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Evidence That Evolution of the Diabetes Susceptibility Gene *SLC30A8* that Encodes the Zinc Transporter ZnT8 Drives Variations in Pancreatic Islet Zinc Content in Multiple Species

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Abstract

Pancreatic islet zinc levels vary widely between species. Very low islet zinc levels in Guinea pigs were thought to be driven by evolution of the *INS* gene that resulted in the generation of an isoform lacking a histidine at amino acid 10 in the B chain of insulin that is unable to bind zinc. However, we recently showed that the *SLC30A8* gene, that encodes the zinc transporter ZnT8, is a pseudogene in Guinea pigs, providing an alternate mechanism to potentially explain the low zinc levels. We show here that the *SLC30A8* gene is also inactivated in sheep, cows, chinchillas and naked mole rats but in all four species a histidine is retained at amino acid 10 in the B chain of insulin. Zinc levels are known to be very low in sheep and cow islets. These data suggest that evolution of *SLC30A8* rather than *INS* drives variation in pancreatic islet zinc content in multiple species.

Keywords

Islet; Zinc; Diabetes; Insulin; Evolution

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Contributions

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

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In most mammals pancreatic islet beta cells have very high zinc levels that are thought to be important for 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, in Guinea pigs (*Cavia porcellus*) islet zinc content is very low (Havu et al. 1977). This was thought to be because insulin in Guinea pigs does not bind zinc due to the replacement of a histidine residue at amino acid 10 in the B chain of the insulin molecule with asparagine (Beintema and Campagne 1987; Blundell et al. 1972; Smith 1966; Zimmerman and Yip 1974). This observation gave rise to the neutralist and selectionist theories of Guinea pig insulin evolution (Chan et al. 1984). However, we recently showed that the *SLC30A8* gene, which has been linked with altered diabetes susceptibility in humans (Flannick et al. 2014; Sladek et al. 2007) and encodes the zinc transporter ZnT8, is a pseudogene in Guinea pigs (Syring et al. 2018). Since deletion of the *Slc30a8* gene markedly reduces islet zinc content (Davidson et al. 2014; Rutter and Chimienti 2015) we speculated that inactivation of the *SLC30A8* gene in Guinea pigs led to low islet zinc content that subsequently allowed for the evolution of insulin that no longer bound zinc (Syring et al. 2018). To address the hypothesis that evolution of *SLC30A8* rather than *INS* drove variation in pancreatic islet zinc content we compared the structure of the *SLC30A8* and *INS* genes with islet zinc content in multiple species.

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 was performed to identify the precise location of the exons in the chinchilla and naked mole rat *SLC30A8* genes (Table 1; Supplemental Figure 1). Potential exon/intron splice junctions in the putative chinchilla and naked mole rat *SLC30A8* genes were determined by comparison with the human (Chimienti et al. 2004), mouse (Pound et al. 2009) and Guinea pig (Syring et al. 2018) *SLC30A8* genes and the consensus sequence for RNA splicing (Jackson 1991) (Table 1). As with the human (Chimienti et al. 2004), mouse *Slc30a8* (Pound et al. 2009) and Guinea pig (Syring et al. 2018) *SLC30A8* genes, the chinchilla and naked mole rat *SLC30A8* genes appeared to contain 8 exons (Table 1). The sizes of these exons in chinchillas and naked mole rats match those in humans for 7/8 and 4/8 exons, respectively (Table 1). However, the chinchilla *SLC30A8* gene contains a single base pair deletion in exon 6 (Supplemental Figure 1) that results in a frame shift and premature stop codon and consequently a mRNA that would encode a truncated ZnT8 variant (Supplemental Figure 2). A similar analysis of the naked mole rat *SLC30A8* gene revealed the presence of multiple mutations, namely an insertion in exon 2, a five base pair deletion in exon 4, a fifteen base pair deletion in exon 7, and a 25 base pair deletion plus the absence of the TAG stop codon that is normally present in exon 8 (Supplemental Figure 1). These mutations would result in

the generation of a mRNA that would encode a truncated ZnT8 variant (Supplemental Figure 2). In addition, comparison with the splice junctions identified in the human *SLC30A8* gene (Chimienti et al. 2004) reveals changes away from the consensus at two junctions that would be predicted to impair splicing (Table 1). PCR using chinchilla and naked mole rat genomic DNA as the template was used to amplify exons 6 and 4, respectively. In both cases DNA sequencing (Accession numbers MK984684 and MK984685, respectively) confirmed the existence of mutations indicating that they were not sequencing errors. These observations indicated that *SLC30A8* is also a pseudogene not only in Guinea pigs but also in evolutionarily-related chinchillas and naked mole rats. Even though Guinea pigs, chinchillas and naked mole rats are evolutionarily related, an inspection of the location of mutations in their *SLC30A8* pseudogenes does not reveal the presence of a shared mutation that might have been the primary event in gene inactivation.

