ transplant. Because of the large difference in blood pressure between the donor and recipient animals, we transplanted tilapia hearts heterotopically on the venous side of the circulation by end-to-side anastomosis of the donor aorta to the recipient inferior vena cava (IVC) and end-to-end anastomosis of the donor sinus venous (i.e., vascular inlet to the fish atrium) to the recipient left renal vein (n.b., the venous oxygen tension in a mammal far exceeds the arterial oxygen tension in a tilapia and so hypoxia was not an issue [20]). We were able to show a mean graft function >8 h and a maximum graft function up to ~20 h. Histologic examination at the time of rejection shows unequivocal HAR characterized by thrombosis, fibrin disposition, hemorrhage, edema, massive neutrophilic infiltration, and in some instances multifocal infarction. Immunofluorescent staining demonstrated dense

deposits of rat IgM and rat C3 on graft endothelium as well as less dense deposits of rat IgG [16]. We later developed a method to perform directly vascularized BB transplants in athymic nude mice, which confirmed that tilapia-to-mouse is a discordant combination. In these transplants, the vascular pedicle supplying and draining the BB region was isolated by a microvascular surgeon and other BB region structures were excluded with the aid of an operating microscope. Once again, grafts were placed on the venous side of the circulation to avoid marked "graft hypertension". The BB cluster graft was interposed between the recipient left renal vein and the recipient IVC by microvascular anastomosis of the graft common mesentery artery to the distal end of the mouse renal vein and the anastomosis of the graft common mesentery vein to the mouse IVC below the renal veins. Histologic evaluation at 3 h post-transplantation showed marked congestion, extensive fibrin thrombi in BB capillaries, neutrophils in vessel walls, and interstitial hemorrhage while at 12 h showed extensive BB graft coagulative necrosis and early venous infarction of the recipient kidney. C3, IgM, and IgG deposits were identified on graft endothelial cells. Treatment with cobra venum factor was protective as identical BB cluster grafts showed only mild congestion and no evidence of HAR at 3 or 12 h [15].

As alluded to above, our initial work with the tilapia-to-mouse model focused on testing various modalities for their ability to prevent islet xenograft rejection. This work has been reviewed elsewhere [10,11]. In general, we found that methods directed at decreasing graft immunogenicity prior to transplantation (e.g., various culture protocols, cryopreservation, etc. [29,30]) were totally ineffective but that chronic high-dose pharmacologic immunosuppression (e.g., cyclosporin-A, leflunomide, 15-deoxyspergualin, tacrolimus) was reasonably effective at prolonging islet xenograft survival for up to a month or rarely more [10,11]; however, if the high-dose immunosuppression was stopped, the BB grafts rejected immediately. Furthermore, we found instances in which high-dose immunosuppression induced post-transplant lymphoproliferative disorder in mice with functional grafts [34]. After these studies, our primary focus changed to encapsulation, studying piscine islet physiology/glucose homeostasis, and genetic engineering.

Xenotransplantation with encapsulation

Encapsulation devices are small semipermeable chambers designed to surround islet grafts and protect them from the host's immune system [35,36]. These devices achieve a degree of immunoprotection by creating a barrier with "pore" sizes small enough to prevent leukocytes and antibodies from damaging the graft but large enough for oxygen, insulin, glucose, and nutrients to pass freely. Although encapsulation devices have real and theoretical problems, probably the most significant one is that hypoxia causes graft attrition over time. Relative to mammalian islets, tilapia BBs are exceedingly resistant to hypoxia [20] making them ideal for encapsulation. We have previously shown that calcium alginate encapsulation markedly prolongs BB xenograft survival in small animal recipients with STZ-induced diabetes [37,38] and that co-encapsulation with allogenic or xenogeneic Sertoli cells prolongs graft survival even further [39]. We also found that the protective effect of Sertoli cells is not mediated by their Fas-ligand expression because Fas-L-deficient Sertoli cells still conferred protection [40].

However, like with mammalian islet xenografts, encapsulation did not significantly prolong BB graft survival in spontaneously diabetic NOD mice. Although empty capsules transplanted intraperitoneally were fully biocompatible, calcium alginate capsules containing BB tissue evoked a massive peritoneal infiltrate of mostly macrophages and eosinophils which quickly enveloped each capsule. Like with unencapsulated BBs [9], immune destruction of encapsulated BBs was CD4 dependent [41]. Very recently, using co-stimulatory blockade combined with barium alginate microencapsulation, we have shown prolongation of BB graft function in excess of 150 days in spontaneously diabetic NOD mice [42].

