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SHORT COMMUNICATION

Post-translational protein modifications in type 1 diabetes: a role for the repair enzyme protein-L-isoaspartate (D-aspartate) *O*-methyltransferase?

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Abstract
Aims/hypothesis Post-translational modifications, such as isomerisation of native proteins, may create new antigenic epitopes and play a role in the development of the autoimmune response. Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase (PIMT), encoded by the gene *PCMT1*, is an enzyme that recognises and repairs isomerised Asn and Asp residues in proteins. The aim of this study was to assess the role of PIMT in the development of type 1 diabetes.
Materials and methods Immunohistochemical analysis of 59 normal human tissues was performed with a monoclonal PIMT antibody. CGP3466B, which induces expression of *Pcmt1*, was tested on MIN6 and INS1 cells, to assess its effect on *Pcmt1* mRNA and PIMT levels (RT-PCR and western blot) and apoptosis. Forty-five diabetes-prone BioBreeding (BB) Ottawa Karlsburg (OK) rats were randomised to receive 0, 14 or 500 μg/kg (denoted as the control, low-dose and high-dose group, respectively) of CGP3466B from week 5 to week 20.
Results A high level of PIMT protein was detected in beta cells. CGP3466B induced a two- to threefold increase in *Pcmt1* mRNA levels and reduced apoptosis by 10% in MIN6 cells. No significant effect was seen on cytokine-induced apoptosis or PIMT protein levels in INS1 cells. The onset of diabetes in the BB/OK rats was significantly delayed (85.6±9.0 vs 84.3±6.8 vs 106.6±13.5 days, respectively; $p<0.01$ for high-dose vs low-dose and control groups), the severity of the disease was reduced (glucose 22.2±3.2 vs 16.9±2.6 vs 15.8±2.7 mmol; $p<0.01$ for high- and low-dose groups vs control group) and residual beta cells were more frequently identified (43% vs 71% vs 86%; $p<0.05$ for high-dose vs control group) in the treated animals.
Conclusions/interpretation The results support a role for post-translational modifications and PIMT in the development of type 1 diabetes in the diabetes-prone BB rat, and perhaps also in humans.

Keywords BB rat · INS1 cells · MIN6 cells · Prevention

Abbreviations

BB	BioBreeding
OK	Ottawa Karlsburg
PIMT	protein-L-isoaspartate (D-aspartate) *O*-methyltransferase

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Introduction

The list of putative autoantigens involved in the development of type 1 diabetes is long, but none, maybe not even insulin, is found in beta cells alone. An explanation for this apparent paradox is lacking. We proposed that post-translational protein modifications in the islet trigger multiple antigen-specific B and T lymphocyte responses in the initiation phase of the pathogenesis of type 1 diabetes [1]. Post-translational protein modifications can potentially create new antigenic epitopes, which may trigger the production of autoantibodies and T lymphocyte reactivity. Indeed, many post-translational modifications of target autoantigens can generate autoimmune responses in human disease and animal models [2, 3]. Oxidative modifications of islet GAD, one of the major beta cell autoantigens, enhance the recognition of this protein by serum antibodies in humans [4], whereas recognition of an insulin epitope by $CD4^+$ T cells requires the formation of a disulfide bond between adjacent cysteine residues [5]. Furthermore, insulin is prone to in vitro isomerisation [6], i.e. spontaneous conversion of an Asp or Asn (Asx) residue into an isoaspartyl residue at susceptible protein sites.

The highly conserved repair enzyme protein-L-isoaspartate (D-aspartate) *O*-methyltransferase (PIMT), encoded by the gene *PCMT1*, recognises isomerised Asx residues and catalyses the conversion of isoaspartyl to normal aspartyl residues. *Pcmt1* knock-out mice have increased T cell proliferative responses, and if their bone marrow is transferred to wild-type mice, the latter develop anti-DNA autoantibodies [7]. To assess the potential role of post-translational protein modifications in the development of type 1 diabetes, as an example, we assessed PIMT production in normal human tissues and evaluated the effect of CGP3466B in vitro on PIMT expression and apoptosis in a murine insulinoma cell line, as well as in vivo on the development of the disease in the diabetes-prone BioBreeding (BB) rat. Treatment with the *R*-(–)-deprenyl-related compound CGP3466 has been shown to upregulate *Pcmt1* expression in vitro, which, in turn, has been demonstrated to have a protective effect against apoptosis [8].

