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Thyroid hormone and Diabetes: an anti-apoptotic pro-survival opportunity for pancreatic β cells.

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Introduction

Diabetes

As one of the major chronic metabolic diseases, diabetes mellitus affects at least 200 million people worldwide and it is predicted to increase to 440 million adults by 2030 (Shaw JE. *et al*. 2010). It is characterized by a failure of glucose omeostasis, resulting in a variety of severe complications. Compelling evidences suggest that the key factor in the pathogenesis of both type I and type II diabetes is the loss of the insulin producing β cells of pancreatic islet of Langherans.

In type I diabetes, accounting for approximately 10% of diabetic patients, the autoimmune response completely reduce pancreatic β cell mass, resulting in inadequate insulin secretion and consequently in abnormally high blood glucose levels. Because of the destruction of β cells, type I patient became dependent on daily injections of insulin, routing monitoring of blood glucose levels and strict diet control all lifelong.

The most common form, classified as type II diabetes, is associated with asymptomatic peripheral resistance to insulin and impaired insulin secretion. However, at late stages of this disease β cell mass decreases significantly leading to hyperglycemia.

One of the main processes involved in insulin producing β death is apoptosis which leads to insulin deficiency. Therefore, it is conceivable that a valuable approach to treat or even to prevent the onset of diabetes, may imply an anti-apoptotic pro-survival therapy of β cell.

Akt pathway and β cell

The entire β cell mass is dictated by a dynamic balance of neogenesis, proliferation, cell size and apoptosis.

The molecular IRS/PI3K pathway plays a critical role in the regulation of β cell mass and the Akt kinase is one of the most promising downstream molecules of this pathway. Akt has been implicated as a critical mediator of insulin-stimulated glucose uptake, suppression of apoptosis, stimulation of glycolysis and activation of glycogen and protein synthesis in various cell-culture systems (Coffer PJ *et al*. 1998).

It has recently become evident that PKB/Akt activation plays a pivotal role in β-cell survival (Lingor MK. *et al*. 2003) and growth; moreover, recent evidences reviewed in Elghazi L. *et al*. (2006) underscore the importance of Akt in the regulation of β cell mass and function. In pancreatic β-cells, PKB/Akt can be activated by different factors, such as IGF-1 and GLP-1 (Buteau J. *et al*. 2001; Giannoukakis N. *et al.* 2000), and it is directly activated by glucose (Dickson LM. and Rhodes CJ. 2004). When activated, via a cAMP-dependent or independent mechanism, Akt mediates a large number of cellular processes, including mitogenesis, survival and differentiation.

Activation of Akt/PKB results in phosphorylation of many substrates that control various biological signalling cascades including insulin-mediated glucose transport, protein and glycogen synthesis, cell proliferation, growth, differentiation, and survival (Woodgett JR. 2005). Experiments in mouse models have assessed the role of Akt in glucose omeostasis. Akt1/PKBα deficient mice show normal glucose homeostasis but impaired foetal and postnatal growth (Cho H. *et al*. 2001b). In contrast, Akt2/PKBβ-deficient mice develop diabetes due to reduction in insulin-stimulated glucose uptake in peripheral tissue and β-cell failure (Cho H. *et al*. 2001a). These results suggest that Akt/PKB could play a role in β-cell adaptation to insulin resistance states. Akt1/Akt2 double-knockout mice

showed severe growth deficiency, impaired skin development, and skeletal muscle atrophy, and died shortly after birth (Peng XD. *et al*. 2003). The generalized defect in conventional knockouts and the expression of different Akt/PKB isoforms with similar biochemical characteristics render β-cell function alterations difficult to interpret. Moreover, overexpression of constitutively active Akt/PKB in β-cells in transgenic mice resulted in augmented β-cell mass by increase in proliferation and cell size and in β cell resistance to streptozotocin-induced death (Bernal-Mizrachi E. *et al*. 2001; Tuttle RC. *et al*. 2001). Transgenic mice with reduction of Akt/PKB activity in β-cells exhibited β cell mass but glucose intolerance, reduced basal insulin levels and defective insulin response to glucose and potassium (Bernal-Mizrachi E. *et al*. 2004). Based on these results and experiments in cell lines, it is likely that Akt/PKB is a major mediator of the responses to insulin, insulin-like growth factor (IGFI), incretins, and glucose. *In vivo* and *in vitro* experiments suggest that Akt/PKB could mediate the proliferative signals induced by activation of Irs2 signalling (Kitamura T. *et al*. 2002).

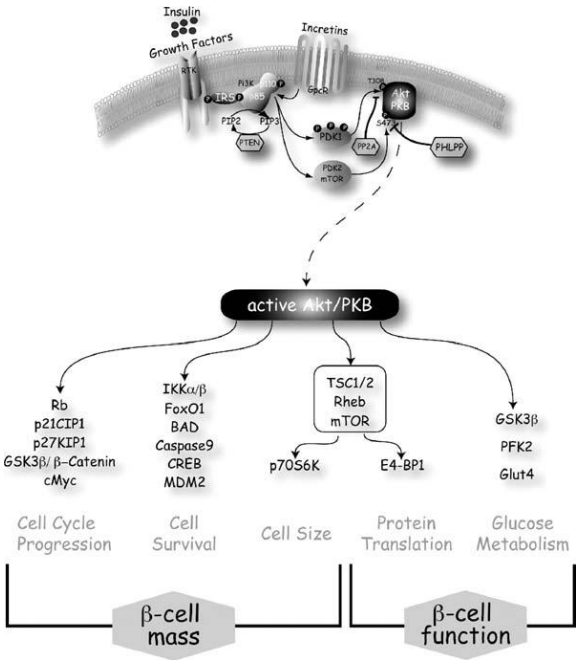
Akt/PKB also regulates cell cycle molecules phosphorylation and inactivation of the Foxo family of forkhead transcription factors (AFX/Foxo4, FKHR/Foxo1, and FKHR-L1/Foxo3a) (Martinez-Gac L. *et al*. 2004). In particular the PI3K-Akt/PKB pathway elicits events that reduce the abundance of $p27^{Kip1}$ by regulation of transcription, translocation and protein levels, indicating that this cell cycle inhibitor can contribute to β-cell failure during the development of type II diabetes in insulin resistant models (Uchida C. *et al.* 2005). Similar to $p27^{Kip1}$, Akt/PKB phosphorylates $p21^{CIP}$, thereby inducing cytoplasmic translocation and stabilization (Liang J. *et al.* 2002, Chang F. *et al*. 2003).

Akt has also implicated in β cell regeneration. In mice after pancreatectomy, Akt activation was observed in proliferating ducts (Jetton TL. *et al*. 2001). This may be mediated by phosphorylating the transcription factor CREB and the forkhead transcription factor Foxo-1. Phosphorylation of CREB has been associated with regulation of insulin and IRS-2 gene expression required for β cell differentiation and survival.

The Akt/PKB signalling is one of the critical pathways regulating cell survival, and its importance in β-cells has been suggested by increased apoptosis observed in $Irs2^{-/-}$ mice. In β-cells, Akt/PKB signalling mediates anti-apoptotic effects induced by diverse agents such as glucose, GLP-1, IGF-1, and insulin (Dickson LM. and Rhodes CJ. 2001; Brubaker PL. and Drucker DJ. 2004;); moreover the activation of Akt/PKB signalling protects β-cells against fatty acid-induced apoptosis and modulates survival to endoplasmic reticulum stress (Srinivasan S. *et al*. 2005, Wrede CE. *et al*. 2002). Glucose activation of Akt/PKB results in part by a paracrine/autocrine stimulation of the insulin receptor suggesting that glucose can be an important modulator of β-cell survival (Ohsugi M. *et al.* 2005). Akt/PKB affects survival by directly regulating members of the Bcl-2 family. Phosphorylation of BAD inhibits the pro-apoptotic activity by releasing it from the Bcl-2/Bcl-X complex and binding to 14-3-3 proteins. Increased apoptosis was associated with decreased Bcl-X_L and Bcl-2 expression in $pdx1^{+/-}$ islets, suggesting a potential link between Akt, pdx1, and survival (Johnson JD. *et al.* 2003).

The regulation of cell size by Akt/PKB is mediated by activation of the mammalian target of rapamycin (mTOR), which targets ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1), key

regulators of protein translation and cell size. Mice deficient in S6k1 exhibited glucose intolerance and hypoinsulinemia associated with a 15% reduction in β-cell size, similar to the phenotype in dS6K-null Drosophila (Pende M. *et al*. 2000). Moreover, overexpression of a constitutively active form of Akt/PKB resulted in increased cell size suggesting that S6K1 relates some of the growth signals induced by Akt/PKB. The potential role of mTOR/S6K signalling in proliferation remains controversial. There is substantial evidence for a role of insulin/IGF signalling in insulin secretion. Experiments in insulinoma cells suggest that the regulation of insulin secretion by PI3K/Akt/PKB is complex and no consensus has yet been achieved, however reduction in Akt/PKB activity in β-cells by overexpression of a kinase-dead Akt/PKB mutant results in an insulin secretory defect (Bernal-Mizrachi E. *et al*. 2004).



(Elghazi L. 2006)

Figure 1. *Schematic representation of Akt/protein kinase B (PKB) signaling*.

Current treatment options for diabetes are mainly based on the exogenous supply of insulin, an approach not fully capable of mimicking the tight control of endogenously produced insulin released from pancreatic β cells.

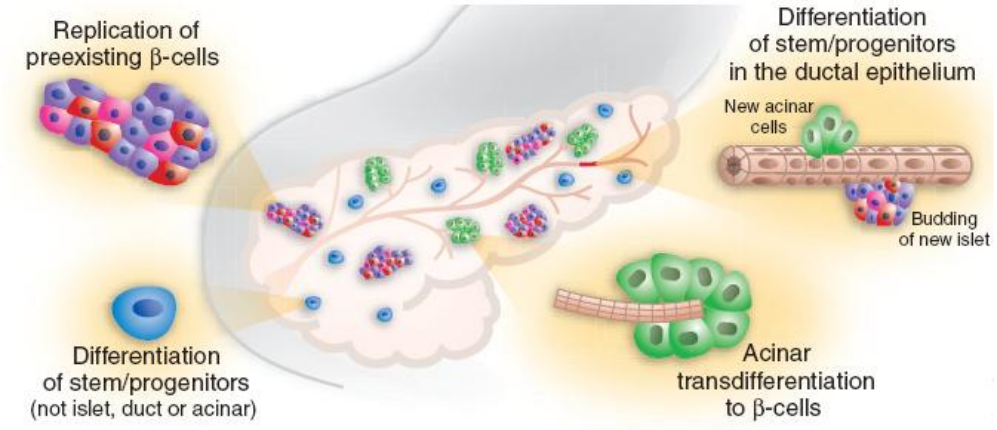
An ideal cure for type I diabetes (and for some cases of type II diabetes) requires a device capable of performing the two essential functions of the missing β cells: sensing blood glucose levels and secreting appropriate levels of insulin to the blood stream.

The pancreas has a remarkable capacity to regenerate and repair tissue damage and itself is likely to be the main source of new insulin- producing β cells and of cells that can regenerate the acini and ducts.

Over the past four decades whole pancreas organ transplantation has been the most effective treatment for diabetes patient but with serious complications (Bonner-Weir S. and Weir GC. 2005). Whole pancreas transplantation, first performed in 1966 in combination with kidney was capable of producing a sustained, euglycemic state, reducing the incidences of hypoglycemia and offering the possible benefit of reducing microvascular, macrovascular and neurologic complications. Pancreas transplantation however, is a major, complex surgical procedure associated with significant risk and cost that may limit its general acceptability, especially when a potential diabetic recipient has little evidence of renal impairment and does not need a kidney transplant.

Therefore, much attention has been focused on the potential of bioengineered insulin-producing surrogate cells (Guo T. *et al*. 2009; Halban PA. *et al.* 2010; Aguayo-Mazzucato C. *et al*. 2010). Several sources have been considered for the *in vitro* generation of insulin-producing cells including *ex vivo* expanded β cell

(Lechner A. *et al.* 2005), endocrine progenitor cells (Yatoh S. *et al*. 2007), transdifferentiated or transduced liver or intestinal cells (Elsner M. *et al*. 2008) , bone marrow mesenchymal stem cells (Sordi V. *et al*. 2008) and pluripotent embryonic stem cells (Assady S. *et al*. 2001; Lumelsky N. *et al*. 2001).



(*Bonner-Weir S. et al. 2005)*

Figure 2. *Pancreas as a source of β cells.*

The most prominent and promising cell source for β cell progenitors are embryonic stem cells (ESCs) derived from the inner cell mass of blastocystis during early stages of embryogenesis. The most important characteristics of ESCs include the capacity to self-renew and the potential to differentiate into all embryonic cell type, a potential termed pluripotency, under *in vivo* and *in vitro* conditions (McKay R. 2000). Another advantage of ESCs is the ability to maintain their stem cell properties upon proliferation under certain cell culture

conditions, thus allowing almost unlimited expansion without compromising their differentiation capacity. The substantial advances in studies on human ESC differentiation have raised the vision for new strategies aimed at generating large amounts of glucose-responsive, insulin-producing β cells for therapeutic purposes.

Numerous approaches have been used to generate insulin- producing β cells from hESCs. Many reported the generation of cells with some degree of insulin production from mouse (Lumelsky N. *et al*. 2001; Soria B. *et al*. 2000), monkey (Lester LB. *et al*. 2004) and human ESCs but none of the studies has affirmed the *in vitro* production of fully functional β cell that can secrete physiologically sufficient amounts of insulin in response to glucose. One reason of this failure is the fact that although pancreatic β cells are the main source of insulin production in mammals they are not the only cell type that can synthesize and release insulin. Although several studies provided evidences that cells containing insulin and various other β cell markers could be generate from embryonic stem cells, a recent report demonstrated that insulin staining could be artifact, reflecting insulin uptake by apoptotic cells from culture media containing high concentrations of insulin (Hansson M. *et al*. 2004).

β cells have long been known to have a substantial capacity for replication, as best shown in rodents with various *ex vivo* and *in vivo* model system (Bonner-Weir S. *et al*. 2000). The replication rate of human β cells, however, is much lower that of rodent β cells although it can be modestly stimulated by transplantation of β cells into insulin resistant mice (Tyrberg B. *et al*. 2001). Considerable attention is now being focused enhancing β cell replication to generate cells for clinical application. Recent data suggest that β cells of human cultured islets can

dedifferentiate and expand, and then be directed to re-differentiate back toward a β cell phenotype (epithelial-mesenchimal transition) (Gershengorn MC. *et al.* 2004). As cells expand from cultured human islet preparations they develop a serpiginous appearance and express nestin and the mesenchymal marker vimentin but not islet hormones. A variety of maneuvers were used to force re-differentiation, including serum-free media, nicotinamide, glucagon like peptide1 and aggregation cells which led to expression of an assortment of islets markers at low levels. A caveat of these studies is the difficulty to distinguish between the dedifferentiation of β cells and the expansion of pancreatic stem progenitor cell that are not of β cell origin.

It is interesting to compare these studies with those of PANC1 cell, a human pancreatic duct cell line, which have been shown to undergo a similar morphological transition with changes in culture conditions. When aggregated in serum-free media with a high glucose concentration, the cells express very low level of islet hormone mRNA and protein (Hardikar AA. *et al.* 2004).

Moreover Hui H. *et al*. (2001) indicated that treatment with GLP-1 is indeed able to induce differentiation of rat (ARIP) and human (PANC-1) cell lines both derived from the pancreatic ductal epithelium, into insulin synthesizing cells.

Misiti S. *et al*. (2005) suggested that thyroid hormone T3 treatment of human pancreatic ductal cell lines is capable to convert them towards a β cell phenotype.

A recent study has challenged the view that neogenesis from ducts or any other progenitor cell take place. Using genetic marking for lineage tracing with the insulin promoter, Dor Y. *et al*. (2004) concluded that no new islets were formed in mice after birth or following 70% pancreatectomy, but that new β cells could generated by replication of existing β cells. Although this study supports the

concept that β cell replication is the dominant mechanism for β cell expansion in adult mice, it remains controversial because it does not convincingly prove that new islets are not formed during neonatal life or after regeneration-inducing maneuvers such as partial pancreatectomy or duct ligation.

Attempts to restore the β cell deficiency occurring in diabetes has been pursued with many strategies; although all these avenues are promising, one presents some difficulties and none is clearly better than the others.

Within the past 30 years, pancreatic islet transplantation has became a clinical practice and an option in the treatment of diabetes. Islet transplantation has a distinct advantage over whole organ transplantation in regards to reduced peri-procedural morbidity. The procedure avoids major surgery and the risk of associated post-operative complications, re-lapatomy and acute graft loss. In 1967 Lacy's group described a novel collagenase-based method (later modified by Dr. Camillo Ricordi) to isolate islets, paving the way for future *in vitro* and *in vivo* islet experiment. Subsequent studies showed that transplanted islets could reverse diabetes in both rodents and non-human primates (Kemp CB. *et al*. 1973, Scharp DW. *et al*. 1975). In a summary of the 1977 workshop on pancreatic islet cell transplantation in diabetes, Lacy commented on the feasibility of islet cell transplantation as a therapeutic approach for the possible prevention of the complications of diabetes in man (Lacy PE. 1978). Improvements in isolation techniques and immunosuppressive regiments ushered in the first human islet transplantation clinical trials in the mid-1980. In 1999 Dr. James Shapiro and his team at University of Alberta in Canada pioneered the "Edmonton protocol": a new set of procedures for islet transplantation. It has been hailed as an historic breakthrough and a major scientific "proof of principle".