In 1994, Dionne et al. described the "ideal" islet for encapsulation as follows. "The ideal tissue has a high insulin output, is correctly regulated by glucose and other secretagogues, has low metabolic demand, and is capable of functioning for extended periods without replacement. In addition, the cells must be procurable in high yield at reasonable cost with protocol meeting FDA standards" [43]. Tilapia BBs meet these criteria, but we would further add that the ideal tissue should secrete human insulin.

Potential clinical islet xenotransplantation using tilapia BBs

We began our BB transplantation research expecting to use tilapia simply as an inexpensive donor source of islets from a discordant species that could be used to study xenograft rejection. The limited literature on piscine islet physiology at that time led us to believe that BBs would not be highly

glucose responsive. We fully expected them to behave sluggishly after xenotransplantation. However, we soon discovered that tilapia BBs were as glucose responsive as rat or mouse islets, as documented by transplantation of equal volumes into STZ-diabetic nude mice and then comparing glucose tolerance testing results [28]. We quickly became enamored with the tilapia BB's ability to provide long-term normoglycemia and human-like glucose tolerance profiles in mice and since that time have speculated that they could be used for future clinical islet xenotransplantation—mostly likely as part of an encapsulation device, for which they are uniquely suited because of their extreme hypoxia resistance [20]. However, there are some significant issues/barriers.

Issue 1: Glucose homeostasis and β-cell function in tilapia

Before one could seriously consider transplanting tilapia BBs into humans, tilapia glucose homeostasis and β-cell function would need to be examined. Although tilapia and humans have extremely similar fasting (75.4/63 mg/dl) and non-fasting (91.9/90 mg/dl) blood glucose levels [44], at the time we began our piscine islet xenotransplantation studies, little was known about piscine glucose homeostasis. In fact, only a few obscure fish species consume glucose as a significant component of their natural diets, and it was dogma that fish islets were not glucose responsive [44,45]. However, the following tilapia BB xenotransplantation study disproved this. Glucose tolerance tests were performed in intact tilapia. Tilapia BBs were then transplanted into STZ-diabetic nude mice. While it took ~3 days for tilapia to dispose of glucose loads, thus confirming extreme glucose intolerance as in other fish species, mice bearing established tilapia BB grafts disposed of an equivalent glucose load in <30 min, demonstrating that tilapia BB insulin secretion was highly glucose responsive and that piscine glucose intolerance was a result of an extreme peripheral resistance to the glucostatic effects of insulin; interestingly, the glucose tolerance profiles of these recipient mice demonstrated significantly better glucose responsiveness than the responses seen in non-diabetic nude mice that had not been treated with STZ [46]. We have since confirmed the glucose responsiveness of tilapia islets in vitro and have dissected the regulation of insulin gene expression and insulin production in tilapia islets [47]. Interestingly, tilapia brain and pituitary also express insulin [48].

As in mammalians, the glucose sensor in the tilapia β-cell is glucokinase [49]. To maintain glucose homeostasis, the sensor must partner with a glucose transporter. In rodent islets, the primary transporter is GLUT-2, but in human islets, it is GLUT-1. Both of these transporters are highly expressed in tilapia BBs ([50]; unpublished data); however, circumstantial evidence favors GLUT-1. STZ and alloxan, known to enter β-cells via GLUT-2, are highly toxic to rodent β-cells, while human β-cells, which preferentially utilize GLUT-1, are highly resistant to both drugs. Tilapia β-cells are also exceedingly resistant to the diabetogenic effects of STZ and alloxan [21–23,45].

Issue 2: Islets are composed of multiple endocrine cell types: What about the other islet peptides?

Islets are comprised of multiple cell types and produce hormones other than insulin. In the context of xenotransplantation, it is possible that these foreign peptides could either have undesirable biological activity or serve as antigens promoting immune complex formation.

