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**METALLOTHIONEIN OVEREXPRESSION PROLONGS GRAFTS SURVIVAL
IN THE EARLY PHASE OF PANCREATIC ISLET TRANSPLANTATION**

By

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A Thesis
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ABSTRACT

Metallothionein overexpression prolongs grafts survival in the early phase of pancreatic islet transplantation

Xiaoyan Li

December 17, 2002

Pancreatic islet transplantation is a very promising treatment for type I diabetes. Many clinical trials have failed due to early islet loss and immune rejection. Reactive oxygen species (ROS) have been demonstrated to be involved in graft damage during transplantation. Metallothionein (MT) is an inducible antioxidant protein. Prior studies in our laboratory have shown that overexpression of MT in beta-cells reduces DNA damage and diabetes induced by streptozotocin (STZ), which damages beta cells by generating ROS. Therefore in this study we examined whether overexpression of MT in beta cells is beneficial to pancreatic islet transplantation. Isolated MT transgenic and normal FVB islets were transplanted under the kidney capsule of Balb/c mice that were treated with STZ to induce severe diabetes. We found that diabetic mice transplanted with MT islets maintained near normal glucose levels for 16.2 ± 2.52 days while those animals transplanted with control islets maintained normal glucose values for only 8.36 ± 1.67 days ($p < 0.01$). To determine whether the early benefit of MT was due to protection from early islet loss or from immune rejection, islets were transplanted into same strain mice, thereby free of immune rejection. Under these conditions MT islets maintained much

higher insulin content than control islets. To assess the possible mechanism of MT benefit isolated FVB and MT islets were exposed to hypoxia (1% O₂, 5% CO₂ and 94% N₂). Compared to control islets, MT decreased cell death and reduced ROS production during hypoxia. Moreover, after isolated islets were treated with a nitric oxide donor (S-nitro-N-acetyl-penicillamine) MT reduced NO induced cell death. By immunohistochemistry in allografts of FVB and MT islets MT was found to markedly reduce the production of nitrotyrosine. These data demonstrated that MT could prolong graft survival in pancreatic islet transplantation. The protective mechanism may be reduction of ROS production or protection from ROS induced beta cell death.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CM-H ₂ DCFDA	5-(6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate
DAB	diaminobenzidine
FBS	fetal bovine serum
FRs	free radicals
HBSS	Hanks' balanced salt solution
KRBB	Krebs-Ringer bicarbonate buffer
MT	metallothionein
PNF	primary non-function
SNAP	S-nitro-N-acetyl-penicillamine
ROS	reactive oxygen species
SOD	superoxide dismutases
STZ	streptozotocin

CHAPTER I

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by glucose intolerance and hyperglycemia. In the United States, between 6%-7% of population equating to about 16 million people are suffering from this disease and every year over 105 billion dollars are spent on its treatment (Olefsky 2001). It is projected that the prevalence of diabetes in the United States will increase to approximately 9% by 2025. Elevated blood glucose level in diabetes is associated with an increased risk of developing microvascular diseases, principally retinopathy, nephropathy, and neuropathy. Due to these long-term complications, diabetes has caused an enormous burden of morbidity. To date diabetes is the leading cause of blindness, amputations and end-stage renal disease and the sixth leading cause of death in the United States. But unfortunately all current drug therapies for diabetes including intensive insulin therapy do not provide sufficiently tight control of blood glucose to avoid the late devastating complications of this disease(1998; 1993).

Islet transplantation has become accepted as an alternative therapy for treatment of type 1 diabetes. Compared to whole pancreas transplantation, islet transplantation is not only a much less invasive procedure but also a procedure much safer and less costly. It may be possible to induce immune tolerance of the engrafted islets by pretreating them in culture or by pretreating the recipient with donor antigen. It is also possible to use cryopreserved pancreatic islets (Warnock *et al.* 1991) rather than fresh islets or whole

pancreas which normally are very limited resources. It has been proven, that clinical islet transplantation, when successful, is capable of maintaining excellent metabolic control in the absence of hypoglycemia and stopping the progression of clinical long-term complications(Boker *et al.* 2001). But over the years this approach has had a poor record of accomplishment in achieving independence from exogenous insulin – approximately 12% of allotransplanted patients achieved insulin independence for over 7 days(White *et al.* 2001). It has been noticed that the percentage of long term graft survival in islet transplantation is much lower than whole-pancreas transplantation. Pancreas graft survival for more than one year is respectively 82%, 74% and 76% when the organ was implanted after the kidney, simultaneously with kidney, or alone while the islet graft survival after one year is less than 10%(White *et al.* 2001). However a more recently published study illustrated that islet transplantation is still a very encouraging approach for diabetes treatment. Shapiro and his colleagues (Ryan *et al.* 2002) established the Edmonton protocol. This method relied on long term application of immunosuppressors and required more than one donor pancreas for one recipient, however this method was more successful-80% of transplanted patients were insulin independent for more than 1 year.

The consistent achievement of long-term success in clinical islet transplantation is limited by the islet mass that can be effectively engrafted and that can maintain function. Two important factors: early islet loss immediately after transplantation and relatively late-occurred immune rejection may account for the islet graft failure after islet allotransplantation. The early events leading to graft loss are termed primary non-function (PNF) or early islet loss. This might be an essential determinant for the success

of islet transplantation (Brendel *et al.* 1999) because a majority of islet grafts are damaged immediately after transplantation. PNF could result from implantation of an inadequate number of functional islets or inflammatory reactions elicited by the interaction between the implanted islets and components of the blood stream (Bennet *et al.* 2000; Bennet *et al.* 1999) or immune components residing in the local organ (Bottino *et al.* 1998). This interaction leads to generation of proinflammatory cytokines, nitric oxide and reactive oxygen species (ROS), and thereby cytotoxic destruction to the islet graft (Davalli *et al.* 1996). It has been clearly demonstrated that local activation of liver macrophages (Steiner *et al.* 1997; Kroncke *et al.* 1993) and endothelial cells (Steiner *et al.* 1997; Kroncke *et al.* 1993) can mediate PNF by releasing cytokines and nitric oxide in intraportal islet allotransplantation. In the rat islet transplantation model, nitric oxide is considered the main islet-toxic agent from allogeneic or syngeneic macrophages (Stevens *et al.* 1995). In addition to local nonspecific inflammatory reactions, islet graft also suffers from an initial period of hypoxic ischemia after transplantation. The engrafted islets are essentially avascular tissue that won't have enough blood supply before the completion of revascularization to the host organ. Revascularization requires over one week (Menger *et al.* 1992b). Therefore the oxygen tension within the islet graft is relatively low (Carlsson *et al.* 2001c) in the early stage of islet transplantation. PNF might be initiated by this ischemic microenvironment because ischemia followed by reperfusion is a condition known to produce detrimental ROS in transplanted organs (Li *et al.* 1997; Petrowsky *et al.* 1995; Land *et al.* 1994; Nakao *et al.* 1994).