The Edmonton protocol involves isolating islets from a cadaveric donor pancreas using an improve method of islets purification and isolation. Each recipient receives islets from one to as many as three donors. The islets are infused into the patient's portal vein. Patients were maintained on a glucocorticoid-free immunosuppression protocol.

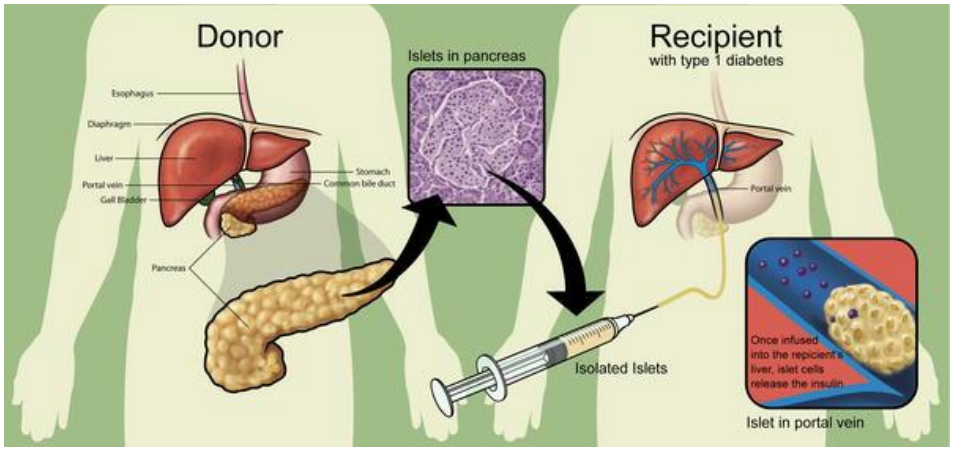


Figure 3. *Islets transplantation practice.*

The clinical outcomes of islet transplantation and the Edmonton protocol are encouraging. The main goal has historically been insulin independence for same years (Ryan EA, Shapiro AM. 2005). Although long-term insulin independence was not achieved in the majority of the patients, islet transplantation did help control blood glucose level more easily when combined with insulin injections.

One major hurdle that precludes islets transplantation from being widely applied is the lack of adequate sources of donor islets and β cells due to the limited availability of cadaveric tissue. The discrepancy between the effectiveness of cell therapy and the limited amount of transplantable material has urged to explore new strategies in a search for renewable source of high quality β cells for transplantation.

This requirement may in part due to poor survival of transplanted islets in the first few days after transplantation, perhaps because of glucotoxicity, hypoxia and/or inflammation (Davalli AM. *et al*. 1996). Thus, a better understanding of the mechanisms controlling isolated islet cell death may lead to the development of strategies to improve islet survival following isolation.

Many laboratories around the world have been focused their studies on developing methods and strategies for increasing the availability of β cells that could be transplanted into patients with TID. Moreover, *ex vivo* culture of β cells provide a good model to study the effect of new promising drugs or factors on the β cell function, proliferation and survival. Among these strategies *in vitro* expansion of islet cell mass by increasing β cell proliferation and survival (Garcia-Ocana A. *et al*. 2001; Hayek A, Beattie GM, 2002) has gained some relevance. For many years scientists believed that pancreatic β cells were terminally differentiated cells incapable of undergoing proliferation. However, multiple studies have clearly shown that pancreatic β cells do normally replicate in basal condition and that proliferation rates can be enhanced in response to different physiological and patho-physiological environments (Sorenson RL, Brejle TC. 1997). Several studies have shown that the major source of new β cells in adult mice life is through proliferation of pre-existing β cells, rather than differentiation of β cell precursors (Bonner Weir S. 2000; Dor Y.*et al*. 2004).

It has became apparent that a number of strategies to counteract the apoptosis of islets and to prevent β cell functions may have therapeutic relevance in preventing diabetes. The IGF1/IGF BP3 complex has recognized to enhance β cell replication and β cell survival after exposure to pro-apoptotic agents, which indicated this complex as a survival factor for β cell undergoing apoptosis (Chen W. 2004).

Glucagon like peptide 1 (GLP1) is a growth factor for β cell and it also has a powerful anti-apoptotic action (Hui H. 2003), the role of glucose is not still clear. Several growth factors and pathways have been deeply studied and Akt kinase has been demonstrated as crucial to drive β cell replication and survival.

It has in fact recently been evidenced that insulin released by the islets in culture activates Akt in an autocrine manner to mediate islet survival thus improving cell culture condition (Aikin R. *et al*. 2006). It has also been outlined an autocrine survival pathway in isolated human islets where secreted insulin improves islet survival by activating Akt, thus enhancing the chances of improving graft survival. Indeed, treatments which activate Akt during islet culture can improve graft survival (Contreras *et al*. 2002), indicating that elevated Akt activity could render islets less susceptible to injury during the immediate post-transplantation period. Moreover, Akt has gained relevance as a viable target designing molecular approaches to treat diabetes, both promoting islet cell mass up-regulation and promoting insulin secretion thus gaining a key role in regulation of β cell function.

Thyroid hormone

Thyroid hormones, 3,5,3'-triiodotyronine (T3) and 3,5,3',5'-tetraiodothyronine (T4) are widely known to influence a variety of physiological processes, including cell growth, differentiation and metabolism in mammals, metamorphosis in amphibia, and development of the vertebrate nervous system (Shi YB. *et al.* 1998; Koibuchi N, Chin WW. 2000). Both T3 and T4 are synthesized by the thyroid gland; however, T4 is the major secreted hormone. Within the cell, T3 is the most potent thyroid hormone as it binds to TH receptors (TRs) with 10-fold higher affinity. The major pathway for the production of circulating T3 is via 5′ deiodination of the outer ring of T4 by selenoproteins known as deiodinases (Kohrle J. 2000). Type I deiodinase is found in peripheral tissues such as liver and kidney and converts circulating T4 to T3. Type II deiodinase has high affinity for T4 (K_m in the nanomolar range) and is found primarily in the pituitary gland, brain, and brown fat, and contributes to both peripheral and intracellular conversion of T4 to T3. Thus, tissues that contain type II deiodinase can potentially respond differently to a given circulating concentration of T4 (by intracellular conversion to T3) than tissues that only can respond to T3. Type III deiodinase is found primarily in placenta, brain, and skin and, together with type I deiodinase, converts T4 to reverse T3 (rT3), an inactive metabolite of TH. Recently, TH transporters that are located in the plasma membrane and which can regulate TH uptake into cells have been described (Jansen J. *et al*. 2005). Thus the intracellular level of T3 is dependent on the relative activities of these three deiodinases.

The classical genomic actions of Thyroid hormones

Cellular action of thyroid hormone may be initiated within the cell nucleus, at the plasma membrane, in the cytoplasm and at the mitochondrion. Thyroid hormone T3 regulates nuclear gene expression by binding the *thyroid hormone receptors* (TRs). Two TR genes, α and β, encode four T3 - binding receptor isoforms (α1, β1, β2 and β3). TR is found in the nucleus as a heterodimer with retinoic acid X receptor (RXR). The heterodimeric complex sheds corepressor protein when T3 is bound and recruits coactivators that facilitate binding of the heterodimer T3 complex to the *thyroid hormone response elements* (TREs) of hormone-responsive genes and consequent gene transcription (Zhang J. and Lazar MA. 2000). This genomic mechanism of thyroid hormone action has been demonstrated in several thyroid hormone responsive cells and leads to modulation of transcription of a hundred or more genes (Feng X. *et al*. 2000; Miller LD. *et al*. 2004). Characteristics of genomic actions of the hormone include the requirement for access of the hormone to the cell interior, translocation and changes in cell content or secretion of specific gene products. Several or more hours are usually required for genomic mechanisms to be manifest.

L-thyroixine (T4) can act via nuclear TR, but the affinity of receptor is much lower than that for T3. Thus T3 is the natural ligand of TRs. In the genomic concept of hormone action, T4 is viewed as a pro-hormone that yields the more metabolically active T3 via action of tissue deiodinase activities.

Although T3 is known to exert many of its actions through the classical genomic regulation of gene transcription, a number of T3 effects occur rapidly and are unaffected by inhibitors of transcription and protein synthesis.

For more than two decades, actions of thyroid hormones in a variety of cells have been described that do not primarily involve nuclear TR (Bussett H. *et al.* 2003; Davis P.J. *et al.* 2005) and thus are 'nongenomic.' The mechanisms of several of these nongenomic actions of thyroid hormone are now understood, at least in part, and depend upon cellular signal transduction systems and either novel cell surface receptors for thyroid hormone (Berg JJ. *et al*. 2005) or extranuclear TRβ (Lei J. *et al*. 2003; Moeller LC. *et al*. 2006) or derivatives of TRα (Davis PJ. *et al*.2007).

Non-genomic or non-classical nuclear hormone effects encompass any actions that do not directly effect nuclear gene expression. Such non-genomic actions frequently have a short latency, are unaffected by inhibitors of transcription and translation, have agonist and antagonist affinity and kinetics divergent from the classical nuclear receptor and persist in genetically modified mice that lack the classical nuclear receptors. These non-genomic responses are frequently associated with secondary messenger signalling pathways including the phospholipase C (PLC). It has been suggested that such non-genomic actions might inositol triphosphate (IP3), diacyl glycerol (DAG), protein kinase C (PKC) and $Ca^{2+}{}_{I}$ pathway, the adenylyl cyclase, protein kinase A (PKA) and the cyclic AMP-response element binding protein (CREB) pathway and the Ras, Raf1 serine/threonine kinase, mitogen activated protein kinase (MEK) and the mitogen activated protein kinase (MAPK) pathway be mediated by either membrane associated isoforms of the classical nuclear receptors or by novel membrane

receptors with significantly different agonist/antagonist affinities (Losel R. and Wehling M. 2003). The recent identification of such receptors for progestins and estrogens has highlighted the non-genomic actions of steroid hormone (Li L. *et al*. 2003; Zhu Y. *et al*. 2003).

Non-genomic actions of thyroid hormones have been described at the plasma membrane, in the cytoplasm and in cellular organelles (Henneman G. *et al.* 2001). Thyroid hormone non genomic action has been related to various second messenger signalling pathways. They have included the modulation of Na^+, K^+, Ca^{2+} and glucose transport, activation of PKC, PKA and ERK/MAPK and regulation of phospholipid metabolism by activation of PLC and PLD (Kavok NS. *et al.* 2001).

In vitro, independent of protein synthesis, T4 induces IP3 and calcium signalling and augments the effects of IFNγ via PKC and PKA (Davis PJ. *et al*. 1989; Lakatos P. and Stern PH. 1991; Lin HY. *et al.* 1997). In addition, T4-linked to agarose, which does not cross the plasma membrane, has been shown to activate MAPK by a pertussis toxin sensitive mechanism suggesting the actions of a G protein-coupled thyroid hormone membrane receptor (Lin HY. *et al.* 1999).

In vivo, T4 regulates thermogenesis and the lipolytic activities of catecholamines within 30 min (Lynch MA. *et al.* 1985; Wrutniak C. and Cabello G. 1989).

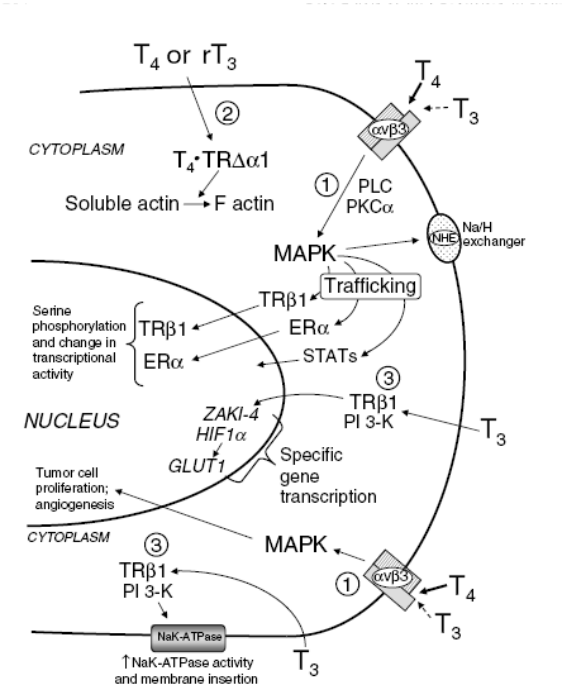
Non-genomic effects of thyroid hormone have also been reported in the myocardium and vasculature. T3 enhances cardiac output and reduces systemic vascular resistance in normal adult males within 3 min (Schmidt BM. *et al.* 2002) and cell culture studies suggest that thyroid hormones rapidly, and non-genomically, regulate the Ca^{2+}ATPase enzyme, the Na^+ channel (I_{Na}) via PKC, the

K^+ channel (I_K) via PI3-kinase, the Na^+/H^+ anti-porter via PKC and MAPK and the inward rectifying potassium channel (I_{K1}) (Davis PJ. and Davis FB. 2002).

T3 also increases sarcoplasmic reticulum Ca^{2+}, cell shortening, contractility and calcium mediated arrhythmic activity suggesting that T3 has a non-genomic, positive ionotropic and arrhythmogenic effect (Wang YG. *et al.* 2003).

In pituitary cells, T3 has been shown to non-genomically stimulate the ether-a-go-go related gene potassium channel (ERG/KCNH2), which reduces endocrine neuronal excitability, via PI3-kinase and the Rac GTPase, whereas TRH (thyroid releasing hormone) inhibits ERG activity via PKC and the Rho GTPase to increase neuronal excitability (Storey NM. *et al.* 2002). These observations suggest a possible role for non-genomic T3 signalling in the hypothalamic/pituitary feedback loop.

Thyroid hormone T3 is also involved in the PI3K pathway: it has recently been reported to regulate the Na,K-ATPase activity via PI3K in alveolar epithelial cells (Lei J. *et al.* 2004) and to activate the protein kinase B via PI3K, in human fibroblasts (Cao X. *et al.* 2005). Estrogen and retinoic acid have also been recognized to activate PI3K rapidly through the nontranscriptional action of their receptors (Simoncini T. *et al*. 2000; Sun M. *et al*. 2001; Haynes MP. *et al.* 2003; Lopez-Carballo G. *et al.* 2002). Furthermore, estrogen receptor-α was demonstrated to activate PI3K through binding with p85α either in a ligand-dependent manner in endothelial cells (Simoncini T. *et al.* 2000) or in a ligand-independent manner in epithelial cells (Sun M. *et al*. 2001).



(Davis PJ. 2007).

Figure 4. *Schematic representation of non genomic actions of T3 and T4.*

Might thyroid hormone influence glucose homeostasis?

Thyroid dysfunctions and Diabetes are the two most common endocrinopathies encountered in clinical practice. Both conditions frequently co-exist and the prevalence of thyroid dysfunctions in diabetic patients is higher than in the general population (Perros P. *et al.* 1995). Thyroid hormones contribute to the regulation of glucose metabolism and pancreas function and it is reported that treatment of thyroid dysfunction in diabetic can improve the glycemic control, to attenuate the cardiovascular risk and, to promote the general well-being (Kadiyala R. *et al*. 2010).

Molecular studies showed that thyroid hormone T3, binding to the thyroid hormone receptor, regulates the phosphatidylinositol 3 kinase /Akt pathway in several cellular system (Cao x. *et al*. 2005; Furuya F. *et al*. 2006)

Pancreatic islets and, in particular, the β cell express thyroid hormone receptor, α and β isoforms, in addition to other nuclear receptors implicated in the regulation of insulin signaling (Malaisse WJ. *et al*.1967; Chuang JC. *et al*. 2008).

We have previously demonstrated that treatment of human pancreatic duct cells with T3 promotes cell differentiation into insulin producing β cells, upregulates insulin and glucose transporter-2 transcripts and increases the insulin release into the medium (Misiti S. *et al.* 2005).

Moreover, Verga Falzacappa C. *et al* (2007) reported that T3 activates Akt in the islets β cell line rRINm5F and hCM through the interaction between thyroid hormone receptor TRβ1 and PI3K-kinase p85α. In particular T3 induced the PI3K signalling thus activating the kinase Akt. Therefore, T3 not only regulates pancreatic β cell proliferation and survival, but also induces the increase of cell size and granulosity cell protein synthesis and, most importantly, c-peptide production in our *in vitro* models.

In conclusion we evidenced (Verga Falzacappa C. *et al.* 2009) that thyroid hormone action on PI3K/Akt signalling leads to the positive regulation of the main β cell features these being preferential targets in the designing of new strategies against β cell loss in diabetic disease.

Taken these considerations I, firstly, hypothesized, for my thesis, that thyroid hormone T3 could act as a survival factor in a freshly isolated rat islets culture, and in particular that T3, being able to counteract physiological and

pharmacological β cell death, could promote islets viability to finally improve transplantation success.

Moreover, the crucial endpoint of my study, linking thyroid hormone and β cell function, was to verify whether T3 treatment is able to preserve and protect functional β cell mass in a *in vivo* model.