Like mammalian islets, tilapia BBs mostly consist of insulin-producing β-cells, glucagon-producing α-cells, and somatostatin (SST)-producing δ-cells, but there are significant differences. First, the percentages of the cell types differ. In mammalian islets, the β-cells represent ~70%, α-cells represent ~20%, and δ-cells comprise <10%; in tilapia, the percentage of β, α, δ-1, and δ-2 cells are 42.3, 11.5, 21.8, and 23.1% [31]. Second, BBs possess two different δ-cells, one producing SST-1, a 14-amino acid peptide the sequence of which is identical in all vertebrates, and the other a "large" SST, the product of the piscine preproSST-II gene which is not seen in mammals. Third, piscine α-cells simultaneously produce glucagon and glucagon-like peptide (GLP)-l, while in mammals, GLP-1 is produced in the intestinal L-cells (unlike mammals, fish do not make GLP-2). Fourth, islet topography is different. In mammals, most islets are composed of a central core of β-cells surrounded by a peripheral mantle of non-β-cells; in contrast, tilapia BBs are comprised of many repetitive units containing a central core of β-cells surrounded by a thin layer of SST-1 δ-cells which are surrounded by SST-2 δ-cells; α-cells are interspersed [31]. Because of this highly repetitive nature, fragmented BBs contain all four major cell types. Finally, the fourth mammalian hormone is pancreatic polypeptide, while in fish, it is Peptide-YY; these cell types each represent 1–2% of islet parenchymal cells in their respective taxonomic classes.

These differences have potential relevance for xenotransplantation. First of all, based upon structure and/or functional data, fish insulins, glucagons, GLP-1s, and SST-1 should all be biologically

active in man. In fact, tilapia SST-1 is 100% homologous. On the other hand, tilapia's large SST would be biologically irrelevant and likely antigenic; if it were to be secreted, it could potentially precipitate immune complexes. However, after xenotransplantation into STZ-diabetic nude mice, the SST-2 secreting δ-2-cells decreased from roughly 25% to negligible numbers in <2 months, apparently due to apoptosis secondary to the lack of any piscine trophic stimulation [51]. Furthermore, after transplantation into a mammalian environment, the percentage of the various endocrine cell types in the grafts became increasingly mammalian-like at each time point examined [51]. Finally, after >2 months, the graft α-cells continued to express ample GLP-1 (unpublished data), a peptide which promotes growth of endogenous β-cell mass in mammals.

Issue 3: Tilapia insulin structure

Although fish insulins are functional in humans [1,2], their amino acid sequences and relative biological activities differ from that of human insulin. Tilapia insulin structure differs from human insulin by 17 amino acids [52], and we suspected this would preclude using tilapia as clinical donors. Therefore, we produced transgenic tilapia expressing a "humanized" tilapia insulin gene by cloning and sequencing the tilapia insulin gene including the 1.5 kb promoter/enhancer region [53], "humanizing" the gene by site directed mutagenesis (i.e., changing only the codons representing the 17 discrepant amino acids), and then microinjecting fertilized tilapia eggs at the near single cell stage via the micropile with the "humanized" tilapia insulin transgene [54,55]. The resulting hatchlings were screened by PCR using humanized tilapia insulin specific primers. One founder, later determined to be a mosaic [56], demonstrated germ line expression, and his positive offspring showed physiologic levels of human insulin in their serum. Like the expression of native tilapia insulin in wild-type tilapia, human insulin is primarily expressed in BB tissue. Histologic sections of BBs from wild-type and transgenic tilapia were stained for human insulin; there were abundant clusters of human insulin-positive β-cells in transgenic BBs and none in wild-type BBs; islet architecture (i.e., distribution of other endocrine cell types) was unchanged. Human insulin extracted and sequenced from transgenic BBs is [desThrB30] human insulin (n.b., Our transgenic human insulin is missing the terminal Thr as the terminal amino acid on the B-chain was omitted from our humanized gene to maintain appropriate cleavage by the endopeptidases). This should not adversely affect activity as porcine insulin, which differs from human insulin by substitution of an Ala for the terminal Thr in position 30 on the B-chain, is fully active.

Our transgenic tilapia were patented in the USA as a new life form in November 2002 [54]. However, the team working on tilapia BBs moved in 2005 from Halifax, where there were extensive aquatic housing facilities, to Calgary, where there were not, and for this and other reasons [57,58], it proved not possible to continue the research. One pilot study performed at that time merits brief mention. A small collaborative study in which BBs harvested and encapsulated in Halifax, shipped to Chicago by courier, and transplanted into STZ-diabetic immunosuppressed cynomolgus monkeys demonstrated that non-transgenic tilapia islets secreting native tilapia insulin function in this primate species [59]. Since that time, we have breed our transgenic tilapia to homozygosity [60] and have now demonstrated lifelong transgene expression [61].

