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Alteration of ZnT5-Mediated Zinc Import into the Early Secretory Pathway Affects the Secretion of Growth Hormone from Rat Pituitary Cells

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Key Words
Growth hormone · Zinc · Regulated GH secretion · ZnT5 transporter

Abstract
Background: Aggregation of growth hormone (GH) required for its proper storage in granules is facilitated by zinc (Zn^{2+}) transported by specific zinc transporters in and out of the regulated secretory pathway. *Slc30a5* (ZnT5) was reported to have the highest gene expression among all zinc transporters in primary mouse pituitary cells while *ZnT5*-null mice presented with abnormal bone development and impaired growth compared to wild-type counterparts. ***Methods:*** In vitro studies performed in GH3 cells, a rat pituitary cell line that endogenously produces rat GH (rGH), included analysis of: cytoplasmic Zn^{2+} pool changes after altering rSlc30a5 expression (luciferase assay), rZnT5 association with different compartments of the regulated secretory pathway (confocal microscopy), and the rGH secretion after rSlc30a5 knock-down (Western blot). ***Results:*** Confocal microscopy demonstrated high co-localization of rZnT5 with ER and Golgi (early secretory pathway) while siRNA-mediated knock-down of rSlc30a5 gene expression led to a significant reduction in rGH secretion. Furthermore, altered expression of rSlc30a5 (knock-down/overexpression) evoked changes in the cytoplasmic Zn^{2+} pool indicating its important role in mediating Zn^{2+} influx into intracellular compartments of the regulated secretory pathway. ***Conclusion:*** Taken together, these results suggest that ZnT5 might play an important role in regulated GH secretion that is much greater than previously anticipated.
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Introduction

Human growth hormone (GH) plays an important role in a variety of physiological and metabolic processes in humans, especially in the process of normal somatic growth. In somatotroph cells of the anterior pituitary, GH is stored in concentrated forms in secretory granules allowing its rapid release in the bloodstream upon GH-releasing hormone (GHRH) stimulation. The complex process of secretory granule formation (biogenesis) begins with self-association (aggregation) of hormones into a reversible aggregate that occurs at the specific location in the early secretory pathway (in the lumen of the *trans*-Golgi layer) [1]. The main principles of this process as well as conditions required have been extensively investigated in the case of prolactin (PRL) [2, 3]. Zn^{2+} has been found to play an important role in facilitating PRL aggregation, which is considered to be crucial for its intracellular processing and normal secretion [4, 5].

A high concentration of Zn^{2+} was reported to be localized in GH-containing secretory granules and to a lesser

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extent in Golgi complex of rat anterior pituitary cells [6], thus confirming its presence in the regulated secretory pathway of GH. Furthermore, Cunningham et al. [7] reported that two Zn^{2+} are required for GH dimers to form and association occurs through binding at high-affinity residues in GH (His18, His21 and Glu174). Size-exclusion chromatography and sedimentation equilibrium analysis performed within the same study demonstrated that mutation of these Zn^{2+}-binding residues to alanine caused reductions of dimeric GH formation. However, it still remains to be elucidated whether a dimer or an oligomer is the final storage form of GH in secretory granules.

At the cellular level, most of Zn^{2+} is tightly bound by various proteins playing an important structural and/or catalytic role in transcription factors and in numerous secretory, membrane-bound, as well as endosome/lysosome-resident enzymes [8–10]. On the other hand, a small pool of the total Zn^{2+} called free Zn^{2+} is loosely to moderately bound to ligands and thus is considered as rapidly exchangeable. It is mainly found in secretory and synaptic vesicles and is required for protein packaging, secretion or neuronal signal transduction [11–13]. Cellular Zn^{2+} homeostasis is tightly regulated and because of its high charge density, transporter proteins are required to move it across cellular membranes. More than 20 zinc transporters classified into two families (Slc30a/ZnT and Slc39a/Zip) mediate Zn^{2+} homeostasis and dynamics at the cellular level but also within the secretory pathway [14, 15]. Nearly half of them are localized in the early secretory (comprising the endoplasmic reticulum (ER) and the Golgi apparatus) and regulated secretory pathways. The role of ZnTs is to reduce intracellular Zn^{2+} by transporting it from the cytoplasm into various intracellular organelles or by moving Zn^{2+} into extracellular space [16] while Zips increase intracellular Zn^{2+} by transporting it in the opposite direction.