The damaging effects of ROS on pancreatic islets have been widely investigated in diabetes (Xenos *et al.* 1994; Horio *et al.* 1994; Brenner *et al.* 1993; Suarez-Pinzon *et al.* 1997; Xu *et al.* 1999) as well as in islet transplantation (Stevens *et al.* 1996; Karsten *et al.* 2001; Mendola *et al.* 1989). It has been shown that the exposure of isolated human (Hadjivassiliou *et al.* 1998) and rodent islets (Malaisse *et al.* 1982d; Xu *et al.* 1999b; Hadjivassiliou *et al.* 1998) or beta cell lines (Zhang *et al.* 1995) to ROS markedly inhibits cell functions including beta cell insulin biosynthesis and secretion, or results in beta cell apoptosis and necrosis. Pancreatic beta cells are much more susceptible to ROS destruction than other cell types such as those in kidney and liver. The most striking evidence is that pancreatic islets are highly sensitive to the destruction of two beta-cell toxins, alloxan and streptozocin (STZ). Both compounds are widely used to produce diabetes and their relatively selective damage to beta cells is thought to act through generation of ROS including superoxide radicals, nitric oxide radicals and hydrogen peroxide (Grankvist *et al.* 1981a; Takasu *et al.* 1991). Some other studies also proved that pancreatic beta cells have several times higher sensitivity than kidney, muscle, or liver cells to the damage of superoxide radical (Burkart *et al.* 1992), hydrogen peroxide (Brunk *et al.* 1995) and nitric oxide (Eizirik *et al.* 1994). Further, quantitative studies of ROS scavengers in pancreatic beta cells indicate that the sensitivity of islets is due to inadequacy of ROS detoxifying systems. Compared with liver, the total islet superoxide dismutases (SOD) is less than 30% (Grankvist *et al.* 1981b). Glutathione peroxidase and catalase in the beta cell are less than 2% of liver (Grankvist *et al.* 1981b; Welsh *et al.* 1995; Malaisse *et al.* 1982; Johansson & Borg 1988). This deficit of ROS detoxifying systems may contribute significantly to the deterioration of islet cells in type 1 diabetes

and probably to the islet graft loss after transplantation. Recent data have shown the successful improvement of early islet transplant survival by various antioxidant combinations such as α -tocopherol (Burkart *et al.* 1995) and other vitamins (Winter *et al.* 2002). Consequently we hypothesize that increasing ROS defenses specifically in beta cells may benefit islet transplantation.

To test our hypothesis, we selected metallothionein (MT) as our candidate antioxidant because MT is an efficient ROS scavenger. MT is a cysteine-rich protein involved in zinc homeostasis and metal detoxification (Klaassen & Liu 1997). MT also appears to play important roles in ROS protection. MT has been shown to be an extraordinarily efficient ROS scavenger protecting from hydroxyl radicals (Malaisse *et al.* 1982; Schnedl *et al.* 1994), nitric oxide radicals (Schwarz *et al.* 1995) and superoxide radicals (Rocic *et al.* 1997; Kumari *et al.* 1998; Schwarz *et al.* 1995). MT protects against many agents known to act through ROS, including hydrogen peroxide, radiation, glutathione depletors, adriamycin, and xanthine oxidase (Sato & Bremner 1993). By using this molecule, we have produced two lines of transgenic mice with overexpression of MT in pancreatic beta cells on the FVB mouse strain. We found that the MT transgene had no deleterious effect on beta cell structure or function. But overexpression of MT was very effective in protecting FVB mice from diabetes induced by streptozotocin (Chen *et al.* 2001). Therefore in the present study we are further testing the potential effect of MT on islet transplantation. We want to know whether an increased ROS defense system in beta cells is beneficial for graft survival in islet transplantation.

CHAPTER II

MATERIALS and METHODS

Animals

Transgenic mice were established on FVB mice with the overexpression of human MT II gene by standard transgenic techniques. This transgene was specifically expressed in pancreatic beta cells because it was driven by a human insulin promoter. The transgenic mice or islets were designated as HMT-1 and the control FVB mice or islets were designated as FVB.

Chemicals

Streptozotocin, collagenase (type V), Histopaque 1077 and metallothionein were obtained from Sigma (St. Louis, MO). Hank's balanced salt solution (HBSS), RPMI 1640 medium, and fetal bovine serum (FBS) were supplied by Gibco BRL (Rockville, MD). Rat insulin standard was bought from Linco (St. Charles, MO). Rabbit antiserum to guinea pig insulin, was purchased from BioGenex (San Ramon, CA). Monoclonal anti-Nitrotyrosine antibody was supplied by Cayman (Ann Arbor, MI). 5-(6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was purchased from Molecular Probes (Eugene, OR). Alarmblue was purchased from Biosource International (Camarillo, CA).

The isolation procedure was based on a modification of the method of Gotoh *et al* (Gotoh *et al.* 1985). Mice were anesthetized with avertin at the dose of 6mg/g body weight via intraperitoneal injection. The pancreas was inflated with 3 ml of 1.55 mg/ml collagenase Type V and incubated at 37°C for 20 min. The digest was resuspended in 10 mL Histopaque 1077, overlaid with 10 ml of HBSS and centrifuged at 700 × g for 20 min at 10°C. Most of the gradient, excluding the pellet was collected and washed 3 times with cold HBSS. Islets were then handpicked in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin. Islets were maintained in suspension culture in RPMI 1640 medium at 37°C and 5 % CO₂.

Allotransplantation

400 FVB or HMT-1 islets were transplanted under both kidney capsules (200 each) according to a modification of the procedure of Montana *et al* (Montana *et al.* 1993b). Recipient mice were male Balb/c mice purchased from Jackson Laboratory (Bar Harbor, Maine). The Balb/c mice, at the age between 8-12 weeks, were injected with STZ (ip. 220mg/kg) for 1 to 3 days to induce diabetes. Only the mice with blood glucose range from 350mg/dl to 500mg/dl were selected as recipients for following transplantation surgery. After recipient mice were anesthetized via ip. injection with 10 ul/g of a solution containing 10 mg/ml ketamine and 3.2 mg/ml xylazine, the left side mouse kidney was first externalized through a small incision and kept moist with saline. An incision was made near the posterior pole of the kidney. 200 islets were picked into a gel-loading pipette tip (0.5 mm diameter) mounted on a 1-cc Hamilton syringe (Reno, NV, USA) and allowed to settle. The tip was inserted through an incision beneath the kidney capsule and

the islets were gently forced out of the tip. The body wall and the skin were closed with sutures. Then the transplantation to the right side kidney was performed by the same procedure. After transplantation, the tail blood glucose levels of the transplanted mice were monitored every other day. Graft failure was defined as a return of hyperglycemia (blood glucose > 250 mg/dl) on two consecutive measurements. Grafts in some recipients were recovered 5 days after transplantation and sectioned for H&E and nitrotyrosine staining.

Syngeneic transplantation

50 FVB and HMT-1 islets were transplanted separately under each kidney capsule in a same normal FVB mouse. 6 days later, grafts were recovered and homogenized in acid ethanol (23 ethanol: 2 HCl: 75 H₂O, v/v/v) for insulin extraction. The preserved insulin content in the islet grafts was measured by an anti-insulin antibody coated tube RIA kit (Diagnostic Products) and rat insulin standard.

Immunohistochemistry for nitrotyrosine and insulin

Islet graft was fixed in 10% formaldehyde in 0.1 mol/l phosphate buffer (pH 7.2), dehydrated in an ascending graded series of ethanol, and subsequently infiltrated with paraffin. Serial section were cut at 5 µm, mounted on polylysine-coated slides, and then deparaffinized in xylenes and a descending graded series of ethanol. For nitrotyrosine staining, slides were treated with target retrieval solution (Dako corporation), followed by M.O.M mouse Ig blocking reagent (Vector Laboratories). Nitrotyrosine monoclonal antibody (Cayman) was added on the slides at the concentration of 10 µg/ml and

incubated overnight at room temperature. After 3 washes in phosphate-buffered saline, slides were incubated with the biotinylated anti-mouse IgG reagent, followed by ABC reagent and developed with DAB as chromagen. The slides without primary antibody treatment were used as negative control.

For insulin staining, sections were treated with rabbit anti-guinea pig insulin antibody diluted 1 to 100 and incubated for 90 min at 37°C. After 4 washes in phosphate buffered saline, slides were incubated with the appropriate biotin-labeled second antibody followed by peroxidase labeled streptavidin and developed with DAB as chromagen.

Hypoxia in vitro studies

Isolated FVB and HMT-1 islets were cultured in a 96-well plate placed in a sealed hypoxic chamber saturated with 1% O₂, 5% CO₂ and 94% N₂ for 24h, 48h, and 72h respectively. Cell viability was assessed by measuring islet metabolism as indicated by alamar blue absorbance. The data for cell viability were represented as percentages of the viabilities of control cells which were cultured under normal condition. ROS production in islets after hypoxia 2 or 7 hr was measured with fluorescence dye (CM-H₂DCFDA) (Molecular Probes). Islets treated with 24h hypoxia were picked for immunohistochemistry for insulin and nitrotyrosine.

