

## 01 Metabolic and Immunological Comparisons Between Autoimmune and Nonautoimmune Diabetes

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Diabetes in humans is a heterogeneous disorder classified clinically into two types: type 1 and type 2 diabetes. Most type 1 diabetic patients are diagnosed in childhood or young adulthood before the age of 35 years and are considered to have autoimmune disease. In contrast, type 2 diabetes is "classically" designated as diabetes of adults and is considered nonautoimmune. However, there is a group of phenotypic adult type 2 diabetic patients (10% of patients) that exhibit autoimmunity. These patients are said to have latent autoimmune diabetes in adults (LADA) or type 1.5 diabetes. Autoantibodies and T-cells reacting with islet proteins have been demonstrated in type 1 and type 1.5 diabetic patients. In contrast, classic autoantibody type 2 diabetic patients are negative for T-cell responses to islet proteins. Therefore, we questioned whether type 1 and type 1.5 diabetes are similar or pathogenically different autoimmune diseases. We have investigated the immunological and metabolic differences between type 1, type 1.5, and classic type 2 diabetic patients. We have identified autoantibody differences, differences in islet proteins recognized by T-cell responses, and differences in insulin resistance between type 1 and type 1.5 diabetic patients. We have also identified a small group of patients ( $n = 6$ ) who have T-cells responsive to islet proteins but are autoantibody negative. These patients appear to be similar to type 1.5 diabetic patients with regard to their T-cell responses to islet proteins, BMI, and stimulated C-peptide values, but appear to be later in the disease process. These data suggest that measurement of T-cell responses to islet proteins in addition to detection of islet autoantibodies may aid in identifying patients with autoimmune diabetes and more severe  $\beta$ -cell dysfunction. Moreover, although type 1 and type 1.5 diabetes appear to be similar clinically, the pathogenesis of type 1 and type 1.5 diabetes appear to be distinct.

## 02 Characteristics of Humoral Islet Autoimmunity Associated With Progression to Type 1 Diabetes

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Type 1 diabetes is associated with the development of islet autoantibodies before disease onset. Autoantibodies to insulin (IAAs), GAD (GADAs), and the protein tyrosine phosphatase-related molecule IA-2 (IA-2As) can be used to stage type 1 diabetes risk in nondiabetic individuals. However, even the presence of multiple autoantibodies is not always followed by progression to type 1 diabetes. We have measured islet autoantibody characteristics such as affinity, titer, subclass, and epitope reactivity in cohorts of first-degree relatives of patients with type 1 diabetes from the Bart's Oxford and the Munich family studies and in offspring of diabetic parents followed from birth in the BABYDIAB study to better stratify type 1 diabetes risk. In offspring, IAAs of high affinity ( $>10^9$  l/mol) were found to be associated with high risk for progression to multiple autoantibodies (91% within 4 years) and diabetes (50% within 6 years). High-affinity IAAs required conservation of insulin A-chain residues 8-13 for epitope recognition and were proinsulin reactive. In relatives, rapid progression to type 1 diabetes was associated with broad IA-2A and IAA responses expressed as high-antibody titer; IgG1 plus IgG2, IgG3, and/or IgG4 subclasses; and spreading of IA-2A reactivity to the related molecule IA- $\beta$ . Using models based on these antibody characteristics, type 1 diabetes risk could be stratified from  $<10\%$  to around 90% within 5 years. The findings enable more accurate risk assessment and improved selection of subjects for intervention.

## 03 HLA-DQB1 Alleles and Genotypes, Islet Antibodies, and $\beta$ -Cell Function in Young Adult Diabetic Patients

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**Background and aims:** Although it is well known that HLA is associated with type 1 diabetes, it is unclear how HLA contributes to the pathogenesis of this disease. The aim of the current study was to relate HLA-DQB1 loci to islet antibodies and  $\beta$ -cell function in 1,789 recently diagnosed young adults with diabetes. **Material and methods:** During a 5-year period, 1963 young adults 15–34 years of age were included in the Diabetes Incidence Study in Sweden (DISS). At diagnosis, blood samples were taken for assessment of islet antibodies (islet cell antibodies [ICAs] and autoantibodies to GAD [GADAs] and IA-2 [IA-2As]), plasma C-peptide ( $<0.25$  nmol/l low and  $<0.10$  nmol/l unmeasurable), and HLA-DQB1 typing. **Results:** Islet antibodies were present in 1,199 (67%) of 1,789 patients. HLA-DQB1 \*02, \*0302, and \*0604 alleles were significantly more frequent in antibody ( $Ab^+$ ) versus  $Ab^-$  patients (24 vs. 17%,  $P < 0.0001$ ; 35 vs. 16%,  $P < 0.0001$ ; 7 vs. 4%,  $P = 0.0018$ , respectively). Comparing  $Ab^+$  with  $Ab^-$  patients, GADAs were closely associated with \*02 ( $P < 0.0001$ ), \*0302 ( $P < 0.0001$ ), and \*0604 ( $P = 0.0035$ ) alleles, whereas IA-2As were closely associated with \*0302 ( $P < 0.0001$ ) and \*0604 ( $P = 0.0008$ ) alleles. Compared with patients with normal C-peptide levels, patients with low C-peptide levels had significantly higher frequencies of \*02 (24 vs. 21%,  $P = 0.04$ ), \*0302 (34 vs. 26%,  $P < 0.0001$ ), and \*0604 (7 vs. 5%,  $P = 0.01$ ) alleles, whereas the frequency of the \*0302 allele (32 vs. 26%,  $P = 0.023$ ) was significantly increased in patients with unmeasurable plasma C-peptide. HLA-DQB1 \*02/0302, \*0302/0604, and \*0302/X (X = either homozygous allele or any allele other than 02, 0302, or 0602-03-04) were significantly more frequent in  $Ab^+$  patients than in  $Ab^-$  patients (27 vs. 8%,  $P < 0.0001$ ; 7 vs. 2%,  $P < 0.0001$ ; 26 vs. 12%,  $P < 0.0001$ , respectively). Compared with patients with normal C-peptide levels, patients with low C-peptide levels had significantly higher frequencies of \*02/0302 (24 vs. 18%,  $P = 0.0027$ ), \*0302/0604 (7 vs. 4%,  $P = 0.0085$ ), and \*0302/X (26 vs. 18%,  $P = 0.0008$ ) genotypes, whereas patients with unmeasurable plasma C-peptide had increased frequency of the \*0302/X genotype (26 vs. 18%,  $P = 0.019$ ). **Conclusions:** Islet antibodies were associated with HLA-DQB1 \*02, \*0302, and \*0604 alleles; GADAs with \*02, \*0302, and \*0604; and IA-2As with \*0302 and \*0604. HLA-DQB1 genotypes comprising the \*0302 allele were associated with IA-2As and severe  $\beta$ -cell destruction at diagnosis of diabetes.