The cellular distribution of ZnT transporters in various endocrine organs in mice investigated by quantitative real-time PCR and immunohistochemistry revealed that from all ZnT transporters analyzed (*Slc30a1-10* genes encoding for ZnT1-10), Slc30a1, Slc30a9 and Slc30a10 displayed higher and Slc30a5 the highest expression level in the pituitary gland [17]. Furthermore, *ZnT5*-null mice are characterized by abnormal bone development, significantly impaired growth when compared to the wild-type animals and a high degree of osteopenia due to systemic decrease in bone density as a result of the reduced activity of osteoblasts [18]. Taken together, this highlights the potential role of ZnT5 in transporting Zn^{2+} in the early secretory pathway, which is important for the proper storage of GH within secretory granules found to be crucial for normal GH secretion.

In the present study, the impact of altered rSlc30a5 expression (knock-down/overexpression) on the cytoplasmic Zn^{2+} pool changes was studied in GH3 cells. Furthermore, by attenuating rSlc30a5 expression in rat pituitary cells using gene-specific siRNAs, the specific role of rZnT5 in the process of rat GH (rGH) secretion was investigated more in detail. In addition, confocal microscopy studies were used to compare differences in co-localization (association) of the rZnT5 with different intracellular compartments (ER, Golgi and secretory granules) within the regulated secretory pathway of rGH.

Materials and Methods

Cell Culture and Treatment

Rat pituitary cell line GH3 cells (a gift from Prof. Roberto Salvatori, Johns Hopkins University School of Medicine, Baltimore, Md., USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose) supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal calf serum, 10 mM Na-pyruvate (Life Technologies/Invitrogen AG, Basel, Switzerland) and 100 U/l penicillin/streptomycin.

Expression Vectors

Full-length rSlc30a5 cDNA (approx. 2.4 kb) was obtained by RT-PCR using total RNA isolated from GC cells (rat pituitary cell line). RNA was extracted using a Qiagen RNeasy kit (Qiagen AG, Basel, Switzerland) including deoxyribonuclease treatment and reverse transcribed (1 μg of total RNA) in 25 μl RT reaction using oligo(deoxythymidine)18 primers, 20 mM of each deoxynucleotide triphosphate, 10× first-strand buffer solution, Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals) and diethylpyrocarbonate.

Generated cDNA was further used as a template for PCR of rSlc30a5 cDNA using primers corresponding to sequences in the 5′- and 3′-untranslated regions (FP 5′-GT<u>CTCGAG</u>CGGCTCCT GAGCCGGCATGGA-3′ and RP 5′-CA<u>GAATTC</u>CCGCTGGG GCT CATCTCACATG-3′). rSlc30a5 cDNA fragment was cloned in pcDNA3.1(–) neo vector (Invitrogen, La Jolla, Calif., USA) using *Xho*I and *Eco*RI restriction sites and the correct nucleotide sequence was confirmed by sequencing across both strands.

For confocal microscopy studies, rZnT5-pEGFP-C3 was generated by cloning rSlc30a5 cDNA in the pEGFP-C3 vector (BD Biosciences/Clontech, Franklin Lakes, N.J., USA) using *Eco*RI and *Xba*I restriction sites.

siRNA-Mediated Gene Silencing and Quantitative Real-Time PCR (SYBR Green)

GH3 cells were transfected using nucleofection (Nucleofector; Amaxa Biosystems GmbH, Cologne, Germany) according to the manufacturer's protocol. Cells (1×10^6/0.100 ml) were transfected without or with 200 pmol of rat Slc30a5-specific siRNA 28 (sense: 5′-GCCUUAUCCCAGCUUGUUU-3′, antisense: 5′-AAACAA