Aim

The pancreatic β cell mass plays an essential role in determining the amount of insulin that is secreted by pancreatic β cells to maintain blood glucose levels within a narrow range. Pancreatic β cell loss is the key factor in the pathogenesis of both type 1 and type 2 diabetes. One of the main process involved in the β cell death is apoptosis, which leads to insulin deficiency. Therefore, it is conceivable that a valuable approach to treat or even to prevent the onset of diabetes may imply an anti-apoptotic pro-survival therapy of insulin producing cells.

To this aim several factors (IGF 1, IGF 2, GLP-1, etc.), known for their proliferative properties and/or their action on cell differentiation, have been shown to interfere with the sequence of events leading to cell apoptosis and of promoting cell survival (Giannoukakis N. *et al.* 2000; Jill DJ. *et al*. 2000; Perfetti R. *et al* 2000). Some pathways have been deeply studied and recent advances have indicated the signal transduction via insulin receptor substrate-2 (IRS-2) and downstream protein kinase B as crucial to the β cell function. In particular AKT kinase has been demonstrated to play an important role in the β cell proliferation, survival, size and insulin secretion.

Our previously evidences suggest that thyroid hormone T3 can regulate cell proliferation, survival, size, protein synthesis and insulin production in the insulin secreting cells rRINm5F and hCM. In particular we have shown that T3 is able to induce the phosphoinositol 3-kinase activity, resulting in the activation of the AKT kinase in the cited cell line models (Verga Falzacappa C. *et al.* 2006, 2007).

β cell replacement by transplantation of islet cells is currently regarded as acceptable therapeutic option for diabetic patients. Nevertheless, extremely large number of islets requiring two to four cadaveric pancreases are usually necessary to restore insulin independence. A critical problem is how islet cells can survive in

a new environment, particularly with regard to revascularization and reinnervation. Shortly after implantation islets grafts function poorly and many transplanted β cells undergo apoptosis prior to full engraftment. Today, *ex vivo* islets cell culture prior transplantation in presence of stimulating factors is considered a good strategy to contrast the short outcoming of islets transplantation.

Taken all these considerations, the aim of my thesis was to investigate the role of thyroid hormone treatment to improve islets transplantation strategy in freshly isolated rat islets during an *in vitro* culture period.

Moreover, the crucial endpoint of my study, linking thyroid hormone and β cell function, was to verify whether T3 treatment was able to preserve and protect functional β cell mass in pharmacological diabetic mice.

Materials and Methods

Chemical

Crude collagenase type 4 was obtained from Worthington Biochemicals Corporation (Lakewood, NJ); 3,5,3'-Triiodothyronine (T3), Polysucrose 400 and Streptozotocin (STZ) and 5-Bromo-2'-deoxyuridine (BrdU)were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Animals studies

Wistar rats male adult (about 12 weeks old) were used as islets donors. The animals had free access to tap water and pelleted food throughout the course of the study. The local animal ethics committee approved all experiments.

Balb/c male mice (about 6 week old) were maintained in a pathogen-free environment in isolator caging system in air conditioned room at 23±1°C in the La Cattolica University's animal facilities (Rome, Italy) in accordance to the institutional guidelines.

Isolation and culture of rat islets

Pancreatic islets were isolated from 300 g weighting male adult *Wistar* rats by standard surgical procurement followed by intraductal collagenase distension, mechanical dissociation and Euroficoll purification. In brief animals anesthetized with ketamine 70 mg/Kg + domitor 0.5 mg/Kg injected intraperitoneally, were sacrified by CO2 inhalation. For the exposure of the whole pancreas, the abdominal wall was opened via a midline incision and the pancreas ductal connection to the intestine clamped. The pancreas was cannulated in situ via the common bile duct using a polyethilen tube (BD, Franklin Lakes, NJ) and distended by pumping a cold solution of collagenase (0.2%) prepared in a specific

isolation medium KRHB, containing (in mmol/l) NaCl 134, KC 4,7, CaCl2 1, MgSO4 1.2, KH2PO4 1.2, HEPES 10, BSA 0.5% pH 7.35. The whole pancreas was excised and transferred to a centrifugal tube and incubed for 20 min with gentle tumbling, at 37°C. Islets were purified on a discontinuous Euroficoll gradient, handpicked under a light stereomicroscope, pooled and then separated into study group and control group for the subsequent culture period. The islets were cultured in CMRL 1066 medium (GIBCO,Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum, L-Glutamine 2 mmol/L and Penicillin 100 μg/ml-Streptomycin 50 μg/ml in not coated plates (BD) with or without T3 (10-7 M) at 37°C in a humidified atmosphere of 5% CO2.

Microscopic monitoring of cultured islets

Morphological changes of isolated islets were monitored during 96 hours of culture. Images were recorded by a Canon digital camera and processed by Image J software. To evaluate the core cell damage of isolated islets, light microscopic analyses were performed at different time points (72 and 96 h) during the culture.

Viability of cultured islets

Batches of 15 islets were cultured in the presence or not of fresh aliquots of T3 (10-7 M) added every day for 72 h and 96 h. Islets viability was tested by using *live-dead cell viability test* (MBL International, 4 H Constitution Way Woburn, MA, US) under manufacturer's instruction. The assay utilizes two different dyes which can differently pass through cell membrane, the Propidium Iodide (PI, red Fluorescence) can only pass damaged membrane (death cells), while the Green Dye can pass the intact membrane (live cells). Images were visualized with a

Leica (D-35606 Burgsolms, Solms, Germany) epifluorescence microscope and taken by a Canon digital Camera. Secondly images were processed by ImageJ software. Nuclei were counted and percentages of live vs. death cells were visualized on histograms.

MTT assay of cultured islets

Islets were cultured in 96 multiwells for 72 and 96 h and treated as previously described. A solution of a tetrazolium salt was added to the culture medium and, after 4 h, the metabolic formazan product was solubilized in an organic solution. After 1 h of solubilization, the absorbances at 570 and 630 nm were recorded by using a 96 well plate reader.

BrdU labeling for cultured islets

Cell proliferation was determined additionally by BrdU staining. Islets were cultured for 48 h in the presence and not of T3 (10-7 M); during the last 24 h of culture BrdU 10 μM (Sigma-Aldrich) was added. The islets were cytospun on polarized slides. Slides were then washed in PBS 1x (Lonza) and incubated with HCl 3N for 25 min at RT; the reaction was then neutralized with borax-borate buffer (pH 9.1), and slides were washed in PBS 1x. Slides were incubated sequentially with PBS 1x, Goat serum 15% (Sigma-Aldrich), Triton X100 0.3% (Sigma) for 15 min at RT, and then with mouse monoclonal antibody anti-BrdU (Roche Diagnostic) 1:200, for 1 h at room temperature. After three washes in PBS 1x, slides were incubated with the secondary antibody Alexafluor 546 anti-mouse (Invitrogen) 1:1,000, for 1 h at room temperature in dark. Slides were then washed in PBS 1x twice and stained with Hoechst 1 μg/ml for nuclear detection.

Localization and intensity of fluorescence were evaluated by optical sections obtained using an Axiovert 200M microscope (Zeiss, Oberkochen, Germany) with ApoTOME© device connected with a CCD camera Zeiss Axiocam. Negative controls including omission of the primary antibody were also performed.

Induction of diabetes in mouse model

Animals were divided into three experimental groups: CTR, STZ, STZ+T3. Diabetes was induced by multiple (for 2 consecutive days) low dose (150mg/kg of body weight) intraperitoneal injection of Streptozotocin (STZ), freshly dissolved in 10mM Na-citrate buffer (ph4,5). STZ-T3 mice were divided into 3 groups and co-treated with 3 different doses of 3,5,3'-Triiodothyronine (T3) (75-100-150 μg of kg body weight, Saint Louis, Missouri, USA) every 24hours for 2 consecutive days. The T3 treatment began contemporary to Streptozotocin.

Evaluation of pancreta tissue and immunostaining

Mice were anesthetized with ketamine (70mg/kg) and domitor (1mg/kg) injected intra peritoneally and then sacrified by cervical dislocation. Pancreata were removed and embedded in killik cryostat embedding medium (Bio-optica, Milan, Italy). Five sections (7μm thick) per pancreata were examined.

Cross sectional islet area was determined on a total of five slides per pancreas stained with hematoxilin/eosin. Images were analysed using the Image J software.

For immunohistochemical analysis crysections were fixed in cold acetone for 1minute, air-dried and fixed in 4% paraformaldehyde for 10 minutes. Endogenous peroxidase activity was blocked by incubating slides in a solution of 3% hydrogen peroxide for 10minutes. Unspecific binding was blocked incubating the slides in

5%goat serum in PBS Ca-Mg Free for 45 minutes and then for 15 minutes in 1%BSA. Sections were incubated in humidified chambers overnight at 4°C with the appropriate primary antibody (insulin 1:100, pAkt 1:50, Akt 1:50 Cell Signalling) followed by 1 h incubation with secondary biotinylated anti-mouse or anti-rabbit (Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA) after which the sections were incubated for 1 more hour with Horseadish Peroxidase Avidin D (Vector Lab.). Immunoreactivity was revealed using 3,3'-diamonobenzidine (DAB, DAKO, north America, Carpinteria, CA) as the chromogen. Sections were counterstained with hematoxylin.

Measurement of insulin mRna by Real Time PCR

Pancreata RNA was extracted by SV Total RNA isolation System (Promega, 2800 Woods Hollow Road Madison, WI) under manufacture's instruction and 1 μg used to synthetize cDNA using Omniscript RT Kit (Qiagen, Chatsworth, CA). cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler Realtime PCR apparatus (Roche Inc., Nutley, NJ). Quantitative PCR was performed using SYBR Premix Ex TAq II (perfect Real Time)(TAKARA BIO INC. 2 Avenue du President Kennedy, 78100 St Germain en Laye, France) as described by the manufacturer. The reactions started with a denaturation step at 95° C for 10 seconds, followed by annealing at 56° to 66° C for 5 seconds and elongation at 72° C for 7 to 13 seconds. The reaction was then heated for 3 seconds at 2° C lower than the melting temperature of the DNA fragment. Oligoprimer pairs that allow the amplification of ~200 bp were designed and their specificity was verified by blasting in the GenBank database. Reading of the fluorescence signal

was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal. mRNA expression levels are expressed as number of copies/µg total RNA using a standard curve of the crossing point vs. logarithm of the quantity. The standard curve was established using known cDNA amounts of 0, 102, 103, 104, 105, and 106 copies of 18s and a LightCycler 3.5 program provided by the manufacturer (Roche Inc.). PCR products were analyzed on a 1,5% agarose gel. The expression levels of target genes were quantified and normalized by 18S level. Primers used for PCR amplification were for INS for: 5'-TGGCTTCTTCTACACACCCA-3'and rev:5'-TGCAGTAGTTCTCCAGCTGG-3'and for 18S for: 5'-GGAGAGGGAGCCTGAGAAA-3'and 18S rev:5'-AAAGAGTCCTGTATTGTTATTTT-3'.

Immunofluorescence analysis

Rat's islets were cytospun on polarized slides and immunofluorescence analysis was performed in the various experimental condition (specifically described within this section) to detect insulin signal. In addition the thyroid receptor b 1 was detected by the same procedure. Islets were stained with primary antibodies, rabbit anti-insulin (Cell Signaling Technology, Inc., 3 Trask lane, Dansvers, MA) and anti-TRβ1 (Santa Cruz Biotechnology Inc., San Diego, CA). After washing in PBS 1x (Lonza), islets were incubated with secondary antibodies fluorochrome conjugated (Alexafluor 488 anti-rabbit; Alexafluor 546 anti-mouse). Hoechst dye (1 µg/ml) was used for nuclear detection. Fluorescence was detected with an epifluorescence microscope (Leica, Germany), images were captured by a Canon

digital camera and images were processed with ImageJ software (Wayne Rusband, National Institute of Health), where no differently specified.

For immunofluorescence analysis in mouse model, slides were fixed and blocked as described previously. Primary antibodies (insulin and glucagon, Cell Signalling,1:100 and Glut2 and TRβ1, Santa Cruz, 1:50) were incubated for 1h at room temperature in humidified chambers. After 3 washes in BSA 1% in CMF slides were incubated with secondary antibodies (Polyclonal swine anti-rabbit Immunoglobulin FITC coniugated, DAKO, Denmark, 1:200) for 1h at room temperature in dark. Nuclei were counter-stained with 1μg/ml Hoechst dye diluited in CMF.

Measurement of apoptosis

Groups of 15 islets were cultured for 48 h in medium with and without T3 (10-7 M) and exposed to STZ (5 mmol/L and 2 mmol/L), to H2O2 (100 μM) for the last 2 h of treatment (before assessment of apoptosis) and cultured in serum-free CMRL medium for 48 h after a sensitization period (24 h) with CMRL completed medium. At the end of the treatment period, islets were fixed for 1 h with 4% paraformaldehyde, washed with cold PBS, incubated in Triton 0.1% in Sodium Citrate 0.1% for 2 min. on ice. After washes, apoptotic cells within islets were detected by the TdT-mediated dUTP biotin nick end labeling (TUNEL) method using an in situ *Cell Death detection kit* (Roche, D-68305 Waldhof, Mannheim, Germany) for 1 h, at RT, in dark, in according to manufacturer's procedures. Islets were counterstained for Insulin reveled by indirect fluorescence and nuclei were counter-stained with 1 μg/ml Hoecht dye diluited in PBS. TUNEL positivity signal was evaluated by optical sections obtained using an Axiovert 200M

microscope (Zeiss, Oberkochen, Germany) with ApoTOME© device connected with a CCD camera Zeiss Axiocam.

TUNEL ASSAY in pancreata tissue were detected by an incubation with Converted- POD in an humidified chamber for 30 min at 37° C and for 5 min with DAB substrate.

CASPASE ACTIVITY ASSAY. The caspGLOWTM red active caspase staining kit (Biovision Middlefield Way, CA) was used to quantify caspase activity. Freshly-prepared seven-micrometer cryosections were incubated with a Red-VAD-FMK at 1:300 diluition in PBS Ca-Mg free at 37°C for 45 min. and washed twice with the provided washing buffer for 10min. Nuclei were counter-stained with 1μg/ml Hoechst dye diluited in PBS.

Western blot analyses

For total protein extraction, islets and pancreata were lysed for 10 min in ice-cold lysis buffer containing 1% Tween 20, 10% glycerol, 150 mmol/L NaCl, 50 mmol/L HEPES pH 7, 1 mmol/L MgCl2, 1 mmol/L CaCl2, 1 mmol/L NaF, 10 mmol/L Na4P2O7, 2 mmol/L NaVO3, 1 mmol/L phenylmethylsulfonylfluoride, protease inhibitors. The lysates were sonicated and centrifugated at 12,000 rpm for 30 min. and the total cellular protein content was measured using Bradford method (Bio-rad, Richmond, CA). 50 μg of total extract per sample were loaded onto an 10% SDS-polyacrilamide gel, electrophoresed, and then blotted onto nitrocellulose membranes (Bio-Rad). Filters were blocked for non specific reactivity by incubation for 1 h at RT in 5% non-fat dry milk dissolved in PBS 1X, Tween 20 0.1% and then incubated for 16 h at 4°C with with the appropriate primary antibody. Antibodies: anti-Akt and anti-pSer473Akt (Cell Signalling,

Danvers, MA, USA, 1:1000), anti-Glut-2 (Santa Cruz 1:500), anti Caspase 3 (Millipore,290 Concord Road, Billerica,MA, USA, 1:200), anti Bax (Santa Cruz 1:250), anti Tra/b (Santa Cruz 1:200).

At the end the membranes were washed and incubated for 45 min with the appropriate HRP-conjugated secondary antibody (anti-mouse, anti-rabbit; Sigma-Aldrich 1:4000) in milk 5%, PBS 1X, Tween 20 0,1% for 45 min at RT.

Immunoreactivity was visualized by the ECL immune-detection system (Amersham Corp, Arlington Heights, IL) in according to manufacturer's instructions. The relative band intensity was evaluated by densitometric analysis (Image J, Wayne Rusband, National Institute of Health, USA) and normalized to total B-actin.

Trasmission electron microscopy

Tissue fragments were fixed with 2% glutaraldehyde in PBS for 2 h at 4°C. Samples were postfixed in 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 1 h at 25°C and stained with uranyl acetate (5 mg/ml) for 1 h at 25°C, dehydrated in acetone, and embedded in Epon 812. Ultra-thin sections were examined unstained or poststained with uranyl acetate and lead hydroxide under a Morgagni 268D electron microscope (Fei, Hillsboro, OR, USA).

Measurements of biochemical parameters

INSULIN SECRETION FOR CULTURED ISLETS. 15 Islets cultured in presence or in absence of T3 were stimulated with two different glucose concentrations ranging from 2.8 to 28 mmol/L in isolation medium and incubated for 45 min at 37°C. Insulin releasing was measured on supernatants by Mercodia

Ultrasensitive Rat Insulin Elisa (Mercodia AB, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden) under manufacture's protocol. Results were presented in pg/ml after normalization with total protein content.

In mouse model all measurements were performed after 8h fast.

INTRAPERITONEAL GLUCOSE TOLERANCE TEST. Glucose tolerance test was carried out 48 hours after STZ and T3 treatment by an intraperitoneal injection of glucose (3g/kg of body weight) to overnight fasted mice. Glucose and insulin concentrations were determinated in tail vein blood at 0, 30 and 120 min after glucose injection by using Ascenzia Breeze (Bayer AG,51368 Leverkusen, Germany) and Mercodia ultrasensitive mouse insulin ELISA kit (Mercodia, Uppsala, Sweden).

