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From Cell Biology to Tissue Engineering

Co-localization of the zinc transporter ZnT8 (slc30A8) with ghrelin and motilin in the gastrointestinal tract of pigs

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Summary. Zinc is an important co-factor for insulin storage in pancreatic β-cells of different species and the uptake of this ion into insulin containing secretory vesicles is managed by the zinc transporter, ZnT8, a member of the slc30A gene family. Recent studies indicate that this protein is a major autoimmune target in human type 1A diabetes and has also been implicated by genome-wide association studies in type 2 diabetes. Since individuals suffering from type 1 diabetes often develop gastrointestinal motility disorders, we investigated the expression of ZnT8 in the porcine gastrointestinal tract. For this purpose, we studied the cell-type specific expression of ZnT8 in the gut and its co-expression with endocrine hormones that are closely linked to intestinal motility regulation. Nested RT-PCR and immunostaining of sequential serial sections, as well as double-immunostaining using antibodies directed against ZnT8, ghrelin, motilin, neurotensin, serotonin and glucagon-like peptide 1, indicated that ZnT8 is co-localized with ghrelin and motilin. Our findings provide important information about the cell-type specific expression of ZnT8 in the porcine gastrointestinal system. The selective and exclusive expression of ZnT8 in two endocrine cell-types that are engaged in motility functions may be of particular interest for further investigations into type I diabetes-associated gastrointestinal dysfunctions.

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Introduction

Diabetes mellitus is associated with hyperglycemia during fasting and postprandial periods, mostly induced by physical and functional loss of pancreatic β-cell activities, such as insulin secretion and function. The resulting diseases are divided into four classes and two of them, type 1 and 2 diabetes, are the most widely distributed diseases in the world (Maraschin, 2012). Type 1 diabetes (T1DM) is in most cases an autoimmune disease characterized by gradual destruction of pancreatic β-cells, whereas type 2 diabetes (T2DM) is an age-dependent disease characterized by insulin resistance, abnormal β-cell function and reduced β-cell mass (Canivell and Gomis, 2014; Kahn et al., 2014). Moreover, the insulin homeostasis in pancreatic β-cells may be influenced by genetic and environmental factors (Kahn et al., 2014).

For T1DM, numerous autoantibodies have been found that target proteins expressed by islet cells, such as insulin, glutamic acid decarboxylase 65, insulinoma-associated antigen-2, heat shock protein, islet-specific glucose-6-phosphatase catalytic subunit related protein and imogen-38 (Taplin and Barker, 2008; Arvan et al., 2012; Han et al., 2013). Recently, additional autoimmune targets for islet cells were identified, such as pancreatic duodenal homeobox factor 1, chromogranin A, islet amyloid polypeptide and zinc transporter 8 (ZnT8) (Han et al., 2013). ZnT8 has attained significant attention in the context of diabetes as

it has been demonstrated to be expressed in pancreatic islet β-cells in different species (Chimienti et al., 2004; Chimienti et al., 2005; Nicolson et al., 2009; Mohanasundaram et al., 2011; Schweiger et al., 2013).

ZnT8 is a zinc transporter belonging to the slc30A gene family, which controls intracellular zinc levels. Zinc levels are regulated either by efflux from cells or by influx into intracellular vesicles (Chimienti, 2013; Huang and Tepaamorndech, 2013). In pancreatic islet β-cells, ZnT8 is responsible for the intracellular uptake of zinc into secretory vesicles that contain insulin, in order to support the crystallization and high packaging of insulin (Dodson and Steiner, 1998; Chimienti et al., 2005; Wijesekara et al., 2010).

Investigations into the auto-antigenicity of ZnT8 show that the main epitopes for autoantibodies reside approximately in the last 100 amino acids of this protein. The epitope R325 plays an important role in ZnT8 autoimmunity and structural defects in the epitope effects the accumulation of zinc ions into secretory granules containing insulin (Wenzlau et al., 2007; Achenbach et al., 2009). Consequently, the release and function of insulin is disturbed. This implies that people carrying an R325 allele in their genes possess an increased risk for developing T2DM (Huang and Tepaamorndech, 2013). Moreover, it has been found that CD8+ T cells also play a crucial role in ZnT8 autoimmunity by targeting distinct epitopes located in the first 200 amino acids of the ZnT8 protein (Énée et al., 2012; Scotto et al., 2012; Wu et al., 2012). The expression of ZnT8 is not restricted to pancreatic tissue, as it has also been described in adipose tissue, in thyroid and adrenal glands (Smidt et al., 2007; Murgia et al., 2009). However, disorders in these organs caused by autoimmunity against ZnT8 have currently not been reported for people with T1DM or T2DM.