Materials and methods

Human histological studies Fifty-nine tissue samples from 38 organs (multi-tissue array AA7; Superbiochips Laboratories, Seoul, Korea) were analysed. A monoclonal PIMT antibody (Cat. No. P 62620/lot 1; BD Biosciences, Heidelberg, Germany), EnVision (peroxidase, Cat. No. K0625; Dako, Glostrup, Denmark), 3,3′-diaminobenzidine (DAB+; Dako) staining and Erlich's haematoxylin counter-staining were used. Control incubation with excess human PIMT completely abolished antibody binding (Fig. 1b), supporting specific PIMT staining. For co-localisation with insulin, the PIMT antibody was detected using mouse EnVision Fuchsin+ (Dako) or biotin-conjugated donkey anti-mouse IgG combined with streptavidin conjugated to gold clusters (NANOGOLD streptavidin; Molecular Probes, Leiden, the Netherlands) and silver enhancement (Cat. No. L-24919; Molecular Probes). For insulin detection, a polyclonal guinea pig anti-insulin antibody (Cat. No. N1542; Dako), horseradish peroxidase-conjugated donkey anti-guinea pig IgG (Cat. No. 706-035-148; Jackson Immunoresearch Laboratories, West Grove, PA, USA) and DAB+ staining were used (Fig. 1).

Studies in MIN6 cell lines We examined the effect of CGP3466B on serum-deprived mouse insulinoma (MIN6) cells. Cells were seeded in 96-well plates in high-glucose DMEM and 10% FCS. After 24 h, they were washed and maintained in serum-free medium in the presence of 5, 50, 500 nmol/l or 5 μmol/l CGP3466B or vehicle for 3 days (replaced daily). Apoptotic cells were detected with the fluorochrome inhibitor of caspases (FLICA; Chemicon International, Temecula, CA, USA), and all cells were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma-Aldrich, Steinheim, Germany). Apoptosis was expressed as the percentage of FLICA-labelled cells. The experiment was repeated twice and each condition was analysed in quadruplicate.

The expression of *Pcmt1* in MIN6 cells was assessed using real-time RT-PCR. Total RNA was isolated from cells cultured in the presence of 0, 50 or 500 nmol/l CGP3466B for 24 h. RT-PCR was carried out on cDNA using a Light-Cycler System and SYBR Green I (Roche Diagnostics, Mannheim, Germany). Transcription levels were normalised to *Gapdh* and *Min51* and total RNA. The experiment was repeated twice and each condition was analysed in triplicate.

Studies in INS1 cell lines We examined the effect of CGP3466B on cytokine-induced apoptosis in rat insulinoma (INS1) cells. Cells (passage numbers 37–98) were seeded in 96-well plates (20,000 per well) and then exposed (or not) to 150 pg/ml IL-1β (Cat. No. 554577; Becton Dickinson, Franklin Lakes, NJ, USA) and to three different concentrations of CGP3466B (50 nmol/l, 5 μmol/l and 200 μmol/l) for 24 h. Apoptosis was quantified using the fluorescence-based Apo-One Homogeneous Caspase 3/7 Assay (Cat No. G7790; Promega, Madison, WI, USA). Fluorescence was measured in a Victor Wallac 1,420 multi-label counter (Perkin Elmer, Boston, MA, USA). The results are expressed relative to the control conditions (no IL-1 or CGP3466B exposure). The experiment was