INSULIN TOLERANCE TEST. Mice received an intraperitoneal insulin injection (0,75U/Kg of bodyweight) under fast condition. Blood glucose concentration was determinated at 0, 30 and 120 min after injection by using Ascenzia Breeze (Bayer AG,51368 Leverkusen, Germany).

Liver Rna isolation and RT-PCR analysis

Total cellular RNA was isolated from livers by using SV Total RNA Isolation kit (Promega, Madison, WI), according to manufacturer's instructions. RNA (1 μg) was subjected to reverse transcription (RT) by using a cDNA synthesis kit OmniScript (QIAGEN, Chatsworth, CA). cDNA was amplified to determine desiodase I expression using the following primer pairs : (mDioI) 5'-AAGAGGCTCTGGGTGCTCTTGG-3' and 5'-GGTTCTGGTGATTTCTGATGTC-3'. Amplification was performed for 30 cycles, after a first denaturing step at 94°C for 5 min, at a denaturing temperature

of 94°C for 30 sec, at annealing temperature of 55°C for 30 sec and at an extension temperature of 72°C for 30 sec. PCR products were electrophoresed onto a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) and visualized under UV light. 18s expression levels were analyzed as a control for RNA quality using the following primers for the amplification5'-GGAGAGGGAGCCTGAGAAA-3'and:5'-GAAAGAGTCCTGTATTGTTATTTT-3'.The relative intensity of the bands was quantitated by densitometric analysis (ImageJ, Wayne Rusband, National Instutite of health, USA) and normalized to the co-amplified 18s cDNA fragments.

All primers were synthesized by MWG Oligo Synthesis Report (Eurofins MWG Operon, Edersberg, Germany)

Statistical analysis

Different statistical analyses were performed depending on the experimental type and are indicated in the relative Figure legends.

Results

Thyroid hormone receptor β1 is highly expressed in the cytoplasm of rat islets

Thyroid receptor β1 resulted to be the main mediator of T3 action on pancreatic β cells (Verga Falzacappa C. *et al*. 2009). To evaluate if TRβ1 is expressed in the rat islets, immunofluorescence experiments have been performed. As shown in figure 5, the staining for TRβ1, revealed that the receptor is highly expressed in the islets cells and that is mainly located in the cytoplasm. In addition, when the islets were counterstained for insulin, it was possible to observe that the two signals were superimposable, indicating that the rat islets β cells do express the thyroid receptor β1.

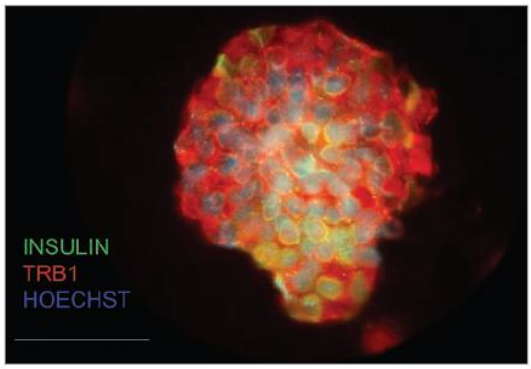


Figure 5. Thyroid hormone receptor β1 immunofluorescence.
Islets were cultured for 24 h and then indirect immunofluorescence was performed for thyroid receptor β1 (red) and for insulin (green). Nuclei were counterstained with Hoechst (blue).

Islets viability in vitro is augmented by T3 treatment

In order to demonstrate a pro-survival role of Thyroid hormone T3 in freshly isolated rat islets, the islets were cultured in the presence or not of T3 (10^{-7} M) for 24, 48, 72 and 96h. Our previous works on cell lines (hCM ad rRINm5F) indicated that the 10^{-7} M dose of thyroid hormone was able to influence β cell viability, proliferation, survival and function *in vitro*; considering that data, we decided to utilize the same dose for our *ex vivo* experiments. Fluorescence microscopy was utilized to determine the proportion of dead cells within the islets. As shown in figure 6A, already at 72h of culture the viability of islets culture without T3 was reduced as evident by the considerable number of PI positive cells. T3 treatment sensibly improved the percentage of viable islets from 50% to 65% (Fig. 6A). Importantly, after 96 hours culture without T3, 80% of islets were decomposed, compared to 50% of islet cells cultured in the presence of T3.

In accordance with the microscopic observations, MTT assay (Fig.6 B) confirmed that the T3 treated islets have cell viability values higher than untreated islets, confirming that the hormone treatment could preserve islets vitality.

Core cell damage is reduced by T3 treatment in rat islets

During *in vitro* culture, necrosis of the cells occurs within the centre of the islets; islet core is primarily constituted by β cells. In this experiment we investigated whether T3 could contrast the core cell damage in isolated islets. Under light microscopy, freshly isolated pancreatic islets from rat had a smooth appearance with compact, differently spherical shapes, varied in size (Fig. 6C). After 48h of *in vitro* culture, the islets began to present some cell damage. It was usually

located in the centre of the islets and characterized by a zone of dark cells that was separated from the surrounding viable tissue. By 96h of culture an extensive damaged area appeared in the centre of the islets. The damaged area was even larger, being extended throughout the islet. The observed results were consistent with the live/death cell analysis, whether they were different from the TUNEL assay. In the TUNEL assay, in fact, apoptotic cell death of single damaged cells within the core of a 24h cultured islet could be identified; however, many cells within the damaged area were also TUNEL negative, indicating that both necrotic and apoptotic cell deaths were involved in the process of core cell damage in cultured isolated rat islets. Strikingly, when the islets were exposed to T3 the damaged area resulted sensibly reduced. As shown in the Figure 6, after 72 and 96h of *in vitro* tissue culture, T3 treated group (right) indicated a significant higher recovery of islets than control group (left panels) . Islets treated with T3 exhibited excellent morphology and did not lead to core cell damage. These data demonstrate that T3 treatment was effective to reduce core cell damage of islets during the *in vitro* culture period.

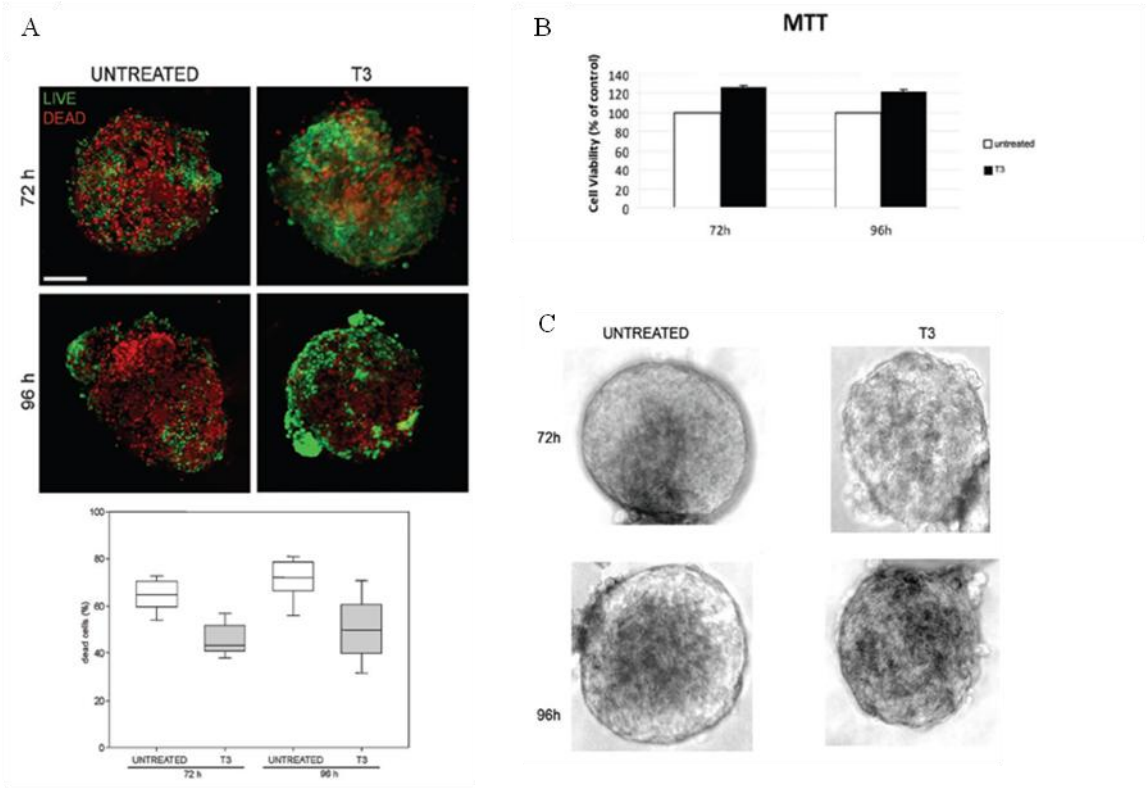


Figure 6.

(A) Islets viability.

Islets were cultured in presence or not of T3 (10^{-7} M). Assessment of islets cell viability using fluorescence microscopy with propidium iodide (dead cell, red) and Live cell dye (MBL) (living cells, green) staining. Representative merged pictures are shown. Islets cultured without T3 showed the presence of numerous dead cells at the centre of the islets cultured with T3 showed fewer dead cells. The percentage of green and red cells was calculated by counting up to a minimum of 200 cells for ten optical fields (200X) for each sample, randomly taken from two different experiments. P-value was calculated using a kruskal-Wallis test. the boxplot showed the median and the 10^{th}, 25^{th} , 75^{th} and 90^{th} percentile. Bar 50µm; $p< 0.005$.

(B) MTT assay.

Cell viability has been evaluated by MTT assay performed on islets cultured in the presence or the absence of T3 for the indicated time. Data presented (B) are the OD values (570 nm) expressed as percentage of control on the axis, as means ± SD, are the results of at least five experiments. Control has been taken as 100%. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Dunnett post-hoc test $p< 0,005$.

(C) Core cell damage.

Islets were cultured in the presence or not of T3. The core cell damage was visualized under light microscopy and representative images are shown. Islets cultured in the presence of thyroid hormone showed a reduced core cell damage compared with islets cultured without T3. Magnification 100X. Bar:50µm.

T3 induces BrdU incorporation in rat islets cells

Our previous demonstration in β cell lines revealed a mitogenic role for T3; although it is well known that the proliferation rate in islets is slight, we decided to investigate the T3 effect on islets cell proliferation via BrdU incorporation. Surprisingly, as shown in figure 7, in the core of the islets some BrdU positive nuclei were detectable in both the untreated and the treated samples. The T3 treated BrdU nuclei number was increased. As shown, the counterstaining for insulin (green fluorescence) confirmed that the core of the islets is mainly populated by β cells, which were the ones positive for the BrdU staining.

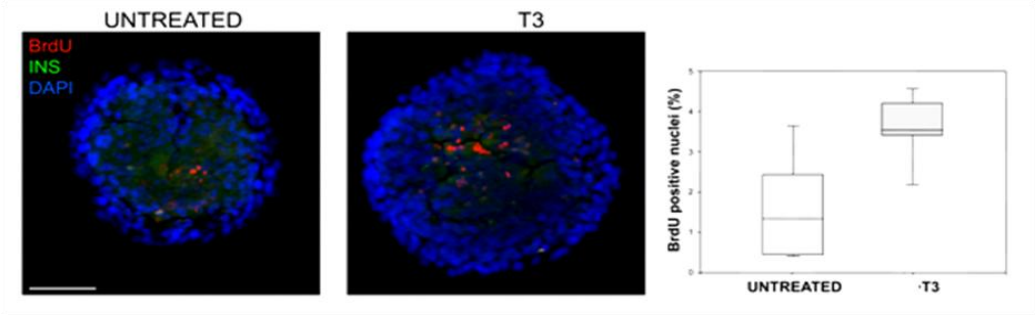


Figure 7. BrdU labelling.
Islets were cultured in the presence or absence of T3 for 48h and exposed to BrdU (10µM) for the last 48h. BrdU incorporation was evidenced with indirect immunofluorescence for BrdU (red). Islets were counterstained for Hoechst (blue). The percentage of BrdU positive cells was calculated by counting up to minimum of 200 cells for ten optical fields (200 X).

T3 protects rat islets from STZ induced apoptosis

To assess whether T3 could also affect the survival of the islets exposed to pro-apoptotic agents, the apoptotic process was induced by Streptozotocin treatment in the islets exposed to T3 or to vehicle alone. Islets were treated with Streptozotocin 2 or 5mmol/L for 2h. As shown in the Figure 8, for the 5 mmol/L dose, the percentage of TUNEL positive cells was high in the islets treated only with STZ, which demonstrates the presence of apoptosis (80%), while in the islets treated with T3 the TUNEL positive cells were highly reduced (30%), indicating that the hormone T3 is able to counteract the pro-apoptotic action of the drug. To evidence the β cells inside the islets, counterstaining with insulin has been performed. As shown the insulin signal was mainly present in the core of the islets and it was superimposable with the TUNEL positivity, indicating that Streptozotocin could induce apoptosis specifically in the β cells. As shown, the untreated islets also show a low number of TUNEL positive cells, indicating that a physiological apoptosis was anyway present in the cultured islets.

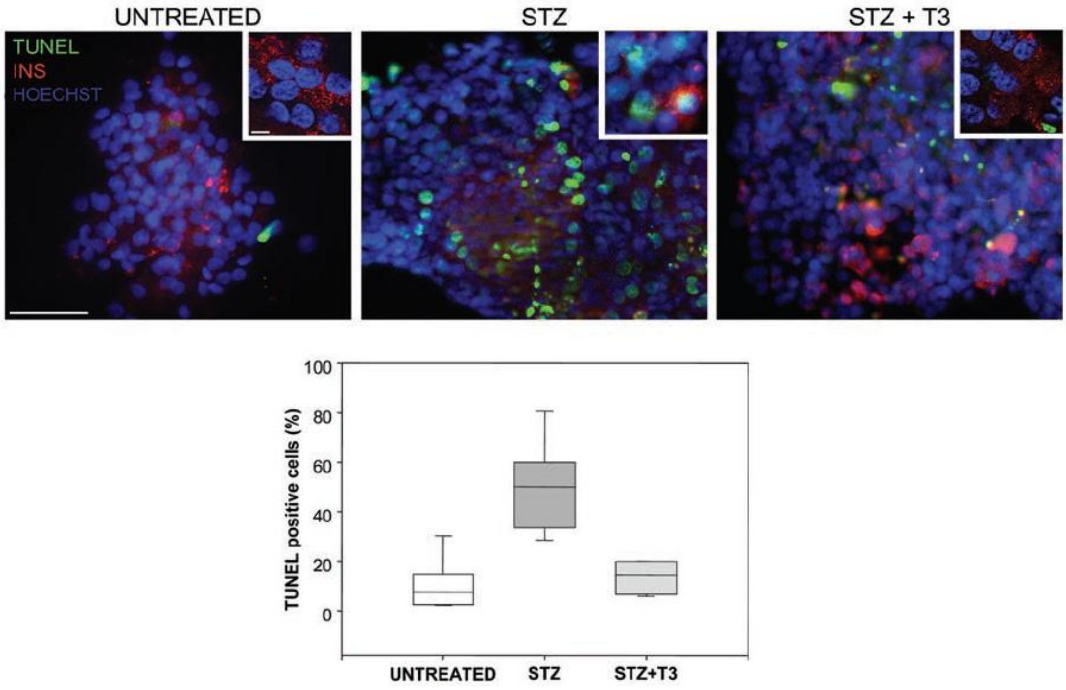


Fig 8. TUNEL assay.

Islets were exposed to two different doses (2mmol/L and 5 mmol/L) of Streptozotocin or not (untreated) and cultured in the presence (T3) or the absence (untreated) of T3 10^{-7}M, as described in Materials and Methods. Apoptotic nuclei were detected as TUNEL-positive, nuclei were counter-stained with Hoechst and merged images from a representative field (5 mmol/L dose) are shown. The mean ± SE percent of TUNEL positive cells was calculated by counting up to a minimum of 300 cells from ten optical fields (200X) for each sample, randomly taken from two different experiments.

P-values were calculated using Kruscal-Wallis test the box plot showed the median and the 10^{th}, 25^{th} , 75^{th} and 90^{th} percentile. Bar: 50µM (10µM in the inset); p=0,0005.

The thyroid hormone treatment preserved basal glucose responsiveness and insulin secretory function in rat islets. Isolated islets were incubated with or without T3 for 72h and 96h. A static glucose challenge assay (Figure 9) was performed and indicated that T3 was able to preserve β cell glucose responsiveness and insulin secretion in both basal (2.8 mmol/L) and stimulated (28 mmol/L) glucose condition. Moreover a significant increase in insulin secretion was observed in both the utilized glucose condition, thus suggesting that the thyroid hormone treatment improves the islets ability to secrete insulin.