The enteric nerve system controls and stimulates complex gastrointestinal motility. Impairments of this nerve system lead to abnormalities in gastrointestinal motor functions, which occur in gastroparesis (Thazhath et al., 2013; Vanormelingen et al., 2013). Interestingly, gastroparesis is quite disseminated among people with long-standing T1DM and T2DM (Thazhath et al., 2013), but it is not described as an additional autoimmune disease, such as Hashimoto syndrome, Addison syndrome and celiac disease (Maraschin, Jorge de Faria 2012; Han et al., 2013). Furthermore, no correlation with distinct zinc importer or transporter molecules, such as ZnT8, was observed.

Because of the potential link between auto-antigenicity of the ZnT8 protein and gastrointestinal motility disorders, the aim of this study was to clarify whether this transporter molecule is also expressed in distinct cell-types of the gastrointestinal tract, and if so, to further investigate whether a correlation exists between ZnT8 expression and gut motility regulation. ZnT8 was identified in pig stomach and intestinal tissue using immunohistochemistry on Bouin-fixed, paraffin-embedded serial sections. Furthermore, our double-immunostainings on sequential serial sections with antibodies against ghrelin, motilin, neurotensin, serotonin and glucagon-like peptide 1 (GLP-1) revealed co-localization of ZnT8 with ghrelin in the stomach and with motilin in the intestines of pigs. Furthermore, we confirmed by RT-PCR the expression of ZnT8 in both the stomach and intestine of pigs.

Materials and methods

Animals and tissue preparation

Six pigs of the German Landrace breed, ranging in age from 4 to 12 weeks, were obtained from the experimental station of animal husbandry, animal breeding and small animal breeding at the University of Hohenheim, Germany. All animals were housed and fed under the same conditions and were slaughtered at different ages. Immediately after slaughter, the stomach and intestinal tissue of each animal was removed, sectioned and divided into two compartments. One part was placed in RNAlater solution (Life technologies, Darmstadt, Germany) for RT-PCR analysis and the other one was immersion-fixed in Bouin's solution for 48 h and embedded in paraffin. For immunohistochemical staining, serial sections of 2-4 μm were cut and mounted on Superfrost glass slides (R. Langenbrinck, Emmendingen, Germany).

Immunostaining

Sets of serial sections were prepared to determine the local expression of ZnT8 in the gastrointestinal tract and for co-localization studies of the ZnT8 molecule with ghrelin, motilin, neurotensin, serotonin and GLP-1. The dilutions of the primary and secondary antibodies are listed in Table 1.

Immunostaining of pig stomach and intestinal tissues was performed as follows. Sections were deparaffinized in clean xylene, rehydrated through a series of graded ethanol solutions and incubated with 10% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After rinsing in PBS, specimens

Table 1. Details of the antibodies used for immunohistochemistry.

Antigen	Host	Dilution	Manufacturer
ZnT8	Rabbit	1:2000	Own production*
Ghrelin	Goat	1:100	SantaCruz, Heidelberg, Germany
Motilin	Rabbit	1:1000	Biotrend, Cologne, Germany
Serotonin	Mouse	1:1000	Dako, Hamburg, Germany
Neurotensin	Rabbit	1:200	Acris, Herford, Germany
GLP-1	Rabbit	1:600	Enzo Life Science, Loerrach, Germany
Anti-Rabbit	Goat	1:400	Dako, Hamburg, Germany
Anti-Mouse	Rabbit	1:300	Dako, Hamburg, Germany
Anti-Goat	Rabbit	1:400	Biotrend, Cologne, Germany

* Generation of the anti-ZnT8 antibody was described and characterized previously (Schweiger et al., 2013).

were incubated with a 10% goat or rabbit serum blocking solution (Dako, Hamburg, Germany) for 30 min to reduce non-specific immunoreactions. Samples were then incubated overnight in a humidity chamber at 5°C with the antibodies listed in Table 1. Slides were then rinsed and treated with the appropriate biotinylated secondary antibody for 30 min at room temperature. The slides were washed with PBS and then incubated with streptavidin-biotin-horseradish-peroxidase (Merck-Millipore, Darmstadt, Germany) for 30 min at room temperature. The reaction product was visualized with 3,3'-diaminobenzidine (DAB) chromogen (Biotrend, Cologne, Germany).