The β-cells in our transgenic tilapia currently co-express both humanized and tilapia insulin, and it may eventually be desirable to knockout the native tilapia insulin gene. For many years, the gold standard was to first knockout the gene using embryonic stem cells. This required us to characterize tilapia embryogenesis [62,63] and also develop a methodology to make chimeras by microinjecting blastula cells [58]. Recently, many newer methods exist for genome modification (e.g., ZFN, TALEN, and CRISPR) that are simpler and faster [64]. The use of CRISPR in zebrafish is now well established (8 papers in 2013!) and can be used quickly and efficiently to knockout multiple genes simultaneously including biallelic loci [65]. We do not expect that silencing the native insulin gene would be lethal as "isletectomized" fish thrive on bovine insulin [66].

Issue 4: Fish have two non-allelic insulin genes

After we began our transgenic studies, it became clear that ancestral fish underwent a genomic duplication several 100 million yrs ago and that many or all modern-day fish possess a second non-allelic insulin gene [67]. It is believed that genome duplication played a major role in evolution as natural selection acting on necessary genes cannot explain "big leaps" which required redundant gene loci that could accumulate forbidden mutations [68]. Although little is known about the insulin-2 gene in fish, such duplication is not unique as it has been known for over three decades that rat and mouse islets express two non-allelic insulin genes [69].

To determine whether tilapia have two non-allelic insulin genes, degenerative primers were designed based upon the alignment of the available sequences for fish insulin 2 genes. We cloned most of the NTins2 (Nile tilapia insulin 2) gene and studied tissue-specific expression. Insulin 2 gene expression occurs in essentially all tilapia tissues (including BBs) at exceedingly low levels [70]. Therefore, the insulin 2 product may not need to be silenced in transgenic tilapia islets.

It may also not be necessary to silence the native tilapia insulin gene, at least from the perspective of function, as Scatchard plots show that the binding of purified tilapia insulin to isolated human insulin receptors is surprisingly almost identical to that of human insulin (unpublished data). Therefore, it is also possible that there is no need to knockout the native insulin gene in our transgenic fish or even that wild-type tilapia could serve as clinical donors.

Economic advantages of tilapia BBs as a cell therapy product and as an experimental model

We published a very detailed cost projection in 2004, which is now somewhat "dated" as some of the regulations related to housing of porcine source animals have changed; nevertheless, we believe that, based upon animal husbandry and islet procurement costs, transgenic tilapia BBs can still be conservatively estimated to be at least 100-fold less expensive than adult porcine islets on a per clinical transplant basis [12]. Regulatory agencies will require that source animals for xenotransplantation be raised in indoor factories under designated pathogen-free (DPF) conditions; therefore, animal husbandry per diem costs will comprise a very significant component of total islet costs. Compared to pigs, tilapia are 2.5-fold more efficient at converting food into body mass (this results in savings on both food and waste management), have shorter "generation intervals" (conception to sexual maturity is 6 months vs. 1 yr), have larger litter sizes (~1000 vs. <10), have shorter minimum intervals between litters (2 weeks vs. 6 months), and require much less space for housing. We conservatively estimate per diem costs per clinical transplant, without taking into account providing DPF conditions, to be at least 30-fold cheaper. It is difficult to estimate the cost of raising both source animal species under DPF conditions, but the costs clearly favor the tilapia by a very wide margin. Pigs are prone to vertical transmission of disease, which can occur in utero, at delivery, or while nursing. In contrast, tilapia eggs are fertilized externally, develop externally, and can be chemically treated with FDA-approved antibacterial, antifungal, and antiviral agents. While it is fully recognized that these topical agents are only FDA-approved for subsequent food consumption and not for xenotransplantation source animals, no one has ever requested approval for the latter use. These agents are administered to the surface of fertilized eggs enveloped within a soft "shell" (i.e., the chorion [63]) and then are rinsed off afterward. The decontaminated eggs could then be transferred to DPF water or even sterile water to hatch. Furthermore, the surface chorion is shed when the fish hatches [62] and then the hatchlings could be transferred again to new DPF or sterile water. On a per transplant basis, providing DPF conditions for tilapia donors should be many-fold less expensive. Finally, on a per transplant basis, we estimated the islet isolation costs to be about 90-fold less for tilapia. Even if some of our assumptions or estimates are wrong or dated, the total donor costs should still be 100-fold less expensive [12].