Nitric oxide in vitro studies

Isolated FVB and HMT-1 islets were exposed to different concentrations of a nitric oxide donor, S-nitro-N-acetyl-penicillamine (SNAP). Glucose stimulated insulin secretion was tested by static assay on 96-well tissue culture microplates and insulin

levels were measured with anti-insulin antibody coated RIA kit. Apoptotic and necrotic DNA were detected by an anti-histone biotin/anti-DNA POD ELISApplus kit (Roche). All values were normalized to islet total DNA measured with picogreen DNA quantification kit (Molecular Probes).

Assays of insulin secretion, insulin content and DNA content

Insulin secretion was measured by static assay on 96-well tissue-culture microplates. For each well 6-10 islets were hand picked into modified KRB (Krebs-Ringer bicarbonate buffer containing the designated concentration of glucose and supplemented with 10mM HEPES, pH 7.4 and 0.1% BSA) containing 3 mM glucose at 37°C. After two washes in 3 mM glucose KRB the islets were incubated for consecutive 30 min incubations in 3 and 20 mM glucose KRB. To determine islet insulin content, islets were sonicated in 150 µl 10mM Tris HCl, 1mM EDTA, 1mg/ml RIA grade BSA, pH 7.0 for 30 seconds. Fifty µl of the solution was used to extract islet insulin with 100 µl acid ethanol (75 ethanol / 2 concentrated HCl / 23 H₂O, v/v/v) at 4 °C overnight. The remainder of the sonicate was digested with an equal volume of 200 µg/ml proteinase K in 100 mM Tris HCl, pH 8.5, 5 mM EDTA, 0.2 % SDS at 55 °C for 2 hours and used for islet DNA quantification with picogreen using a kit from Molecular Probes. Insulin was measured with the coated tube RIA kit from Diagnostic Products and rat insulin standards.

Islet fluorescence measurement of ROS

Both hypoxia treated or untreated islets were loaded with 1 μ M 5-(6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Molecular Probes, Eugene, OR) for 30 min. After islets were washed, fluorescence intensity of each islet was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Each islet in different groups was sampled using an Olympus IX70 inverted microscope equipped with a digital cooled CCD camera and ImagePro image analysis software (Media Cybernetics, Silver Spring, MD) under same parameters. Fluorescence was calibrated using Image-Pro Plus software.

Alamarblue assay

Alamarblue assay, which incorporates a redox indicator that changes color or fluorescence in response to cell metabolic activity, is a commonly used method to assess cell viability and/or proliferation of mammalian cells(Gonzalez & Tarloff 2001) and micro-organisms(Byth *et al.* 2001). In our studies, 15 overnight cultured islets either control FVB islets or transgenic HMT-1 islets were hand picked into 200ul fresh culture medium containing 1:20 diluted AlamarBlue on a 96-well plate. Islets were cultured for 4hr and the developed AlamarBlue absorbance was measured on a fluorescent microplate reader (Tecan) at the excitation wavelength of 535nm and the emission wavelength of 595nm. After three washes of 200ul fresh culture medium, islets were cultured and treated under different oxygen conditions for specified times. At the end of treatment, the culture medium was replaced with new 200ul fresh culture medium containing 1:20 diluted Alamarblue. The color was developed for another 4hr and the absorbance was

measured again. The islet cell viability was calculated from the ratio of the absorbance value after treatment over the absorbance value before treatment.

Apoptotic and Necrotic DNA measurement

The measurement of apoptotic and necrotic DNA in SNAP treated islets was performed by a cell death detection ELISA (Roche) based on the manufacturer instruction manual. Basically, 40 to 50 islets were cultured in 500ul fresh culture medium in a 1.5ml microtube with or without SNAP treatment for 24hrs. After treatment the microtube was centrifuge at 200g for 10 minutes at 4°C. The supernatant was removed as the necrosis DNA sample. The pellet was lysed by 100µl lysis buffer from the kit for 30 minutes at room temperature. The microtube was centrifuge again at 200g for 10 minutes at 4°C. The supernatant was removed as apoptotic DNA sample. To quantify the necrotic and apoptotic DNA, both DNA samples were added to the streptavidin-coated microplate offered in the kit. With the addition of a mixture of anti-histone-biotin and peroxidase-conjugated anti-DNA antibody followed by 2hrs incubation at room temperature, the DNA samples were captured to the microplate by biotinylation. After washing off unbound components, the quantitative determination of sample DNA content was carried out by measuring conjugated peroxidase activity with ABTS as substrate at 405nm.

Data analysis

Data are presented as the mean \pm standard error. Statistical significance for two-group analysis was performed by student's t-test (2-tailed) and for three group analysis was performed by ANOVA and Dunnet's post hoc (2-tailed) test. Kaplan-Meier survival

analysis and Mantel-Cox Log-rank test were used to analyze islet graft survival time. Computations were done using the statistical program from SPSS (version 10.0).

CHAPTER III

RESULTS

Transplantation

Islet transplantation has been carried in many different sites in recipients, such as liver intraportal route, kidney subcapsular site, intraperitoneal space and small bowel subserosal space. In our studies we successfully set up a transplantation method by placing the islet graft under kidney capsule. This site is optimal for islet survival in that the site beneath the kidney capsule is expected to have sufficient blood supply but have less direct interactions with immune system than liver intraportal site does (Brandhorst *et al.* 2001). As shown in Figure 1, the isolated islets from FVB mouse were implanted under FVB mouse kidney capsule. Thirty days after transplantation, we recovered the islet graft and examined the islet morphology and insulin content by H&E staining and immunohistochemistry. We found that the 30-day islet graft still maintained relatively normal islet structure and insulin staining.

To test whether overexpression of MT in the beta cells would improve islet graft survival, we first performed an allotransplantation (allotransplantation indicates that the grafts were transplanted on to a different strain of mice) study by implanting 400 FVB control islets or MT transgenic islets under the renal capsule of STZ induced diabetic Balb/c mice. We found that the Balb/c diabetic mice that received a graft of 400 FVB islets were brought to euglycemia for a short period of time. However, within about one week they returned to hyperglycemia. The average period of euglycemia with an FVB

islet graft was 8.36 ± 1.67 days. However, the Balb/c mice that received MT transgenic islet grafts remained euglycemic for a much longer time. The period of euglycemia for recipients of an MT islet graft is markedly increased to 16.2 ± 2.52 days (Figure 2). We also examined nitrotyrosine staining in both MT and FVB islet grafts recovered after 5 days of transplantation. Nitrotyrosine is recognized as an indicator of protein oxidative damage when the tyrosine residues are oxidized by peroxynitrite, a highly potent ROS formed by superoxide radical and nitric oxide. In transplantation the production of peroxynitrite was greatly increased in the grafts suggesting that peroxynitrite may play an important role in graft rejection and loss (MacMillan-Crow *et al.* 1996). Our data confirmed this finding. In Figure 3, the nitrotyrosine staining in control FVB islet graft was marked elevated after transplantation. But the antioxidant MT transgene markedly reduced the nitrotyrosine production. These data indicate that ROS may be involved in the graft damage, at least in the early stage of allotransplantation and the protective effect of MT to prolong islet graft survival may be through the elimination of oxidative damage by scavenging ROS.