## 04 Diabetes Family History, Autoimmunity, and Metabolic Features in Children With Diabetes in the SEARCH Study

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Type 1 and type 2 diabetes are considered distinct etiological entities. However, individuals often present with mixed features of autoimmunity and insulin resistance, making classification of type 1 or type 2 diabetes difficult. In this study, we investigated the effect of a family history (FH) of diabetes on metabolic features in 3,404 subjects, 0–22 years old, participating in the SEARCH study, a population-based, multicenter study of childhood diabetes. Diabetes FH involving first-degree relatives and/or grandparents was assessed by administered questionnaire, with FH categories defined by the relatives' age at diagnosis. For this analysis, we compared subjects with an FH that included relatives diagnosed at  $\geq 25$  years of age (later FH<sup>+</sup>) with subjects who had no FH of diabetes (FH<sup>-</sup>). Diabetes autoantibodies (DAAs) to GAD65, IA-2, and insulin were used to classify subjects as DAA<sup>+</sup> or DAA<sup>-</sup>. Fasting C-peptide levels were higher in children with later FH<sup>+</sup>. Metabolic syndrome features associated with later FH<sup>+</sup> included higher BMI, higher triglycerides, lower HDL, higher blood pressure, and increased frequency of acanthosis nigricans. The association between later FH<sup>+</sup> and fasting C-peptide, BMI, triglycerides, and acanthosis remained significant in a subgroup of DAA<sup>+</sup> subjects. Thus, later FH<sup>+</sup> (implying type 2 diabetes genetics) confers features of the metabolic syndrome in children and youth with type 2 diabetes as well as those with islet cell autoimmunity (DAA<sup>+</sup>).

## 05 Frequency of Type 1 and Type 2 Diabetes Among Relatives of Children With Type 1 Diabetes

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**Background:** Familial clustering of type 1 diabetes is well established, but it is less clear whether type 2 diabetes is increased in families of children with type 1 diabetes. **Aim:** To define frequencies of type 1 and type 2 diabetes among relatives of children with and without type 1 diabetes. **Subjects and methods:** Extended family history of diabetes was obtained from 300 children with type 1 diabetes and 381 control children matched for age, sex, and geographical location of residence. A structured interview of the parents was performed. Type 1 diabetes was classified as insulin-dependent diabetes diagnosed before the age of 30 years. Gestational and unclassified diabetes were not included. The  $\chi^2$  statistics were used to analyze the frequency of diabetes among parents and siblings (A); grandparents, aunts, and uncles (B); and cousins (C). **Results:** Frequency of type 1 diabetes was higher among the relatives of children with type 1 diabetes than among relatives of the control children (A: 14.7 vs. 2.4%,  $P < 0.001$ ; A+B: 28.8 vs. 10.3%,  $P < 0.001$ ; and A+B+C: 39.2 vs. 15.2%,  $P < 0.001$ ). Frequency of type 2 diabetes was similar among the relatives of type 1 diabetic and control children (A: 0.7 vs. 1.6%, NS, and A+B: 48.0 vs. 54.1%, NS). **Conclusions:** These results confirm that type 1 diabetes is increased in families of children with type 1 diabetes. No evidence of increased frequency of type 2 diabetes among relatives of type 1 diabetic children was obtained.

06

#### Association of Systemic Chemokine Concentrations With Impaired Glucose Tolerance and Type 2 Diabetes: Results From the Cooperative Health Research in the Region of Augsburg Survey S4 (KORA S4)

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Chemokines are crucial immune mediators in many physiological and pathophysiological conditions. Chemokines have been hypothesized to be involved in macrophage infiltration into adipose tissue in obesity and might therefore play an important role in the development of obesity-related disorders such as type 2 diabetes. Out of 1,653 individuals aged 55–74 years participating in a population-based survey in southern Germany (KORA S4, 1999–2001), 236 individuals with type 2 diabetes, 242 subjects with impaired glucose tolerance (IGT), and 244 normoglycemic control subjects were analyzed for circulating concentrations of interleukin (IL)-8; interferon- $\gamma$ -inducible protein-10 (IP-10); regulated on activation, normal T-cell expressed and secreted (RANTES); and eotaxin. Systemic concentrations of RANTES were higher in individuals with IGT or type 2 diabetes than in control subjects, whereas IL-8 levels were elevated in type 2 diabetic patients only ( $P < 0.001$  for all comparisons). IP-10 and eotaxin were not significantly associated with IGT or type 2 diabetes. Adjustment for age, sex, BMI, hypertension, LDL cholesterol, HDL cholesterol, uric acid, C-reactive protein, and IL-6 did not alter these findings. Our analyses indicate that RANTES and IL-8 may be involved in the development of type 2 diabetes, independently of metabolic syndrome-related risk factors and of each other. There is no general upregulation of chemokine production in type 2 diabetes, but rather an association of the disease with specific members of the chemokine family.