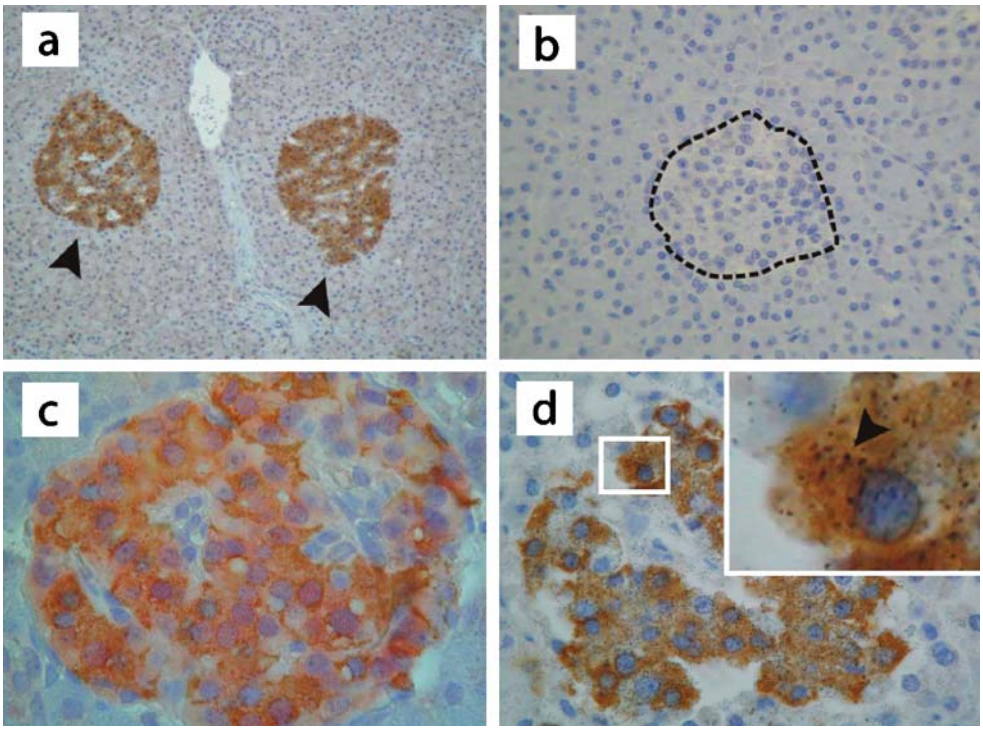

Fig. 1 Immunohistochemistry of normal human pancreas showing the expression of PIMT. **a** Visualisation of PIMT using DAB+ as the chromogen (*brown staining*) in two islets (*arrows*). Only a very low level of staining is seen in cells of the exocrine pancreas. **b** PIMT monoclonal antibody was blocked with a 100-fold molar excess of human recombinant PIMT before being applied to the tissue section (*negative control*). The *broken line* indicates the contours of an islet. **c** Double staining of insulin (*DAB+, brown*) and PIMT (*Fuchsin, red*). **d** Double staining of insulin (*DAB+, brown*) and PIMT (*gold label, black dots*). *Insert* shows the indicated area magnified. PIMT (*arrow*) is primarily localised to insulin-positive areas

performed five times and each condition was analysed in duplicate.

To assess PIMT levels, INS1 cells (passage numbers 37–98) were seeded in 24-well plates (250,000 per well) and then exposed to IL-1β and CGP3466B as described above. The cells were lysed for 30 min on ice and then spun (12,000 *g*, 5 min, 4°C), and the protein concentration was measured in the supernatant fraction by the Bradford method, following the manufacturer's instructions (BioRad, Hercules, CA, USA). Volumes with equivalent protein content were loaded onto NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA), and the proteins were separated by electrophoresis and transferred onto nitrocellulose membranes according to the manufacturer's instructions. Rabbit anti-(human) PIMT polyclonal antibody (1:1,000 dilution; Proteintech Group, Chicago, IL, USA) and mouse anti-β-actin (1:10,000 dilution; Abcam, Cambridge, UK) were used as primary antibodies. The corresponding horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG [1:4,000 dilution; Cell Signaling, Beverly, MA, USA] and anti-mouse IgG [1:1,000 dilution; Oncogene, Cambridge, MA, USA]) were then used. Human SAOS-2 cell lysate was used as a positive control.

The proteins of interest were detected by a chemiluminescence detection system (lumiGLO; Cell Signaling) and visualised using an LAS3000 imaging system (Fuji Film, Tokyo, Japan). PIMT levels were quantified and normalised against β-actin using Multi Gauge software v2.0 (FUJI Film). The ratio of PIMT:β-actin under control conditions was arbitrarily set to 1. Values obtained from each condition were compared using the two-tailed Mann–Whitney *U* test. A p value of <0.05 was considered significant.