During allotransplantation the islet grafts are exposed to an environment containing a variety of stressors, including immune rejection, glucose toxicity, ischemic damage and reperfusion injury. All these processes can induce ROS production. In order to differentiate whether the early benefit of the MT transgene is due to the protection against ischemic/reperfusion injury or against immune rejection or glucotoxicity, we did further syngeneic transplantation studies. In this transplantation model we used FVB mice with normal blood glucose levels as recipients, thereby excluding the influence of immune rejection and glucose toxicity. Six days after transplantation islet graft was

removed from the kidney and the graft insulin content was measured. Graft insulin content is a widely used parameter to assess the response of transplanted islets (Montana *et al.* 1993; Jansson *et al.* 1995). As shown in Figure 4, both FVB and MT islet grafts after 6 days of syngeneic transplantation lost part of their insulin contents compared to their initial values. However, FVB grafts lost much more insulin than MT islet grafts did. MT overexpression protected beta cells by preserving more than 50% of original islet insulin, whereas FVB graft lost almost 80% of original insulin. This finding that islet grafts lose insulin very much in the early stages of syngeneic transplantation is consistent with other reports (Davalli *et al.* 1996; Xu *et al.* 1999). During this early stage of transplantation, the islet cells in the graft dynamically change their metabolism rate and gene expression pattern characterized by substantial islet cell dysfunction and death. Ischemia after the transplantation and the following reperfusion during revascularization may contribute to this early islet graft failure. Our result that MT protected from early islet graft insulin loss indicates that the MT transgene may protect from ischemia/reperfusion damage after transplantation. This is because ischemia followed by reperfusion is known to induce ROS and antioxidants are known to be beneficial for preventing reperfusion injury (Li *et al.* 1997; Wang *et al.* 2001).

Hypoxia in vitro studies

To assess the possible mechanism of MT protection, we carried out *in vitro* studies by exposing isolated FVB and MT islets to hypoxic conditions in a sealed chamber. In this chamber only 1% O₂ was included, which is close to the environment that the transplanted islets suffer from in the *in vivo* condition after transplantation. We

chose 1% O₂ because Carlsson et al. (Carlsson *et al.* 2001) recently reported a mean PO₂ level was 5-10 mmHg (about 1% O₂) in syngeneically transplanted islets. After exposure to the hypoxia condition for 24, 48, and 72hrs, the cell viability of both FVB and MT islets was measured with Alamar Blue assay. As shown in Figure 5, the islet cell viability in FVB islets was markedly decreased by hypoxia treatment. However the overexpression of MT in beta cells significantly inhibited hypoxia induced reduction of cell viability at all time points we analyzed.

Hypoxia and hypoxia followed by reoxygenation is known to induce ROS production(Li & Jackson 2002; Duranteau *et al.* 1998). Therefore it may also be true that overexpression of the ROS scavenger, MT, would decrease hypoxia induced ROS production in the islet cells. We measured ROS production by a redox state sensitive fluorescence dye (CM-H₂DCFDA) in both FVB and MT islets treated with hypoxia for 2 and 7 hr. The data in the Figure 6 demonstrated that hypoxia induced ROS generation in both FVB and MT islets. But the ROS levels in MT islets were dramatically lowered by the MT transgene. Hence, the protective effect of MT against hypoxia induced cell damage may be through a decrease of ROS production.

Nitric oxide in vitro studies

The association between NO production and early islet graft damage has been previously described in xenotransplantation(Ketchum *et al.* 2000). Application of selective iNOS inhibitors has been shown to protect from early islet graft failure in rat intraportal islet transplantation(Brandhorst *et al.* 2001). Our immunohistochemistry data of nitrotyrosine staining in allotransplantation study support this concept. Our results

suggest that NO may be one of the major ROS involved in the early islet graft damage after transplantation and NO perhaps is one of the main targets that MT acts on. By eliminating the toxic effects of NO, MT is then able to provide protective effect against early islet graft destruction. Therefore, to test this hypothesis both FVB and MT islets were treated *in vitro* with a NO donor, SNAP at different concentrations. After treatment we analyzed the islet cell viability by observing islet morphology and by measuring apoptotic and necrotic islet DNA. We also evaluated the islet function by measuring glucose stimulated insulin secretion.

Visual observation revealed obvious, reproducible differences in the sensitivity of MT and FVB islets (Figure 7) to SNAP. FVB islets were visibly damaged at 1 mM SNAP and generally disintegrated into single cells at 2 and 3 mM. But MT islets did not exhibit a similar degree of damage until the SNAP concentration was raised to 3 mM. The apoptotic and necrotic DNA quantification by a photometric enzyme-immunoassay confirmed this protective effect of MT. As shown in Figure 8, SNAP treatment induced islet DNA cleavage. Both apoptotic DNA and necrotic DNA were greatly increased in FVB control islets after exposure to SNAP. However MT islets had markedly reduced contents of cleaved DNA, relative to FVB islets. This was especially obvious at 3mM SNAP. At this concentration DNA damage in the transgenic MT islets was over 50% less than that in FVB control islets. These results imply that one mechanism of preserved islet mass by the MT transgene was probably through the protection of islet DNA.

However further analysis of islet insulin secretion function failed to confirm this protective effect of MT. In Figure 9 we exposed both FVB and MT islets to different concentrations of SNAP for 12 hrs, and the glucose stimulated insulin secretion was

measured. We found SNAP significantly inhibited insulin secretion on both types of islets. But no difference in insulin secretion was found between FVB and MT islets. Currently we have no good explanation for the discrepancy that MT protected from SNAP induced islet DNA damage but not from the inhibition of islet insulin secretion. It is very possible that these two effects on islet cells may be through two different pathways and MT only has an action on one of them. It is notable that the insulin secretion of FVB islets significantly rose at 2 mM SNAP. Since the increase in released insulin was seen at both low and high glucose concentrations this was probably not due to regulated insulin secretion. This phenomenon might be explained by the fact that at 2mM SNAP structural integrity of FVB islets was severely damaged (Figure 7). Due to this structural damage, beta cell insulin leaked into the media.

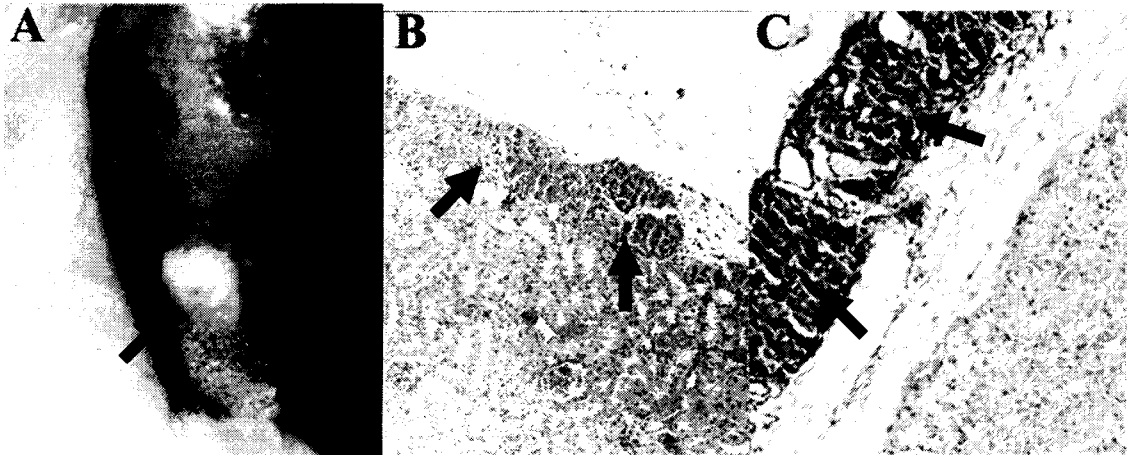


Figure 1. Islet graft under the kidney capsule one month after syngeneic transplantation. In panel A, the white spot is the graft of FVB islets transplanted to FVB recipient mouse. Panel B is H&E staining of the graft. Panel C is insulin staining of the graft (Brown staining). Arrows in each panel point to the graft.

