ANNUAL REPORT SFD/NOVARTIS

Project title:

Impact of deficient tRNA methylation in ß-cell function and survival

Introduction: Type 2 diabetes (T2D) is characterized by the progressive loss of functional β-cell mass as result of environmental insults in genetically predisposed individuals. The critical pathways mediating β-cell loss in T2D are poorly understood. Polymorphisms in CDKAL1, a tRNA methylthiotransferase, have been associated with increased T2D risk. Loss-of-function mutations in TRMT10A, a putative tRNA methytransferase, cause young onset diabetes and microcephaly, suggesting that abnormal tRNA modifications can lead to pancreatic β-cell demise.

Aims of the project: To elucidate the impact of deficient tRNA methylation in β-cell function and survival.

Methods: Lymphoblast cell lines were derived from TRMT10A-deficient patients, heterozygous carriers for the mutations and healthy individuals. TRMT10A was silenced in rat (INS-1E) and human β-cells (EndoC-βH1) using RNA interference approach. Primer extension assays, and a newly setup high sensitive real-time PCR-based method were used to identify TRMT10A substrates. tRNA fragmentation was assessed by Northern blot. tRNA charging was evaluated by radioactive labeled amino acid incorporation. ROS production was measured using DCF and HPF probes and apoptosis by staining with nuclear dyes. Western blots and real-time PCR were used to measure protein and mRNA expression, respectively.

Results: Our preliminary tRNA methylation analysis of tRNAs isolated from controls and TRMT10A-deficient patient confirmed the role of TRMT10A as a mammalian tRNA methyl transferase (Fig. 1A). Primer extension assays, performed to identify TRMT10A tRNA substrates, showed that $tRNA^{Gln}$ and $tRNA^{iniMeth}$ (Fig. 1B-D), but not $tRNA^{Asn}$, $tRNA^{Gly}$ or $tRNA^{His}$ (data not shown) are methylated by TRMT10A at guanosine 9, suggesting that the enzyme has high substrate specificity. These results were confirmed using a newly designed real-time PCR-based assay that we have developed to assess m^1G_9 in specific tRNAs (Fig. 2A-C). This technique is more sensitive than primer extension assays and requires very low amount of sample (50 ng of total RNA). The method relies on the fact that the presence of m^1G_9 disrupts the ability of G residues to form G-C Watson-Crick base pairs, leading to the blockage in the reverse transcription. A schematic representation of the experimental steps is shown in figure (Fig.2A). We also used this method in RNAi-mediated TRMT10A-KD human β-cells, and showed that TRMT10A deficiency also leads to $tRNA^{Gln}$ and $tRNA^{iniMeth}$ hypomethylation in β-cells (Fig. 2D-F).

To evaluate whether tRNA hypomethylation affects trNA charging, we cultured patient and control lymphoblasts in medium containing ^{3}H-glutamine or ^{35}S-methionine. After total RNA extraction in acidic conditions to preserve tRNA charging, radioactivity was measured with a scintillometer. Radioactive signal was normalized by total RNA content. No differences in tRNA charging were detected in patients and controls suggestion that tRNA hypomethylation does not affect TRNA aminoacylation (Fig. 3).

Since hypomodified tRNAs may be degraded or fragmented we performed Northern blot analysis in tRNAs isolated from from control and TRMT10A-deficient patient lymphoblasts, to evaluate whether tRNA hypomethylation leads to tRNA fragmentation, and/or degradation. These experiments showed increased 5' $tRNA^{Gln}$ fragments in patients compared to controls (Fig. 4). The fragmentation pattern suggests that the absence of m^1G_9 leads to specific tRNA processing rather than random tRNA degradation. No fragmentation was observed for $tRNA^{iniMeth}$, $tRNA^{Asn}$ or $tRNA^{His}$ suggesting that the absence of m^1G_9 affects only some tRNA species (Fig.4).