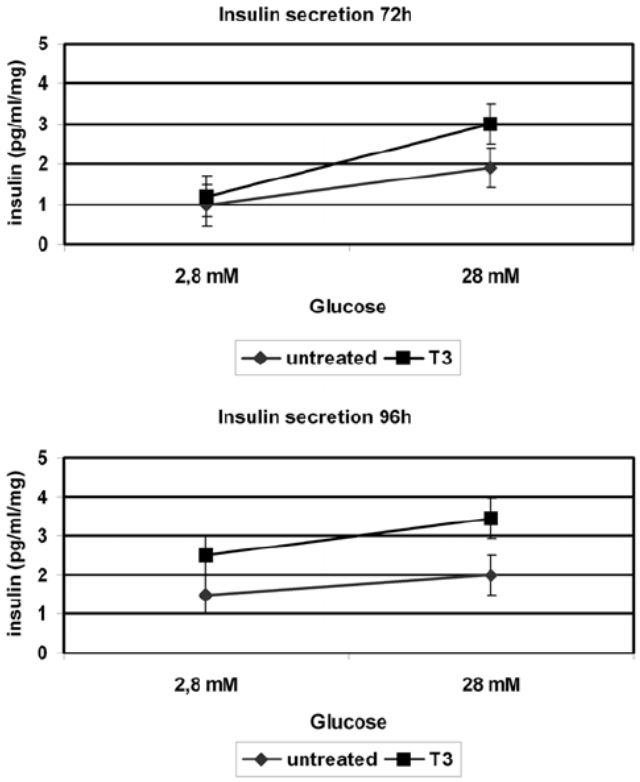


Figure 9. Insulin secretion.
Islets were cultured in the presence or not of T3 (10^{-7}M). Insulin content of medium from islets cultured in the presence or the absence of T3 and exposed to basal (2.8 mmol/L) and stimulating (28 mmol/L) glucose concentration for 1h was assessed by Chemiluminescence. Results represent the mean ± SE of three separate experiments.

T3 upregulates Akt activation in rat islets

Thyroid hormone treatment can induce the Akt phosphorylation in rat islets. As shown in the Figure 9, Western Blot for pAkt (Ser 473) clearly indicated that T3 treatment (24h) was able to induce the activation of the kinase of 8 fold (4 R.D.U. in T3 cells vs 0,2 in control).

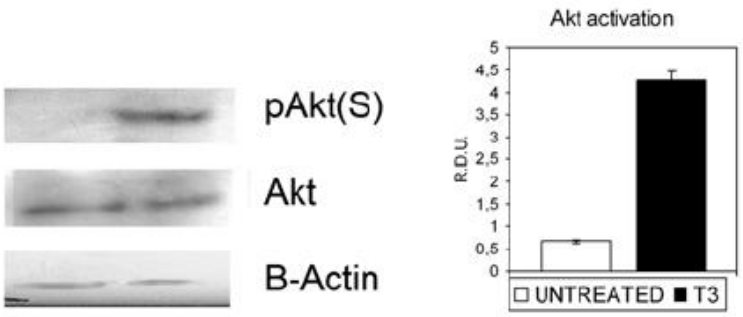


Figure 9. Akt activation.
Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of β-Actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here.
Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to β-actin for equal loading. Data relative to each protein are presented on the right of the Western Blot panel in the histogram as Relative Densitometric Units (y axis). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. p = 0,003.

T3 treatment preserves islets morphology and dimension in Balb/c mice

To study the *in vivo* effects of the thyroid hormone T3, we used Balb/c mice. Diabetes has been induced with a "multiple low dose Streptozotocin injection protocol", as described in the Materials and Methods section. The thyroid hormone action is mainly mediated through two thyroid receptor isoforms, namely TRα and TRβ. Our previous data evidenced that T3 action on pancreatic β cells and islets is predominantly mediated through the thyroid receptor β1 (Verga Falzacappa C. *et al*. 2009). Hence even if the expression of the thyroid hormone receptors α and β in murine pancreatic islets has already been described (Zinke A. *et al*. 2003) we confirmed this in our model by immunofluorescence and western blot analyses (Fig.10). Mice were divided into three separate groups of study, namely the control one (CTR), which received vehicles alone; the Streptozotocin one (STZ), which received 2 intraperitoneal injections of STZ every 24 hours for 2 days; and the Streptozotocin + T3 one (STZ+T3), which received both T3 and STZ, ip every 24 hours, for 2 days. At the end of treatments, mice were sacrificed, and pancreata were excised. Size and shape of the islets were evaluated by hematoxylin/eosin staining, while the expression of insulin as well as of glucagon by immunofluorescence analysis (Fig.11A). At the morphological level, as expected, islets from STZ-treated mice showed disarray of cellular architecture, irregular boundaries, reduced area and diminished β cell mass, when compared to control mice (Fig.11A).

By contrast, T3 administration significantly prevented the decrease in β cell mass and islets area, counteracting the morphological changes induced by STZ. The β cell mass was directly analyzed by morphometry and the crossection area of the islets was measured as described in the Materials and Methods section. In fact, as

shown in Figure 11B while the area of the Streptozotocin islets was significantly reduced, the decrease was completely overcome by the T3 treatment. Moreover, the T3-induced preservation of islets shape and size was not due to growth of undifferentiated mass, since no increase in the number of Ki67 + cells was observed (data not shown), excluding an eventual T3 caused indifferentiated mass growth. To better highlight the beneficial thyroid hormone effects on β cell mass and function, Real Time RT-PCR for Insulin has been performed. Typical standard curves plotted by the Lightcycler were obtained for Ins and rRNA 18s (r=-1). Melting point analysis of PCR products for both genes demonstrated single product formation, as confirmed by gel electrophoresis. As shown in the histogram, (Fig.11C) whether the presence of Insulin mRNA was barely detectable in the STZ treated pancreata, the thyroid hormone treatment was sufficient to maintain the Insulin gene levels comparable to control (65%), strongly overcoming the STZ inhibition.

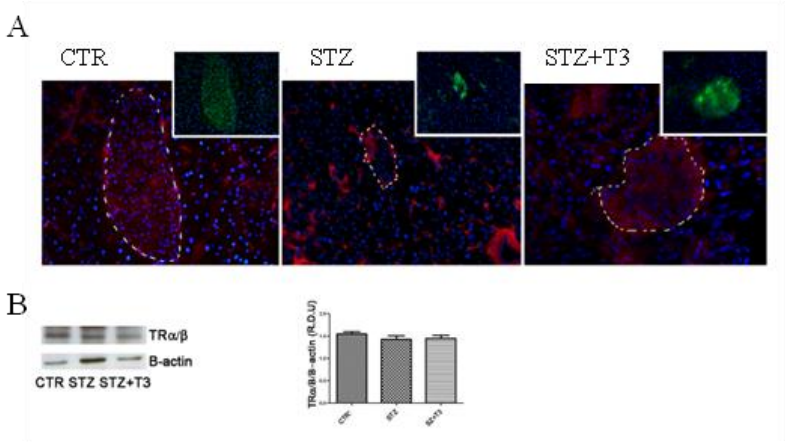
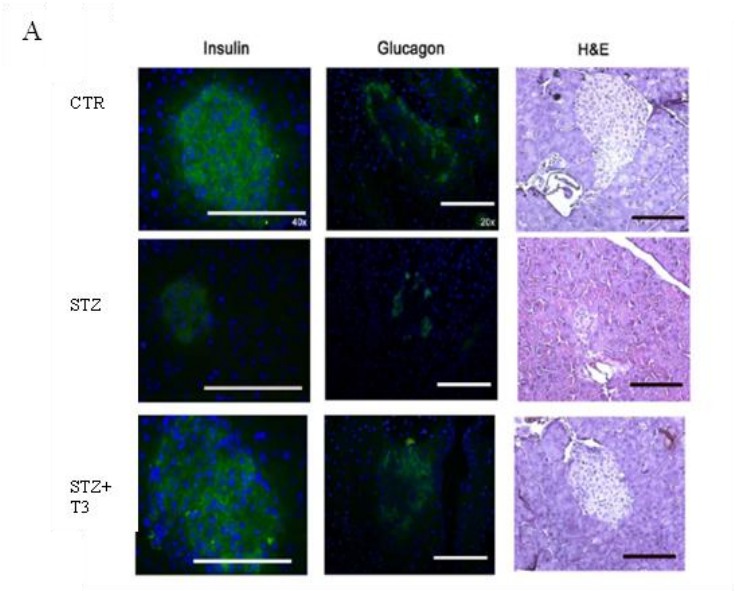
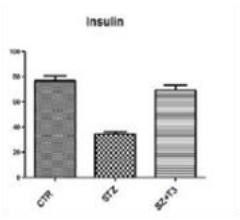
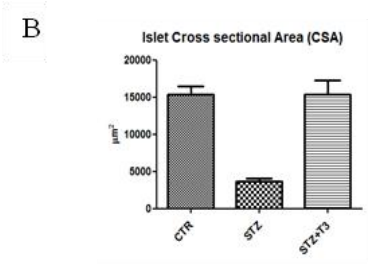


Figure 10. Thyroid hormone receptors expression.
Tissue sections from the different experimental groups of animals have been obtained as described in the Materials and Methods section. Indirect immunofluorescence for Thyroid Receptor (Red) and Insulin (Greeen) revealed the presence of TRα/β within the islets and the pancreatic tissue surrounding. Nuclei were counterstained with Hoechst (blue). Data are from 1 or 2 experiments with similar results (n=5 animals/group). At least ten fields *per* chamber and three independent cultures were examined. Space bar: 100 μm
Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the Thyroid Receptor α/β was detected. The expression of β -actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here.
Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to β -actin for equal loading. Data are presented on the right of the Western Blot panel in the histogram as Relative Densitometric Units (y axis). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. p= 0.003


A
Insulin
Glucagon
H&E
CTR
STZ
STZ+
T3


Insulin
CTR
STZ
STZ+T3


B
Islet Cross sectional Area (CSA)
20000
15000
10000
5000
0
CTR
STZ
STZ+T3

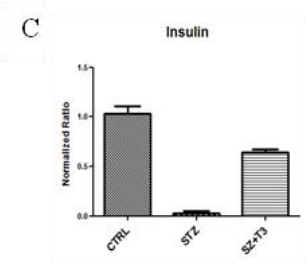

C
Insulin
Normalized Ratio
1.5
1.0
0.5
0.0
CTRL
STZ
SZ+T3

Figure 11.

(A) Histopathology. Tissue section from the different experimental groups of animals have been obtained as described in the Materials and Methods section. Immunofluorescence for Insulin (green, left panels), and Glucagon (green, middle panel), and Hematoxilin and Eosin staining (right panels) were performed to analyze histopathological changes in pancreatic islets compared to control mice (CTR). Nuclei were counterstained with Hoechst (blue) in the IF experiments. Data are from 1 or 2 experiments with similar results (n=5 animals/group). At least ten fields *per* chamber and three independent cultures were examined Space bar: 100 μm. Histogram: The percentage of Insulin positive cells was calculated by counting up to a minimum of 200 cells for ten optical fields (200X) for each sample, randomly taken from two different experiments.

The effect of treatment with T3 was statistically significant versus STZ. Student's *t* test: $p < 0.05$ vs STZ+T3.

(*B) Islet crossection area.*

Crossection area was calculated as described in the Materials and Methods and the results were averaged and represented on the histogram. The presence of T3 significantly counteracts the reduction of islet area and deterioration. At least 10 different islets per sample were analyzed for each experiment. Data are from 1 or 2 experiments with similar results (n=5 animals/group). The effect of treatment with T3 was statistically significant versus STZ+T3. Student's *t* test: $p < 0.01$ vs STZ+T3.

(C) Real Time PCR.

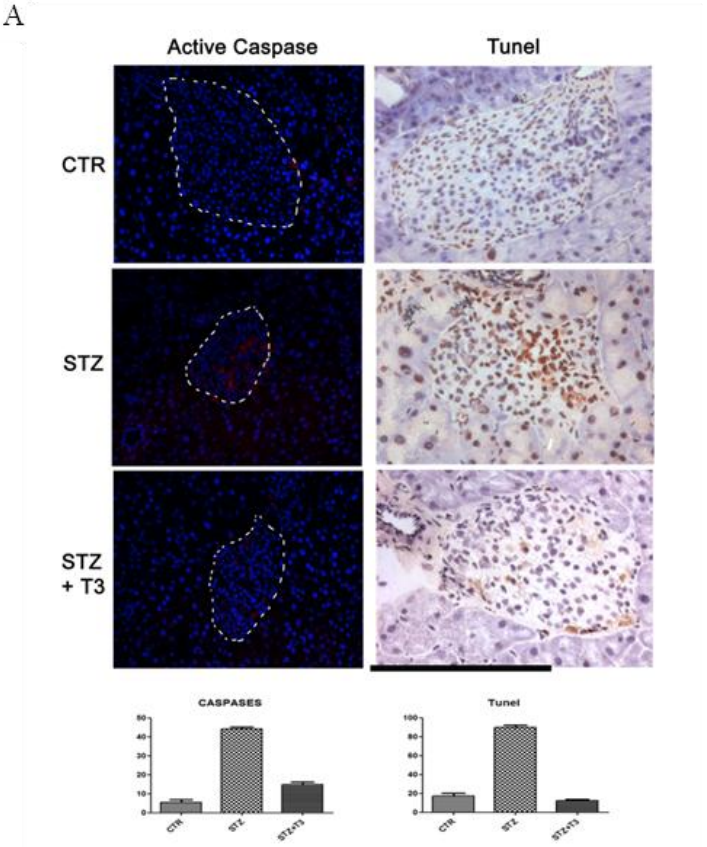
Total RNA was obtained from pancreata from animals of the various experimental groups and RT-qPCR was performed as described in the Materials and Methods section. Melting point analysis of PCR products for both genes demonstrated single product formation, as confirmed by gel electrophoresis (on the right). All PCR products were of the expected size and sequence. Normalized ratios are shown in the histogram; the presence of T3 was able to overcome the STZ inhibition of Insulin gene expression.

T3 counteracts STZ induced islets apoptosis in mice

Based on our previous observations in β cell lines and in *ex vivo* cultured islets, where the presence of T3 was sufficient to completely overcome the ongoing apoptotic process, and considering the evident preservation of the β cell mass in T3 treated mice, we wanted to verify whether T3 treatment could protect the animals from STZ-induced effects by preventing apoptotic cell death. To this aim, TUNEL analyses and active caspases staining were assessed in pancreases from the different groups of mice. As shown in Figure 12, STZ treatment results in a much higher number of TUNEL + and active caspases + cells within the islets, when compared to control. These results demonstrate that the acute oxidative injury caused by STZ treatment induces a high level of cell death. By contrast, T3 co-treatment prevents STZ-induced cell death, lowering the number of TUNEL + and active caspases + cells to levels comparable to control samples, as shown in the histogram of Fig. 12A.

To deepen into the molecular changes induced by T3 in the apoptotic cascade, two major pro-apoptotic molecules have been analyzed by Western Blot, which are moreover both targeted by the Akt action. As shown in the Figure 12B, while STZ clearly induced BAX expression, the T3 can maintain its levels comparable to the control ones. In agreement, even the activation of the caspase3, a Bax downstream, which is clearly evident and strong in the STZ samples, shows basal levels in both the control and the STZ+T3 ones, indicating that T3 can contrast its activation by STZ.

A


Active Caspase
Tunel
CTR
STZ
STZ
+ T3
CASPASES
Tunel
CTR
STZ
STZ+T3

B

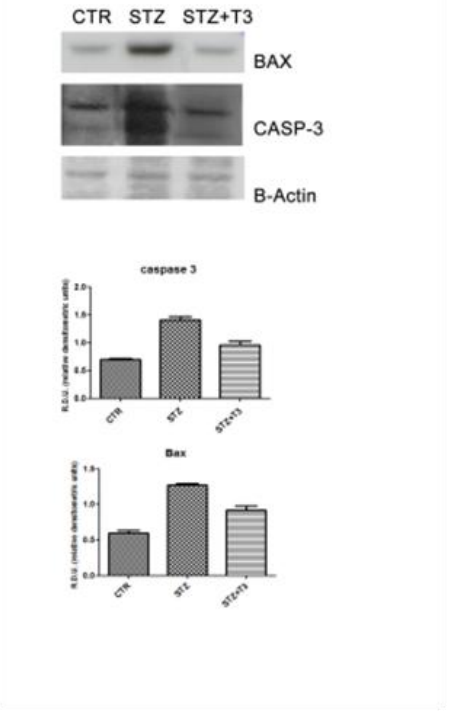

CTR STZ STZ+T3
BAX
CASP-3
B-Actin
caspase 3
Bax

Figure 12.

(A) Apoptosis and survival. Tissue sections from the different experimental groups of animals have been obtained as described in the Materials and Methods section. Caspases activity (left panel, red) was detected by CaspGLOW and TUNEL assay (right panel) was visualized by Immunohistochemistry. At 48h of STZ alone apoptotic nuclei were clearly detectable within the islets, while in the samples exposed contemporary to T3 apoptotic nuclei were hardly detectable within the islets. At least 10 different islets per sample were analyzed for each experiment. Data are from 1 or 2 experiments with similar results (n=5 animals/group). Space bar: 100 μm

Histogram: The percentage of TUNEL or Caspase positive cells was calculated by counting up to a minimum of 200 cells for ten optical fields (200X) for each sample, randomly taken from two different experiments.

The effect of treatment with T3 was statistically significant versus STZ.

Student's *t* test: $p < 0.01$ vs STZ .