07

#### GAD Antibody Positivity in Relatives of Type 2 Diabetic Subjects or Subjects with Latent Autoimmune Diabetes in Adults: Results From the Botnia Study

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GAD antibodies (GADAs) separate an insulin-deficient subgroup of subjects with type 2 diabetes, also called latent autoimmune diabetes in adults (LADA). To study the significance of GADAs in nondiabetic (ND) relatives of patients with type 2 diabetes, we analyzed GADAs in 9,181 subjects from the Botnia Study, including patients with type 1 diabetes ( $n = 370$ ) or type 2 diabetes ( $n = 3,217$ ) and their family members (881 spouses without [ND<sub>CONT</sub>] and 4,713 relatives with [ND<sub>REL</sub>] family history of diabetes). GADAs were found in 47% of type 1 diabetic subjects (median duration >10 years), 8.5% of type 2 diabetic subjects, 5.1% of ND<sub>CONT</sub> subjects, and 4.9% of ND<sub>REL</sub> subjects ( $P < 0.00001$  for type 1 diabetes vs. others and type 2 diabetes vs. ND). The median GADA level was highest in type 1 diabetes followed by type 2 diabetes and ND (228 vs. 80.5 vs. 46 IU/ml, respectively,  $P < 0.00001$ ). Of the GADA<sup>+</sup> ND subjects, 17% had a relative with type 1 diabetes, and 25% had a GADA<sup>+</sup> type 2 diabetic relative. Also, 18.1% of the type 2 diabetic relatives of GADA<sup>+</sup> ND subjects were GADA<sup>+</sup> ( $P < 0.00001$  vs. all type 2 diabetic subjects). Among the 2,293 ND subjects followed for 6 years, GADAs ( $n = 140$ ) were not associated with either any clinical characteristics or deterioration of glucose tolerance. However, those repeatedly GADA<sup>+</sup> during the follow-up ( $n = 51$ ) had a higher risk of developing early-onset (<45 years) diabetes (hazard ratio 5.6, 95% CI 1.2–25.6,  $P = 0.026$ ) compared with GADA<sup>-</sup> and/or GADA<sup>+</sup> at a single occasion. **Conclusion:** GADA positivity clusters in families with type 1 diabetes or LADA. Low-level positivity is not associated with a clinical phenotype. Repeatedly GADA<sup>+</sup> subjects seem to have an increased risk of developing early-onset diabetes.

08

#### HLA Class II Transgenic Mice: How Closely Do They Reproduce Human CD4 T-Cell Immune Responses?

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**Introduction:** The HLA class II transgenic mouse model is designed to provide an experimental *in vivo* animal model in which certain aspects of human CD4 T-cell immune responses can be studied. The question is how well the current version of this model serves this purpose. To address this, we wanted to study the CD4 T-cell immune responses obtained in HLA-DR\*0401 transgenic mice after immunization with recombinant protein autoantigen (human proinsulin [P-Ins]) and compare these to analogous CD4 memory T-cell responses found in HLA-DR\*0401-positive humans after development of type 1 diabetes. **Results and Discussion:** We compared the fine specificities of two murine T-cell hybridomas with a human T-cell clone specific for the same immunodominant CD4 T-cell epitope of P-Ins using T-cell clones restricted by HLA-DR\*0401. Although the two murine T-cell hybridomas recognized this P-Ins epitope in exactly the same binding register as the human T-cell clone, they had rather different T-cell receptor interaction profiles. While amino acid positions P7 and P8 were required for activation of either of the murine T-cell hybridomas, P8 and P9 were the important amino acid residues for activation of the human T-cell clone ULM52C1, which was cloned from an HLA-DR\*0401-positive diabetic patient. NH<sub>2</sub>- and COOH-terminal truncation studies also showed important differences between the murine and the human P-Ins<sub>73-90</sub>-specific CD4 T-cells with respect to the optimal peptide length. A comparison of the amino acid sequence of the human P-Ins versus the two murine P-Ins proteins showed significant variations in the area of this particular peptide epitope. Thus, human P-Ins is in effect a foreign antigen in the HLA class II transgenic mice, and this is likely to affect T-cell recognition as well as T-cell receptor repertoire selection in the thymus of these mice. Related results have previously been reported for other “self” or foreign protein antigens (Patel et al., *Proc Natl Acad Sci U S A* 94:8082–8087, 1997; Eckels et al., *Immunol Cell Biol* 80:106–112, 2002). **Conclusion:** Whereas humans and HLA class II-matched transgenic mice seem to select very similar sets of immunogenic CD4 T-cell epitopes from both self and foreign protein antigens, this *in vivo* model is still troubled by a number of shortcomings associated with crossing the species barrier between mice and humans. To overcome these problems, it will be necessary both to add a given human autoantigen as a transgene and to introduce a naturally regulated human CD4 species into these mice.

### Overexpression of IA-2 Induces G2/M Arrest and Apoptosis

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IA-2 is a major autoantigen in type 1 diabetes. Up to 70% of newly diagnosed patients have autoantibodies to IA-2. These autoantibodies appear years before the development of clinical disease and have become important diagnostic and predictive markers. IA-2 is a transmembrane protein located in dense core secretory vesicles of  $\beta$ -cells and other neuroendocrine cells. In mice, the knockout of IA-2 results in impairment of both insulin secretion and glucose tolerance tests. In cell lines, overexpression of IA-2 results in increased glucose-stimulated insulin secretion. In the present experiments, we show that overexpression of IA-2 also can lead to G2/M arrest and apoptosis under high, but not low, glucose conditions. Treatment with IA-2 siRNA blocked both glucose-induced G2/M arrest and apoptosis. Overexpression of IA-2, under high glucose conditions, also decreased phosphorylation of proteins in the Akt/protein kinase B (PKB) signaling pathway. Treatment with IA-2 siRNA reversed this effect. In addition, overexpression of IA-2 increased the susceptibility of the cells to apoptosis induced by tumor necrosis factor- $\alpha$  and interferon- $\gamma$ . We conclude that overexpression of IA-2 puts cells in a pre-apoptotic state by indirectly decreasing Akt/PKB phosphorylation and making the cells more susceptible to the toxic effects of high glucose and inflammatory cytokines.