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GCUGGGAUAAGGC-3′), siRNA 29 (sense: 5′-GAUAGUAAAC CUUAUUGGA-3′, antisense: 5′-UCCAAUAAGGUUUACUA UC-3′) (Sigma-Aldrich, Buchs, Switzerland) or control siRNA non-targeting pool (Dharmacon; Thermo Fisher Scientific, Wohlen, Switzerland). Transfection efficiency was checked using an EGFP-N1 control plasmid expressing enhanced green fluorescent protein, analyzed by fluorescence microscopy and found to be consistent among independent experiments. Total RNA was extracted 48 h after transfection and RT-PCR was performed as described above.

For quantitative RT-PCR (qRT-PCR), the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, Calif., USA) was used. In brief, PCR reactions were performed in 96-well plates (MicroAmp; Applied Biosystems) using cDNA prepared as described above. We used ABsolute QPCR SYBR Green Mix (ABgene; Thermo Fisher Scientific), 1 μl (20 pmol/μl) specific primers (Microsynth, Balgach, Switzerland) and 40 ng cDNA in a total volume of 25 μl. Relative expression values were determined by the comparative C_t method using 18S rRNA as the reference gene. Amplification curves and the mean C_t values were calculated using the 7500 Fast System SDS software (Applied Biosystems).

Cytoplasmic Zinc Pool Assay (4× Metal Response Element Luciferase Reporter Assay)

The 4× MRE (metal-response element) pGL3-luciferase reporter was kindly provided by Prof. Walter Schaffner (Institute of Molecular Life Sciences, University of Zurich, Switzerland). Cells (1×10^6/0.100 ml) were transfected without or with 200 pmol of rat Slc30a5-specific siRNA 28 or 200 pmol of control siRNA non-targeting pool (Dharmacon; Thermo Fisher Scientific) or with 2 μg of rZnT5-pcDNA3.1 (to overexpress rZnT5) using nucleofection as described above. After transfection, GH3 cells were plated into 12-well plates at density of 0.15×10^6/well in DMEM + 15% HS + 2.5% FCS. At this point, non-transfected GH3 cells were also plated at the same density.

After 48 h of incubation, transfected cells (with siRNA 28, siRNA non-targeting pool or with rZnT5-pcDNA3.1) were re-transfected with pRL-TK renilla vector (0.05 μg) and 4× MRE pGL3 luciferase reporter (0.8 μg) in Opti-MEM medium using Lipofectamine 2000 (Invitrogen AG). Control (mock) included transfection of non-transfected GH3 cells with pRL-TK renilla vector (0.05 μg) and 4× MRE pGL3 luciferase reporter (0.8 μg). All transfections were incubated further for additional 24 h and then treatment with $ZnSO_4$ (1 μM) for 24 h was performed to activate the promoter as previously described [19, 20]. Finally, cells were lysed and assayed for dual luciferase activities as described by the manufacturer (Promega, Dübendorf, Switzerland).

Immunofluorescence Staining and Confocal Microscopy Analysis

GH3 cells were grown on slide flask Nunc delta, article No. Nunc170920 (Falcon; Nalge Nunc International, Naperville, Ill., USA), and were transfected with 1 μg of the rZnT5-pEGFP-C3 using Lipofectamine 2000 (Invitrogen AG). After 24 h incubation, immunofluorescence staining was performed following quantitative co-localization analysis as described [21]. Polyclonal rabbit anti-turbo GFP (green fluorescence protein) (Evrogen/BioCat, Heidelberg, Germany), rat monoclonal anti-Grp94 (glucose-related protein) (MBL; LabForce, Nunningen, Switzerland), mouse monoclonal anti-bCOP (Sigma-Aldrich, St. Louis, Mo., USA) and mouse monoclonal anti-Rab3a (Synaptic Systems, Göttingen, Germany) antibodies were used, respectively. All conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. and Molecular Probes (West Grove, Pa., USA).