(B). Western Blot. Western Blot analyses were performed as described in Materials and Methods on protein extracts from the various experimental groups and a specific band corresponding to Bax and Casapase3 was detected. As shown, while the presence of STZ clearly induced the expression of Bax and the activation of Casp 3, the presence of T3 was able to counteract STZ action, maintaining Bax and Casp 3 levels comparable to the CTR samples. The expression of β-actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here.Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to β-actin for equal loading. Data relative to each protein are presented on the right of the Western Blot panel in the histogram as Relative Densitometric Units (y axis). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. $p = 0.003$

T3 does not influence Glut-2 expression or localization

Since it is known that the glucose-analogue STZ requires the β cell glucose transporter Glut-2 to enter the cell and to exert its apoptotic effect (Schnedl WJ. *et al.* 1994), we investigated Glut-2 expression and localization in the islets by immunofluorescence analysis. As shown in Figure 13, the STZ could completely disarray the Glut-2 expression and localization, consistently with its negative effect on the whole islet structure; on the other hand both control and STZ+T3 islets showed good localization and levels of the Glucose transporter. Interestingly, T3 treatment did not alter either expression or localization of Glut-2, ruling out the possibility that T3 could counteract apoptosis in β cells by preventing STZ entry via Glut-2. In addition, since Glut-2 is the key responsible in the glucose uptake by the β cell, we can hypothesize that T3 effects are not due to any alteration in the glucose entry in the cells.

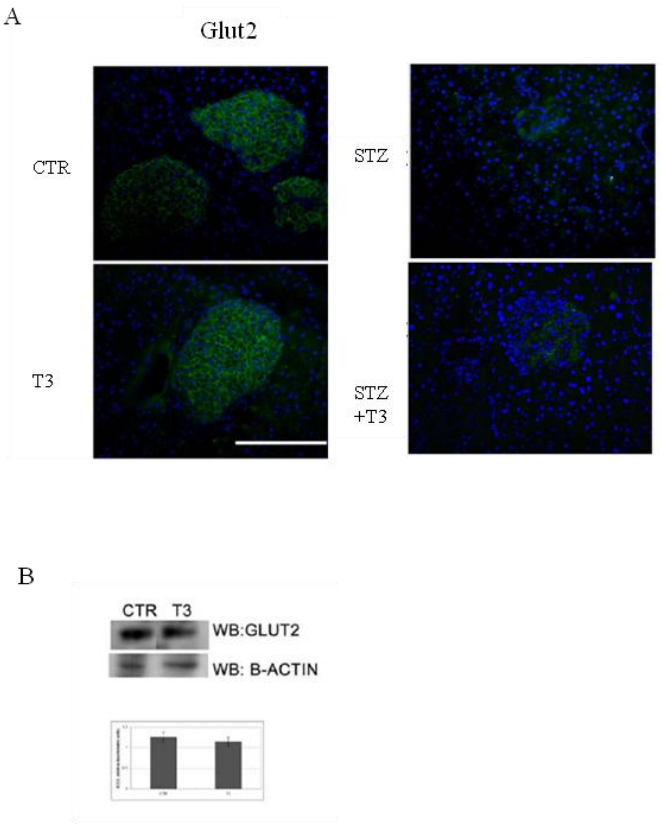


Figure 13.

(A) Immunofluorescence. Tissue sections from the different experimental groups of animals have been obtained as described in the Materials and Methods section. Indirect Immunofluorescence for Glut-2 (green) revealed the presence of the Glucose transporter within the islets, clearly detectable . Nuclei were counterstained with Hoechst (blue). As shown with T3 treatment, no differences either in the expression levels or in the localization of the Glucose transporter Glut-2 was observed. On the other hand, the presence of the STZ was sufficient to cause a strong disarray in both expression and localization of Glut-2 in the islets. Data are from 1 or 2 experiments with similar results (n=5 animals/group). At least ten fields *per* chamber and three independent cultures were examined. Space bar: 100 μm

(B) Western Blot. Western Blot analyses were performed as described in Materials and Methods on protein extracts from the CTR and the T3 treated animals and a specific band corresponding to Glut-2 was detected. The presence of T3 did not provoke any change in the Glut-2 expression, as compared to B-actin expression. The expression of β-actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here.

Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to β-actin for equal loading. Data are presented on the right of the Western Blot panel in the histogram as Relative Densitometric Units (y axis). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. $P<0.003$

T3 preserves ultrastructure of β cells against STZ

To further evaluate the morphological recovery induced by T3 treatment in STZ-treated mouse islets, TEM analysis was performed. Figure 14 contrasts the difference in ultrastructural appearance of well-granulated β cells in an islet of STZ+T3 mouse, as well as a CTR mouse, versus the extensively degranulated appearance of an islet from a STZ mouse. The marked reduction in insulin storage granules was generally associated with a dilatation of the rough endoplasmic reticulum. β cells containing mitochondria with a less dense matrix and partly damaged cristae were also observed, while large vacuoles, swollen cisternae of endoplasmic reticulum and myelinic bodies were also evident. These ultrastructural features are consistent with extreme secretory stress placed on residual β cells. In contrast, the β-cells of STZ+T3 treated islets were characterized by an overall unaltered ultrastructural morphology, which was similar to control islets. The cytoplasm contained numerous granules of the round medium-dense core type or with angular or round crystalline material surrounded by a large clear halo.

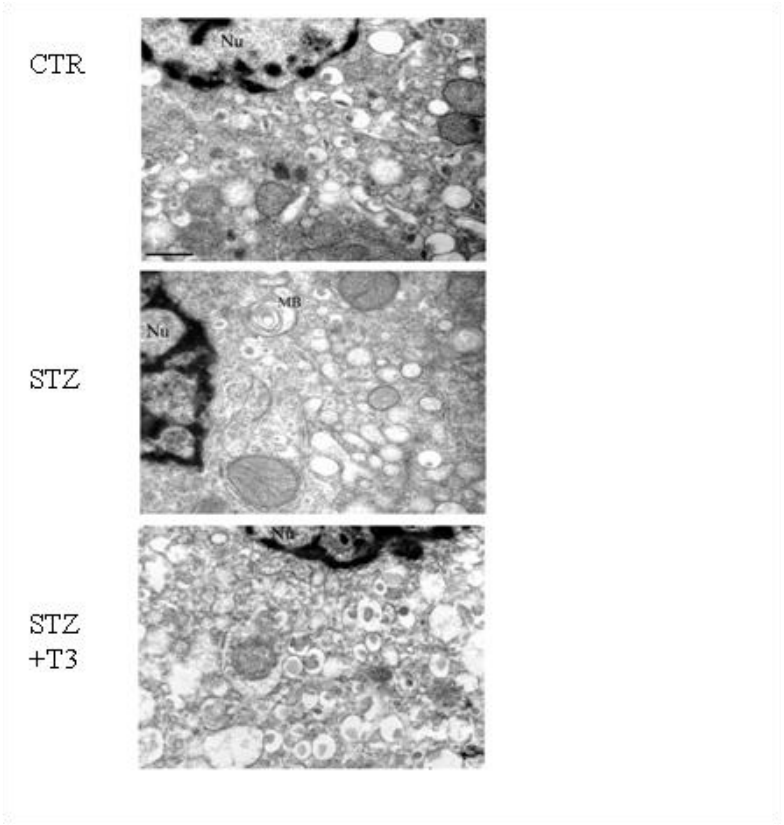


Figure 14. Ultrastructure of β cell.
Trasmission electron micrographs of STZ and STZ+T3 treated pancreatic islets compared to control (Uranyl acetate/lead citrate; space bar 1μm). Nu, nucleus; MB, myelinic bodies. The ultrastructure of the beta cells was affected by STZ and maintained unaltered by the addition of T3.
Data are from 1 or 2 experiments with similar results (n=5 animals/group).

T3 stimulates islets Akt activation in mice

Previously, we demonstrated that T3 is able to activate the Akt signalling in pancreatic β cells and, most importantly, that this activation is the key event in the T3 action on pancreatic β cell function and survival; hence, we sought to verify whether the observed T3-induced anti-apoptotic action may depend on Akt activation/phosphorylation. As shown in Figure 15, immunostaining for pAkt (Ser473) demonstrated that, while in STZ-treated islets Akt activation was significantly inhibited, when T3 was additionally administered, the levels of Akt phosphorylation were actually maintained comparable to control, untreated islets. This observation was further confirmed by Western blot analysis (Fig. 15, right panel). Furthermore considering that Akt survival action includes the regulation of some pro-apoptotic factors, as Bax and caspase 3, the evidence for Akt activation can easily be related to the shown Bax and Casp 3 inhibition by T3.

These results, together with our previous evidences in β cell lines and in islets, strongly suggest that T3 exerts its β cell protective effect at least in part through the Akt signalling.

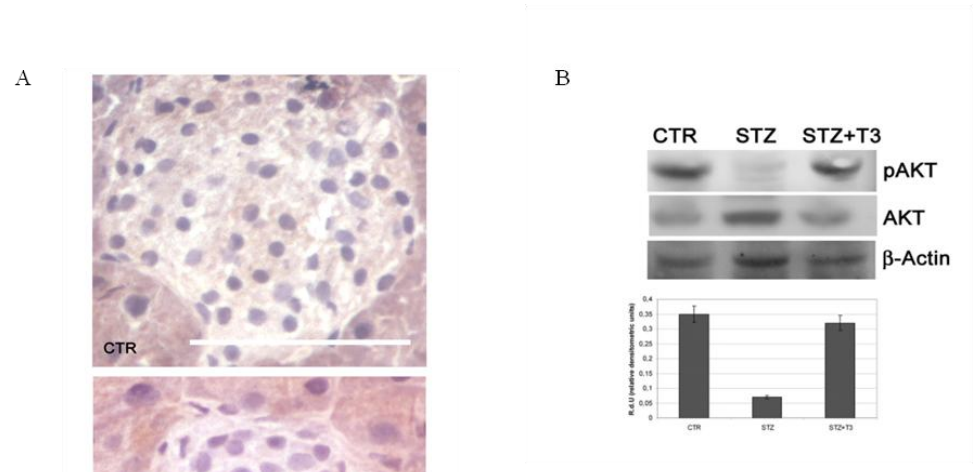


Figure 15.

*(A) Immunohistochemistry for pAkt.*Tissue section from the different experimental groups of animals have been obtained as described in the Materials and Methods section. Immunohistochemistry for pAKT (Ser 473) was performed as described in teh Materials and Methods. The presence of T3 clearly provoked an increment in Akt activation, as compared to total Akt expression (data not shown). Data are from 1 or 2 experiments with similar results (n=5 animals/group). At least ten fields *per* chamber and three independent cultures were examined Space bar: 100 μm.

(B) Western Blot. Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of total Akt was analyzed as a control for gel loading. The presence of T3 clearly provoked an increment in Akt activation(Ser 473), as compared to total Akt expression. At least three different experiments were performed, and a representative one is shown here. Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to Akt for equal loading. Data relative to each protein are presented on the right of the Western Blot panel in the histogram as Relative Densitometric Units (y axis). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. P< 0.001

T3 preserves glucose responsiveness in STZ treated mice

Metabolic parameters were assessed in mice. After STZ injection mice were diabetic with significant fasting hyperglycemia, as described below, glycosuria and hypoinsulinemia when compared with age-matched control mice and STZ+T3 mice. At the time of sacrifice body weight was still similar between mice injected with either STZ or STZ+T3, showing a little decrease only in few case of STZ injected animals. This was presumably due to dehydration and protein wasting associated with diabetes. Thus, the STZ injection protocol we used generated an experimental model of type 1 diabetes, as expected.

Considering that T3 increases β cell function and survival, as we previously demonstrated, and that it maintains β cell mass in STZ-treated mice, it is conceivable that T3 may act as an anti-diabetic factor, ensuring euglycemic status by preserving β cell mass. We thus analyzed the ability of the different groups of mice to respond to an ip glucose tolerance test. Among the 25 mice receiving STZ, 21 became overlay diabetic (blood glucose >250 mg/dl); while 4 showed borderline elevated blood glucose (300>200 mg/dl). In contrast, among the 25 mice treated with T3 together with STZ, only 4 became diabetic; the remaining 18 maintained normal blood glucose levels (<150 mg/dl), while 3 died. In addition, glucose tolerance test evidenced that while the Streptozotocin treated mice completely lost their ability to normally respond to glucose loading , the presence of T3 preserves the ability of mice to restore their normal glycemia 120 min after glucose loading and maintains the serum glucose levels in the euglycemic range (Fig.16)

T3 preserves islets function in STZ treated mice

Finally, we assayed serum insulin levels to analyze the effect of T3 treatment on islets function. As shown in Figure 16 (A), STZ treatment induced a significant decrease in the insulin response, as showed by the lower levels of serum insulin at the different time points, according to the affected ability of control glucose blood levels; on the other hand when T3 was administered at the same time of STZ, serum insulin levels were comparable to the control (Fig. 16B), suggesting that T3 treatment preserves insulin production, preventing STZ effects. These final observations supported the hypothesis on that T3 acts as an anti-diabetic *in vivo*, preserving β cell mass, counteracting β cell apoptosis and regulating the insulin response, via the Akt signalling.

To better characterize the physiology of our mice, we decided to exclude the occurrence of Insulin intolerance by an Insulin Tolerance test. As shown in the histogram in Figure 16C, all animals showed an adequate Insulin responsive, although, as expected, glucose blood levels were higher in the animals treated with STZ.

In all the experiments described above, the serum levels of FT4 and FT3 were evaluated by chemiluminescence to exclude the presence of hyperthyroidism in the animals; moreover the expression of the deiodinase 1 in liver was not altered by the treatment, as shown in Figure 16D, indicating a condition of euthyroidism in the T3 treated mice.

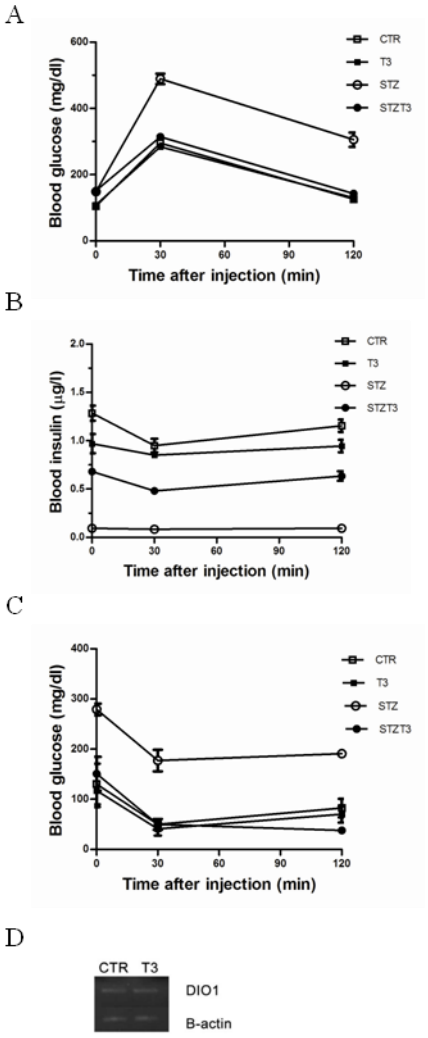


Figure 16.

(A, B) Physiological parameters. Analysis of blood glucose and Insulin levels after intra-peritoneal glucose tolerance test (upper panels). Glycemia was measured by glucometer, while Insulin concentration was assessed by ELISA assay, as described in the Materials and Methods section.

Oral administration of T3 significantly reduces severity and progression of STZ-induced diabetes in Balb/c mice and assured normal Insulin responsiveness.

(C) ITT: Insulin tolerance was performed (lower panel) after intra peritoneal glucose injection. Insulin was injected intraperitoneally after glucose to the different experimental groups of animals. Glycemia was measured by glucometer. Results represent the mean 6 SE of three separate experiments. Grey: control black:STZ white:STZ+T3.

(D) RT-PCR: Total RNA was extracted from liver from mice of the different experimental groups and RTPCR was performed as described in the Materials and Methods section. A single product was obtained for each gene, as showed by agarose electrophoresis. All PCR products were of the expected size and sequence. The presence of T3 did not induce any change in the DIO1 expression, as normalized to 18s.

Discussion

Apoptosis plays an essential role in diabetic pathology, nonetheless being involved in the β cell death usually occurring in islet transplantation, it is also critical for diabetes treatment (Butler AE. *et al*. 2003). Factors that can augment the β cell mass are of particular interest in the field of the diabetes treatment, and to date many growth factors have been already investigated as potential agents able to increase or preserve the islet β cell mass (Nielsen JH. *et al*. 2001). The ability of thyroid hormone T3 to influence pancreatic β cell has recently been investigated by my group. Our evidences (Verga Falzacappa C. *et al*. 2006; 2007) clearly demonstrated that T3 can be considered a mitogenic and survival factor for pancreatic β cell *in vitro*.

In this study I investigated the effects of the thyroid hormone T3 treatment on rat pancreatic islets during an *in vitro* culture and on β cell *in vivo* in a murine model.

Thyroid hormone T3 improves function and survival of rat pancreatic islets during an in vitro culture

The present study was undertaken to determine the effects of thyroid hormone T3 on the survival and function of rat primary islets and resulted in four major findings.

First, the addition of T3 to the culture medium can *per se* enhance islets viability and counteract the ongoing of core cell damage. Second, we demonstrated that thyroid hormone protects rat islets from apoptosis that occurs after streptozotocin exposure. Third the insulin secretion of islets is augmented in the islets cultured in the presence of the drug. Fourth T3 induced a significant increment in the activation of kinase Akt in rat islets.