If one is fortunate enough to have access to an appropriate aquatic animal facility, wild-type tilapia are a very economical and practical donor source for experimental islet xenotransplantation studies such as studying the mechanism of xenograft rejection [9] or whether different treatments prolong encapsulated islet xenograft function [42]. When relying upon human or adult porcine donor islets for experimental studies in murine recipients, there is a tendency toward feast or famine. Either very large numbers of islets are available, or there are none, making it difficult to plan experiments. It is much easier to plan when using tilapia BBs as one can decide how many transplants one intends to perform and then easily harvest the right amount of BB tissue on a daily basis simply by weighing donor fish [12].

Conclusions

Advantages of the tilapia islet xenotransplantation model include ease of harvesting BBs, total procurement costs, and the ability to transplant fragmented BBs into multiple body sites with immediate function. The major disadvantage is the need for specialized aquarium facilities for housing.

We have produced homozygous transgenic tilapia with islets expressing a humanized tilapia insulin gene, which after extensive characterization, could serve as a platform for further development into a cell therapy product for treatment of diabetes (see Special Comment below).

Special comment

After most of the authors moved to Calgary, the absence of an aquatic housing facility and other logistical issues precluded moving tilapia to Calgary and performing preclinical testing of the transgenic tilapia BBs. We are looking for established islet transplanters, working in institutions with aquatic housing facilities capable of maintaining large tropical freshwater fish species, who can take over this research. These fish are free to collaborators who will continue this work. We can also provide species-specific reagents.

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Authors contributions

JRW—drafting article, critical revision of article, approval of article; HY—critical revision of article, approval of article; OH—critical revision of article, approval of article; B-YX—critical revision of article, approval of article; WY—critical revision of article, approval of article; BP—critical revision of article, approval of article. All authors played critical roles in generating data over the past two decades upon which the review is based.

References

1. Wright JR Jr. From ugly fish to conquer death: JJR Macleod's fish insulin research, 1922–24. Lancet 2002; 359: 1238–1242.
2. Wright JR Jr. Almost famous: E. Clark Noble, the common thread in the discovery of insulin and vinblastine. CMAJ 2002; 167: 1391–1396.
3. Wright JR Jr, Polvi S, Maclean H. Experimental transplantation with principal islets of teleost fish (Brockmann bodies): long-term function of tilapia islet tissue in diabetic nude mice. Diabetes 1992; 41: 1528–1532.
4. Wright JR Jr. Experimental transplantation using principal islets of teleost fish (Brockmann bodies). In: Ricordi C, ed. Pancreatic Islet Cell Transplantation: 1892–1992—One Century of Transplantation for Diabetes. Austin: RG Landes Co, 1992: 336–351.
5. Morsiani E, Lebow LT, Rozga J et al. Teleost fish islets: a potential source of endocrine tissue for the treatment of diabetes. J Surg Res 1995; 1995: 583–591.
6. Leventhal JR, Sun JD, Zhang J et al. Evidence that tilapia islets do not express a(l,3) Gal: implications for islet xenotransplantation. Xenotransplantation 2004; 11: 276–283.
7. Wright JR Jr, Kearns H, Polvi S et al. Experimental xenotransplantation using principal islets of teleost fish (Brockmann bodies): graft survival in selected strains of inbred mice. Transplant Proc 1994; 26: 770.
8. Wright JR Jr, Keams H, Yang H et al. Immunophenotyping fish-to-mouse islet xenograft rejection: a time course study. Ann Transplant 1997; 2: 12–16.
9. Dickson BC, Yang H, Savelkoul HFJ et al. Islet transplantation in the discordant tilapia-to-mouse model: a novel application of alginate microencapsulation in the study of xenograft rejection. Transplantation 2003; 75: 599–606.
10. Wright JR Jr, Yang H. Tilapia Brockmann bodies: an inexpensive, simple model for discordant islet xenotransplantation. Ann Transplant 1997; 2: 72–76.
11. Wright JR Jr, Pohajdak B. Cell therapy for diabetes using piscine islet tissue. Cell Transplant 2001; 10: 125–143.
12. Wright JR Jr, Pohajdak B, Xu B-Y et al. Piscine islet xenotransplantation. ILAR J 2004; 45: 314–323.
13. Al-Jazaeri A, Xu B-Y, Yang H et al. Effect of glucose toxicity on intraportal tilapia islet xenotransplantation in nude mice. Xenotransplantation 2005; 12: 189–196.
14. Coddington DA, Lawen JG, Yang H et al. Xenotransplantation of fish islets into the non-cryptorchid testis. Transplant Proc 1997; 29: 2083–2085.
15. Yu W, Xu B-Y, Wright JR Jr. Directly Vascularized Pancreatic Islet Xenotransplantation: Is Tilapia-To-Nude Mouse Discordant? 7th International Xenotransplantation Congress, Glasgow, UK, 10/03/03 (abstract).
16. Yu W, Wright JR Jr. Heterotopic cardiac xenotransplantation: fish-to-rat. Xenotransplantation 1999; 6: 213–219.
17. Korsgren O, Jansson L, Eizirik D et al. Functional and morphological differentiation of fetal porcine islet-like cell clusters after transplantation into nude mice. Diabetologia 1991; 34: 379–386.
18. Korbutt GS, Elliot JF, Ao Z et al. Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119–2129.
19. Ricordi C, Socci C, Davalli AM et al. Isolation of the elusive pig islet. Surgery 1990; 107: 688–694.