## 10

### The Growth Arrest and DNA Damage-Inducible Gene (*gadd45*) Inhibits Proapoptotic Jun NH<sub>2</sub>-Terminal Kinase Signaling in Pancreatic $\beta$ -Cells Exposed to Interleukin-1 $\beta$

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By the induction of apoptosis in pancreatic  $\beta$ -cells, interleukin (IL)-1 $\beta$  is believed to be an important mediator of the  $\beta$ -cell destruction leading to type 1 diabetes. The apoptotic effect of IL-1 $\beta$  is primarily mediated by Jun NH<sub>2</sub>-terminal kinase (JNK), because inhibition of JNK activity completely blocks IL-1 $\beta$ -induced  $\beta$ -cell apoptosis. The growth arrest and DNA damage-inducible gene (*gadd45*) has been reported to inhibit JNK activation via inhibition of the upstream kinase MKK7. The aim was to investigate *gadd45* regulation by IL-1 $\beta$  in  $\beta$ -cells and non- $\beta$ -cells and to evaluate the involvement of *gadd45* in  $\beta$ -cell JNK signaling. The methods used were quantitative RT-PCR on RNA isolated from cells exposed to cytokines and Western blotting. IL-1 $\beta$  caused a minor induction of *gadd45* mRNA in two  $\beta$ -cell lines (INS-1E and  $\beta$ TC3) compared with IL-1 $\beta$ -exposed NIH-3T3 fibroblasts (11- and 4-fold vs. 120-fold, respectively), indicating insufficient *gadd45* induction by IL-1 $\beta$  in  $\beta$ -cells. As a preliminary evaluation of *gadd45* involvement in  $\beta$ -cell JNK signaling, pools of overexpressing INS-1E and  $\beta$ TC3 cells (transfection efficiency ~50%) were exposed to IL-1 $\beta$ . *gadd45* overexpression reduced IL-1 $\beta$ -induced JNK activation by 42 and 63% in INS-1E and  $\beta$ TC3 cells, respectively. This identifies *gadd45* as a  $\beta$ -cell JNK inhibitor, and the impact of this inhibition on cytokine-induced  $\beta$ -cell apoptosis will be further investigated.

## 11

### Suppressor of Cytokine Signaling (SOCS)-3 Inhibits Cytokine-Mediated Mitogen-Activated Protein Kinase/Nuclear Factor- $\kappa$ B Activation, Nitrogen Oxide Production, and Apoptosis in Rat Pancreatic $\beta$ -Cells

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The cytokines interleukin (IL)-1 and interferon (IFN)- $\gamma$  are believed to be mediators of immune-mediated  $\beta$ -cell destruction in type 1 diabetes. Inhibition of the nuclear factor (NF)- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways protects  $\beta$ -cells against cytokine-induced apoptosis, partly because of inhibition of inducible nitric oxide synthase

expression and nitric oxide (NO) generation. We have previously shown that suppressor of cytokine signaling (SOCS)-3 prevents cytokine-induced NO production and apoptosis in a  $\beta$ -cell line. The aim of this study was to investigate by microarray the gene pattern induced by IL-1 and its suppression by SOCS-3 in a  $\beta$ -cell line. Furthermore, we also investigated whether SOCS-3 inhibits the NF- $\kappa$ B and MAPK pathways, NO production, and apoptosis induction in primary rat  $\beta$ -cells. By microarray analysis (Affymetrix U34A Array), we found that IL-1 stimulation (150 pg/ml, 6 h) upregulated 23 annotated genes, 15 of which were inhibited by SOCS-3. Interestingly, of these 15 genes, 9 have previously been reported to be NF- $\kappa$ B dependent. Using an adenovirus expression approach, we found by Western blotting that SOCS-3 inhibited I $\kappa$ B degradation and MAPK activation in dispersed rat islets cultures exposed to IL-1 (250 pg/ml, 15 min). In addition, both NO production and apoptosis induction by IL-1 + IFN- $\gamma$  (250 and 10 ng/ml, 24 h) were suppressed 50% by SOCS-3. In conclusion, we show that expression of SOCS-3 inhibits NF- $\kappa$ B-dependent gene transcription in a  $\beta$ -cell line and inhibits I $\kappa$ B degradation and MAPK activation in primary rat  $\beta$ -cells. These findings elucidate the signaling pathways involved in the mechanism by which SOCS-3 reduces cytokine-induced toxicity in  $\beta$ -cells.

## 12

### Increased $\beta$ -Cell Replication With Onset of Diabetes and After Induction of Immune Tolerance in the NOD Mouse

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Recent studies concerning  $\beta$ -cell turnover suggest that there is a continual process of  $\beta$ -cell death and renewal. The effects of autoimmunity on this process have not been well studied. We have examined changes in  $\beta$ -cell mass and replication in the presence of islet inflammation and during tolerance induction with CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells in NOD mice. The percentage of replicating  $\beta$ -cells was higher in female pre-diabetic NOD mice (age 9–14 weeks) than in age-matched NOD/Scid mice (1.62  $\pm$  0.9%,  $n$  = 6, vs. 0.19  $\pm$  0.1%,  $n$  = 5;  $P$  < 0.01). There was no significant difference in  $\beta$ -cell mass between the two groups (0.07  $\pm$  0.04 mg,  $n$  = 4, vs. 0.09  $\pm$  0.05,  $n$  = 4; NS). Upon diabetes onset in female NOD mice, the percentage of replicating  $\beta$ -cells increased to 3.56  $\pm$  0.9% ( $n$  = 3).  $\beta$ -Cell replication was stimulated in NOD/Scid mice by transfer of diabetogenic splenocytes (1.93  $\pm$  0.4%,  $n$  = 3,  $P$  < 0.05, 4–5 weeks after transfer). Cotransfer of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells (Tregs), from NOD mice treated with anti-CD3 monoclonal antibodies, prevented the transfer of diabetes to NOD/Scid mice. In mice protected by Tregs,  $\beta$ -cell mass was significantly greater than that in animals that received CD4<sup>+</sup>CD25<sup>-</sup> cells (0.02  $\pm$  0.02 mg,  $n$  = 5, vs. 0.00011  $\pm$  0.00001 mg,  $n$  = 5, respectively;  $P$  = 0.03). The percentage of replicating  $\beta$ -cells in mice that received Tregs was 1.97  $\pm$  0.6% ( $n$  = 5). These findings suggest that, in NOD mice, islet inflammatory lesions can play a role in stimulating  $\beta$ -cell replication. Heightened  $\beta$ -cell replication, in response to autoimmunity, is insufficient to overcome autoimmune-mediated  $\beta$ -cell destruction. Cotransfer of Tregs leads to improved  $\beta$ -cell mass with maintenance of heightened  $\beta$ -cell replication and prevents the development of diabetes in NOD mice.

