Executive summary

The two main forms of diabetes mellitus are T1D and T2D. They are a major cause of morbidity and mortality, decreasing both life quality and life expectancy of nearly 30 million affected individuals in Europe. T1D is characterized by a near complete lack of insulin production due to specific destruction of the pancreatic beta cells that typically develops over several years. Although some immune-related biomarkers can identify individuals at risk to develop T1D, the process by which the beta cells are destroyed is not well understood. As a consequence, there are no adequate strategies for preservation of beta cell mass and prevention of the disease. Accumulating evidence suggests that beta cell loss in T1D is the result of an autoimmune mediated process, where a chronic inflammation called insulitis causes beta cell destruction. This is mediated by cytokines and other mediators released by the activated immune cells invading the islets, which activate secondary pathways of cell death in the target beta cells. T2D results from a reduced ability of the pancreatic beta cells to secrete enough insulin to stimulate glucose utilization by peripheral tissues. Initially, this causes impaired glucose tolerance, i.e. a reduced capacity to clear glucose from the blood following a glucose load. As beta cell mass decreases and beta cell secretory capacity further deteriorates, there is a progressive increase in the fasting glucose concentration, eventually culminating in overt hyperglycaemia. Defects in both insulin secretion and action contribute to the pathogenesis of T2D, but it is now acknowledged that T2D is an insulin deficiency syndrome associated with a progressive reduction in beta cell mass. The loss of beta cell mass in T2D is probably secondary to chronic exposure to high glucose and free fatty acid (FFA) levels (glucolipotoxicity). In conclusion, a reduction in beta cell mass is a key component of diabetes mellitus and the molecular mechanisms underlying beta cell loss remain to be clarified.
This Specific Targeted Research Project aims to utilize functional genomics to identify pathways responsible for the reduction of beta cell mass in diabetes, and use this knowledge to define targets for intervention to preserve beta cell mass. This will be reached through the following steps:

- Identification of the regulatory molecular pathways that control physiological beta cell mass through regeneration, differentiation and apoptosis.
- Use of functional genomics to identify key pathophysiological events in the above pathways that are responsible for reduction of beta cell mass in diabetes, with focus on the mechanisms regulating cytokine- and glucolipotoxicity-induced beta cell apoptosis.
- Intervention in the defective signal transduction pathways identified above using genetically modified mice, long-acting viral vectors and small interfering RNAs. This step should identify and validate targets to preserve beta cell mass in diabetes.

To achieve this ambitious goal, we have established a consortium of leading European experts from seven European countries in the fields of pancreatic beta cell diabetes research, functional genomics and bioinformatics (listed below). The results to be obtained will foster the development of cutting-edge functional genomics technology and will contribute to the development of novel therapies to prevent diabetes.

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Several important and novel findings were obtained by partners of SAVEBETA during the course of the project. These findings validate the proposed systems biology approach to understand the molecular pathways underlying decreased beta cell mass in diabetes mellitus and have provided important information for the development of novel alternatives to preserve beta cell mass in early diabetes. Some of the most important findings by the SAVEBETA project are listed below (detailed information on these and other novel findings are provided in Section 2; *the main discoveries made in the second part of the project, i.e. from months 19-36, are indicated in italics all over the text):*

a. The role of EGFR signalling in beta cell expansion has been characterized using a Pdx-1-EGFR-dominant negative mouse, developed by members of the consortium. It was shown that these mice fail to increase their beta cell mass in response to a high fat diet and develop diabetes. This indicates that EGFR signalling is essential for compensatory beta cell growth in response to a form of metabolic demand highly prevalent in Western societies, namely high fat- (HF) and calorie-rich diets. *Interestingly, beta cell mass growth during pancreatic regeneration following partial duct ligation, was not affected by the EGFR signalling defect. These results indicate that EGFR signalling is required for beta cell mass expansion in HF-diet induced insulin resistance but not in islet neogenesis after partial duct ligation (Hakonen, Miettinen & Otonkoski, manuscript in preparation). EGFR is clearly involved in beta cell mass regulation but it also may regulate beta cell functionality. We have shown that inhibition of EGFR impairs insulin secretion (human beta cells, MIN6 cells and the islets from Pdx-1-EGFR mouse). Further analysis of this is concentrating on changes in intracellular calcium-fluxes and potential mislocation of the important beta cell transcription factor FoxO1. Using a mouse cDNA array (PancChip) we could show that FoxO1 mRNA expression is down-regulated in the Pdx-1-EGFR mice by two-fold, although these results are compromised by alpha cell contamination. Translocation of FoxO1 after EGFR inhibition was studied using a GFP-FoxO1 adenovirus provided by Dr. Rutter in the context of the SAVEBETA project (Otonkoski, Eizirik & Rutter, manuscript in preparation).*