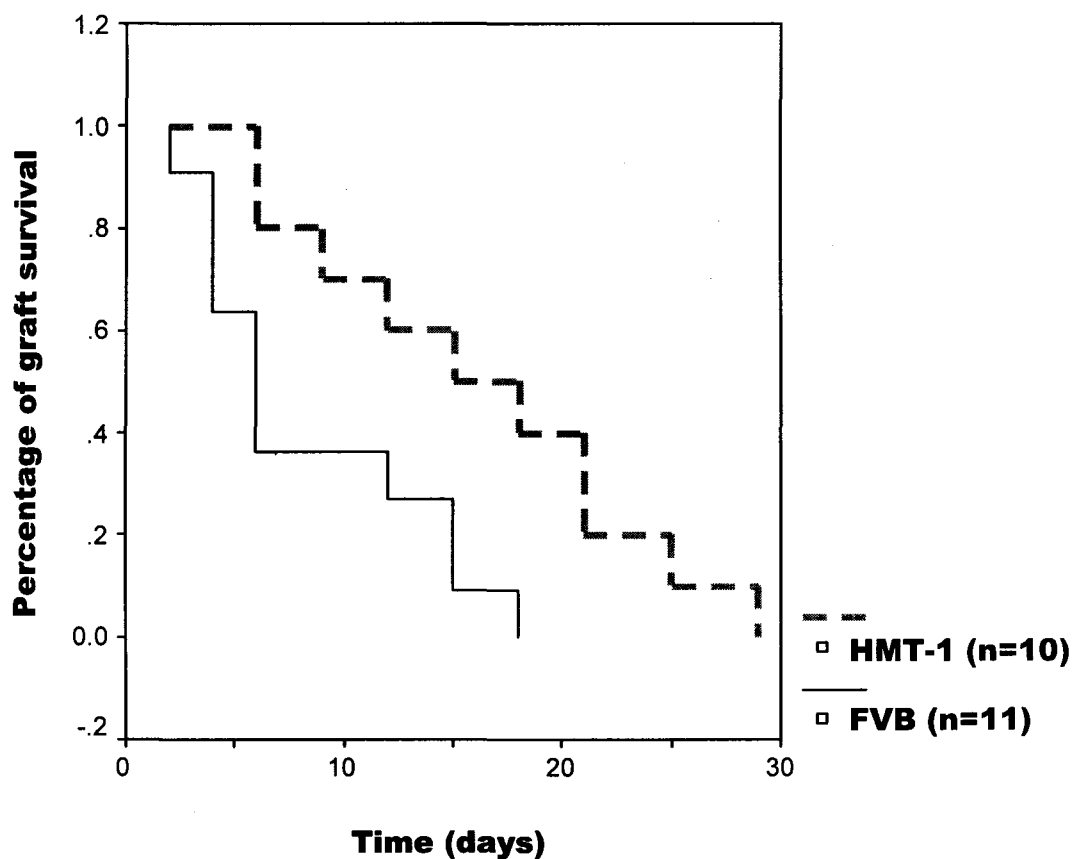


Figure 2. Graft survival of FVB and HMT-1 islets transplanted into diabetic Balb/c mice. MT overexpression prolongs islet graft survival in allotransplantation. 400 islets were transplanted under both kidney capsules of STZ induced diabetic Balb/c mice. Graft survival was defined in Methods. The survival time of HMT-1 grafts (16.2 ± 2.52 days) is significantly longer than that of FVB grafts (8.36 ± 1.67 days), $P < 0.01$ by Mantel-Cox Log-rank test.

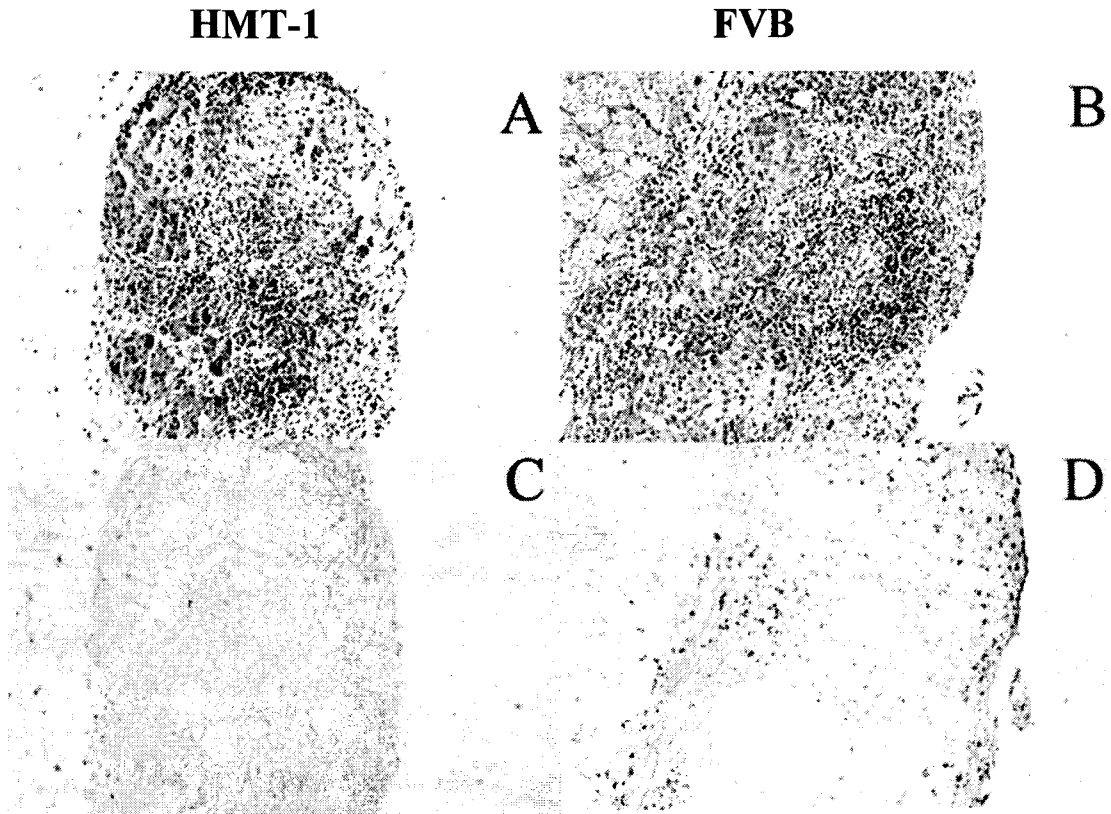


Figure 3. H&E staining and immunohistochemistry staining of nitrotyrosine in FVB and HMT-1 allografts 6 days after transplantation. MT overexpression reduced nitrotyrosine production in allotransplantation. A and B show the H&E staining of grafts. Monoclonal anti-nitrotyrosine primary antibody was used for nitrotyrosine staining (C, D). Similar results were obtained from three independent recipient transplanted mice. Magnification $\times 100$.