20. Wright JR Jr, Yang H, Dooley KC. Tilapia—A source of hypoxia-resistant islets for encapsulation. Cell Transplant 1998; 7: 299–307.
21. Xu B-Y, Morrison CM, Yang H et al. Tilapia islets grafts are highly alloxan-resistant. Gen Comp Endocrinol 2004; 137: 132–140.
22. Wright JR Jr, Abraham C, Dickson BC et al. Streptozotocin dose response curve in tilapia, a glucose-responsive teleost fish. Gen Comp Endocrinol 1999; 114: 431–440.
23. Yang H, Wright JR Jr. Human beta cells are exceedingly resistant to streptozotocin in vivo. Endocrinology 2002; 143: 2491–2495.
24. Wright JR Jr. Procurement of fish islets (Brockmann bodies). In: Lanza RP, Chick WL, eds. Pancreatic Islet Transplantation Series. Volume 1: Procurement of Pancreatic Islets. Austin: RG Landes Co., 1994: 125–135.
25. Wright JR Jr, Schrezenmeir J. Transplantation of fish islets. In: Ricordi C, eds. Methods in Cell Transplantation. Austin: RG Landes Co., 1995: 533–545.
26. Morrison CM, Pohajdak B, Tam J et al. Development of the islets, exocrine pancreas and related ducts in the Nile tilapia, *Oreochromis niloticus* (Pisces: Cichlidae). J Morphol 2004; 261: 377–389.
27. Yang H, Wright JR Jr. A method for mass harvesting islets (Brockmann bodies) from teleost fish. Cell Transplant 1995; 4: 621–628.
28. Yang H, Dickson B, O'Hali W et al. Functional comparison of mouse, rat, and fish islet grafts transplanted into diabetic nude mice. Gen Comp Endocrinol 1997; 106: 384–388.
29. Wright JR Jr, Kearns H. Long-term culture, low temperature culture, and hyperoxic culture do not prolong fish-to-mouse islet xenograft survival. Xenotransplantation 1995; 2: 19–25.
30. O'Hali W, Yang H, Pohajdak B et al. Cryopreservation of fish islets: the effect on function and islet xenograft survival. Transplant Proc 1997; 29: 1990–1991.
31. Yang H, Morrison CM, Conlon JM et al. Immunocytochemical characterization of the pancreatic islet cells of the tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 1999; 114: 47–56.
32. Dickson B, Yang H, Pohajdak B et al. Quantification of tilapia islets: a direct relationship between islet cell number and body mass. Transplant Proc 1998; 30: 621–622.
33. Hering BJ. Islet xenotransplantation. In: Ricordi C, eds. Pancreatic Islet Cell Transplantation: 1892–1992—One Century of Transplantation for Diabetes. Austin: RG Landes Co., 1992: 313–335.
34. Yang H, McAlister VC, Al-Jazaeri A et al. Liposomal encapsulation significantly enhances the immunosuppressive effect of tacrolimus in a discordant islet xenotransplant model. Transplantation 2002; 73: 710–713.
35. de Vos P, Spasojevic M, Faas MM. Treatment of diabetes with encapsulated islets. Adv Exp Med Biol 2010; 670: 38–53.
36. Vaithilingam V, Tuch BE. Islet transplantation and encapsulation: an update on recent developments. Rev Diabet Stud 2011; 8: 51–67.
37. Yang H, O'Hali W, Kearns H et al. Long-term function of fish islet xenografts in mice by alginate encapsulation. Transplantation 1997; 64: 28–32.
38. Yang H, Wright JR Jr. Microencapsulation methods: alginate (Ca^{+2}-induced gelation). In: Atala A, Lanza R, eds. Methods of Tissue Engineering. New York: Academic Press, 2002: 787–801.
39. Yang H, Wright JR Jr. Co-encapsulation of Sertoli enriched testicular cell fractions further prolongs fish-to-mouse islet xenograft survival. Transplantation 1999; 67: 815–820.