## 13

### Tolerance to Proinsulin Is Due to Expression by the Thymic Epithelium: Implications for Diabetes Immunotherapy

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Autoimmune diabetes is due to the failure of self-tolerance to  $\beta$ -cell antigens, in particular proinsulin. The relative contribution of central and peripheral mechanisms is not well established in T-cell tolerance to proinsulin. In human type 1 diabetes, susceptibility alleles of the insulin gene promoter (5'VNTR) are associated with lower promiscuous thymic insulin expression. In the mouse, insulin is encoded by two genes, with

proinsulin-2 (pro2) highly expressed in the thymus. The aim of our study was to decipher the mechanisms of T-cell tolerance to proinsulin in normal (nonautoimmune) mice, focusing on the role of the proinsulin-2 gene in the immune recognition by autoreactive T-cells. We have previously shown that nonautoimmune mice knocked-out for the pro2 gene (pro2KO) are intolerant to pro2, whereas wild-type (wt) mice are tolerant. We have now investigated the site of expression of pro2 relevant to tolerance induction using thymus and bone marrow chimeras. CD4<sup>+</sup> T-cells from wt animals transplanted with a pro2KO bone marrow are tolerant to pro2, whereas CD4<sup>+</sup> T-cells from pro2KO animals transplanted with a wt bone marrow respond to pro2. This rules out a role for expression of pro2 in radiation-sensitive bone marrow-derived cells. CD4<sup>+</sup> T-cells from pro2KO mice, thymectomized and grafted with a wt thymus and a pro2KO bone marrow, become fully tolerant to pro2, i.e., they do not respond to pro2 after immunization. Conversely, CD4<sup>+</sup> T-cells from thymus and bone marrow chimeras resulting in a pro2KO thymus in a wt environment are intolerant to pro2, i.e., they respond in vitro after antigen immunization. However, these animals do not display autoimmune response to proinsulin-2 expressed in the islets, since no leukocyte infiltrate or insulin antibodies are detected. To further evaluate the role of peripheral mechanisms involved in the regulation of pro2 self-tolerance, we transferred activated anti-pro2 CD4<sup>+</sup> T-cells from pro2KO mice to wt immunodeficient mice (CD3εKO recipient mice); no leukocyte infiltrate or insulin antibodies were detected in recipients. However, transferred CD4<sup>+</sup> T-cells were not deleted or anergized, since they were still fully activated by immunization of the recipient mice several months after transfer. These results demonstrate the functional role of proinsulin-2 expression by radio-resistant thymic cells in induction of self-tolerance and the existence of additional factors involved in the induction of islet pathology. They suggest that islet proinsulin-2 is ignored by autoreactive T-cell in our model and argue against peripheral mechanisms of T-cell tolerance to proinsulin-2. Future research in diabetes immunotherapy should aim at restoring central (thymic) T-cell tolerance rather than manipulating peripheral tolerance.

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#### Transcriptional Effects of the Insulin Variable Number of Tandem Repeats (VNTR) Polymorphism

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The *IDDM2* diabetes susceptibility locus maps to a variable number of tandem repeats (VNTR) situated 596 bp upstream of the insulin gene (*INS*). The short class I VNTR alleles (26–63 repeats) predispose to type 1 diabetes, whereas class III alleles (140–210 repeats) are dominantly protective. Evidence from our laboratory and others has suggested that the protective effect of this noncoding polymorphism may be explained by higher levels of thymic *INS* expression, facilitating immune tolerance induction. To test this hypothesis, we established a NOD transgenic mouse model, carrying the human *INS* promoter, with the class I or class III VNTR allele, fused to the whole human *INS* gene knocked into the *HPRT* locus. The relative levels of human *INS* mRNA transcripts in each transgenic mouse recapitulate what was previously reported in human tissues: lower levels of *INS* mRNA from class III than from class I in the pancreas and twofold higher from class III than from class I in the thymus. The difference of expression observed between the two alleles in the mouse pancreas is higher than that observed in humans. This could be explained by an endogenous insulin competition and a possible role of the VNTR in the metabolic regulation of insulin transcription. The opposite results obtained in the pancreas and the thymus suggest that insulin expression in the thymus is regulated by a mechanism different from that of the pancreas.

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#### Rapid Opposite Effects of Glucose on XBP1 mRNA Unconventional Splicing and GADD153 Expression in Cultured Rat Islets

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Excessive activation of the endoplasmic reticulum (ER) stress pathway by chronic hyperglycemia may contribute to β-cell apoptosis in type

2 diabetes. To test whether glucose induces ER stress in β-cells, XBP1 mRNA unconventional splicing and GADD153 mRNA and protein levels were measured in rat islets precultured for 1 week in 10 mmol/l glucose (G10) and further cultured for 18 h in G2, G5, G10, or G30. As expected, 1 μmol/l thapsigargin, an inhibitor of ER Ca<sup>2+</sup>-ATPases that triggers ER stress in various cell types, increased islet XBP1 mRNA splicing and GADD153 expression (3- and 10-fold, respectively) in G10. In contrast, glucose exerted opposite effects on these two ER stress markers: a two- and threefold increase in XBP1 mRNA splicing after culture in G10 and G30 versus G2–G5 and an ~40 and ~90% reduction in GADD153 expression after culture in G5 and G10–G30 versus G2. After an overnight culture in G5 and further culture in G30, the increase in XBP1 mRNA splicing and decrease in GADD153 mRNA levels occurred within 2 h of stimulation. In conclusion, in contrast with thapsigargin, glucose activates only part of the ER stress pathway while reducing expression of the pro-apoptotic factor GADD153. These rapid glucose effects may play a physiological role in nutrient-induced maintenance of the β-cell phenotype.