For co-localization double-immunostaining studies, sections were immunostained for ZnT8 and visualized by DAB reaction (brown staining) as described above. After several rinses in PBS, specimens were incubated twice with LinBlock for 2 min (Linaris. Dossenheim, Germany). This substrate was used to remove the antibodies from the first stain and to block the enzyme system before the same section was treated with another chromogen. Slides were then rinsed in PBS and immunostained for ghrelin or motilin using the ABC-method and visualized by Histogreen (green staining; Linaris. Dossenheim, Germany). Additionally, single stained control sections were made to better illustrate the double-staining. For this purpose, stomach and small intestine sections were stained with either DAB (brown staining) for ZnT8 or with Histogreen (green staining) for ghrelin or motilin.

The specificity of the staining reaction was demonstrated by serial dilution of the primary antigen, which resulted in a gradual decrease in signal intensity. Finally, all specimens were counterstained with hematoxylin, dehydrated, cleared with xylene and mounted in Entellan (Merck, Darmstadt, Germany) for light microscopy examination.

Immunohistochemical controls

Control slides were also developed, which included either substitution of the primary antibodies with goat or rabbit serum, omitting the primary antibodies, incubation with the secondary antibodies alone or incubation with the diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. No specific staining was observed in these control experiments.

RNA extraction

Total RNA was isolated from 20 mg of stomach and small intestine tissues using an RNA isolation kit (Promega, Mannheim, Germany). To rule out contamination by genomic DNA, isolated RNA was treated with RNAse-free DNAse according to the manufacturer's protocol (Promega, Mannheim, Germany). Thereafter, RNA was quantified by measuring the A260/280. The RNA yields ranged between 1-2 μg per extraction. RNA quality was assessed by polyacrylamide gel electrophoresis (12,5% gel) and silver staining (GE Healthcare, Freiburg, Germany).

Primer sequences

The primers used in this study for the nested RT-PCR were designed according to the known genomic DNA sequence of the pig ZnT8 protein (NBCI nucleotide database number: FJ588029). ZnT8-0 forward (nucleotide 79-102), 5'-CTCCAGCAGA AACCCTTGAATAAA-3', reverse (nucleotide 536-557) 5'-ACTGCGCAGCTTGAAACGATAA-3' and ZnT8-1 forward (nucleotide 135-156) 5'-CGAGTCGGGAG CCATCAATCAC-3', reverse (nucleotide 486-507) 5'-TGGGTACAGCAGGCGTTCACAA-3'. These primers were previously used for detection of ZnT8 expression in porcine pancreases (Schweiger et al., 2013).

Performing nested RT-PCR

Total RNA (1-2 μg) was denatured with a 500 ng mixture of oligo-dN and -dT primer by incubation at 70°C for 5 min and then was slowly chilled to 25°C and stored on ice. This mixture was used for reverse transcription in a 20 μL volume according to the manufacturer's protocol (Promega, Mannheim, Germany). The solution was incubated at 42°C for 45 min followed by a 70°C incubation for 15 min and then was chilled on ice.

The two-step nested PCR was carried out in 50 μL containing the ZnT8-0 primer pair and all PCR reagents. The PCR consisted of denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 1.5 min. The last step was followed by a final extension at 72°C for 7 min. Thereafter, the probes were purified using a PCR purification kit (Macherey-Nagel, Düren, Germany) and used for the second PCR step. This step was performed as described for the first PCR step using the ZnT8-1 instead of ZnT8-0 primer pair.

PCR amplification products were visualized after electrophoresis on a 12.5% polyacrylamide gel and staining with silver (GE Healthcare, Freiburg, Germany).

To rule out possible contamination by genomic DNA in RNA probes, the RT-PCR was performed without a reverse transcriptase enzyme or without RNA as well as a PCR without cDNA. No spurious DNA fragments were detected.