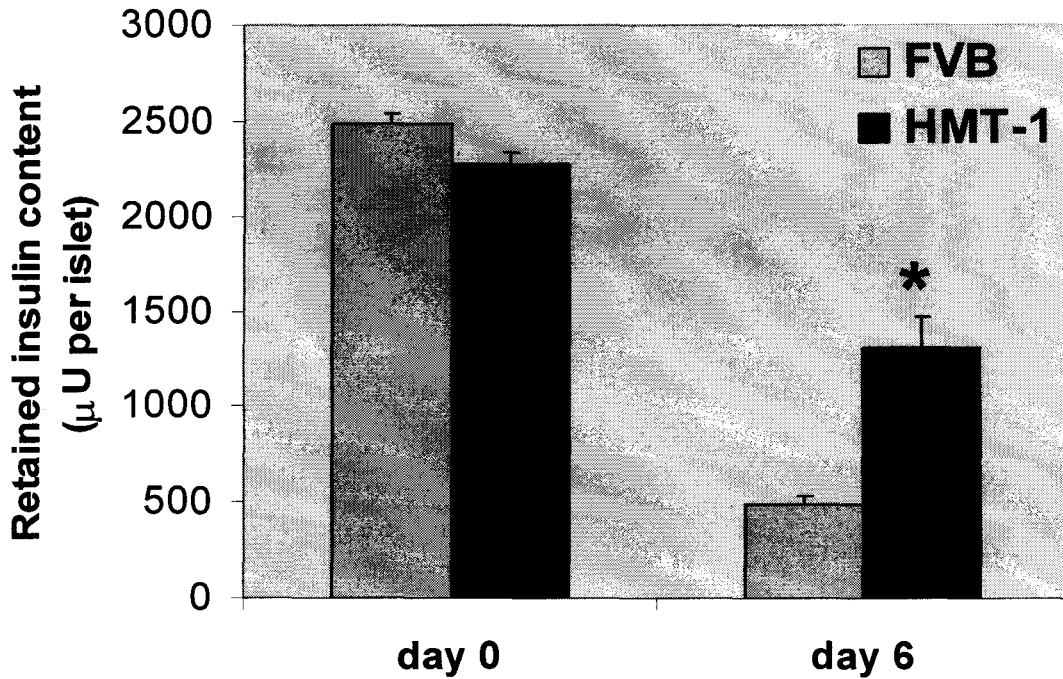


Figure 4. Retained insulin contents in grafts of FVB and HMT-1 islets after syngeneic transplantation. MT overexpression protects from graft insulin loss during syngeneic transplantation. Islets were transplanted into normal FVB mice and the recovered graft insulin levels were measured as described in Methods. Data at 6 days was collected from 12 recipient FVB mice. The value of MT graft is different from the value of FVB graft, * P<0.01 by two tailed Student's t-test. Vertical bars indicate standard error of mean.

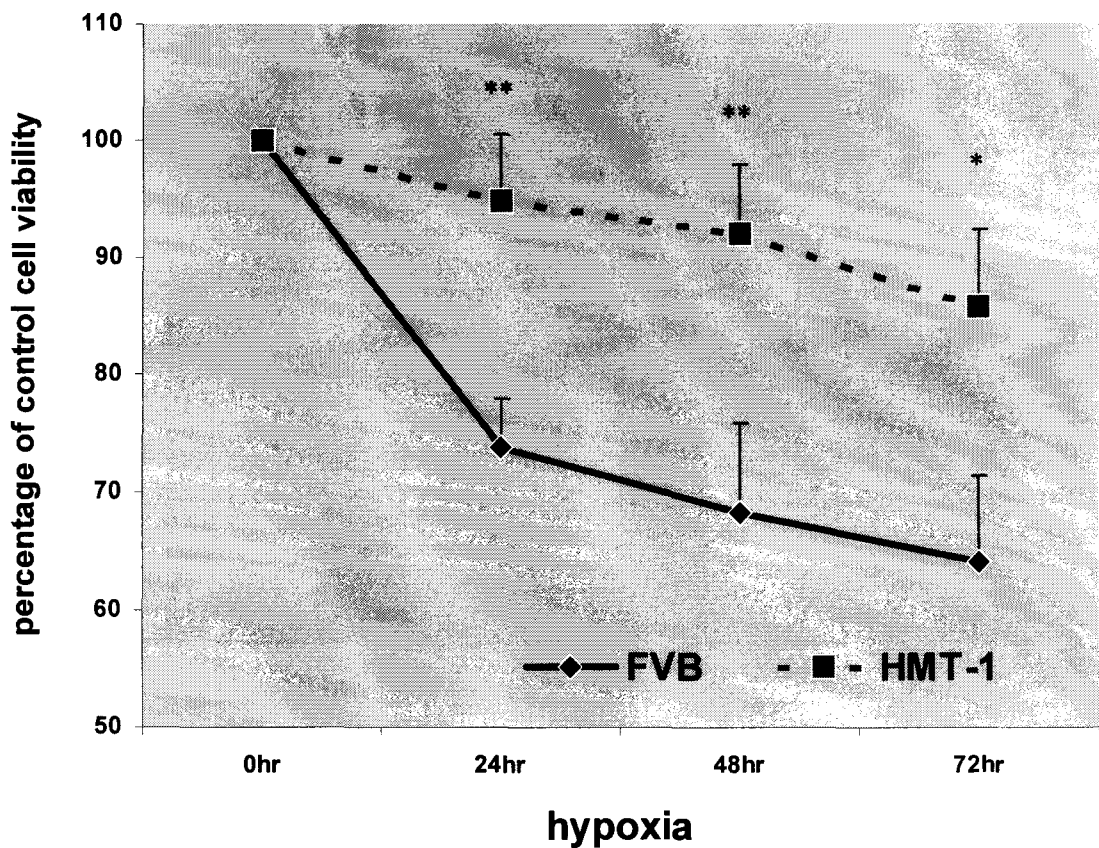


Figure 5. The MT transgene increased cell viability during hypoxia. FVB and HMT-1 islets were treated with hypoxia for 24h, 48h, and 72h. Cell viability was measured by alamarblue. Data were calculated from three independent experiments. Where HMT-1 values are different from the corresponding FVB values, * $P < 0.05$ and ** $P < 0.01$ by two tailed Student's t-test. Vertical bars indicate standard error of mean.

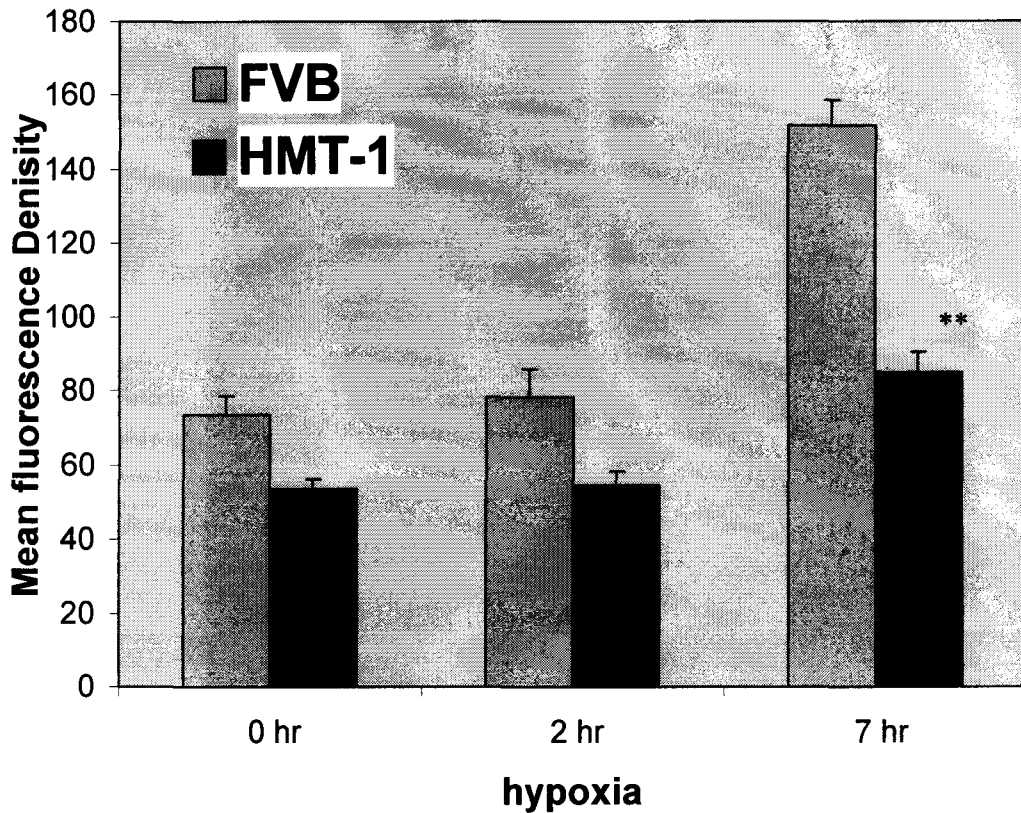


Figure 6. ROS production in FVB and HMT-1 islets exposed to hypoxia. MT overexpression reduced ROS production during hypoxia. FVB and HMT-1 islets were treated with hypoxia for 2h or 7h. ROS production was measured using fluorescence dye (CM-H₂DCFDA). Where HMT-1 values are different from the corresponding FVB values, * P<0.05 and ** P<0.01 by two tailed Student's t-test. Vertical bars indicate standard error of mean.

