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The Diabetes Susceptibility Gene *SLC30A8* that Encodes the Zinc Transporter ZnT8 is a Pseudogene in Guinea Pigs Potentially Contributing to Low Guinea Pig Islet Zinc Content

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Abstract

In most mammals pancreatic islet beta cells have very high zinc levels that promote the crystallization and storage of insulin. Guinea pigs are unusual amongst mammals in that their islets have very low zinc content. The selectionist theory of insulin evolution proposes that low environmental zinc led to the selection of a mutation in Guinea pig insulin that negated the requirement for zinc binding. In mice deletion of the *Slc30a8* gene, that encodes the zinc transporter ZnT8, markedly reduces islet zinc content. We show here that *SLC30A8* is a pseudogene in Guinea pigs. We hypothesize that inactivation of the *SLC30A8* gene led to low islet zinc content that allowed for the evolution of insulin that no longer bound zinc.

Keywords

Islet; Zinc; Diabetes; Insulin; Evolution

High islet zinc levels have been reported to be important for multiple aspects of pancreatic islet beta cell function including paracrine signaling from beta to alpha cells (Hardy et al. 2011; Robertson et al. 2011), proinsulin processing (Dunn 2005), crystallization of insulin hexamers (Chausmer 1998; Dunn 2005) and promotion of insulin aggregation (Xu et al. 2012), which may be protective against proteolysis (Zimmerman and Yip 1974). However, the importance of islet zinc appears at odds with the observation that in some mammalian species, for example Guinea pigs (*Cavia porcellus*), islet zinc content is very low (Havu et al. 1977; Zimmerman and Yip 1974). This is thought to be because in these species insulin does not bind zinc due to the absence of a histidine residue in the B10 position of the insulin molecule (Beintema and Campagne 1987; Blundell et al. 1972; Smith 1966). The observation that deletion of *Slc30a8* in mice markedly lowers islet zinc, with little effect on

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Author Contributions

K.E.S, K.J.B, J.K.O all contributed to gene expression analyses and wrote parts of the manuscript. M.S. and R.O'B. designed experiments and wrote parts of the manuscript.

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glucose metabolism (Davidson et al. 2014; Rutter and Chimienti 2015), not only further increased doubt about the importance of zinc for islet biology but also raised the question as to whether Guinea pigs still retained high islet expression of *SLC30A8* despite having low zinc levels. A positive result would imply that both ZnT8 and the ability of insulin to bind zinc are required for high islet zinc levels. It would also suggest an important role of ZnT8 in Guinea pigs islets despite the low zinc content.

As a first step towards determining whether Guinea pig islets still maintain high *SLC30A8* expression, despite low zinc content, we attempted to isolate a Guinea pig *SLC30A8* cDNA. A BLAST search of the NCBI nr and refseq_rna databases using the human *SLC30A8* cDNA sequence (NM_173851) as the query failed to identify any clones. We therefore searched for the Guinea pig *SLC30A8* gene by performing a BLAST search of the NCBI refseq_genomic database using the human *SLC30A8* cDNA sequence as the query. This identified a genomic scaffold that contains the entire Guinea pig *SLC30A8* gene as a contiguous sequence (NT_176419).

Potential exon/intron splice junctions in the putative Guinea pig *SLC30A8* gene were determined by comparison with the human *SLC30A8* (Chimienti et al. 2004) and mouse *Slc30a8* (Pound et al. 2009) genes and the consensus sequence for RNA splicing (Jackson 1991) (Table I). As with the human *SLC30A8* (Chimienti et al. 2004) and mouse *Slc30a8* (Pound et al. 2009) genes, the Guinea pig *SLC30A8* gene appeared to contain 8 exons, 7/8 of which matched the sizes of the human and/or mouse exons (Table I). However, closer inspection of the sequence while attempting to re-construct a putative Guinea pig *SLC30A8* cDNA revealed that the Guinea pig *SLC30A8* gene is a pseudogene (Fig. 1). Notable differences with the human *SLC30A8* open reading frame include a switch from a starting methionine to a valine, the presence of two in-frame stop codons and the deletion of 11 base pairs in exon 7 (Table I) that would result in a frame-shift (Fig. 1). In addition, comparison with the splice junctions identified in the human *SLC30A8* (Chimienti et al. 2004) and mouse *Slc30a8* (Pound et al. 2009) genes reveals changes away from the consensus at three junctions that would be predicted to impair splicing (Table I). Since sequencing of the Guinea pig genome is complete, the possibility that the *SLC30A8* gene underwent duplication in Guinea pigs and that we have failed to identify an intact *SLC30A8* variant appears remote. In addition, this pseudogene is flanked on one side by Guinea pig homologs of *EIF3H, UTP23* and *RAD21* and on the other by homologs of *MED30* and *EXT1,* a relationship and orientation that are consistent with other known mammalian *SLC30A8* genes. The discovery that *SLC30A8* is a pseudogene in Guinea pigs suggests that the inability of Guinea pig insulin to bind zinc and the absence of ZnT8 may both contribute to low islet zinc content.