b. Additional and novel information was obtained regarding the human diabetes syndrome 6q24 transient neonatal diabetes mellitus (6q24 TNDM), a rare form of diabetes caused by over-expression on imprinted chromosome 6q24. Members of the consortium have previously developed a transgenic mouse (ZAC1) that partially recapitulates the TNDM phenotype, and have now completed the analysis of the four genes flanking *Zac1* in mouse embryo and adult mouse tissues, besides screening the 300 kb TNDM for additional imprinted genes. A new upstream promoter has been identified (*Zac1c/ZAC1c*), and additional analysis is ongoing in the human material. *The incompletely penetrant diabetes phenotype of TNDM29 mice prompted us to reinvestigate whether there were additional imprinted genes closely linked to ZAC1 that could contribute to the development of T2D in this syndrome. The minimal genetic interval for TNDM encompasses several genes (Sf3b5, Fam164b, Ltv1) for which allelic expression status has not yet been*

described. Using tissues from C57BL/6J x SPRET/EiJ (Mus spretus) hybrids and mice with uniparental duplication of proximal chromosome 10 (MatDp(prox10) and PatDp(prox10)), where the Zac1 maps, we found these 3 genes to be biallelically expressed and non-imprinted. Similar findings have been made by collaborating groups in the homologous human genes (I. K. Temple, Univ. of Southampton). These results confirm ZAC1 as the likely diabetes-causing gene when over-expressed. Recent data by ourselves and others have shown that ZAC1 does not only function as a classic transcription factor by binding to the target DNA, but also as a co-activator using a protein domain independent of the zinc finger domain that is less conserved between human and mouse. We have also determined the organisation of the ZAC1 gene in more detail and by 5'RACE and RT-PCR identified the existence of a new upstream promoter conserved in mouse and human, denoted Zac1c/ZAC1c. Zac1c/ZAC1c is expressed biallelically, i.e., non-imprinted, and expressed in most cell types, but at a lower level than the imprinted Zac1/ZAC1 promoter. Interestingly, analysis of human pancreas material (provided by T. Otonkoski in the context of SAVEBETA) showed that transcripts from the canonical ZAC1 promoter are detected with similar abundance in islets and exocrine cell populations but transcripts from the novel ZAC1c promoter are restricted to islets.

c. Major advances were made in the characterization of neogenesis in human pancreatic tissue, with the identification of a double-layered basement membrane organization of human pancreatic islets and its role in human beta cell differentiation and proliferation. Furthermore, cell sorting methods have been optimized to purify human CK19 positive duct cells that may differentiate into an islet lineage *and adult human beta cells, which serve as "gold standard" for comparisons against differentiating cells. The proliferation and plasticity of these cell types was then studied on the laminin isoforms, which form the natural niche for human islet cells. The results revealed that certain laminin isoforms, which are found in the islet basement membrane (particularly LM511), favour the proliferation and inhibit the de-differentiation of human beta cells (Banerjee and Otonkoski, submitted).*

d. Viral infections may contribute to the pathogenesis of T1D, but the mechanisms by which viruses or viral products such as double stranded RNA (dsRNA) affect beta cell survival and trigger autoimmunity remain unknown. Members of SAVEBETA have now discovered that dsRNA triggers apoptosis and chemokine production by both activation of the toll like receptor 3 (TLR3), *MDA5 and RIG signalling*, leading to massive production of interferons; blocking interferon α/β action by use of mice knockout (KO) for the IFN receptor, or by using antibodies, prevented beta cell apoptosis. *Of particular importance was the recent identification by Eizirik that the candidate gene for T1D, MDA5, plays a key role for chemokine production by pancreatic beta cells confronted with a viral infection.*