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#### The Fas Pathway Is Involved in β-Cell Secretory Function

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Fas is thought to be a death receptor involved in apoptosis, but in the presence of the caspase-8 inhibitor FLICE inhibitory protein (FLIP), Fas engagement may switch from a death to a proliferative signal. Increased extracellular glucose induces Fas expression in pancreatic β-cells leading to apoptosis or proliferation, depending on the presence of FLIP. Chronic hyperglycemia also impairs insulin secretion in addition to changes in β-cell mass. Here we show that the Fas pathway not only mediates glucose-induced changes in cell turnover, but also regulates β-cell secretory function. We observed impaired glucose tolerance both in Fas-deficient and β-cell-specific Fas knockout mice, despite normal insulin sensitivity. In situ pancreas perfusion of Fas-deficient mice displayed a delayed and decreased insulin secretory pattern in response to glucose, and in isolated islets, the stimulatory index of glucose-induced insulin secretion was reduced. Insulin and ATP production of Fas-deficient β-cells was severely impaired and partly compensated by a dramatic increase in β-cell mass. Upregulation of FLIP induced insulin production independent of changes in cell turnover and protected the β-cell from the toxic effects of chronic exposure to high glucose. In vivo, Fas-deficient mice were resistant to the diabetogenic effect of a high-calorie diet, supporting the idea that Fas is involved in mediating nutrient-induced β-cell toxicity. The results support a new role for the Fas-pathway in regulating insulin production and release.

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#### Role of Nitric Oxide on Glucose- and Arginine-Induced Insulin Secretion in INS-1E Cells

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Nitric oxide (NO) synthase is expressed in INS-1E, and intracellular NO is generated in response to glucose and arginine. The role of NO in the modulation of insulin secretion is controversial. We evaluated the effect of chronic (48-h) and acute (1-h) intracellular NO supplementation with hydroxylamine (HA) and NO synthase inhibition with N-nitro-L-arginine-methyl ester (L-NAME) on glucose-induced insulin secretion (GIIS) (glucose = 3.3, 11, and 20 mmol/l) and arginine-induced insulin secretion (AIIS) (glucose = 20 mmol/l + arginine = 10 mmol/l). Unstimulated insulin secretion was unaffected by either acute or chronic exposure to HA or L-NAME. Acute 1 mmol/l L-NAME increased GIIS by 53, 40, and 100% (for the three glucose concentrations, respectively, *P* < 0.002

by ANOVA), whereas 2 mmol/l L-NAME had no effect (21, -7, and 10%, NS). Similarly, chronic 1 mmol/l L-NAME (15, 68, and 80%,  $P < 0.02$ ), but not 2 mmol/l L-NAME (-18, 2, and 39%, NS), potentiated GIIS. Acute 0.4 mmol/l HA had no effect (-9, -11, and -8%, NS), whereas 0.8 mmol/l HA significantly depressed GIIS (-23, -15, and -49%,  $P < 0.002$ ). Chronic HA, at both 0.4 and 0.8 mmol/l, had a negligible negative effect on GIIS (-10, -19, and -7%, NS, and -14, and -17%,  $P = 0.11$ ). AIIS was completely prevented by acute L-NAME (1 and 2 mmol/l) and unaltered by chronic L-NAME. Acute exposure to 0.4 mmol/l HA had no effect on AIIS, whereas 0.8 mmol/l HA potentiated AIIS by 43% ( $P < 0.003$ ). Chronic incubation with HA had no effect. In conclusion, intracellular NO synthesis inhibits GIIS, while potentiating AIIS.

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### The Endoplasmic Reticulum Stress Response in $\beta$ -Cells: Differential Effects of Free Fatty Acids, Glucose, and Cytokines

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$\beta$ -Cell apoptosis may be a common feature of the pathogenesis of both type 1 and type 2 diabetes. We presently compared the molecular pathways contributing to  $\beta$ -cell death in in vitro models of the two forms of diabetes. The cytokine interleukin (IL)-1 $\beta$  (30 units/ml; model for type 1 diabetes) and the free fatty acids (FFAs) oleate and palmitate (0.5 mmol/l in the presence of 1% albumin; type 2 diabetes model) induced INS-1E cell apoptosis after 72 h. FFA toxicity was not potentiated by tumor necrosis factor (TNF)- $\alpha$  (1,000 units/ml), an adipocyte-derived cytokine, but it was increased twofold by high glucose (28 mmol/l). Both IL-1 $\beta$  and TNF- $\alpha$  activated the transcription factor nuclear factor (NF)- $\kappa$ B, but FFA and/or high glucose did not. In the case of IL-1 $\beta$ , NF- $\kappa$ B-mediated nitric oxide (NO) formation led to the downregulation of the endoplasmic reticulum (ER) pump SERCA2b and the depletion of ER Ca<sup>2+</sup>, triggering an NO-dependent ER stress response via activation of the ER stress transducers inositol-requiring ER-to-nucleus signal kinase 1 $\alpha$  (IRE1 $\alpha$ ) and RNA-dependent protein kinase-like ER kinase (PERK). Cytokines did not activate activating transcription factor (ATF)-6 or induce immunoglobulin heavy-chain-binding protein (BiP) expression. Oleate and palmitate, which did not induce iNOS and NO, activated the IRE1 $\alpha$ -XBP-1, PERK-ATF-4, and ATF-6-ER stress pathways and upregulated the ER chaperone BiP. The ER stress response activated by both cytokines and FFAs culminated in the induction of the proapoptotic transcription factor CHOP. In conclusion, the mechanisms leading to FFA- and cytokine-induced  $\beta$ -cell apoptosis are different. Whereas cytokines induce NF- $\kappa$ B activation, NO production, and ER stress, FFAs and/or glucose activate a different ER stress pattern via an NF- $\kappa$ B- and NO-independent mechanism.