We have previously shown that TRMT10A deficiency induces β-cell apoptosis. Our preliminary

data indicates that TRMT10A β-cell silencing leads to oxidative stress measured by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Fig. 5B). We also showed that TRM10A deficiency activates the intrinsic pathway of apoptosis, assessed by caspase-9 cleavage and cytochrome c release (Fig. 5C and D), and enhances the expression and splicing of the pro-apoptotic protein Bim (Fig. 5E and F). Moreover, our results suggest that Bim is a key mediator of TRMT10A deficiency-induced β-cell death since Bim silencing prevents TRMT10A deficiency-mediated apoptosis (Fig. 5G).
Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted by intestinal endocrine cells in response to food intake. In β-cells it stimulates cAMP formation, enhances insulin secretion and promotes β-cell survival. Long-acting GLP-1 analogs e.g. exendin-4, have been developed for the treatment of T2D. Our unpublished data shows that the cAMP inducer forskolin and exendin-4 are protective for TRMT10A-deficient β-cells (Fig.6) suggesting that GLP-1 analogs may have therapeutic potential for TRMT10A-deficient patients.

Conclusions and ongoing experiments: We confirmed that TRMT10A is a tRNA methyltransferase that modifies G_9 residues in mammalian tRNAs. We have identified $tRNA^{Gln}$ and $tRNA^{iniMeth}$ as TRMT10A substrates. We have developed a new real-time PCR-based method for studying tRNA G_9 methylation and we confirmed our findings in TRMT10A-knowkdown β-cells. We showed that TRMT10A-mediated tRNA methylation does not influence tRNA charging, but it is necessary to prevent fragmentation of specific tRNAs. Since tRNA fragments are a novel class or non-coding RNAs, that may regulate cellular processes by modulating gene expression and cell survival, we are currently evaluating whether the identified tRNA fragments are responsible for the activation of the intrinsic pathway of apoptosis observed in TRMT10A-deficient β-cells. We have shown that GLP-1 analogs may have therapeutic potential for TRMT10A deficient patients. We are presently studying the molecular mechanisms underlying this protection. The proteomic studies of TRMT10A-deficient β-cells are presently ongoing (in collaboration with Dr. Kris Gevaert, Ghent University, Belgium), and we are also actively working on the differentiation of iPSC from control individuals and TRMT10A-deficient patients into β-cells. We expect to have results regarding this issues in the next reporting period.

- ***This work has been or will be presented in the following international and national meetings as oral or poster presentations:***

1. **Igoillo-Esteve M**, Cosentino C, Atta M, Ravanat JL, Eizirik DL, Cnop M. Elucidation des mécanismes pathogènes du diabète induit par une déficience en TRMT10A et identification des nouvelles approches thérapeutiques. Congrés Annuel de la Societé Francophone du diabète. 2017. Lille, France.
2. Cosentino C, Atta M, Ravanat JL, Diaz Villamil E, Toivonen S, Pachera N, Eizirik DL, Cnop M, **Igoillo-Esteve M**. TRMT10A deficiency causes tRNA hypomethylation and tRNA fragmentation – a novel mechanism for human and rodent β-cell demise. Islet Study Group and Beta Cell workshop. **2017**. Dresden, Germany.
3. Cosentino C, Oltean T, Atta M, Ravanat JL, Eizirik DL, Miriam Cnop, **Igoillo-Esteve M**. Deficiency in the tRNA methyltransferase TRMT10A activates the intrinsic pathway of apoptosis in pancreatic β-cells. 52nd annual meeting, European association for the study of Diabetes (EASD) **2016**. Munich, Germany.
4. **Igoillo-Esteve M.** tRNA modifications and disease: "Studying the role of TRMT10A deficiency in diabetes and microcephaly" Interdisciplinary Biomedical Research Seminars, IRIBHM. *Invited lecture*. September 22nd **2016**. Campus Erasme. ULB.

- **A manuscript related with this project is presently in preparation:**
  TRMT10A deficiency leads to tRNA hypomethylation and tRNA fragmentation - a novel mechanism for human and rodent β-cell demise. Cosentino C, Atta M, Ravanat JL, Diaz Villamil E, Deglasse JP, Jonas JC, Eizirik DL, Cnop M, **Igoillo-Esteve M**.