rGH Secretion and Western Blot

Secreted proteins in 48 h conditioned media from siRNA-treated cells were extracted as previously described: 0.8 ml of methanol was added to an aliquot of 0.2 ml of culture medium and the procedure was followed as previously described [22]. At the end, dried protein pellet was solubilized in 50 mM Tris and protein concentration was measured with protein DC Protein Assay (Bio-Rad, Reinach, Switzerland). 50 μg of total protein extract/lane were loaded and separated on 15% SDS-PAGE gel and blotted on Immobilon P transfer membranes (Millipore, Bedford, Mass., USA) using a Trans-Blot Semi-Dry apparatus (Bio-Rad Laboratories, Hercules, Calif., USA). 10 ng of the recombinant rat GH (Prospec; Chemie Brunschwig AG, Basel, Switzerland) was used as control. Membranes were probed with monoclonal mouse anti-rat GH antibody (R&D Systems, Abingdon, UK) and as a secondary antibody, anti-mouse immunoglobulin (Santa Cruz Biotechnology/Labforce AG, Nunningen, Switzerland) was used. Protein bands were visualized by ECL substrate reagent and exposed on ECL Plus films (Amersham Pharmacia Biotech, Dübendorf, Switzerland) and densitometrically quantified using Quantity One software (Bio-Rad Laboratories, Mississauga, Ont., Canada) as follows: densitometrically determined values were first normalized to the total intracellular protein amount and then compared to rrGH. In the second step, the results were normalized to the exact volume of culture medium in which rGH was secreted giving at the end the secretion of rGH in pg/μg of total intracellular protein.

Statistical Analysis

The statistical significance was assessed using two-sided one-way ANOVA test followed by Bonferroni's post hoc comparison tests (* $p < 0.05$, ** $p < 0.01$).

Results

Intracellular Localization of rZnT5 within the Regulated Secretory Pathway

To investigate and analyze possible differences in rZnT5 association with different compartments of the regulated secretory pathway, co-localization of the rZnT5 with ER, Golgi and secretory granules was studied in GH3 cells using confocal microscopy analysis (fig. 1). Cells transiently expressing rZnT5 (with GFP tag) for 24 h were fixed, co-stained with antibodies against GFP and specific organelle markers either for ER, Golgi or secretory granules and confocal microscopy images of independent cells were taken and analyzed. Panel I shows the overall distribution of rZnT5 with the heaviest staining in a perinuclear region, consistent with accumulation in the ER and Golgi. Punc-

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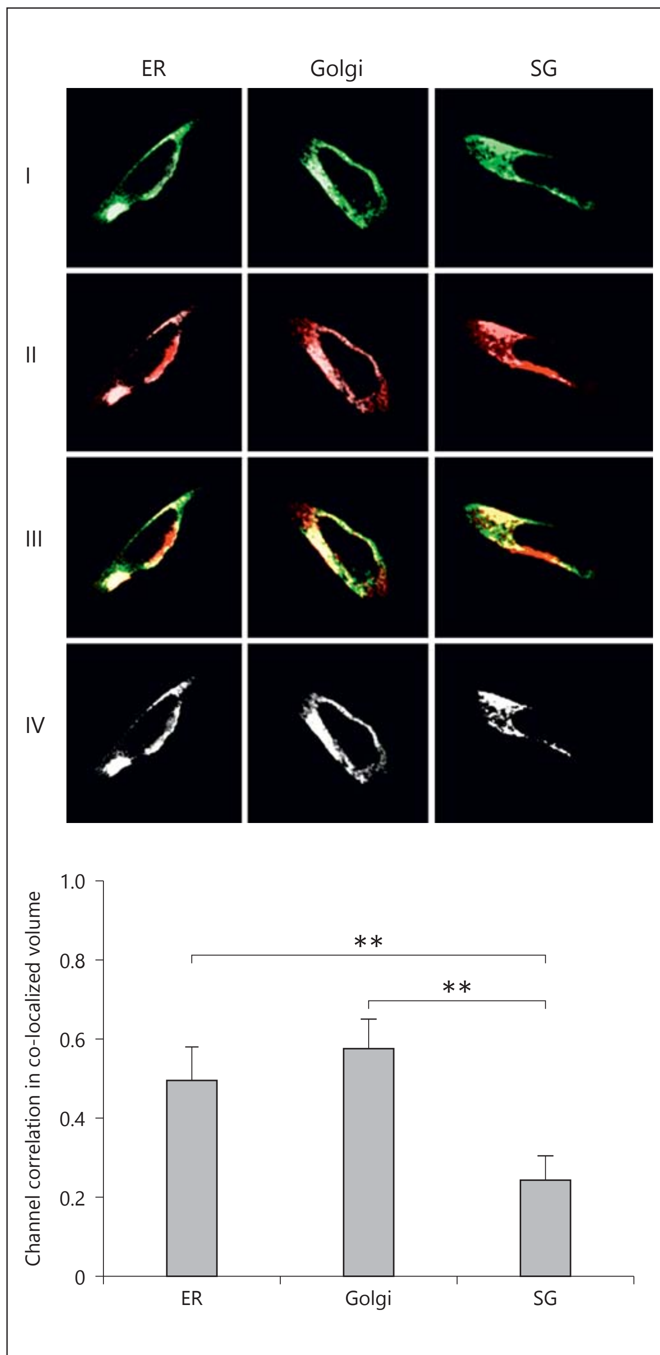