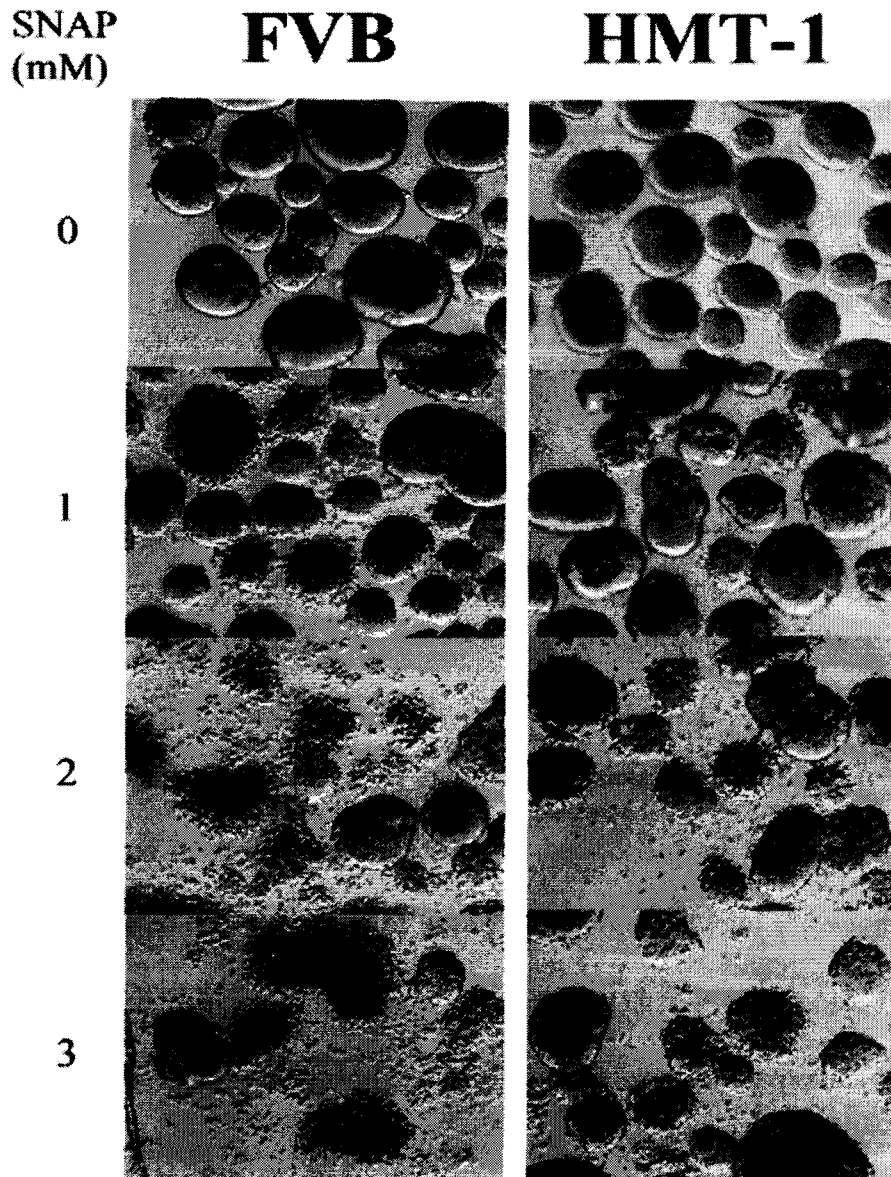


Figure 7. Representative photomicrographs of FVB and HMT-1 islets after SNAP treatment *in vitro*. Overexpression of MT in HMT-1 islets reduced the disruption to morphology. FVB and HMT-1 islets were exposed to SNAP for 22 h. The concentrations of SNAP are shown on the left. Similar results were obtained in four independent experiments. Magnification X 100.

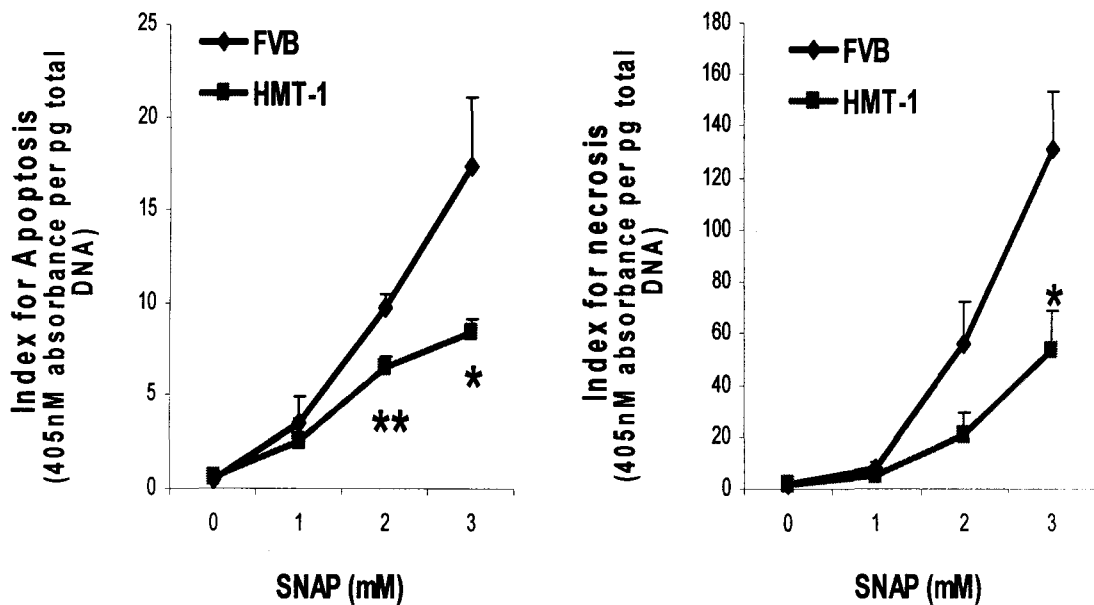


Figure 8. Apoptosis and necrosis in FVB and HMT-1 islets exposed to SNAP *in vitro*. MT transgene reduced both apoptotic and necrotic beta cell death. Isolated islets were exposed to the indicated concentrations of SNAP for 22 h. Apoptotic and necrotic DNA levels were measured using an anti-histone biotin/anti-DNA POD ELISAP^{plus} kit as described in Methods. Data were calculated from four independent experiments. Where HMT-1 values are different from the corresponding FVB values, * P<0.05 and ** P<0.01 by two tailed Student's t-test. Vertical bars indicate standard error of mean.