Preliminary data

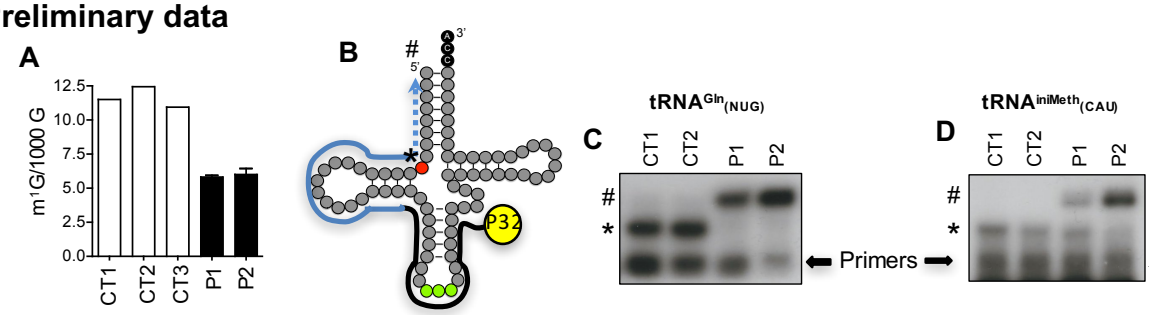

Fig. 1. TRMT10A deficiency impairs tRNA^{Gln} and tRNA^{iniMeth} guanosine methylation. tRNAs were purified from lymphoblasts from three controls (CT1-3), and two TRMT10A-deficient patients (P1-2). (A) Analysis of guanosine methylation (m^1G) on the isolated tRNAs. m^1G was quantified by ultra performance liquid chromatography coupled to mass spectrometry. The results are expressed as number of methylated guanosines per 1000 guanosines (m^1G/1000G), n=1-2. (B-D) Assessment of guanosine 9 methylation (m^1G_9) in specific tRNAs by primer extension assay. Purified tRNAs were reverse transcribed using P^{32} radiolabeled primers targeting tRNA^{Gln} and tRNA^{iniMeth} both bearing G_9. The amplification products were separated in denaturing polyacrylamide gels and the radioactive signal was detected by autoradiography. (B) Schematic representation of the assay. The red circle represents G_9, green circles represent the anticodon. The black line represents the radiolabeled oligonucleotides used as primers. m^1G_9 stops the reverse transcription generating short amplicons (*). In the absense of m^1G_9 the reverse transcription continues up to the 5' end of the tRNA (blue dotted lines) generating longer amplification products (#). (C and D) Representative autoradiography for each tRNA n=3-4.