Results

Immunohistochemical staining of pig stomach paraffin sections revealed that ZnT8 expression was regularly found in entero-endocrine cells localized between gastric gland cells, particularly in the fundus region (Fig. 1A1). These cells were integrated between chief or parietal cells, were ovoid, triangular or

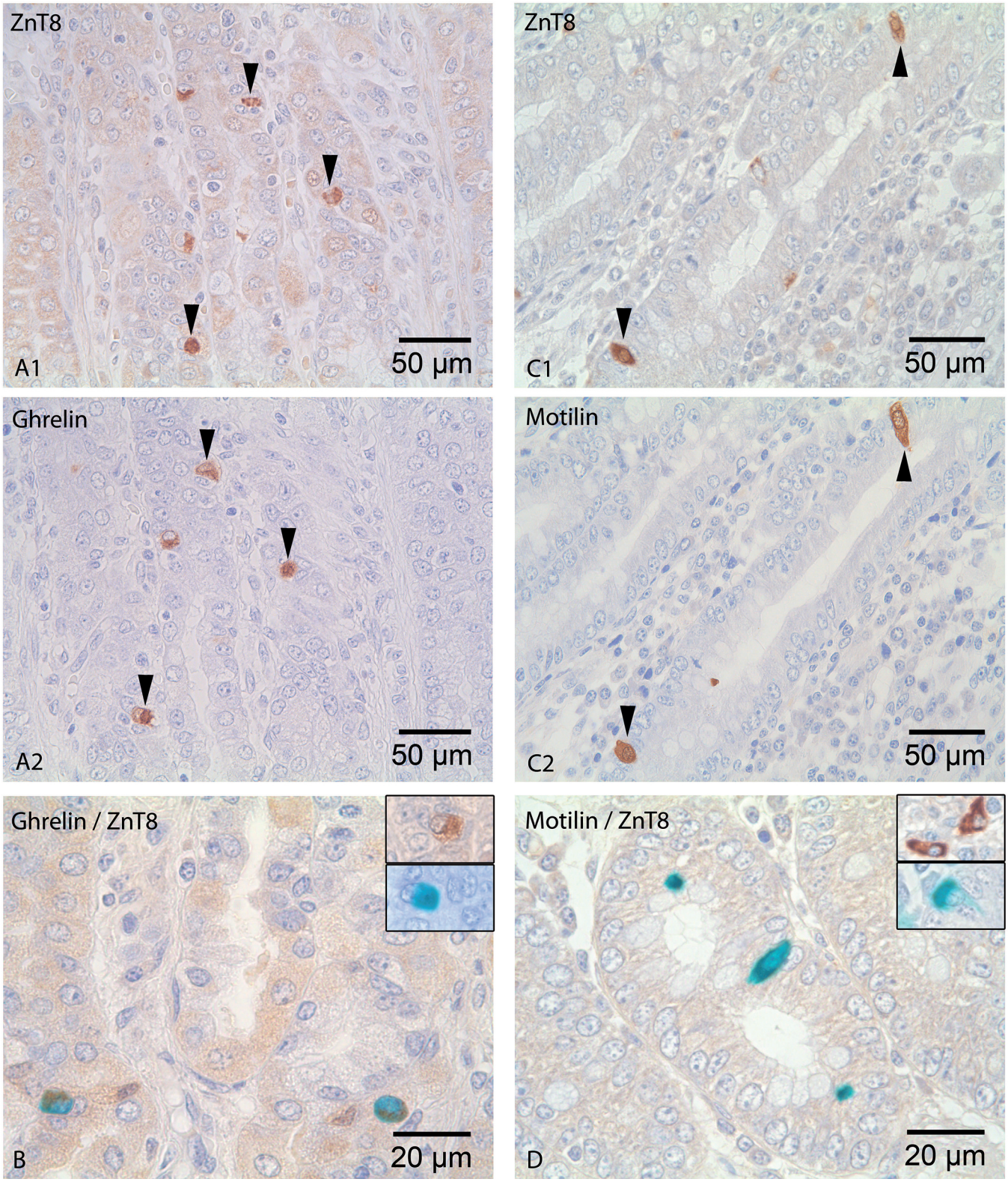

Fig. 1. Cell-specific immunolocalization of ZnT8, ghrelin and motilin in porcine stomach and small intestine serial sections. In the stomach section, the cellular pattern and localization of ZnT8-immunoreactive cells (**A1**, arrowheads) closely resembled those stained by ghrelin-antibodies (**A2**, arrowheads). Similar observations were found in cells of the small intestine when immunostained with ZnT8 antibodies (**C1**, arrowheads) and motilin antibodies (**C2**, arrowheads). Double-immunostaining of stomach and small intestine sections identify cell-specific co-expressions of ZnT8 and ghrelin in the stomach **(B)** as well as ZnT8 and motilin in the small intestine (**D)**. Both inserts show single stained control sections either for ZnT8 (brown) or ghrelin (green) in B and either for ZnT8 (brown) or motilin (green) in D.