Fig. 1. Subcellular co-localization of rZnT5 with different organelle markers in fixed GH3 cells. Row I: specific staining for rZnT5 is indicated by fluorescent green color (GFP). Row II: specific staining for cell organelles (Grp94-ER marker, βCOP-Golgi marker and Rab3a-secretory granules marker) shown by fluorescent red color (CY3) in the same microscopic field as in row I. Row III: merged images. Row IV: masked areas, in which the intensity of green and red fluorescence was measured in the co-localized area. The degree of co-localization between rZnT5 and a specific organelle is plotted as average of Pearson coefficients of at least 20 independent cells (means ± SD) (** $p < 0.01$).

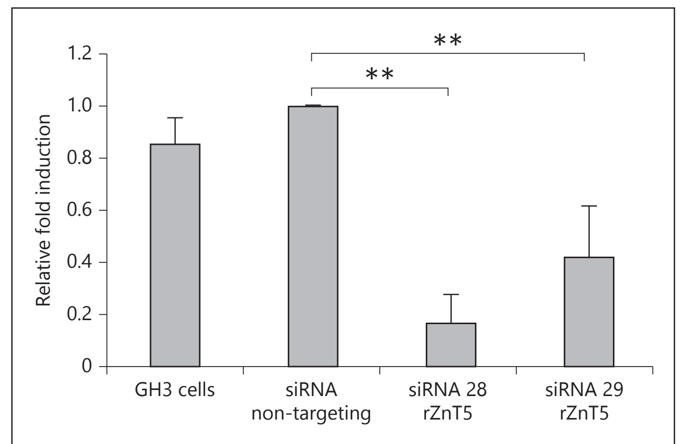

Fig. 2. Knock-down of rSlc30a5 using gene-specific siRNAs analyzed by quantitative real-time PCR (SYBR green). Relative values of rSlc30a5 mRNA expression were compared in GH3 cells transfected with rSlc30a5-specific siRNAs to those transfected with control (siRNA non-targeting). The 18S ribosomal RNA was used to normalize cDNA concentration. The levels of expression were calculated by comparative C_t method relative to 18S. Results are given as individual fold induction over cells transfected with control siRNA. Results are given as the means ± SD of four independent experiments (* $p < 0.05$, ** $p < 0.01$).

tuate staining near the plasma membrane is consistent with accumulation of rZnT5 in secretory granules. The degree of co-localization of two fluorochrome signals (green for rZnT5; red for a given intracellular compartment) was measured from a single cell. The average Pearson correlation coefficients revealed a significantly increased ($p < 0.01$) subcellular co-localization of rZnT5 with ER and Golgi when compared to that of secretory granules. Thus, our confocal microscopy data suggest a higher association of rZnT5 with compartments of the early secretory pathway.