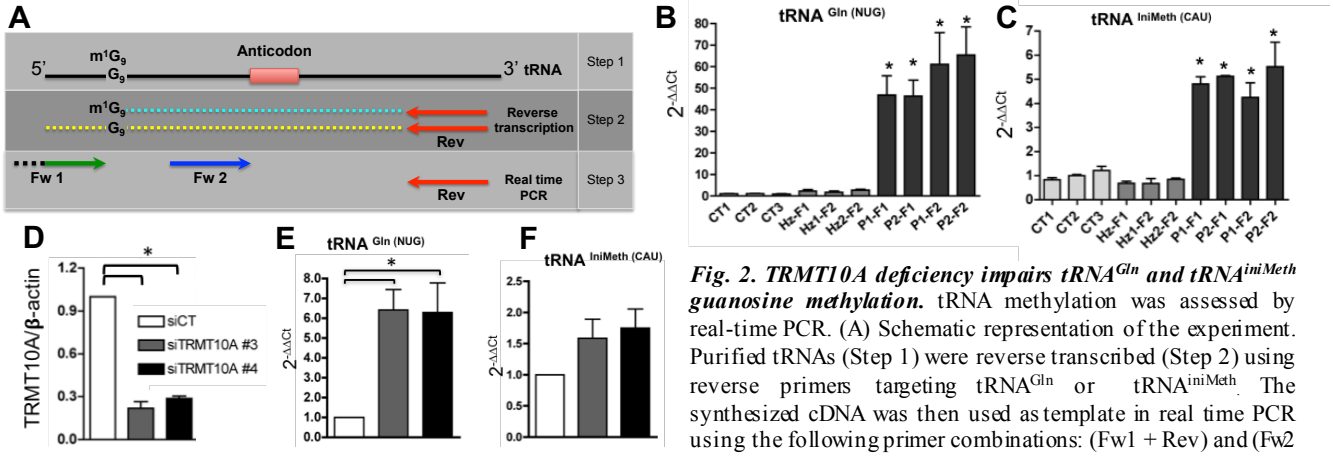

Fig. 2. TRMT10A deficiency impairs tRNA^{Gln} and tRNA^{iniMeth} guanosine methylation. tRNA methylation was assessed by real-time PCR. (A) Schematic representation of the experiment. Purified tRNAs (Step 1) were reverse transcribed (Step 2) using reverse primers targeting tRNA^{Gln} or tRNA^{iniMeth}. The synthesized cDNA was then used as template in real time PCR using the following primer combinations: (Fw1 + Rev) and (Fw2 + Rev) (Step 3). The presence of m^1G_9 blocs the reverse transcription leading to a short amplicon (blue dotted line in panel H), and no PCR amplification is obtained when Fw1 + Rev primers are used. In the absence of m^1G_9 the reverse transcription continues until the 5' end of the tRNA (yellow dotted line) allowing PCR amplification with Fw1 + Rev primers. The PCR amplification with Fw2 + Rev is not modified by the presence m^1G_9. (B and C) real-time PCR data for the specified tRNAs on tRNAs-enriched total RNA isolated from lymphoblasts from three controls (CT1-3), three heterozygous carriers (Hz) for TRMT10A nonsense mutations from two independent families (F1 and F2), and two TRMT10A-deficient patients from each family (P1-2 F1) and (P1-2 F2). D) TRMT10A mRNA expression in EndoC-βH1cells. Two different siRNAs targeting human TRMT10A (siTRMT10A #3 and #4) were used to silence TRMT10A gene expression, n=3. Gene expression was normalized to the reference gene GAPDH. (E and F) real-time PCR data for the specified tRNAs on tRNAs-enriched total RNA isolated from RNAi-mediated TRMT10A-deficient EndoC-βH1 cells. The results are expressed as $2^{-\Delta\Delta Ct}$ and are means ± SE of n=3-4 independent experiments.

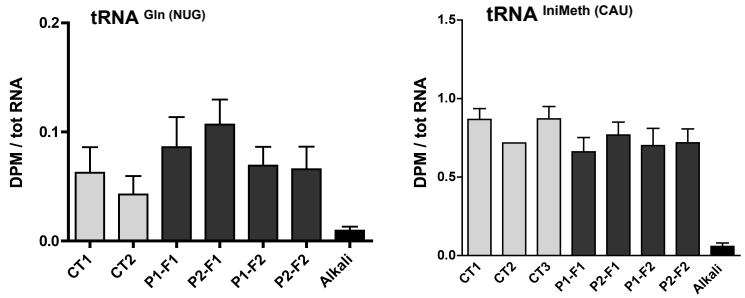

Fig.3. TRMT10A-mediated tRNA methylation does not modify tRNA charging. Lymphoblasts from controls (CT 1-3) and TRMT10A-deficient patients (P1 and 2) from two different families (F1 and F2) were incubated in glutamine or methionine-free medium for 15 min to induce tRNA uncharging. Then the samples were incubated for 10 min in in the presence of radiolabelled ^{35}S-Methionine or ^{3}H-Glutamine. The cells were then collected by centrifugation, washed to eliminate the unincorporated radioactive amino acids, and lysed for RNA extraction in acidic conditions (pH 4.5). This allows tRNA charging preservation. One sample was alkali-treated (pH 9) for 30 min to complete uncharge the tRNAs. This sample was used as negative control (Alkali). Radioactivity was measured by radiometric analysis. The results means ± SE and are expressed as DPM

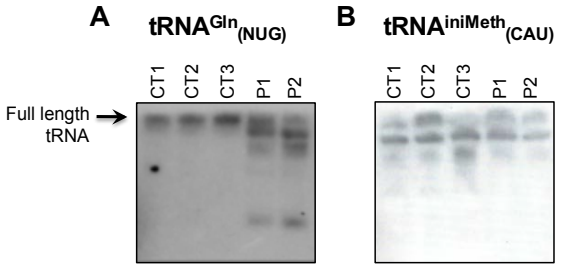

Fig. 4. TRMT10A deficiency leads to tRNA$_{Gln}$ fragmentation. Processing of both pre-tRNA and mature tRNA can give rise to small RNA fragments, a process enhanced by deficient tRNA modifications. tRNA fragmentation was analyzed by northern blot using radiolabeled probes designed to target 5' halves. tRNA^{Gln} was differentially fragmented in patient samples compared to controls (A), while no difference in fragmentation was observed for tRNA^{iniMeth} (B). Panels show representative autoradiographies, n=2-3 independent experiments.