polygonal in shape and were sparsely distributed from the neck to the base of the tubular glands. Distinct ZnT8 immunoreactivity was found in the cell cytoplasm, which was clearly localized to secretory granules. All other gastric glandular cell types, such as chief, parietal, neck mucous or surface mucous cells were negative for ZnT8 staining.

Sequential serial sections stained with ghrelin antibodies displayed an identical staining pattern as ZnT8, in terms of both number and localization pattern of cells (Fig. 1A1,A2). Double-immunostaining of stomach sections with ghrelin and ZnT8 antibodies confirmed the cell-specific co-expression of ghrelin and ZnT8 in cytoplasmatic secretory granules (Fig. 1B).

In the small intestine, ZnT8 immunohistochemical staining was restricted to a subset of entero-endocrine cells, preferentially localized to the crypts and to the base of the villous system (Fig. 1C1). These positively labelled cells were ovoid or triangular in shape and resembled open entero-endocrine cells.

Our immunohistochemical staining of sequential serial sections with antibodies against ZnT8, motilin, neurotensin, serotonin and GLP-1 revealed that motilin positive cells have an identical distribution pattern as ZnT8 positive cells (Fig. 1C1,C2). Furthermore, this result is supported by our double immunostaining approach, which also identified co-localization of ZnT8 and motilin within cells (Fig. 1D).

To confirm ZnT8 expression in the porcine gastrointestinal tract, we performed a nested RT-PCR. The analysis of the PCR products revealed the presence of ZnT8-specific mRNA in the pig stomach and intestine. The PCR fragments were of the expected size of 373 bp.

Discussion

In pancreatic β-cells, ZnT8 is involved in the intracellular uptake of zinc into secretory vesicles containing insulin and consequently ZnT8 supports the biosynthesis and secretion of insulin (Lemaire et al., 2009; Pound et al., 2009; Wijesekara et al., 2009; Wijesekara et al., 2010). Moreover, this zinc transporter molecule exhibits antigenic properties and therefore is a potential risk factor for developing T1DM and T2DM (Wenzlau et al., 2007; Achenbach et al., 2009; Énée et al., 2012; Huang and Tepaamorndech, 2013). The expression of ZnT8 outside of the pancreas was found only in a few organs, such as the thyroid and adrenal glands, but not in other tissues (Murgia et al., 2009).

In this study, we demonstrate for the first time that ZnT8 is expressed in different cell-types of the