Within the past 30 years, pancreatic islet transplantation has became a clinical practice and an option in the treatment of diabetes. Islet transplantation has a distinct advantage over whole organ transplantation attempting to reduce peri-procedural morbidity. The major obstacles for successful clinical transplantation are the isolation of sufficient mass of islets together with the management of graft rejection (Shapiro AM. et al. 2006). The fatal outcoming, which is not related to immune rejection, has been thought to be due to insufficient or non-established vascularization of transplanted islets (Jansson L. *et al*. 2002). It has been demonstrated that during the first two days after transplantation, islets are not vascularized, leading to processes that impairs the inner β cell mass of the islets (Ono J. *et al*. 1979; Metrakos P. *et al.* 1994). As demonstrated, the main causative mechanisms involved in core damage might be necrosis and apoptosis (Rosemberg L. *et al*. 1999). Necrotic cell death may depend on the limitation of nutrition diffusion, while apoptosis is generally caused by pathological atmosphere arising from the isolation procedure.

In this study, we evidenced the presence of a relevant core cell damage, which occurs mainly in the first day of culture and it is predominantly due to necrosis, as clearly demonstrated by TUNEL assay. Thyroid hormone T3 was able to counteract the ongoing of this process, thus preserving islets viability. Although at the present moment it is not precisely known which molecular mechanisms are involved in the core cell damage and no evidences exist about the link between thyroid hormones and necrosis, our previous works have unambiguously evidenced that T3 can promote the β cell proliferation, viability and survival by regulating mainly Akt pathway. We thus can hypothesize that a general impulse

from T3 to improve islets status might be due to its ability of regulating the main β cell features via Akt.

An additional advantage of the pre-transplantation culture consists in practicing interventional strategies to prevent the profound β cell loss occurring via apoptosis, which has been estimated to cause up to 70% of the transplanted β cell mass destruction (Van der Windt DJ. *et al*. 2007). Hence, another approach has been to directly inhibit the apoptotic cascade, thus improving the survival capability of the islets (Emamaulle JA. *et al.* 2006). Wide spread apoptosis in the implanted tissue may also have long term deleterious consequences in islets transplantation, since the recipient's immune system is challenged with a large amount of apoptotic tissue, possessing both allo and auto-antigens from two or more donors.

Taking these consideration, using protective factors to enhance β cell survival and prevent islets apoptosis is today widely explored. In accordance with our previous findings about T3 pro-survival effect, we herein demonstrated that the apoptotic process, induced by streptozotocin, could be counteracted by the T3 presence in a relevant manner.

We previously demonstrated the pro survival action of T3 against STZ induced apoptosis in a rat insulinoma cell line (rRINm5f) and elucidated the molecular mechanisms underlying this effect, which involved, once again, the Akt signalling. It is conceivable that T3 might exert its survival action on STZ induced apoptosis mainly involving the same mechanism.

The activation of Akt during islets culture might improve graft survival, indicating that Akt activity could render islets less susceptible to injury during the immediately post transplantation period. Therefore, it is plausible that adding T3

to the islets culture medium and thus activating Akt during an *in vitro* culture period prior to transplantation could yield islets that are more likely to survive the insults encountered immediately after transplantation. In this study we sought how T3 is able to induce a 60% increment in Akt activation. We have previously deeply examined the key role that Akt plays in T3 action on pancreatic β cells. It is well established that Akt signalling, involving PI3K, is implied in cell cycle progression and survival even in pancreatic β cells, thus rendering the link between this molecule and the modulation of β cell mass, function and plasticity a critical subject for the intervention against diabetes. T3 is able to induce cell proliferation and survival; moreover, T3 can increases pancreatic β cell size, protein synthesis and insulin secretion. All the cited effects appeared to be Akt mediated, thus confirming the relevance of this molecule in pancreatic β cell.

In this study, we evidenced that thyroid hormone treatment can, indeed, induce islets survival and function. Infact, as shown, T3 can also increase insulin secretion. It has been demonstrated an autocrine effect of insulin on Akt activation, which results in an increment of survival and vitality of islets in culture (Aikin R. *et al*. 2006). Here, we demonstrated that T3 can increase insulin secretion and we also made evidence that the hormone caused an increment of 8 fold in Akt activation already after 48 hours of treatment. Considered our previous evidences, we suggested that the observed Akt activation is directly dependent on T3 presence, however, considering the data about insulin autocrine action, we can speculate that Akt phosphorylation resulted from both a direct and indirect action on the signaling, thus involving also the insulin action. It has been demonstrated that the addition of exogenous insulin immediately following isolation was not able to improve short-term islet survival, while autocrine regulation via Akt was.

In particular, exogenous insulin can upregulate Akt during the first day of culture, but the effect is lost lately. It is thus plausible that stimulating insulin secretion involving the already seen mechanism, mainly through direct Akt activation. However, given the increment in insulin secretion due to thyroid hormone presence, we can hypothesize that also the autocrine insulin action might play a role in the survival effect of T3 on the islets.

In conclusion, the presented observations, in the first part of my study, propose thyroid hormone T3 as a suitable factor to optimize and stimulate recovery and subsequent function of islets during *in vitro* culture indicating that thyroid hormone could play an important physiological role in pancreatic islets.

Thyroid hormone T3 counteracts streptozotocin induced diabetes in mouse

The crucial endpoint of my study was to describe a novel protective action of thyroid hormone T3 from streptozotocin-induced diabetes *in vivo.*

Recent clinical evidences indicate that thyroid hormone treatment can ameliorate diabetic condition (Ortega E. *et al*. 2008; Skarulis MC. *et al.* 2010). Our study is the first one to focus on β cell function in a diabetic animal model (STZ-induced diabetes mice) in presence of T3 administration.

At first, T3 treatment rescued STZ-induced islets deterioration, as shown by the maintenance of the islet structure, size and consistency. Indeed, while STZ treatment induced reduction in islets size and cell number, the morphology of the islets, the abundance and distribution of insulin-, as well as of glucagon-, expressing cells in the animals treated with T3 and STZ, remained comparable to islets derived from control mice. Preservation of islets morphology was also confirmed at the ultrastructural level, where the presence of T3 prevented the

induction of STZ-induced features of cell damage (clumped chromatin, disorganized insulin-containing granules, altered mitochondria, endoplasmatic reticulum and vacuoles morphology). The observed ability to preserve the islets appearance was associated with protective role of T3 on STZ-induced β cell death, as shown by TUNEL analysis and caspase activation. The STZ-induced cell death observed within the islets was almost completely prevented by T3, in accordance with our previous data. It is known that streptozotocin enters β cells via the glucose transporter Glut-2 and induces islets deterioration by inducing β cell apoptosis (Shnedl WJ. *el al*. 1994). We sought that neither expression or localization of Glut-2 was altered by T3 treatment, ruling out that the observed effects may be dependent on impairment in streptozotocin internalization. These results are consistent with our previous observations where we showed that T3 may be considered a mitogenic and survival factor for pancreatic β cell *in vitro* it rescued, indeed, survival and function in freshly isolated islets in culture and protect cultured β cells from pharmacological induced apoptosis.

Taken together our results show that the main mechanism leading to the increase in β cells mass, survival and function, when T3 is administered contemporary with STZ, is based on the prevention of STZ-induced β cells apoptosis.

Increasing evidences indicate that the decrease of the functional β cells mass is the hallmark of both type I and type II diabetes, resulting in the absolute or relative insulin insufficiency in both conditions.

In this context, β cells apoptosis and impaired proliferation, consequences of hyperglycemia, are features that may be present in all forms of diabetes, suggesting that the classification of diabetes should be revalued.

β cells death can thus be considered has the key event of such diseases, highlighting the urgency to identify factors able to specifically target the β cells mass, avoiding any β cells toxic side effects.

Moreover, we showed in this study that the observed T3 induced anti-apoptotic effects are associated with activation of the kinase Akt, the most important signaling pathway in the islets. This is consistent with recent data showing the ability of thyroid hormone T3 to stimulate Akt in neurons (Cao X. *et al*. 2009), in vascular myocytes (Carrillo-Sepulveda MA. *et al*. 2010) and with our previous data showing T3 stimulates Akt in pancreatic β cells *in vitro*.

Considering these evidences, it is conceivable that the survival action of T3 in mice might involve the same mechanism, so that we did not deepen into Akt pathway.

The important outcome of the observed T3 protective effects in β cells survival and function is the preservation of pancreatic metabolic activity. Indeed, we showed that T3 administration actually preserves any intact response to glucose, and keeps plasma insulin levels in STZ-treated mice comparable to those in control mice; moreover we showed that both STZ and STZ+T3 treated mice do not develop insulin resistance.

While β cell loss by apoptosis is a recognized feature of both type I and type II diabetes, approaches to block this process are limited, so far. Currently, the main goal for diabetes treatment is the maintenance of glucose homeostasis as close to normal as possible in order to avoid the devastating complications of this disease. These treatments include oral hypoglycemics and insulin sensitizers, different insulin preparations administered daily by multiple injections, continuous insulin pumps and, in some TID patients, transplantation of the whole pancreas or islets.

None of these approaches is focused on the maintenance of endogenous β cell mass, though it has been shown that even a small amount of preserved endogenous insulin secretion has great benefits in terms of clinical outcome. Therefore, finding a molecule that could be useful to block β cell apoptosis and thereby preserve and enhance endogenous β cell mass would represent a major breakthrough. The results presented in this study suggest that T3 may actually be a good candidate.

To this aim, however, therapeutic protocol should be accurately designed, in terms of both doses and time intervals, to avoid side effects. It is known indeed that excess of thyroid hormones production by the thyroid gland or by exogenous thyroid hormones administration, results in hyperthyroidism or thyrotoxicosis, characterized by tachycardia, with possible atrial arrythmias and heart failure, muscle wasting, osteoporosis in post-menopausal women, and other symptoms (Webb P. *et al*. 2004). However, thyroid hormone excess also results in beneficial effects, including the metabolic ones. Given the widespread effects of thyroid hormones on the physiology of multiple organs, the chance to use them in a therapeutical fashion remains attractive. In this context, it has been recognized that the induction of a subclinical hyperthyroidism, especially if temporary induced, might be well tolerated by patients and could be accepted in some clinical cases. As reported by Kaptein EM. *et al*. (2009), thyroid hormone treatment in obese patients with nonthyroidal illnesses provoked only a subclinical risk and no significant side effects concerning either weight loss or heart rate; not even mortality was worsened by THs treatment. In the present study, animals, which received thyroid hormone T3 for 48 hours, did not show any significant risky alterations in the thyroid status and did not develop any hyperthyroidism.

Next to the possibility to use T3 (or analogues) *in vivo* to counteract diabetes, given the pro-survival and anti-apoptotic activity on β cells described, T3 administration may also be considered to improve setting for islets transplantation. A major impediment indeed to islet transplantation is the large number of islets required in order to confer insulin independence, resulting in the need of several organ donors (Harlan DM. *et al*. 2009). This fact is in contrast with the known small amount of β cell mass necessary for the maintenance of glucose homeostasis *in vivo*. It is therefore assumed that a large fraction of transplanted islets undergoes apoptosis and is lost. We previously demonstrated that the administration of T3 to the islets in culture preserves their vitality against both physiological and pharmacological cell death. T3 treatment makes islets less susceptible to stress during the transplantation, preventing β cell loss, reducing the number of the required islets and thereby improving the outcome of islets transplantation.

In conclusion, our findings demonstrate for the first time that T3 administration counteracts STZ-induced diabetes, as being a pro-survival, anti-apoptotic factor for β cells, and thus preserves glucose sensing machinery. Altogether these results suggest that T3 can be considered for diabetes supportive therapy.

As both type I and type II diabetes are diseases where deficiency in β cell mass and function is pathogenic, the ability of thyroid hormone to preserve islet mass without loss of β cell differentiated function makes T3 an attractive factor for future therapies for diabetes.

References

Aguayo-Mazzucato C, Bonner-Weir S. stem cell therapy for type 1 diabetes mellitus. *Nat Rev Endocrinol* 2010; **6**: 139-148

Aikin R., Hanley S., Maysinger D., Lipsett M., Castellarin M., Paraskevas S., Rosemberg L. Autocrine insulin action activates Akt and increase survival of isolated human islets. *Diabetologia.* 2006. **49:** 2900-2909.

Assady s, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. insulin production by human embryonic stem cell. *Diabetes* 2001. **50:** 1691-1697.

Bassett JH. Harvey CB. Williams GR. Mechanism of thyroid hormone receptor-specific nuclear and extranuclear actions. *Mol cell Endocrnol* 2003. **213**: 1-11.

Bergh JJ, Lin H-Y, Lansing L, Mohamed SN, Mousa S, Davis FB, Mousa S, Davis PJ. Integrin αvβ3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. *Endocrinology* 2005.**146**:2864-2871

Bernal-Mizrachi E, Fatrai S, Johnson JD. , Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA . Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest* 2004; **114**: 928-936.

Bernal-Mizrachi E, Wen W, StahlhutS, Welling CM, PermuttMA. Islet beta xell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia and hyperinsulinemia. J Clin Invest 2001;**108**:1631-1638.

Bonner-Weir S, Weir GC. New sources of pancreatic β cells. *Nat Biotechnol* 2005. **23**: 857-861.

Bonner-Weir S. Islet growth and development in the adult. *J Mol Endocrinol* 2000. **24**:1-6.

Brubaker PL, Drucker DJ. Minireview: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology*. 2004. **145**(6):2653-9.

Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ. Prentki M. Protein kinase czeta activation mediated glucagon-luke peptide-1 induced pancreatic beta cell proliferation. *Diabetes.* 2001.**50**: 2237-2243

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003. 52:102-110

Cao X, Fukushi K, Moeller LC, Refetoff S, Seo H. Thyroid hormone induces rapid activation of Akt/PKB-m TOR-p70^{S6K} cascade through PI3K in human fibroblasts. *Mol Endo Jan* . 2005.**19**(1): 102-12

Cao X, Kambe F, Yamauchi M, Seo H. Thyroid-hormone-dependent activation of the phosphoinositide 3-kinase/Akt cascade requires Src and enhances neuronal survival. *Biochem J.* 2009 **11**;424(2):201-9.

Carrillo-Sepúlveda MA, Ceravolo GS, Fortes ZB, Carvalho MH, Tostes RC et al. Thyroid hormone stimulates NO production via activation of the PI3K/Akt pathway in vascular myocytes. *Cardiovasc Res*. 2010 **1**;85(3):560-70.

Chang F Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, Franklin RA, McCubrey JA. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy.*Leukemia.* 2003.**17**(3):590-603.

Chen W, Salojin KV, Mi QS, Grattan M, Meagher TC, Zucker P, Delovitch TL Insulin-like growth factor (IGF)-I-IGF-Binding Protein-3 complex: therapeutic efficacy and mechanism of protection against type 1 diabetes. *Endocrinology* 2004. **145** (2) :627-638.

Chidakel A, Mentuccia D, Celi FS. Peripheral metabolism of thyroid hormone and glucose homeostasis. *Thyroid* 2005. **15**: 899-903.

Cho H, Mu J, Kim JK et al. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2/PKBβ. *Science* 2001; 292:1728-1731.

Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 2001; 276: 38349-38352.

Coffer PJ, Jin J, Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochemical Journal*. 1998. **1**;335 (Pt 1):1-13.

Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. *Diabetes*. 1996. **45**(9):1161-7.

Davis P.J. and Davis F.B.. Nongenomic actions of thyroid hormone on the heart. *Thyroid.* 2002. **12**: 459–466.

Davis P.J., Davis F.B., Lawrence W.D. Thyroid hormone regulation of membrane $Ca^{2(+)}$-ATPase activity. *Endocr. Res.* 1989. **15**: 651–682.

Davis PJ, Davis FB, Cody V. Membrane receptors mediating thyroid hormone action. *Trends endocrinol. metab.* 2005. **16**:429-435.

Davis PJ, Leonard JL, Davis F B. Mechanisms of nongenomic actions of thyroid hormone. *Front Neuroendocrinol*. 2008. **29**(2):211-8.

Dickson LM and Rhodes CJ. Pancreatic β cell growth and survival is the onset of type 2 diabetes: a role for protein kinase B in the Akt? *Am Jof Physiol Endocrinol and Metab*. 2004. **287**: E192-E198.

Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature*. 2004. **429**: 41-46.

Elghazi L, Balcazar N, Bernal-Mizrachi E. Emerging role of protein kinase B/Akt signaling in pancreatic β-cell mass and function. *Int J Biochem Cell Biol*. 2006. **38**:157-163

Elsner M, jorns A, lenzen S. diabetes therapy by lentiviral hepatic insulin gene expression without transformation of liver. *Diabetologia*. 2008. **51**: 694-5.

Emamauelle JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. *Diabetes*. 2006. **55**:1907-14.

Feng X, Jiang P, Meltzer P, Yen PM. Thyroid hormone regulation of hepatic genes in vivo detected by complimentary DNA microarray. *Mol Endocrinol*. 2000. **14**: 947-955

Garcia-Ocana A, Vasavada RC, Cebrian A, Reddy V, Takane KK, Lopez-Talavera JC, Stewart AF. Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. *Diabetes* 2001. **50**(12):2752-62.