Evolutionary studies suggest that Guinea pigs are closely related to chinchillas (*Chinchilla lanigera)* and naked mole rats *(Heterocephalus glaber)* (Gorbunova and Seluanov 2009). A BLAST search of the NCBI nr database using the human *SLC30A8* cDNA sequence (NM_173851) identified *SLC30A8* homologs of both chinchillas (XM_013512711) and naked mole rats (XM_013074097) that were derived by computer prediction from genomic scaffolds that contain the entire putative chinchilla (NW_004955417) and naked mole rat (NW_004624763) *SLC30A8* genes as contiguous sequences. A BLAST search of the NCBI

refseq_genomic database using the human *SLC30A8* cDNA sequence as the query showed that, even if expressed, chinchilla and naked mole rat *SLC30A8* mRNAs would contain frameshift mutations. This suggests that *SLC30A8* is also a pseudogene not only in Guinea pigs but also in evolutionarily-related chinchillas and naked mole rats. However, this conclusion will only be proven once the complete sequencing of the chinchilla and naked mole rat genomes have ruled out the existence of gene duplication events.

The demonstration that *SLC30A8* is a pseudogene in Guinea pigs has interesting implications for the neutralist and selectionist theories of insulin evolution (Chan et al. 1984). The selectionist theory is based on the idea that a local environmental deficit in zinc availability drove adaptations in the Guinea pig insulin molecule that removed the requirement to bind zinc (Chan et al. 1984). This theory appears weak since zinc is required for the activity of multiple enzymes such that the effects of zinc deprivation would be predicted to be broad and severe. In addition, zinc has a ubiquitous environmental distribution making deprivation unlikely. The demonstration that deletion of *Slc30a8* in mice markedly lowers islet zinc levels (Davidson et al. 2014; Rutter and Chimienti 2015) implies that inactivation of the Guinea pig *SLC30A8* gene could have created a local cellular deficit in zinc that allowed for the evolution of a Guinea pig insulin that no longer bound zinc, consistent with the selectionist theory. On the other hand, if sequencing of the chinchilla and naked mole rat genomes confirm that *SLC30A8* is a pseudogene in those species, and if biochemical studies demonstrate the predicted low islet zinc levels, it would be hard to reconcile this low zinc environmental selectionist theory of insulin evolution with the fact that both chinchilla (XP_005384246) and naked mole rat (XP_004852149) insulin have retained the B10 histidine that is critical for zinc binding.

In mice individual deletion of *Slc30a8* or *Slc30a7,* which encodes ZnT7, markedly reduces islet zinc content but has little effect on glucose-stimulated insulin secretion (GSIS) (Syring et al. 2016). However, deletion of *Slc30a8* in combination with *Slc30a7* abolishes GSIS (Syring et al. 2016). This further suggests that high islet zinc levels are not important for GSIS and that ZnT7 can compensate for the absence of ZnT8 in islets. We therefore next explored the possibility that ZnT7 may be able to compensate for the absence of ZnT8 in Guinea pigs as it can in mice. A BLAST search of the NCBI nr database using the human *SLC30A7* cDNA sequence (NM_133496) as the query identified a computer predicted Guinea pig *SLC30A7* cDNA (XM_003479176). The computer predicted sequence generates a peptide that differs at 4 amino acids that are highly conserved in other species leading us to suspect that the computer prediction was incorrect. We therefore cloned a Guinea pig *SLC30A7* cDNA (KY847522). An alignment of human and Guinea pig ZnT7 shows 96.5% amino acid identity, including conservation of all 4 ambiguous amino acids (data not shown). Similar BLAST searches identified predicted chinchilla (XM_005388805) and naked mole rat (XM_004869139) *SLC30A7* cDNAs that encode proteins showing 95% and 97% amino acid identity with human ZnT7, respectively. Guinea pig *SLC30A7* is expressed in multiple tissues, namely brain, liver, pancreas and testis (data not shown). Mouse *Slc30a7* is expressed in the same tissues (data not shown) whereas mouse *Slc30a8* is predominantly expressed in pancreas (data not shown). No signal was detected using primers to Guinea pig *SLC30A8* as predicted (data not shown). These results suggest that ZnT7 may be able to compensate for the absence of ZnT8 in Guinea pigs as it can in mice.