40. Yang H, Al-Jazaeri A, Wright JR Jr. The immunoprotective effect of Sertoli cells co-encapsulated with islet xenografts is not dependent upon Fas-ligand expression. Cell Transplant 2002; 11: 799–801.
41. Xu B-Y, Yang H, Serreze DV et al. Rapid destruction of encapsulated islet xenografts by NOD mice is CD4 dependent and facilitated by B-cells: innate immunity and autoimmunity do not play significant roles. Transplantation 2005; 80: 402–409.
42. Safley SA, Cui H, Cauffiel SMD et al. Encapsulated piscine (tilapia) islets for diabetes therapy: studies in diabetic NOD and NOD-SCID mice. Xenotransplantation 2014; 21: 127–139.
43. Dionne K, Scharp D, Lysaght M et al. Macroencapsulation of islets for the treatment of diabetes. In: Lanza PR, Chick WL, eds. Pancreatic Islet Transplantation. Vol. 3. Immunoisolation of the Pancreatic Islets. Austin: RG Landes Co., 1994: 119–131.
44. Wright JR Jr, Bonen A, Conlon JM et al. Glucose homeostasis in the teleost fish tilapia: insights from Brockmann body xenotransplantation studies. Am Zool 2000; 40: 234–245.
45. Alexander E, Dooley KC, Pohajdak B et al. Things we've learned from tilapia islet xenotransplantation. Gen Comp Endocrinol 2006; 148: 125–131.
46. Wright JR Jr, O'Hali W, Yang H et al. GLUT-4 deficiency and absolute peripheral resistance to insulin in the teleost fish tilapia. Gen Comp Endocrinol 1998; 111: 20–27.
47. Hrytsenko O, Wright JR Jr, Pohajdak B. Regulation of insulin gene expression and insulin production in Nile tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 2008; 155: 328–340.
48. Hrytsenko O, Wright JR Jr, Morrison CM et al. Insulin expression in the brain and pituitary cells of tilapia (*Oreochromis niloticus*). Brain Res 2007; 1135: 31–40.
49. Joy P. Cloning, Sequencing, and Expression of the Tilapia Glucokinase. MSc Thesis, Dalhousie University, Halifax, Nova Scotia, Canada, 2001.
50. Hrytsenko O, Pohajdak B, Xu B-Y et al. Cloning and molecular characterization of the glucose transporter 1 in tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 2010; 165: 293–303.
51. Morrison CM, Yang H, Al-Jazaeri A et al. Xenogeneic milieu markedly remodels endocrine cell populations after transplantation of fish islets into streptozotocin-diabetic nude mice. Xenotransplantation 2003; 10: 60–65.
52. Nguyen T, Wright JR Jr, Nielsen PF et al. Characterization of the pancreatic hormones from the Brockmann body of the tilapia—implications to islet xenograft studies. Comp Biochem Physiol 1995; 111C: 33–44.
53. Mansour M, Wright JR Jr, Pohajdak B. Cloning, sequencing and characterization of the tilapia insulin gene. Comp Biochem Physiol 1998; 121B: 291–297.
54. Wright JR Jr, Pohajdak B. Transgenic Tilapia Comprising a Humanized Insulin Gene. U.S. Patent No. 6,476,290 Bl—(issued 11/5/2002).
55. Pohajdak B, Mansour M, Hrytsenko O et al. Production of transgenic tilapia with Brockmann bodies secreting [desThrB30] human insulin. Transgenic Res 2004; 13: 313–323.
56. Wright JR Jr, Snowden J, Hrytsenko O et al. Immunohistochemical staining for tilapia and human insulin demonstrates that a tilapia transgenic for humanized insulin is a mosaic. Transgenic Res 2008; 17: 991–992.