Intervention study in diabetes-prone BB Ottawa Karlsburg rats Forty-five male diabetes-prone BB Ottawa Karlsburg (OK) rats were randomised to receive three weekly (Monday, Wednesday, Friday) subcutaneous injections containing 0.2 ml distilled water (control group) or one of two doses of CGP3466B (14 μg/kg [low-dose group] or 500 μg/kg [high-dose group]) dissolved in 0.2 ml distilled water for weeks 5–20. The animals were provided by the Department of Laboratory Animal Science of the University of Greifswald (Karlsburg, Germany). They were kept in groups of three in Macrolon cages (Size 3; Ehret, Emmendingen, Germany), under strict hygienic conditions. The rats had free access to food (Sniff R; Sniff, Soest, Germany) and water, and were maintained on a 12-h light–dark cycle (lights on 05.00–17.00 h). They were cared for according to local guidelines and law, and their appearance and activity were monitored daily. Body weight and blood glucose were measured every other week from week 5 and, after the age of 50 days, animals were inspected daily and screened for glucosuria twice a week (Diabur-Test 5,000; Boehringer, Mannheim, Germany). Diabetes was confirmed by blood glucose >11 mmol/l on two consecutive days, and age of disease onset was defined by the time glucosuria was detected. Survival time (without insulin) was defined as time from onset until worsening of their general condition, with weight loss >10 g/day. At this

point, animals were killed with an overdose of thiopenthal and their pancreas was removed and examined by a pathologist unaware of their status and treatment. Each pancreas was fixed in Bouin's solution and embedded in paraffin. Sections (7 μm thick) were stained with haematoxylin/eosin or immunohistochemically for insulin by the alkaline phosphatase, anti-alkaline phosphatase method. Approximately 35 islets per animal were assessed for the presence of beta cells.

Results

Distribution of PIMT in human tissue PIMT was produced in all studied organs, especially in the brain, lymph nodes and pancreas (data not shown), where it was exclusively confined to the islets of Langerhans (Fig. 1a). Control incubation with excess human PIMT completely abolished antibody binding (Fig. 1b), supporting specific PIMT staining. Most of the PIMT-positive cells in normal islets were also insulin-positive (Fig. 1c,d), indicating that high PIMT levels are present in beta cells.

Effect of CGP3466B on Pcmt1 expression and apoptosis In serum-deprived MIN6 cells, only the highest dose (5 μmol/l) of CGP3466B reduced the rate of apoptosis significantly (10% reduction; $p<0.05$). CGP3466B treatment increased levels of *Pcmt1* mRNA, as assessed by RT-PCR, by two- to threefold (data not shown).

In INS1 cells, the two lowest concentrations of CGP3466B tested (50 nmol/l and 5 μmol/l) produced a non-significant reduction of IL-1β-induced apoptosis of 15–21%. The highest concentration tested (200 μmol/l) was toxic to the cells, increasing apoptosis by 325% ($p<0.05$). There was no significant effect of IL-1 or CGP3466B exposure on PIMT levels (data not shown), except for the complete disappearance of the PIMT band at the highest CGP3466B concentration.

Intervention study in BB/OK rats The three groups had similar weights at baseline and after the treatment period (only non-diabetic rats were included in the latter assessment), and CGP3466B was well tolerated. Overall, the incidence of diabetes was similar in all three groups, but its onset was delayed and its severity was reduced in the treated animals (Table 1, Fig. 2), which showed lower glucose concentrations at diagnosis, and less weight loss and longer survival after diagnosis. In the diabetic animals, most islets displayed heavy lymphocytic infiltration, disrupted architecture and reduced relative islet volume. Complete loss of insulin staining was seen in all groups, albeit less frequently in the high-dose treatment group (Table 1).

Table 1 Main effects of treatment of BB/OK rats with 0 (control), 14 (low-dose) or 500 (high-dose) μg/kg CGP3466B

Treatment group	Control group (n=15)	Low-dose group (n=15)	High-dose group (n=15)
Diabetes (n)	7	7	7
Glucose at diagnosis (mmol/l)	22.2±3.2	16.9±2.6[a]	15.8±2.7[a]
Day of diabetes onset	85.6±9.0	84.3±6.8	106.6±13.5[b]
Weight loss after diagnosis (% body weight/day)	2.5±1.5	1.7±1.2	1.1±1.3[c]
Survival time after onset (days)	2.6±1.8	3.9±2.7	8.9±8.5[c]
Presence of insulin-positive cells (n [%])	3 (43)	5 (71)	6 (86)[d]