siRNA-Mediated Knock-Down of rSlc30a5 Gene Expression in GH3 Cells

In order to investigate the specific role of ZnT5 in mediating Zn^{2+} transport in and out of the secretory pathway and its possible impact on the process of GH secretion, we knocked down rSlc30a5 expression in GH3 cells using gene-specific siRNAs. Cells were transfected with either siRNA control (siRNA non-targeting) or with rSlc30a5-specific siRNAs (siRNA 28 or siRNA 29). After 48 h of incubation, a significant reduction ($p < 0.01$) of *rSlc30a5* gene expression up to 80% (siRNA 28) or 60% (siRNA 29) was observed when compared to control siRNA as detected by qRT-PCR (fig. 2). Differences in effectiveness of

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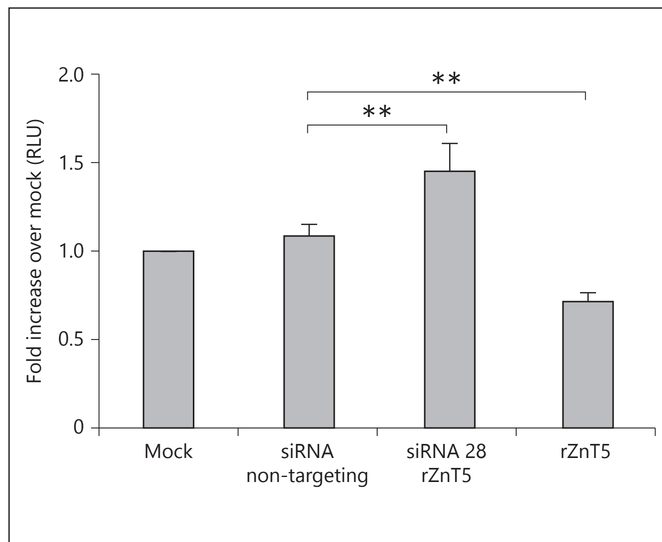

Fig. 3. Alteration of rSlc30a5 expression affects changes in the cytoplasmic Zn^{2+} pool in GH3 cells. Cytoplasmic Zn^{2+} level was measured in GH3 cells with knocked-down or overexpressed rSlc30a5 following transfection with 4× MRE luciferase reporter construct and pRL-TK renilla vector. Mock control corresponds to non-transfected GH3 cells transfected with 4× MRE luciferase reporter construct and pRL-TK renilla vector. 24 h before termination of the experiment, cells were treated with 1 μM $ZnSO_4$ to activate the promoter. Chemiluminescence values were determined using a luminometer (mean relative light units (RLU) determined by the ratio of firefly:renilla luciferase activity). The results are given as the means ± SD of three independent experiments and plotted as a fold change of relative luciferase units (RLU) over mock (* $p < 0.05$, ** $p < 0.01$).