57. Wright JR Jr. Academic uses of GM fish technology: a call for a common sense approach to regulation. In: Devlin RH, ed. Assessment of Environmental and Indirect Human Health Effects of Genetically Modified Aquatic Organisms. Canadian Technical Report of Fisheries and Aquatic Sciences 2581. Ottawa: Department of Fisheries & Oceans Canada, 2006: 111–126.
58. Wright JR Jr, Hrytsenko O, Pohajdak B. Transgenic tilapia for islet xenotransplantation. In: Fletcher G, Rise M, eds. Aquaculture Biotechnology. West Sussex, UK: John Wiley & Sons, 2012: 281–290.
59. Leventhal JR, Kissler H, Xu B-Y et al. Piscine Islets Can Reverse Hyperglycemia in Diabetic Nonhuman Primates. Boston, MA: World Transplant Congress, 7/22-27/06 (abstract).
60. Hrytsenko O, Pohajdak B, Wright JR Jr. Production of transgenic tilapia homozygous for a humanized insulin gene. Transgenic Res 2010; 19: 305–306.
61. Hrytsenko O, Rayat G, Xu B-Y et al. Lifelong stable human insulin expression in transgenic tilapia expressing a humanized tilapia insulin gene. Transgenic Res 2011; 20: 1397–1398.
62. Morrison CM, Miyake T, Wright JR Jr. Development of embryo and early larva of *Oreochromis niloticus* (Pisces: Cichlidae). J Morphol 2001; 247: 172–195.
63. Morrison CM, Pohajdak B, Wright JR Jr. Structure and enzymatic removal of the chorion of embryos of the Nile tilapia *Oreochromis niloticus*. J Fish Biol 2003; 63: 1439–1453.
64. Gaj T, Gersbach CA, Barbas CF III. ZFN, TALEN and CRISPR/case-based methods for genome engineering. Trends Biotechnol 2013; 31: 397–405.
65. Jao L-E, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. PNAS 2013; 110: 13904–13907.
66. Kelley KM. Experimental diabetes mellitus in a teleost fish. I. Effect of isletectomy and subsequent hormonal treatment on metabolism of the goby, *Gillichthys mirabilus*. Endocrinology 1993; 132: 2689–2695.
67. Taylor JS, Braasch I, Frickey T et al. Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Res 2003; 13: 382–390.
68. Conlon JM, Larhammar D. The evolution of neuroendocrine peptides. Gen Comp Endocrinol 2005; 142: 53–59.
69. Lomedico PT, Rosenthal N, Kolodner R et al. The structure of rat preproinsulin genes. Ann N Y Acad Sci 1980; 343: 425–432.
70. Hrytsenko O. Molecular Characterization of the Insulin Genes in Nile Tilapia (*Oreochromis niloticus*). PhD Dissertation, Dalhousie University, Halifax, Nova Scotia, Canada, 2007.
71. Reemtsma K. Experimental islet cell grafting: a transplantation model. Transplant Proc 1970; 2: 513–515.
72. Weber C, Weil R, McIntosh R et al. Xenotransplantation of piscine islets into hyperglycemic rats. Surgery 1975; 77: 208–215.
73. Schrezenmeir J, Stürmer W, Göbel D et al. Brockmann-bodies in hollow fibers may solve availability problems for islet transplantation. Life Support Syst 1985; 3(Suppl. 1): 666–669.
74. Schrezenmeir J, Faust P, Laue C et al. Immunoprotection by biocompatible membranes permits transplantation of piscine islets to rats. Transplant Proc 1989; 21: 2730–2735.
75. Morsiani E, Lacy PE. Effect of low temperature culture and L3T4 antibody on the survival of pancreatic islet xenografts (fish to mouse). Eur Surg Res 1990; 22: 78–85.