Quantitative data are expressed as mean ± SD. [a] $p<0.01$ vs control group; [b] $p<0.01$ vs control and low-dose groups; [c] $p<0.05$ vs control and low-dose groups; [d] $p=0.02$ vs control group

Discussion

In the present study, treating BB/OK rats with CGP3466B led to a delay in the development and a reduction in the severity of diabetes. CGP3466B is a compound related to the anti-Parkinson's drug *R*-(–)-deprenyl, which has anti-apoptotic effects on the human neuroblastoma cell line PUJA [9] and upregulates *Pcmt1* expression in rat astroglial cell cultures [8]. In addition, transfection of mouse cortical cells with *Pcmt1* protects against apoptosis [8]. The effects

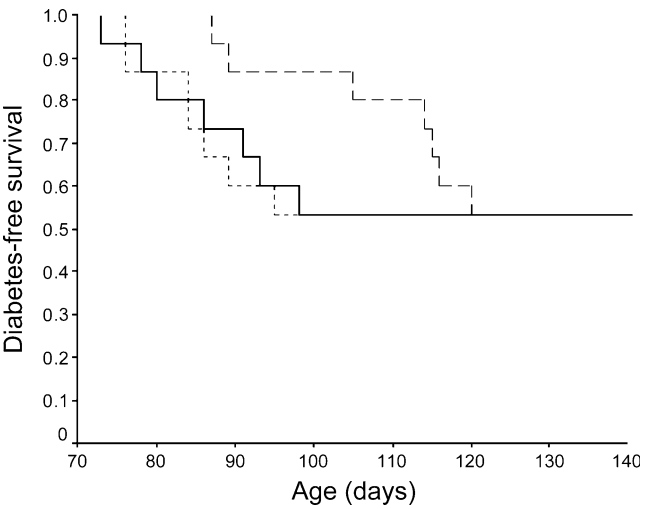

Fig. 2 Disease-free survival (*in days*) after treatment with placebo (*control group, continuous line*), 14 μg/kg CGP3466B (*low-dose group, short broken line*) or 500 μg/kg CGP3466B (*high-dose group, long broken line*). The *y*-axis displays the fraction of rats free from the disease. $p<0.05$ for high-dose group vs control and low-dose groups

described in the BB/OK rats could be due to either a direct effect on apoptosis or an indirect effect mediated by the induction of *Pcmt1*. The latter is supported by the increased transcription of *Pcmt1* in MIN6 cells, despite only a moderate effect on apoptosis at a higher CGP3466B dose than that which induced *Pcmt1* transcription. Furthermore, in the rat INS1 cells, no significant effect was seen on apoptosis. Overexpression of *Pcmt1* has an anti-apoptotic effect similar to that seen in response to the overexpression of *Bcl2* [8], which is in concordance with findings suggesting that isomerisation of anti-apoptotic molecules may have a negative effect on their function [10]. Repair of these post-translational modifications by PIMT possibly mediates the in vivo anti-diabetic effect of CGP3466B. The fact that a high level of PIMT was detected in beta cells supports its role in type 1 diabetes. Indeed, in a cellular model of beta cell maturation, exposure to cytokines led to a 1.5-fold decrease in *Pcmt1* mRNA expression in mature, but not immature, pre-beta cells [11], suggesting that PIMT response may be part of the specific phenotype that makes the beta cell prone to immune-mediated destruction. Proteomic studies of this model suggested that post-translational protein modifications may reflect the acquired sensitivity of the beta cell to cytokines [12]. In fact, major putative autoantigens are prone to isomerisation: insulin can undergo this kind of modification in vitro [6], the GAD65 molecule has four Asx-Gly sites, susceptible to isomerisation, three of which are located within recognised epitopes [13, 14] and the IA2 molecule also contains several susceptible sites (http://www.ncbi.nlm.nih.gov/ accessed 21 October 2005, accession AF007555.1).