Flannick *et al.* (Flannick et al. 2014) have strikingly shown that *SLC30A8* haploinsufficiency is protective against the development of type 2 diabetes (T2D) in humans. Because *SLC30A8* is expressed most highly in islets it has been assumed that this protection against T2D is mediated through an effect on islet function. However, multiple groups have shown that deletion of *Slc30a8* in mice has little or no effect on GSIS (Davidson et al. 2014; Rutter and Chimienti 2015) leading to the hypothesis that ZnT8 is not essential for islet function and that *SLC30A8* haploinsufficiency may affect T2D susceptibility through actions in other tissues where it is expressed at low levels rather than through effects on pancreatic islet function (Syring et al. 2016). The observation that *SLC30A8* is a pseudogene in Guinea pigs (Table I; Fig. 1), naked mole rats and chinchillas supports the hypothesis that ZnT8 is not essential for islet function.

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Abbreviations:

GSIS	glucose-stimulated insulin secretion
T2D	type 2 diabetes
SNP	single nucleotide polymorphism
GWAS	genome-wide association studies

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Guinea Pig  1    VESLERTYLVTDKDIRMYDCTLESMELQRNTANKDRGPGKRSEQ-EPGGIYHCHSSSKAT  59
                 +E LERTYLV DK  +MY  TLES+ELQ+   NKD+ P +R E+ E GG+YHCHS SK T
Human       1    MEFLERTYLVNDKAAKMYAFTLESVELQQKPVNKDQCPRERPEELESGGMYHCHSGSKPT  60

Guinea Pig  60   ENRTKEQVGGRWKLGVAWAICLLLVIAEVVVGHIAGSLAIITGAAHLLVDLTNFLLCLFS  119
                 E    E    +WKL  A AIC + +IAEVV GHIAGSLA++T AAHLL+DLT+FLL LFS
Human       61   EKGANEYAYAKWKLCSASAICFIFMIAEVVGGHIAGSLAVVTDAAHLLIDLTSFLLSLFS  120

Guinea Pig  120  LWLSTRSSSKKLTFGWYQAEIFGALLSILCICVMTRLLMDLVSGHLLCPDWXIQAILLII  179
                 LWLS++  SK+LTFGW++AEI GALLSILCI V+T +L+ L    LL PD+ IQA ++II
Human       121  LWLSSKPPSKRLTFGWHRAEILGALLSILCIWVVTGVLVYLACERLLYPDYQIQATVMII  180

Guinea Pig  180  ISGCAVVADVVINVLLHQRCLGHNHKKVXVNASVRAAFMHTLGDVLDSISVLTSTLVTYF  239
                 +S CAV A++V+ V+LHQRCLGHNHK+V  NASVRAAF+H LGD+  SISVL S L+ YF
Human       181  VSSCAVAANIVLTVVLHQRCLGHNHKEVQANASVRAAFVHALGDLFQSISVLISALIIYF  240

Guinea Pig  240  KTDYRIADLICMFVFTILVLGSTITIVKNFTIVLMEGVAKGLSYSYVKTLILAINGVZZZ  299
                 K +Y+IAD IC F+F+ILVL STITI+K+F+I+LMEGV K L+YS VK LILA++GV
Human       241  KPEYKIADPICTFIFSILVLASTITILKDFSILLMEGVPKSLNYSGVKELILAVDGVLSV  300

Guinea Pig  300  KGLNLCSLTMNQVMLLVKVTAVASWDSQIVWRGVTRALSKSFTIYLLTIQMESLADQNPK  359
                   L++ SLTMNQV+L   V   AS DSQ+V R + +ALSKSFT++ LTIQMES  DQ+P
Human       301  HSLHIWSLTMNQVILSAHVATAASRDSQVVRREIAKALSKSFTMHSLTIQMESPVDQDPD  360

Guinea Pig  360  CFLCEDPKD  368

                          C  CEDP D
Human       361  CLFCEDPCD  369
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Figure 1. Alignment of the Human and Non-Functional Guinea Pig ZnT8 Peptide Sequences.