e. *In vitro* (using siRNAs and adenoviral vectors), *in vivo* (using transgenic and KO mice) and *in silico* studies were used to characterize the role of the transcription factors NF-κB and STAT-1 in the process of beta cell apoptosis (these transcription factors were detected as key pro-apoptotic signals by previous array analysis made by SAVEBETA members). Blockage of each of these transcription factors protected beta cells *in vitro* against cytokines or dsRNA and *in vivo* against diabetes induced by multiple doses of streptozotocin. *Importantly, the single blockade of NF-κB in an allogenic islet transplantation model, was sufficient to induce a delay in time of rejection, and attenuate islet infiltration.* This is a crucial "proof of concept" for the basic premise of the SAVEBETA project, namely to identify targets for intervention by genomics/proteomics analysis and then validate these targets using novel *in vitro* and *in vivo* models. Interestingly, it was found that another transcription factor downstream of STAT-1, namely IRF-1, has a protective role against beta cell death, suggesting a dual mechanism of regulation of the IFN-γ-STAT-1-IRF-1 pathway. *Moreover, we found that PTPN2, a candidate gene for T1D diabetes, has a negative feedback role in STAT-1 activation, and that the transcription factor JunB inhibits both cytokine-induced ER stress and apoptosis, suggesting that it acts as "protective" transcription factor in beta cells. New array, proteomic analysis and in vivo experiments (involving islet transplantation) were completed or are presently ongoing to clarify the mechanisms involved.*

f. AMP-activated protein kinase (AMPK) was identified as a downstream mediator of cytokine- and T cell-mediated apoptosis. Importantly, expression of a dominant negative AMPK protected beta cells against these mediators of apoptosis in T1D, suggesting that AMPK is a novel and interesting target to suppress immune-mediated beta cell death in both early T1D and following islet transplantation. *In addition, other mechanisms through which AMPK activation may contribute to beta cell dysfunction, have also been investigated. Previous work revealed that cells over-expressing AMPK CA blocked the glucose-stimulated*

recruitment of insulin containing granules to the plasma membrane, suggesting that AMPK may modulate insulin granules movement. Insulin granules are transported to the plasma membrane by the microtubule motor protein kinesin, which is a heterotetramer consisting of two heavy chains and two light chains. Both the heavy and light chains were found to contain putative AMPK phosphorylation sites, but only a peptide corresponding to the light chain was phosphorylated by AMPK. Ongoing work has involved the characterisation of: AMPK CA and AMPK DN transgenic mice; AMPK double KO mice (α1 whole body and α2 pancreatic beta cell-specific knock out) and LKB1 KO mice with two manuscripts currently submitted based on these studies. In brief, deletion of both (but not either one) of the catalytic subunits of AMPK in the beta cell and in a small set of hypothalamic neurons using the RIP-Cre transgene (Jackson Labs) leads to abnormal insulin secretion in vivo, likely due to the requirement for a permissive factor derived from the hypothalamus, whereas secretion from isolated islets is somewhat enhanced in this model (Sun, G., Submitted).

g. Endoplasmic reticulum (ER) stress was identified via collaborative work from members of SAVEBETA as a common final pathway by which cytokines (involved in beta cell apoptosis in T1D) and free fatty acids (FFA; involved in beta cell apoptosis in T2D) trigger beta cell apoptosis. *The mechanisms involved were clarified, and point to depletion of ER Ca^{2+} as a main trigger for ER stress, and a downstream activation of the Bcl-2 member DP5 as a key "executioner step" of apoptosis*. Of note, proteomic analysis identified post-translational changes in BiP, a key ER stress chaperone, in cytokine-treated beta cells. This may further hamper ER stress function and contribute for beta cell apoptosis.

h. *In order to identify novel approaches to protect beta cells against ER stress, we (Cnop, Eizirik and Rutter) evaluated the molecular mechanisms involved in the protection of beta cells from lipotoxic ER stress by GLP-1 agonists, utilized in the treatment of type 2 diabetes. INS-1E or FACS-purified primary rat beta cells were exposed to oleate or palmitate with or without the GLP-1 agonist exendin-4 or forskolin. CPA was used as a synthetic ER stressor, while the ATF4-CHOP branch was selectively activated with salubrinal. The ER stress signaling pathways modulated by GLP-1 agonists were studied by real time PCR and Western blot. Knockdown by RNA interference was used to identify mediators of the antiapoptotic GLP-1 effects in the ER stress response and downstream mitochondrial cell death mechanisms. Exendin-4 and forskolin protected beta cells against FFAs via the induction of the ER chaperone BiP and the anti-apoptotic protein JunB that mediate beta cell survival under lipotoxic conditions. On the other hand, exendin-4 and forskolin protected cells against synthetic ER stressors by inactivating caspase 12 and up-regulating Bcl-2 and XIAP, both inhibiting mitochondrial apoptosis. These observations suggest that GLP-1 agonists increase, in a context-dependent way, beta cell defence mechanisms against different pathways involved in ER stress-induced apoptosis. The identification of the pathways modulated by GLP-1 agonists is an important discovery of SAVEBETA, and may allow for targeted approaches to alleviate beta cell ER stress in diabetes.*