Garofalo RS, Orena SJ, Rafidi K et al. Severe diabetes, age dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/ PKBβ. J Clin Invest 2003; 112: 197-208.

Gershengorn MC, Hardikar AA, Wei C, Geras-Raaka E, Marcus-Samuels B, Raaka BM. Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. *Science*. 2004. **306**(5705):2261-4.

Giannoukakis N, Mi Z, Rudert WA, Gambotto A, Trucco M, Robbins P. Prevention of beta-cells dysfunction and apoptosis activation in human islets by adenoviral gene transfer of the insulin-like growth factor I. *Gene Ther*. 2000.**7**: 2015-2022.

Guo T, Hebrok M. stem cells to pancreatic β cell: new sources for diabetes cell therapy. *Endocr Rev* 2009. **30**: 214.227.

Halban PA, German MS, Kahn SE, Weir GC. Current status of islet cell replacement and regeneration therapy. *J Clin Endocrinol Metab* 2010; **95**: 1034-1043.

Hansson M, Tonning A, Frandsen U, Petri A, Rajagopal J, Englund MC, Heller RS, Håkansson J, Fleckner J, Sköld HN, Melton D, Semb H, Serup P. Artifactual insulin release from differentiated embryonic stem cells. *Diabetes*. 2004 **53**(10):2603-9.

Hardikar AA, Marcus-Samuels B, Geras-Raaka E, Raaka BM, Gershengorn MC. Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. *Proc Natl Acad Sci* 2003 **100**(12):7117-22.

Harlan DM, Kenyon NS, Korsgren O, Roep BO. Current advances and travails in islet transplantation. *Diabetes*. Oct. 2009; *58*(10):2175-84.

Hayek A and Beattie GM. Alternatives to unmodified human islets for transplantation. *Curr Diab Rep*. 2002. **2**(4): 371-6.

Haynes MP, Li L, Sinha D, Russell KS, Hisamoto K, Baron R, Collinge M, Sessa WC, Bender JR. Src kinase mediates phosphatidylinoditol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen. *Journal of Biological Chemistry*. 2003. **278**:2118-2123.

Hennemann G, Docter RE, Friesema CH, De Jong M, Krenning EP, Visser TJ. Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Reviews* 2001. **22**:451-476

Hui H, Nourparvar A, Zhao X and Perfetti R. Glucagon-Like Peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5'-adenosine monophosphate-dependent Protein kinase A- and a Phosphatidylinositol 3-kinase-dependent pathway. *Endocrinology*. 2003. **144** (4): 1444-1455.

Jansen J, Friesema EC, Milici C. Visser TJ. Thyroid hormone transporters in heath and desease. *Thyroid* 2005. **15**: 757-768.

Jansson L, Arlsson PO. Graft vascular function after transplantation of pancreatic islets. *Diabetologia* 2002. **45**: 749-63.

Jetton TL, Liu YQ, Trotman We, Nevin PW, Sun XJ, Leahy JL. Enhanced expression of insulin receptor substrate-2 and activation of protein kinase B/Akt in regenerating pancreatic duct epithelium of 60%-partial pancreatectomy rats. *Diabetologia* 2001. **44**: 2056-2065.

Jill DJ, Strutt B, Arany E, Zaina S, Coukell S, Graham CF. Increased and persistent circulating insulin-like growth factor II in neonatal transgenic mice suppresses developmental apoptosis in the pancreatic islets. *Endocrinology* 2000.**141**: 1151-1157.

Johonson JD, Ahmed NT, Luciani Ds, Han Z, Tran H, Fujita J, Misler S, Edlund H, Polonsky KS. Increased islet apoptosis in Pdx1+/- mice. *J Clin Invest*. 2003 **111**: 1147-1160.

Kaptein EM, Beale E and Chan LS. Thyroid hormone therapy for obesity and nonthyroidal illnesses:a systematic review. *J Clin Endocrinol Metab.* 2009. **94** (10):3663-3675

Kavok N.S, Krasilnikova O.A., Babenko N.A. Thyroxine signal transduction in liver cells involves phospholipase C and phospholipase D activation. genomic independent action of thyroid hormone. *BMC Cell. Biol*. 2001. **2**: 5.

Kemp CB, Kinight MJ, Scharp DW, Lacy PE, Ballingher WF. Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. *Nature*. 1973. 244:447.

Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH III, Wright Cv, White MF, Arden KC, Accili D. the forkead transcription factor Foxo 1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest*. 2002. **110**: 1839-1847.

Kohrle J. The selenoenzyme family of deiodinase isozymes controls local thyroid hormone availability. *Rev Endocr metabc Disord* 2000. **I** : 49-58

Koibuchi N And Chin WW. Thyroid hormone action and brain development. *Trends Endocrinol Metab* 2000. **11**:123-128.

Lacy PE. Workshop on Pancreatic islet cell transplantation in diabetes sponsored by the national institute of arthritis, metabolism and digestive diseases and held at the national institute of Health in Bethesda. Maryland November 29-30. *Diabetes* 1977. **27**: 427-429.

Lakatos P., Stern P.H. Evidence for direct non-genomic effects of triiodothyronine on bone rudiments in rats: stimulation of the inositol phosphate second messenger system. *Acta Endocrinol*. 1991. **125**: 603–608.

Lecher A, Nolan AL, Blacken RA, Haber JF. Redifferentiation of insulin-secreting cells after in vitro expansion of adult human pancreatic islet tissue. *Biochem Biophys Res Commun* 2005.**327**: 581-588.

Lei J, Mariash CN, Ingbar DH. 3,3',5-triiodo-L-thyronine up-regulation of Na,K-Atpase activity and cell surface expression in alveolar epithelial cells in Src kinase- and phosphoinositide 3-kinase-depenent. *Journal of Biological Chemistry* 2004. **279**:47589-47600

Lei J, Nowbar S, Mariash C.N, Ingbar D.H. Thyroid hormone stimulates Na–K-ATPase activity and its plasma membrane insertion in rat alveolar epithelial cells. *Am. J. Physiol*. 2003. **285** 762–772.

Lenzen S, Bailey CJ. Thyroid hormone, gonadal and adrenocortical steroids and the function of the islets of Langherans. *Endocr Rev* 1984. **5**: 411-434.

Lester LB, Kuo HC, Andrews L, Nauert B, Wolf DP. Directed differentiation of rhesus monkey Es cell into pancreatic cell phenotypes. *Reprod Biol Endocrinol*. 2004. **2**: 42.

Li L, Haynes M.P. Bender J.R. Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. *Proc. Natl. Acad. Sci.* 2003.**100**: 4807–4812.

Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27-ediated G1 arrest. Nat Med. 2002. 8: 1153-1160.

Lin H.Y, Davis FB, Gordinier JK, Martino LJ, Davis PJ. Thyroid hormone induces activation of mitogen-activated protein kinase in cultured cells. *American Journal of Physiology*. 1999. **276**: C1014–C1024.

Lin H.Y., Yen P.M., Davis F.B. Davis P.J. Protein synthesis-dependent potentiation by thyroxine of antiviral activity of interferon-gamma. *Am. J. Physiol*. 1997. **273**: C1225-C1232.

Lingor MK, Dickinson LM, Wrede CE, Briaud I, McCuaig JF, Myers MG Jr, Rhodes CJ. Decreasing IRS-2 expression in pancreatic β-cells (INS-1) promotes apoptosis, which can be compensated for by introduction of IRS-4 expression. *Molecular and Cellular Endocrinology*. 2003.**209**: 17-31

Lopez-Carballo G, Moreno L, Masia S, Perez P, Barettino D. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. *Journal of Biological Chemistry.* 2002. **277**:25297-25304

Losel R, Wehling M. Nongenomic actions of steroid hormones. *Nature Reviews Molecular and Cellular Biology* 2003 **4**(1):46-56.

Lumenlsky N, Blondel O, Laeng P, Velasco I, Ravin R, Mckay R. Differantiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 2001.**292**: 1389-1394.

Lynch M.A., Andrews J.F., Moore, R.E.. Low doses of T3 induce a rapid metabolic response in young lambs. *Horm. Metab. Res*. 1985. **17**: 63–66.

Malaisse WJ, Malaisse-Lagae F, McCraw EF. Effects of thyroid function upon insulin secretion. Diabetes 1967. **16**. 643-646.

Martínez-Gac L, Marqués M, García Z, Campanero MR, Carrera AC. Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. *Mol Cell Biol*. 2004. **24**(5):2181-9.

McKay R. Stem cells-hype and hope. *Nature*. 2000. **292**: 154-156.

Metrakos P, Yuan S, Qi SJ, Duguid WP, Rosenberg L. Collagen gel matrix promotes islet cell proliferation. *Transplant Proc*. 1994; **26**: 3349-3350

Miller L.D, McPhie P, Suzuki H, Kato Y, Liu E.T, Cheng S.Y. Multi-tissue gene-expression analysis in a mouse model of thyroid hormone resistance. *Genome Biol*. 2004. **5**:31

Misiti S., Anastasi E., Sciacchitano S., Verga Falzacappa C., Panacchia L., Bucci B., Khouri D., D'Acquarica I., Brunetti E., Di Mario U., Toscano V. and Perfetti R. 3,5,3'-triiodo-L-thyronine enhances the differentiation of a human pancreatic duct cell line into endocrine cells. *Journal of Cellular Physiology*, 2005 Jul; **204** (1) :286-96.

Moeller L.C, Cao X, Dumitrescu A.M, Seo H, Refetoff S. Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor beta through the phosphatidylinositol 3-kinase pathway, *Nucl. Recept. Signal* 2006. 4.e020.

Nielsen JH, Galsgaard ED, Moldrup A, Friedrichsen BN, Billestrup N, Hansen JA, Lee YC, Carlsson C. Regulation of beta-cell mass by hormones and growth factors. *Diabetes* 2001; **50** Suppl 1:S25-9.

Ohsugi M. Cras-Meneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA. Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. *J Biol Chem*. 2005 **280**(6):4992-5003.

Ono J, Lacy PE, Michael HE, Greider MH. Studies of the functional and morphologic status of islets maintained at 24°C for four weeks in vitro. *Am J Pathol* 1979; **97**: 489-503

Ortega E, Koska J, Pannacciulli N, Bunt J and Krakoff J. Free triiodothyronine plasma concentrations are positively associated with insulin secretion in euthyroid individuals *Eur J of Endocinol* 2008. **158:**217-221

Pende M, Kozma SC, Jaquet M, Oorschot V, Burcelin R, Le Marchand-Brustel Y, Klumperman J, Thorens B, Thomas G. Hypoinsulinaemia, glucose intolerance and diminished beta-cell size in S6K1-deficient mice. *Nature*. 2000 **408**(6815):994-7.

Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG, Hay N. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2.*Genes Dev*. 2003. 1;**17**(11):1352-65.

Perfetti R, Zhou J, Doyle ME, Egan JM. GLP-1 induces cell proliferation, PDX-1 expression and increases endocrine cell mass in pancreas of old, glucose intolerant rats. *Endocrinology*. 2000.**141**:4600-4605.

Perros P, McCrimmon RJ, Shaw G, Frier BM. Frequency of thyroid dysfunction in diabetes patients: value of annual screening. *Diabetes Med* 1995; **12**: 622-7.

Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005. **54**(7):2060-9.

Rosenberg L, Wang R, Paraskevas S, Maysinger D. Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery* 1999; **126**: 393-398.

Schmidt B.M., Martin N., Georgens A.C., Tillmann H.C., Feuring M., Christ M. Wehling M. Nongenomic cardiovascular effects of triiodothyronine in euthyroid male volunteers. *J. Clin. Endocrinol. Metab*. 2002. **87**: 1681–1686.

Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive. *N Engl J Med*. 2000. **343**(4):230-8.

Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R. et al. international trial of the Edmonton protocol for islet transplantation. *N Engl J Med*. 2006. **355**:1318-30.

Sharp DW, Murphy JJ, Newton WT, Ballinger WF, Lacy PE. Transplantation of islets of Langerhans in diabetic rhesus monkey. *Surgery* **77**. 100-105

Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Pract*. 2010. **87**: 4-14.

Schnedl WJ, Ferber S, Johnson JH, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT-2-expressing cells. *Diabetes* 1994. 43(11): 1326–33.

Shi YB, Su Y, li Q, Damjanovski S. Autoregulation of thyroid hormone receptor genes during metamorphosis: roles in apoptosis and cell proliferation. *Int. J. Dev. Biol.* 1998. **42** : 107-116.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000. **407**: 538-541

Skarulis MC, Celi FS, Mueller E, Zemskova M, Malek R. et al. Thyroid hormone induced brown adipose tissue and amelioration of diabetes in a patient with extreme insulin resistance. *J Clin Endocrinol Metab.* Jan 2010; **95**(1):256-62

Sordi V, Bertuzzi F, Piemonti L. diabetes mellitus: an opportunità for therapy with stem cells? *Reagen Med* 2008. **3**: 377-397.

Sorenson RL, Brelje TC. Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Horm Metab Res*. 1997. **29**(6):301-7.

Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes*. 2000. **49**: 157-162.

Srinivasan S, Bernal-Mizrachi E, Ohsugi M and Permutt MA Glucose promotes pancreatic islet β-cell survival through a PI 3-kinase/Akt signalling pathway Am *J Physiol Endocrin Metabol* 2002. 283: E748-E793

Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Bernal-Mizrachi E, Permutt MA. Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3beta in mouse insulinoma cells. *Diabetes*. 2005 **54**(4):968-75.

Storey N.M., O'Bryan J.P. Armstrong D.L. Rac and Rho mediate opposing hormonal regulation of the ether-a-go-go-related potassium channel. *Curr. Biol*. 2002. **12**: 27–33.

Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV, Cheng JQ. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ERα) via interaction between Erα and PI3K. *Cancer Research* 2001.**61**:5985-5991.

Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furrth EE, Polonsky NS, Naji A, Birnbaum MJ. Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt/PKB alpha. *Nat Med* 2001.**7**: 1133-1137

Tyrberg B, Ustinov J, Otonkoski T, Andersson A. Stimulated endocrine cell proliferation and differentiation in transplanted human pancreatic islets: effects of the ob gene and compensatory growth of the implantation organ. *Diabetes*. 2001 **50**(2):301-7

Uchida T, Nakamura T, Hashimoto N, Matsuda T, Kotani K, Sakaue H, Kido Y, Hayashi Y, Nakayama KI, White MF, Kasuga M. Deletion of Cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. *Nat Med*. 2005. **11**(2):175-82.

Van der Windt DJ, Bottino R, Casu A, Campanile N, Cooper DK. Rapid loss of intraportally transplanted islets: an overview of pathophysiology and preventive strategies. *Xenotransplantation* 2007. **14**:288-97.

Verga Falzacappa C, Panacchia L, Bucci B, Stigliano A, Cavallo MG, Brunetti E, Toscano V, Misiti S. 3,5,3'-triiodothyronine (T3) is a survival factor for pancreatic beta-cells undergoing apoptosis. *Journal of Cellular Physiology* .2006 **206**(2):309-21.

Verga Falzacappa C, Patriarca V, Bucci B, **Mangialardo C**, Michienzi S, Moriggi G, Stigliano A, Brunetti E,Toscano E, Misiti S. TRb1 is essential in mediating T3 action on Akt pathway in human pancreatic insulinoma cells. *J Cell Biochem*. 2009 **106**(5):835-48

Verga Falzacappa C, Petrucci E, Patriarca V, Michienzi S, Stigliano A, Brunetti E, Toscano V and Misiti S. Thyroid hormone receptor β1 mediates Akt activation by T3 in pancreatic β cells. *Journal of Molecular Endocrinology*, 2007 **38**(2):221-33.

Wang Y.G., Dedkova E.N., Fiening J.P., Ojamaa K., Blatter L.A. Lipsius S.L. Acute exposure to thyroid hormone increases Na^+ current and intracellular Ca^{2+} in cat atrial myocytes. *J. Physiol*. 2003. **546**: 491–499.

Woodgett JR. Recent advantages in protein kinase B signaling pathway. *Curr opin cell biol.* 2005. **17**(2):150-7.

Wrede CE, Dickson LM, Lingohr MK, Briaud I, McCuaig JF, Rhodes CJ. Protein kinase B/Akt prevents fatty acid induced apoptosis in pancreatic β cells (INS-1*) J. Biol. Chem*. 2002. **277**: 49676-49684

Wrutniak C. Cabello G. Influence of tri-iodothyronine or lipid administration on the response of the pituitary-thyroid axis to exposure to cold in the newborn lamb. *J. Endocrinol*. 1989.**121**: 361–365.

Yatoh S, Dodge R, Akashi T, Omer A, Sharma A, Weir GC. Differentiation of affinity-purified human pancreatic duct cells to β cells. *Diabetes*. 2007. **56**: 1802-1809.

Zhang J. and Lazar M.A. The mechanism of action of thyroid hormones . *Annu. Rev. Physiol*. 2000. **62** : 439-66.

Zinke A, Schmoll D, Zachmann M, Schmoll J, Junker H, Grempler R, Kirsch G, Walther R. Expression of thyroid hormone receptor isoform a1 in pancreatic islets. Exp Clin Endocrinol Diabetes 2003; 111(4): 198–202.

Zhu Y. Bond J. Thomas P. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc. Natl. Acad. Sci*. U.S.A. 2003. **100.** 2237-2242.