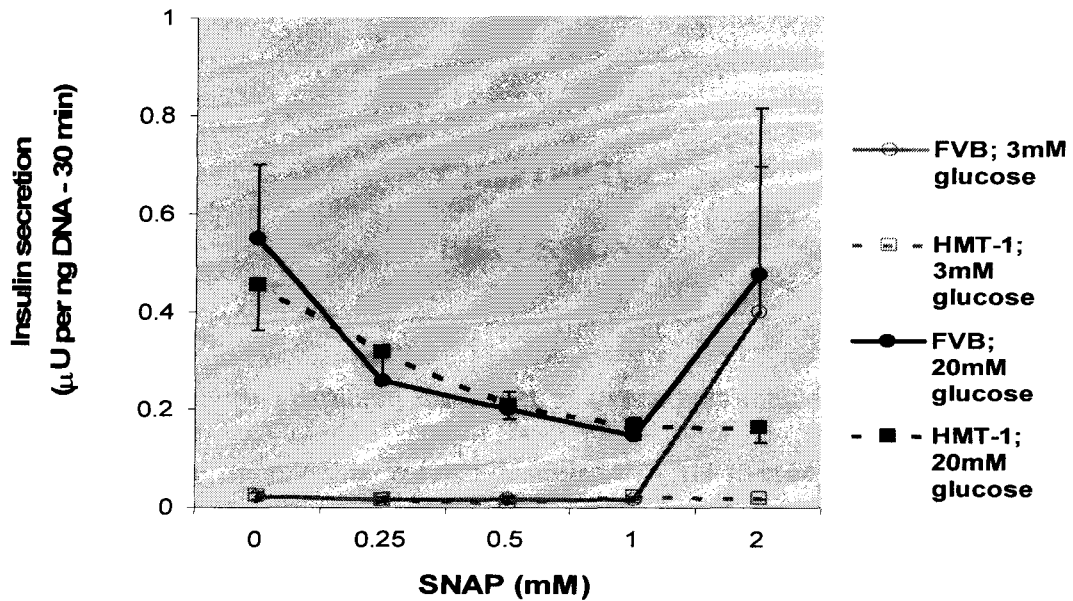


Figure 9. Glucose stimulated insulin secretion from FVB and HMT-1 islets exposed to SNAP *in vitro*. MT overexpression does not protect from the inhibitory effect of nitric oxide on glucose stimulated insulin secretion. After islets were exposed to the indicated concentrations of SNAP for 12 h, basal (3 mM glucose) and high glucose (20 mM) stimulated insulin secretions were measured as described in Methods. Data were calculated from eight independent assays. No significant difference in insulin secretion was found between FVB and HMT-1 islets. It is notable that insulin secretion rose in FVB islets at 2 mM SNAP. This is probably due to the damaging effect of SNAP on FVB islet structural integrity, as seen in Fig 7.

CHAPTER IV

DISCUSSION

In this study, we explored the effect of overexpression MT in beta cells on the outcome of islet transplantation. We demonstrated that MT overexpression could prolong graft survival time. The benefit of MT is probably due to the protection from early islet graft damage not immune rejection. In addition, a set of *in vitro* experiments was performed to reveal the possible mechanisms of MT protection. Hypoxia *in vitro* studies demonstrated that MT could inhibit hypoxia induced beta cell death and ROS production. Further, nitric oxide *in vitro* studies suggested that islets that overexpressed MT were more resistant to nitric oxide induced cytotoxicity, but MT overexpression could not protect from nitric oxide induced insulin secretion dysfunction.

Although the data we presented here only showed that MT doubled islet graft survival time in allotransplantation, the result could still be significant for clinic trials of islet transplantation. To date islet transplantation has been recognized as a very valuable therapy for treatment of type 1 diabetes. Despite a fairly low number of successful procedures, when successful, the islet transplantation is able to tightly control blood glucose level without insulin therapy and stops deleterious long-term complications. However many clinical trials of islet autotransplantation have demonstrated that islet grafts fail very soon, usually in the subsequent weeks and months after a short period of euglycemia immediately after surgery (White *et al.* 1998; Farney *et al.* 1991; Cameron *et*

al. 1980). Since there is no immune rejection during islet autotransplantation, these data indicate that the early phase of transplantation might be an important stage for late islet engraftment and normal beta cell function. In this stage islet grafts are destroyed by many cytotoxic mediators including local activated macrophage, cytokines, nitric oxide and ROS. Therefore the early stage of islet graft loss after transplantation might be one of the main obstacles for the success of islet transplantation. Looking for approaches to overcome this obstacle should be a promising direction of islet transplantation study. In our present study, the finding that MT significantly improved islet graft survival both in allotransplantation and syngeneic transplantation may indicate that this procedure could provide a feasible method to increase the chance of successful islet transplantation.

Understanding the mechanism of the early graft loss is another important step in islet transplantation study. This mechanism could be very complex because after transplantation many factors have been shown to be involved in this process of damage. This loss is unlikely due to immune rejection because this specific immune reaction won't occur normally until two weeks after transplantation. Therefore, other factors may account for the early islet graft damage in our allotransplantation.

After transplantation the islet graft is under a condition of oxidative stress. Chronic hypoxia and ischemia/reperfusion are among the reasons leading to this oxidative damage. It has been measured that the mean PO_2 is 5-10 mmHg (about 1% O_2) in syngeneically transplanted islets 9-12 weeks after implantation, regardless of the implantation site(Carlsson *et al.* 2001). Islet grafts are initially an essentially avascular tissue. They are particularly prone to the destruction caused by ischemia followed by reperfusion during the process of revascularization of the islet grafts(Menger *et al.* 1992).

Low levels of oxygen or a hypoxic environment can impair islet cell function for example by inhibiting beta cell insulin biosynthesis. It has been shown that there is a strong correlation between reduced oxygen supply and the occurrence of apoptosis in isolated human and rat islets(Moritz *et al.* 2002). The hypoxia *in vitro* study in the present study also demonstrated this point.

It has been well documented that various ROS including nitric oxide(Reid *et al.* 2001) and superoxide(Akizuki *et al.* 2000) are produced during the early phase of transplantation. Both chronic hypoxia, the ischemia/reperfusion process (Li & Jackson 2002)and the non-specific infiltration of macrophage in the islet graft may contribute to ROS generation in the early stages of islet transplantation. Systemic measurement of nitrotyrosine production after transplantation indicates that the day for the peak of nitrotyrosine production is 7 days after transplantation(Sakurai *et al.* 1999). Our data confirmed these concepts by detecting the ROS production under hypoxia condition and by immunostaining of nitrotyrosine in the allotransplanted graft. Consequently ROS mediated destruction may be another important factor in the early islet graft loss.

The pancreatic beta cells contain low levels of ROS scavenger systems(Grankvist *et al.* 1981b; Welsh *et al.* 1995; Malaisse *et al.* 1982; Johansson & Borg 1988), therefore any approach to reduce oxidative tension in transplanted islet by enhancing their ROS defense system is expected to improve islet graft survival. In our study we introduced an antioxidant MT transgene specifically in beta cells to increase their ROS scavenging capacity. Our results supported the importance of antioxidants and oxidative stress in transplantation. These data are consistent with clinical antioxidant trials and recent successful reports of antioxidant treatments in islet transplantation(Winter *et al.* 2002;

Bottino *et al.* 2002). In a very recent study(Bottino *et al.* 2002) an antioxidant SOD mimic was found to greatly increase islet graft survival when this drug was used during the isolation of islets. Consequently external application of antioxidants, either by direct exposure or gene transfer into isolated islets, might be a very valuable tool to increase the efficacy of islet transplantation.

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Publications

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Sucrose preference is restored by electroacupuncture combined with chlorimipramine in the depressive rats induced by chronic mild stress. *Caps News Communication* 1998, 17(Suppl 1): 113-114

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Expressions of preproenkephalin mRNA during electroacupuncture analgesia enhanced by fenfluramine. *Acta Pharmacol Sin* 1995;16(5): 431-4

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Alterations of central delta and Kappa opioid receptor binding sites in droperidol enhanced acupuncture analgesia. *Acta Acad Med Shanghai* 1994; 21(suppl): 14-8

Changes of opioid receptor in rat brain during electroacupuncture analgesia potentiated by fenfluramine. *Acta Acad Med Shanghai* 1994; 21(suppl):50-5

C-Fos expression during electroacupuncture analgesia in rats--an immunohistochemical study. *Acupuncture & Electro-Therapeutics Res, INT.J.* 1992; 17:165-176

Honors

Outstanding Student Fellowship, 1988, 1990, 1992, SMU, China

First Prize for the research thesis on the mechanism during electroacupuncture analgesia enhanced by fenfluramine, 1994, The Third East-West Panin Conference, XiAn, China

One of the winners of the First Prize for Scientific Technological Development by the National Education Commission for the research on relationship between opioidnergic and dopaminergic system during electroacupuncture combined with drugs. 1997, National Education Commission, China

One of the winners of the Second Prize for Scientific Technological Development by the National Education Commission for the research on opioid receptor and its molecular mechanism during acupuncture analgesia, 1999, National Education Commission, China

IPIBS Fellowship, 2001-2002, University of Louisville,

Honorable Mention for Graduate Student Division, 2001, Research Louisville 2001

3rd Place Award for Graduate Student Division, 2002, Research Louisville 2002