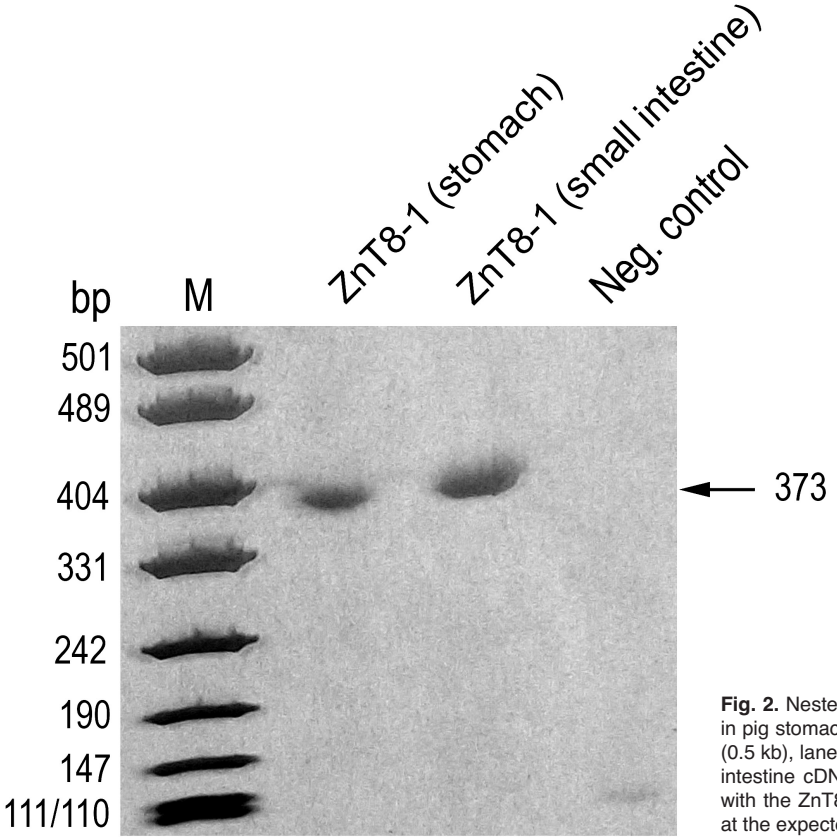

Fig. 2. Nested RT-PCR was used to detect the presence of ZnT8 in pig stomach and small intestine. Lane 1: molecular size marker (0.5 kb), lane 2: stomach cDNA with ZnT8-1 primers, lane 2: small intestine cDNA with ZnT8-1 primers, lane 3: no-template control with the ZnT8-1 primer pair. The PCR product for ZnT8 migrated at the expected size of 373 bp.

gastrointestinal tract, including endocrine cell types of the stomach and intestinal tract. Sequential- and double-immunostaining techniques revealed co-expression of ZnT8 with ghrelin in the stomach and with motilin in the intestinal tract.

These findings indicate that ZnT8 may be involved in the biosynthesis, packaging, storage and releases of ghrelin and motilin. However, it is not known whether ZnT8 is integrated in membranes of secretory vesicles containing zinc alone or in vesicle membranes containing one of the hormones ghrelin or motilin. In the latter case, zinc could possibly be involved in packing, storage and release of these hormones, as it is for insulin in pancreatic β-cells. Otherwise, zinc and the hormones ghrelin or motilin could separately be secreted.

The identification of ZnT8 in ghrelin or motilin producing cells indicates that zinc may also play an essential role in the physiological activities of both of these hormones. The physiological functions of both ghrelin and motilin are similar, as neither are secreted postprandial, their concentration in blood is high during fasting periods, they accelerate gastric emptying and are involved with gastrointestinal motility (Schmidt et al., 2006; Wierup et al., 2007; Chen and Tsai, 2012). The receptors of ghrelin and motilin are most prevalent in the enteric nerve system. Ghrelin receptors are mainly found on excitatory cholinergic neurons and motilin receptors are mainly localized on inhibitory nitrergic and cholinergic neurons (Dass et al., 2003; Xu et al., 2005; Takeshita et al., 2006; Broad et al., 2012. In the CNS, it has been shown that zinc molecules can modulate excitatory and inhibitory neurotransmission, likely as an endogenous allosteric modulator (Smart et al., 2004). This effect was observed in synapses between neurons with zinc-rich synaptic vessels (Sensi et al., 2009). Zinc-rich vesicles are also found in many excitatory cholinergic and inhibitory nitrergic intestinal neurons of the enteric nerve system (Wojtkiewicz et al., 2012a,b). How released zinc from ghrelin or motilin secreting cells modulates the effects of both hormones in the enteric nerve system remains unclear and therefore requires further investigation.

Our RT-PCR results indicate that ZnT8 translation may be tightly regulated in the porcine gastrointestinal tract, as this has already been described in porcine pancreatic tissue (Schweiger et al., 2013). As has been described for porcine β-cells, here we initially found local mRNA expression for ghrelin and motilin (data not shown) but not for ZnT8, although ZnT8, ghrelin and motilin were detected at similar levels with immunohistochemistry. Only after performing a nested RT-PCR was the expected PCR product for ZnT8 obtained. Indeed, strong regulation of genomic DNA is known for zinc-binding proteins, including zinc transporters, which underlie a complex zinc-dependent mechanism (Jackson et al., 2008; Lichten et al., 2011, Coneyworth et al., 2012; Yu et al., 2013). However, such a regulation at the genomic DNA level has not yet been confirmed for ZnT8.

In summary, the results presented here indicate that ZnT8 is a protein cell-type-specific expressed in the porcine gastrointestinal tract with ghrelin or motilin. As ZnT8 is found in endocrine cell-types that are engaged in motility functions and has been reported to exhibit antigenic properties, ZnT8 may be of particular interest for further investigations into T1DM-associated gastrointestinal dysfunctions.

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