The alignment of the peptide sequence of human ZnT8 (Accession Number NM_173851) with the non-functional Guinea pig ZnT8 is shown. Identities are indicated by matching letters and similarities by the '+' symbol. The two stop codons (X) in the Guinea pig peptide sequence are shown in red. An 11 bp deletion in the putative Guinea pig exon 7 deletes 3 amino acids (indicated by ZZZ in blue) and creates a frameshift mutation. If translated, the open reading frame would be maintained 3 ' of this frameshift as shown. Putative transmembrane domains predicted using TOPCONS web server (Tsirigos et al. 2015) are shaded in gray. The locations of the 6 transmembrane domains are somewhat different to those previously predicted (Chimienti et al. 2004).

Table I.

Comparison of the Exon/Intron Boundaries of the Human and Guinea Pig *SLC30A8* Genes.

Intron	Species	5′ Exon/Intron Junction	3′ Intron/Exon Junction	Exon	Exon Size (bp)
A	Human	CACTAGAAAG/gtaatagatg	tcatccatag/TGTGGAACTCCAA	1/2	71/200
	Mouse	CTCTAGACAG/gtaagaagat	tcctcaacag/AGAACTTCGA		71/197
	Guinea Pig	CCCTGGAGAG/gtaagagaca	tcatccacag/TATGGAACTC		71/197
B	Human	GAGGTCGTGG/gtgagtcttt	cattctctag/GTGGGCACAT	2/3	200/147
	Mouse	GAGGTGGTGG/gtgagtactg	catctcacag/GTGGACACGT		197/147
	Guinea Pig	GAAGTCGTGG/gtaagtacgg	acccctgtag/TTGGGCACAT		197/147
C	Human	CACCGAGCAG/gtacggttca	gaattcctag/AGATCCTTGG	3/4	147/154
	Mouse	TATCGAGCAG/gtaacattct	tgactcccag/AGATCCTCGG		147/154
	Guinea Pig	TACCAAGCAG/gtacagttct	gaattctcac/AGATTTTTGG		147/154
D	Human	CCAACATTGT/gtaagtcatc	tctctttcag/ACTAACTGTG	4/5	154/151
	Mouse	CCAACATTGT/gtaagtcata	tctctttcag/ACTAACTATG		154/151
	Guinea Pig	CCGACGTTGT/tgtgtaagta	tctttttcag/AATAAATGTG		154/151
E	Human	CTACTTTAAG/gtgagtttga	ttttttctag/CCAGAGTATA	5/6	151/106
	Mouse	CTACTTTAAG/gtgagtgtgt	tgttttccag/CCTGACTACA		151/106
	Guinea Pig	CTACTTTAAG/gtgaatgtga	tgttttccag/ACAGATTACA		151/106
F	Human	CTCATGGAAG/gtaggagtga	cttttgtcag/GTGTGCCAAA	6/7	106/135
	Mouse	CTCATGGAAG/gtaggactgc	cctttgtcag/GTGTTCCAAA		106/135
	Guinea Pig	CTCATGGAAG/ataggaggga	ccttcatcag/GTGTAGCCAA		106/124
G	Human	GTTGCTACAG/gtcagtgagt	ttatcaacag/CAGCCAGCCG	7/8	135/146
	Mouse	GTTGCTACAG/gtcagtgagt	ttatcaacag/CTGCCAGCCA		135/143
	Guinea Pig	GTCACTGCAG/gtcactgagc	ctaccatcag/TGGCCAGCTG		124/146
	Consensus	(A or T)G/gtaa	cag/G		

Exon and intron sequences are shown in uppercase and lowercase letters, respectively. The 5′ and 3′ consensus splice sequences are from Jackson et al. (Jackson 1991). The sizes of exons 1 and 8 represent just the coding sequence within these exons and do not include untranslated regions. Differences between human and mouse are shown in red, differences between human and Guinea pig are shown in blue. Both mouse and Guinea pig exon 2 encode one less amino acid than human exon 2 but the changes occur in different locations.