In the present series of experiments, PIMT levels were not affected by exposure of INS cells to IL-1 or CGP3466B. There could be different explanations for this, such as regulation of RNA translation by miRNA and the time frame studied in these experiments. Non-coding RNA is an increasingly recognised means of regulating gene expression in mammals and other complex organisms; miRNA binding sites are located in the 3′ UTR region of mRNA molecules [15] and *PCMT1* contains at least three putative binding sites (http://www.genome.ucsc.edu, last accessed in October 2006, NM 005389, May 2004 Build) [16]. Regarding the time scale for gene transcription and protein translation, we know that *PCMT1* mRNA is downregulated within the first 6 h after INS1 cell exposure to cytokines (C. Brorsson, unpublished data), and is upregulated after exposure of MIN6 cells to CGP3466B for 24 h, but it remains to be determined whether the latter takes place at an earlier stage or if changes in protein expression occur much later. The performance of experiments that simultaneously assess *Pcmt1* mRNA and PIMT protein levels should provide an answer to this question.

In conclusion, the present study supports a role for post-translational protein isomerisation and the repair enzyme PIMT in the development of type 1 diabetes in the diabetes-prone BB rat. The findings presented may also support our hypothesis [1] that non-beta cell-specific proteins may become beta cell-specific and antigenic by post-translational modifications induced by changes in the islet microenvironment during the initiation phase of human type 1 diabetes. However, further studies are needed to explain the discordance between mRNA and protein levels.

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Duality of interest The authors are not aware of any conflict of interest relative to the contents of this paper.

References

1. Nerup J, Mandrup-Poulsen T, Helqvist S et al (1994) On the pathogenesis of IDDM. Diabetologia 37(Suppl 2):S82–S89
2. Doyle HA, Mamula MJ (2001) Post-translational protein modifications in antigen recognition and autoimmunity. Trends Immunol 22:443–449
3. Cloos PA, Christgau S (2004) Post-translational modifications of proteins: implications for aging, antigen recognition and autoimmunity. Biogerontology 5:139–158
4. Trigwell SM, Radford PM, Page SR, Loweth AC, James RFL, Morgan NG (2001) Islet glutamic acid decarboxylase modified by reactive oxygen species is recognized by antibodies from patients with type 1 diabetes mellitus. Clin Exp Immunol 126:242–249
5. Mannering SI, Harrison LC, Williamson NA et al (2005) The insulin A-chain epitope recognized by human T cells is post-translationally modified. JEM 202:1191–1197
6. Brange J (1992) Chemical stability of insulin. Mechanisms and kinetics of chemical transformations in pharmaceutical formulation. Acta Pharm Nord 4:209–222
7. Doyle HA, Gee RJ, Mamula MJ (2003) A failure to repair self-proteins leads to T cell hyperproliferation and autoantibody production. J Immunol 171:2840–2847
8. Huebscher KJ, Lee J, Rovelli G et al (1999) Protein isoaspartyl methyltransferase protects from Bax-induced apoptosis. Gene 240:333–341
9. Kragten E, Lalande I, Zimmermann K et al (1998) Glyceraldehyde-3-phosphate dehydrogenase, the putative target of the antiapoptotic compounds CGP3466 and *R*-(–)-deprenyl. J Biol Chem 273:5821–5828
10. Deverman BE, Cook BL, Manson SR et al (2002) Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. Cell 111:51–62
11. Nielsen K, Kruhøffer M, Ørntoft T et al (2004) Gene expression profiles during beta cell maturation and after IL-1β exposure reveal important roles of Pdx-1 and Nkx6.1 for IL-1β sensitivity. Diabetologia 48:2185–2199

12. Nielsen K, Sparre T, Larsen MR et al (2004) Protein expression changes in a cell system of beta-cell maturation reflect an acquired sensitivity to IL-1β. Diabetologia 47:62–74
13. Schlosser M, Banga JP, Madec AM et al (2005) Dynamic changes of GAD65 autoantibody epitope specificities in individuals at risk of developing type 1 diabetes. Diabetologia 48:922–930
14. Al-Bukhari TAMA, Radford PM, Bouras G et al (2002) Distinct antigenic features of linear epitopes at the N-terminus and C-terminus of 65 kDa glutamic acid (GAD65): implications for autoantigen modification during pathogenesis. Clin Exp Immunol 130:131–139
15. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20
16. Mattick JS, Makunin IV (2006) Non-coding RNA. Hum Mol Genet 15:R17–R29