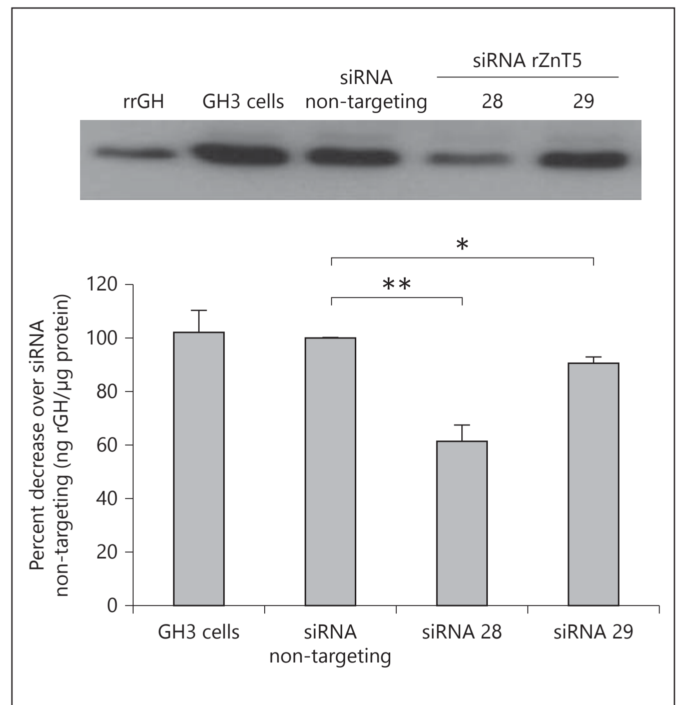

Fig. 4. GH secretion from GH3 cells after rSlc30a5 knock-down. The secretion of GH was measured in culture medium from GH3 cells transfected with rSlc30a5-specific siRNAs, control siRNA or from non-transfected cells. The band corresponding to secreted rGH from cells transfected with control siRNA was quantified and set to 100% and intensity of other bands were compared against this. This experiment was repeated three times and representative blots are shown (* $p < 0.05$).

rSlc30a5 knock-down expression between siRNA 28 and 29 could be attributed to differences in binding to their respective target sequences which can be affected by secondary RNA structure formation or partially masked through binding of various RNA-binding proteins to mRNA.

Alteration of rSlc30a5 Expression Affects Changes in the Cytoplasmic Zn^{2+} Pool

To study whether knock-down of rSlc30a5 or its overexpression affect changes in the cytoplasmic Zn^{2+} pool, a luciferase reporter assay (using 4× MRE luciferase reporter construct) was performed after 48 h of either rSlc30a5 knock-down or overexpression (fig. 3). In this assay an increase in the cytoplasmic Zn^{2+} pool is reflected by higher chemiluminescence activity, arising from increased Zn^{2+} binding to MRE. Accordingly, decreased Zn^{2+} binding to MRE arising from a decrease in the cytoplasmic Zn^{2+} pool would be detected through a lower chemiluminescence activity. Results obtained revealed a significant increase ($p < 0.01$) in luciferase activity in rSlc30a5-attenuated cells while the overexpression of rSlc30a5 led to its significant decrease ($p < 0.01$) compared with mock-transfected cells. Hence, our data indicate that alterations of rSlc30a5 expression have a significant effect on Zn^{2+} uptake and cytoplasmic Zn^{2+} levels in GH3 cells.

Attenuation of rSlc30a5 Expression Affects rGH Secretion from GH3 Cells

Finally, to assess whether attenuated rSlc30a5 expression involved in Zn^{2+} transport within the early secretory pathway has an ultimate impact on the secretion of rGH from GH3 cells, the extracellular secretion of rGH was analyzed by Western blot in proteins extracted from culture medium after rSlc30a5 knock-down using gene-specific siRNAs (fig. 4). Attenuated rSlc30a5 expression evoked 40–45% reduction in extracellular rGH secretion

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($p < 0.01$) when siRNA 28 was used for knock-down and 10–15% reduction ($p < 0.05$) in the case of siRNA 29. Taken together, our secretion data indicate the direct link between attenuated rSlc30a5 expression and decreased rGH secretion, suggesting in that way the direct involvement of ZnT5 in the process of GH secretion.

Discussion

During the process of secretory granule biogenesis, aggregation of a hormone destined for secretion is considered crucial for its proper storage in granules. Protein aggregation takes place in the lumen of *trans*-Golgi layer and studies on PRL aggregation demonstrated that environmental factors like acidic pH and a high concentration of Zn^{2+} are required to induce this process [4, 23]. In general, Zn^{2+} binding to a protein can occur through the low-affinity binding sites (glutamate, aspartate and glutamine residues) [24], or through the high-affinity binding ones (histidine residues) as reported for human GH and PRL [7, 25]. Based on the fact that GH and PRL are two structurally related hormones, there are good predictions that during the process of aggregation, GH might behave in a similar manner as PRL does.