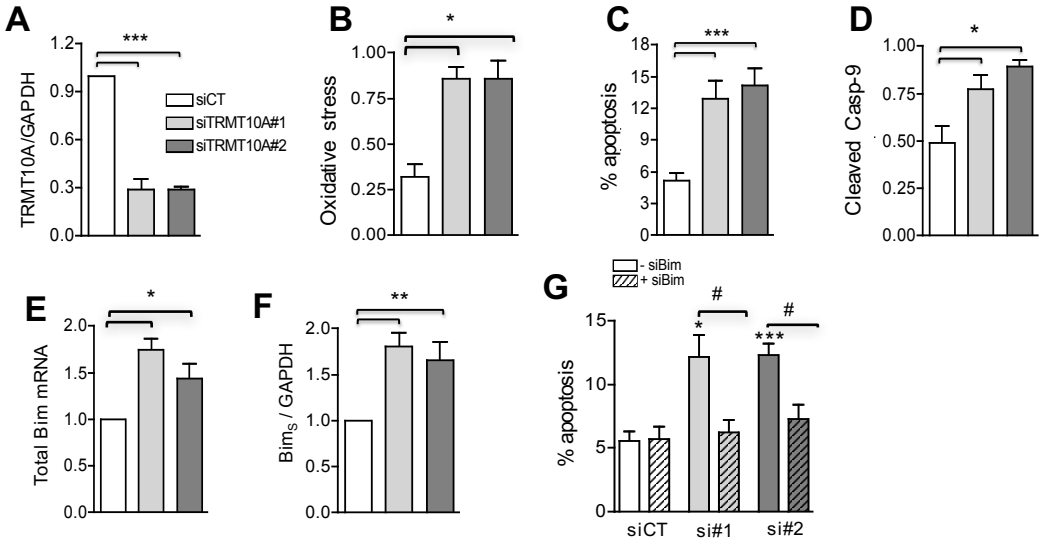

Fig. 5. TRMT10A deficiency activates the intrinsic pathway of apoptosis in β-cells. TRMT10A expression was silenced (siTRMT10A) or not (siCT) for 48h in INS-1E using two siRNAs targeting rat TRMT10A (si#1 and #2). TRMT10A mRNA expression was analyzed by real-time PCR (A). Oxidative stress was assess by DCF (B). β-cell apoptosis was examined by Hoechst/propidium iodide staining (C). Activation of the intrinsic pathway of apoptosis was assessed by Western blots for cleaved caspase-9 (D). Caspase-9 expression was normalized to the reference proteins GAPDH and α-tubulin, respectively. The graphic is the densitometric quantification of the Western blots. (E and F) Total Bim and Bim small mRNA expression examined by real-time PCR 48 h after TRMT10A knockdown. (G) double knockdown of TRMT10A and Bim. INS-1E cells were transfected with siCT, or the two siRNAs targeting rat TRMT10A (si#1 and #2) alone or combined with Bim siRNA (siBim). β-cell apoptosis was examined by Hoechst/propidium iodide staining. Data are shown as means ± SE of n=5-11 independent experiments. *p<0.05, **p<0.01, ***p<0.001, siTRMT10A vs siCT, #p<0.05, siTRMT10A/Bim vs siTRMT10A by paired t test.

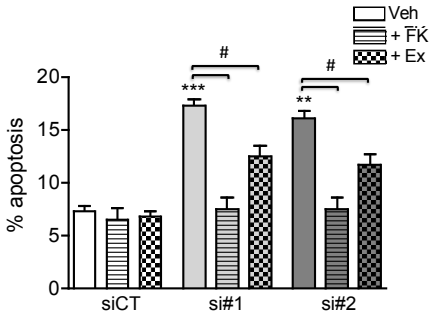

Fig.6. cAMP inducers prevent TRMT10A deficiency-induced apoptosis in β-cells. INS-1E cells were transfected with a control siRNA (siCT), or siRNAs targeting rat TRMT0A (#1, #2) . After transfection the cells were treated or not for 48 h with 20 μM forskolin (FK) or 50 nM exendin-4 (Ex). β-cell viability was assessed by Hoechst/propidium iodide staining. Results are mean±SE of n=3-5 independent experiments. **p<0.01, ***p<0.001 siTRMT10A vs si CT, #p<0.05, siTRMT10A (+ FK or Ex) vs siTRMT10A (Veh).