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### Novel *mt-Nd2* Variant Encoded by the ALR/Lt Mouse Strain Provides Resistance to Free Radical-Induced Mitochondrial Dysfunction

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A significant role for free radical-induced mitochondrial dysfunction has been proposed in the pathogenesis of both type 1 and 2 diabetes as well as diabetes complications. The ALR/Lt mouse strain was derived from the same outbred mouse stock as the autoimmune diabetes-prone NOD mouse, and while these two strains share many diabetes susceptibility alleles, ALR is resistant to both autoimmune and chemically induced type 1 diabetes. When the progeny of reciprocal outcrosses between ALR and NOD was backcrossed to NOD, type 1 diabetes developed at a fourfold lower rate when ALR contributed the mitochondrial genome. Sequencing of the mitochondrial genomes (mtDNA) of ALR and NOD determined that the only difference was a single nucleotide polymorphism unique to ALR in the *mt-Nd2* gene for subunit 2 of complex I (CI-NADH dehydrogenase). The *mt-Nd2* variation resulted in a nonconservative change of leucine to a methionine in ALR. Initial

comparisons of ALR mitochondria (mt) to those isolated from control strains determined that the ALR-encoded allele of *Nd2* did not alter basal mitochondrial respiratory function or free radical production. To simulate conditions of elevated oxidative stress, isolated mitochondria were incubated with nitric oxide (NO) (known to lead to peroxynitrite formation and damage mitochondria) while respiring on both CI (glutamate and malate) and complex II (CII-succinate) substrates. Studies of respiration via both complexes demonstrated that respiration was curtailed in a dose-dependent manner by NO in all control strains. In comparison, mitochondria isolated from ALR liver exhibited significantly improved respiration at all concentrations of NO. As ALR mitochondria maintained improved respiration via both CI and CII substrates, to determine if the ALR *Nd2* allele provided specific protection against free radical mitochondrial dysfunction, reciprocal consomic mice were bred—one with ALR mtDNA and NOD nuclear genome (D.Rmt) and one with NOD mtDNA and ALR nuclear genome (R.Dmt). Under conditions of oxidative stress, isolated mitochondria from D.Rmt were shown to function equivalently to ALR when respiring on complex I substrates and significantly better than NOD, whereas R.Dmt functioned equivalently to NOD. In addition, assays for specific complex I enzymatic activity also demonstrated a strong association of the ALR allele *mt-Nd2* with resistance to NO damage. Further, tests to examine the resistance of the consomics to NO-mediated CII inhibition showed that the nuclear genome and not the mtDNA controlled the resistance phenotype. ALR and R.Dmt respired equivalently and exhibited resistance to impairment compared with NOD or D.Rmt after exposure to increasing concentrations of NO. In summary, the *mt-Nd2* allele of ALR provides clear resistance to free radical-induced mitochondrial complex I dysfunction.

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### Improvement of the Metabolic State in Diabetic *Psammomys obesus* With S 23521: A New Glucagon-Like Peptide 1 Analog

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Glucagon-like peptide (GLP)-1 analogs are considered potential drugs for type 2 diabetes. We studied the effect of a novel GLP-1 analog, S 23521 [(a<sup>8</sup>-des R<sup>36</sup>) GLP-1-(7-37)-NH], on the progression of diabetes in the *Psammomys obesus* model of type 2 diabetes. Animals with marked hyperglycemia were given twice-daily injections of 100  $\mu$ g/kg S 23521 for 15 days. Food intake was decreased in S 23521-treated *P. obesus*; however, there was no significant difference in body weight from controls. Progressive worsening of hyperglycemia was noted in controls, as opposed to maintenance of pretreatment glucose levels in the S 23521 group. Treated animals had higher serum insulin, insulinogenic index, and leptin, whereas plasma triglyceride and FFA levels were decreased. S 23521 induced a fivefold increase in pancreatic insulin content. Immunohistochemical analysis showed islet degranulation with disrupted morphology in untreated animals, whereas islets from S 23521-treated animals appeared intact and filled with insulin;  $\beta$ -cell apoptosis was ~70% reduced, without change in  $\beta$ -cell proliferation, resulting in a twofold increase in relative  $\beta$ -cell volume. Thus, S 23521 prevented the progression of diabetes in *Psammomys obesus* with marked improvement of the metabolic profile, pancreatic insulin reserve, and  $\beta$ -cell mass. These effects emphasize the feasibility of preventing metabolic deterioration over time in type 2 diabetes.

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### Current Diet, Obesity, and Glucose Tolerance in an Adult Cohort

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Although there is an increasing body of data that suggests that the nature of the diet is associated with susceptibility to impaired glucose

tolerance and type 2 diabetes, research continues into the best parameters of a "healthy diet." We investigated the associations of adult diet with obesity and glucose tolerance. We carried out a retrospective cohort study in 1,475 adults in Hertfordshire, U.K., aged 59–70 years. All participants completed a food frequency questionnaire. All subjects had a standard 75-g oral glucose tolerance test (OGTT) with glucose and insulin measurements. We computed two indexes of a "healthy diet": total whole-grain consumption and a "Prudent Diet Score" using the principal components analysis technique. A higher whole-grain intake was associated with a reduced BMI/waist-to-hip ratio in men and women and lower insulin and glucose concentrations during a 75-g OGTT in men. The "Prudent Diet Score" was associated with a reduced waist-to-hip ratio in men and women and borderline improvement in 75-g OGTT in women.