Out of all the ZnT transporters localized to subcellular compartments throughout the secretory pathway, ZnT5-7 have been identified to localize in the ER and Golgi complex [26]. Studies employing Zn^{2+} transport assay using radioactively labelled Zn^{2+} and zinc-staining analysis using fluorescent zinc probes confirmed that ZnT5-7 mediate Zn^{2+} dynamics from the cytoplasm into the early secretory pathway [27, 28] and therefore might be of importance for the process of GH aggregation to occur efficiently. In addition, deletion of ZnT5 gene in mouse leads to abnormal bone development and significantly impaired growth when compared to the wild-type mice [18]. By analyzing specific gene expression patterns of all zinc transporters in GH-producing cells from GH-eGFP transgenic mice using a microarray technique, Robinson and colleagues [29, 30] revealed the expression of ZnT5 to be the strongest in somatotropes [31]. Taken together, these data suggest high involvement of ZnT5 in the processes of GH storage and secretion.

In the present study we aimed first, by using gene-specific siRNAs, to attenuate rSlc30a5 expression in GH3 cells (rat pituitary cell line) and to investigate a possible impact on the secretion of rGH, endogenously produced in these cells. Unlike the human *SLC30A5* gene, which has been reported to have two splice variants (one expressed in the Golgi apparatus and the other expressed throughout the cell, including plasma membrane) [27, 32], rat *Slc30a5* gene encodes for only one rZnT5 variant.

Unlike many other eukaryotic cells, which rapidly release all their secreted proteins immediately after being synthesized, endocrine and neuroendocrine cells store hormones in concentrated forms in secretory granules to be rapidly released when cells are stimulated. Up until now, subcellular localization of ZnT5 has mostly been investigated in non-neuroendocrine cells and found to be primarily associated with the ER and Golgi complex [32–34]. To extend that further to neuroendocrine cells, we studied the association of rZnT5 with the main compartments of the regulated secretory pathway in rat pituitary cells (GH3 cells). Our confocal microscopy analysis performed in neuroendocrine cells confirmed an increased association of rZnT5 with ER and especially with the Golgi complex – the compartments of the early secretory pathway.

In order to fulfil its expected role in the process of GH aggregation, Zn^{2+} needs to be transported in a sufficient amount into the (early) secretory pathway. Hence, it is reasonable to hypothesize that altered expression of rSlc30A5 would consequently evoke changes in the cytoplasmic Zn^{2+} pool. We aimed to test this hypothesis and to detect these changes by using a 4× MRE luciferase reporter construct after rSlc30A5 knock-down or overexpression in our in vitro system. Attenuated rSlc30A5 expression (80% decrease at mRNA level) evoked an increase in the cytoplasmic Zn^{2+} pool as detected through an increased binding of Zn^{2+} to MRE, while rSlc30A5 overexpression led to a decrease in the cytoplasmic Zn^{2+} pool when compared to mock-transfected cells. Thus, our data confirm the important role of rZnT5 in mediating Zn^{2+} influx into intracellular compartments of the regulated secretory pathway.

Considering the important role of rZnT5 in transporting Zn^{2+} from cytoplasm into the compartments of the early secretory pathway, we further investigated the ultimate effect of attenuated rSlc30A5 expression on the extracellular rGH secretion in our in vitro model based on GH3 cells. Despite the fact that the knock-down of rSlc30A5 was not complete (based on qRT-PCR data), these cells displayed reduced rGH secretion (40–45%) when compared to secretion from cells expressing control siRNA. Hence, our data suggest that attenuated expression of rSlc30A5 exerts a direct impact on the secretion of rGH. Of note is that the secretion of rPRL (also endogenously produced and secreted from GH3 cells) in rSlc30A5

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knock-down cells was reduced up to a similar extent as that of rGH (data not shown).

In conclusion, the data presented in this study indicate that the role of ZnT5 in supplying the early secretory pathway with a sufficient amount of Zn^{2+} required for GH aggregation and storage in secretory granules, which is crucial for normal secretion, may be greater than previously anticipated.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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