i. The antioxidant enzyme catalase mediated protection against FFA toxicity, indicating that formation of oxygen free radicals contributes to lipotoxicity. There was also an increased mitochondrial membrane potential after exposure to the saturated FFA palmitate, and the "dialogue" between the ER and the mitochondria in the context of cytokine- and FFA-induced toxicity was studied by targeting free radical scavenging enzymes (catalase, MnSOD etc) to different sub-cellular compartments. *The results obtained indicate that H_2O_2-production in the cytosol and not in the mitochondria has an important role in toxicity.*

j. Gene profiling have been performed in human islets and novel glucose up- and down-regulated genes (90 in total) have been identified. Candidate genes were validated by RT-PCR and their expression compared to that observed in islets isolated from control or T2D patients. *The candidate gene on top of list is the thioredoxin interacting protein Txnip, upregulated by 6-22-fold in glucose-treated islets, as identified by microarray analysis and confirmed by real time PCR. Its expression is 4-fold in T2D patients (n=5 T2D islets preparations) compared to islets from normal individuals (n=6). Thioredoxin-interacting protein (Txnip), is a negative regulator of the antioxidant thioredoxin (Trx). Txnip expression was upregulated by exposure to high glucose concentrations (HG), its overexpression potentiates and Trx attenuates high glucose-mediated apoptosis in beta cells. Knockdown of endogenous Txnip reduces sensitivity to HG-induced apoptosis.*

k. A SREBP1 null mouse was used to characterize the role of this transcription factor for beta cell adaptation to prolonged exposure to high glucose, a condition that prevails in T2D. It was observed that enhanced

lipid synthesis mediated by SREBP1c-dependent genes is required for the adaptive changes in islet gene expression and insulin secretion in the presence of chronic exposure to high glucose.

l. During the 36 months of the project there have been three internal meetings attended by all PIs and other scientists involved in the project, namely the "SAVEBETA Opening Meeting" in Brussels, on December 11-12th, 2006 (attended by 29 participants), the "SAVEBETA Annual Internal Meetings" in London, on December 5th, 2007 (attended by 27 participants) *and in Leuven, on December 1-2, 2008 (attended by 30 participants)*. Of particular relevance, SAVEBETA organized in collaboration with the Biochemical Society, UK, and the Juvenile Diabetes Research Foundation International a two day Focused Meeting entitled *"Pancreatic beta-cell: birth, life and death"* in London, December 3-4, 2007 (see additional information at http://www.biochemistry.org/meetings/programme.cfm?Meeting_No=SA080). This meeting was attended by more than 200 participants, and was a huge success: all available places were filled at the "early bird registration" stage, the meeting was unanimously considered one of the best beta cell meetings in recent years and the Biochemical Society UK has already proposed a similar joint meeting for the end of 2010. Proceedings of the meeting (based on lectures by invited speakers and including several reviews written by members of SAVEBETA; see below list of publications) were published in Biochemical Society Transactions vol. 3, part 3, 2008.

There have been 56 publications supported at least in part by the SAVEBETA project during the 36 months of the project, with 43 original papers and 13 review papers in professional scientific journals. Of the 43 original papers, 13 (28%) were the result of collaborative work between different partners inside the SAVEBETA project. This indicates both an excellent scientific production and, more important, and active and growing collaboration between the different partners of the SAVEBETA consortium. Of note, the number of publications in the first part of the project (months 1-18) was 17 original papers and 7 review papers. Thus, the second part of the project (months 19-36) was even more productive, with 26 original papers and 6 reviews, indicating the progress of the work.

The STREP offers to the public a freely accessible webpage of the project at www.savebeta.eu which contains contact information of the project coordinator office in Brussels, title and detailed aims of the project, a list of all partners and an internal site (accessible only for members of the consortium) where relevant administrative information are posted on a regular basis. Array analysis made by members of the Consortium are posted on an open access website of the Beta Cell Gene Expression Bank (http://www.t1dbase.org/page/BCGB_Enter/display/), an initiative coordinated by D.L. Eizirik, Coordinator of SAVEBETA.

As a whole, the meetings organized (including a public meeting attended by >200 participants), the open access websites of SAVEBETA and the Beta cell Gene Expression Bank, *and the continuous publication of data generated by members of the SAVEBETA consortium in high impact journals (43 original publications, mostly in high impact journals, plus 13 reviews in the course of the project) assured excellent dissemination of the knowledge generated in the project.*