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### Innovative Approaches to Monitoring Insulin Secretion

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Typically, insulin release assays are the gold standard to determine the ability of isolated animal and human islets to respond to glucose concentrations with adequate insulin secretion. In vitro insulin release is measured using static and dynamic incubation techniques. The ability of islet grafts to correct hyperglycemia in streptozotocin-induced diabetic immunodeficient mouse recipients also provides solid information on insulin secretion, but it involves additional engraftment factors. Overall, these procedures are time-consuming. New alternative methodologies are now available to gain information on the secretory ability of isolated human islets. Adenoviral delivery of green fluorescent protein and timer protein in the C-peptide coding region of the insulin gene allows effective expression of fluorescent pro-insulin in the granules of rodent and human islet  $\beta$ -cells. Incubation of human islets expressing ins-C-GFP and ins-C-Timer inserts, with high glucose or exposure to cytokines, induces differences in color and fluorescence intensity between treated and untreated control islets, easily appreciated by real-time microscopic analysis. The results correlate with data accrued using more classic methodologies. This new technical approach helps to better explore the activity of insulin release in isolated human islets and may contribute to identifying markers associated with islet functionality and relevant to islet performance after transplantation.

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### Relationship Between Low Birth Weight and Expression of Insulin Signaling Proteins in Adipose Tissue in Young Adult Men

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Individuals with a low birth weight are at increased risk of developing type 2 diabetes; however, the underlying molecular mechanisms are not known. We have shown previously that low birth weight is associated with changes in insulin signaling protein expression in muscle. The current aim was to determine if low birth weight is associated with changes in insulin signaling proteins in adipose tissue. A total of 17 males aged 22 years were recruited from the Danish Medical Birth Registry. Nine had low birth weights (bottom 10th percentile), and eight had a normal birth weight (50th to 90th percentile). Adipose biopsies were taken from the abdomen using a Bergstrom needle, and protein expression was determined by Western blotting. There were no differences in expression of the insulin receptor, protein kinase C  $\zeta$ , glycogen synthase kinase (GSK)- $3\alpha$ , or GSK $3\beta$  between the two groups. However, there

were reductions in expression of GLUT4 ( $52 \pm 10.9\%$  reduction,  $P < 0.01$ ), p85 $\alpha$  regulatory subunit of phosphatidylinositol (PI) 3-kinase ( $45 \pm 9\%$  reduction,  $P < 0.01$ ), and the p110 $\beta$  subunit of PI 3-kinase ( $48 \pm 17\%$  reduction,  $P = 0.06$ ). These findings suggest that low birth weight is associated with reduced expression of important insulin signaling proteins in adipose tissue that may contribute to an increased risk of type 2 diabetes.

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### Genetic Variation of the Adiponectin (APM1) Gene Is Associated With Type 1 Diabetes in Swedish Caucasians

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Adiponectin is an adipose tissue-derived protein (adipocytokine) that regulates insulin sensitivity. The adiponectin gene (*APM1*) is located on chromosome 3q27. In this region, the susceptibility loci linked to type 2 diabetes, obesity, and coronary heart disease reside. Recently, we have demonstrated that single nucleotide polymorphisms (SNPs) –11426(A/G) and –11377(G/C), located in the proximal promoter of the *APM1* gene, are associated with type 2 diabetes in Swedish Caucasian subjects. To test whether the *APM1* gene confers genetic susceptibility to development of type 1 diabetes, we genotyped for 13 SNPs of the *APM1* gene in 424 patients with type 1 diabetes (175 had diabetic nephropathy) and 483 nondiabetic control subjects by using dynamic allele-specific hybridization (DASH). All subjects were Swedish Caucasian. We found that SNPs 45G15G(T/G) and 276(G/T) are strongly associated with type 1 diabetes ( $P \leq 0.002$ ). The genetic variation of the *APM1* gene was not related to diabetic nephropathy. Furthermore, analysis based on haplotypic genotypes (diplotypes) constructed with SNPs 45G15G(T/G) and 276(G/T) and the two promoter SNPs –11426(A/G) and –11377(G/C) indicated that common haplotypes are strongly associated with type 1 diabetes ( $P < 0.001$ ). The present study provides evidence that genetic variation in the *APM1* gene is strongly associated with type 1 diabetes in Swedish-Caucasian subjects and also suggests that the *APM1* gene confers the susceptibility to both type 1 and type 2 diabetes.

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### Analysis of Ptpvr (OST-PTP/Esp) in Adipose Tissue and Adipocyte Differentiation

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*Ptpvr* is a transmembrane tyrosine phosphatase expressed in embryonic stem cells, gonads, and osteoblast precursors. In bone formation, it has been characterized as a molecular marker of mesenchymal bone collar cells and shown to play a role in osteoblast differentiation. We show for the first time that *Ptpvr* is also expressed in adipose tissue. In this tissue, *Ptpvr* expression is restricted to cells of the stroma vascular fraction, which contain adipocyte precursors, but is not detected in differentiated adipocytes. Moreover, *Ptpvr* expression is regulated. Indeed, both in 3T3L1 cells and mouse embryo fibroblast models of adipocyte differentiation, *Ptpvr* expression increases when cells reach confluence and then decreases upon exposure to adipogenic conditions. However, *Ptpvr* expression patterns do not strictly overlap with Pref-1 and ACLP, two proteins expressed in preadipocytes but not in adipocytes. In addition, *Ptpvr* does not appear as a critical player in adipocyte differentiation, since MEF cells lacking *Ptpvr* are able to differentiate into adipocytes and mice lacking *Ptpvr* have normal adipose tissue development. It is thus likely that this phosphatase does not play an important role in adipose tissue biology. Moreover, we have cloned the human ortholog of *Ptpvr* and shown that this gene (*1*) has no "functional" parent in the human genome, (*2*) has retained an "intron-exon" structure, and (*3*) is transcribed in a regulated manner. Taken together, these results indicate that *Ptpvr* is the only PTP-ase whose function has been lost during the evolution process between rodents and humans.