Other species included in the same evolutionary branch as Guinea pigs, chinchillas and naked mole rats include capybara (*Hydrochoerus hydrochaeris*), North American porcupines (*Erethizon dorsatum*), and nutria (coypu; *Myocastor coypu*). Unfortunately, *SLC30A8* genomic sequence is unavailable for these species. However, because these species are all herbivores we speculated that the nature of the diet in herbivores in some way negates the need for ZnT8 and storage of insulin in a crystalline form with zinc. To test this prediction, we examined the structure of the *SLC30A8* gene in two other groups of herbivores for which genomic sequence is available and that are evolutionarily related, but are on distinct evolutionary branches, to Guinea pigs. One branch includes sheep (*Ovis aries*), bison (*Bison bison*), cows (*Bos taurus*) and alpaca (*Vicugna pacos*). The other branch includes rabbits (*Oryctolagus cuniculus*) and pika (*Ochotona princeps*).

A BLAST search of the NCBI nr database using the human *SLC30A8* cDNA sequence (NM_173851) identified *SLC30A8* homologs of sheep (XM_004011988), bison (XM_010863549) and cows (XM_015474502) that were derived by computer prediction from genomic scaffolds that contain the entire putative sheep (NC_040260), bison (NW_011495050) and cow (NC_037341) *SLC30A8* genes as contiguous sequences. A BLAST search of the NCBI refseq_genomic database using the human *SLC30A8* cDNA sequence as the query was performed to identify the precise location of the exons in the sheep, bison and cow *SLC30A8* genes (Table 1; Supplemental Figure 1). As with the human *SLC30A8* gene (Chimienti et al. 2004), the sheep and cow, but not bison, *SLC30A8* genes appeared to contain 8 exons (Table 1). The sizes of the exons in sheep, bison and cows match those in humans at 6/8, 5/8 and 5/8 exons, respectively (Table 1). However, the sheep *SLC30A8* gene contains an in frame stop codon in exon 3, a single base pair deletion in exon 4, and a single base pair insertion in exon 6 (Supplemental Figure 1). These same mutations are present in two other, independent genomic scaffolds (NC_040260 and NW_020995975). A similar analysis of the bison *SLC30A8* gene revealed the presence of multiple mutations, namely a single base pair deletion in exon 4, a two base pair deletion in exon 7, and the complete absence of exon 3 (Supplemental Figure 1). In addition, comparison with the splice junctions identified in the human *SLC30A8* gene (Chimienti et al. 2004) reveals changes away from the consensus at one junction that would be predicted to impair splicing (Table 1). A similar analysis of the cow *SLC30A8* gene also revealed the presence of multiple mutations, namely single base pair deletions in exons 4 and 8, and a

two base pair deletion in exon 7 (Supplemental Figure 1). PCR using sheep, bison and cow genomic DNA as the template was used to amplify exons 4, 7 and 7, respectively. In each case DNA sequencing (Accession Numbers MK984686, MK984687 and MK984688, respectively) confirmed the existence of the mutations indicating that they were not sequencing errors. For all three species the identified mutations would result in the generation of mRNAs that would encode truncated ZnT8 variants (Supplemental Figure 2). These observations suggest that *SLC30A8* is also a pseudogene not only in Guinea pigs but also in evolutionarily distantly related sheep, bison and cows.

Even though sheep, bison and cows are evolutionarily related an inspection of the mutations in their *SLC30A8* pseudogenes does not reveal the presence of a shared mutation that might have been the primary event in gene inactivation. However, in bison and cows the location of the single base pair deletion in exon 4 and two base pair deletion in exon 7 are identical (Supplemental Figure 1). Supporting evidence for this conclusion that the *SLC30A8* gene is inactivated in sheep and cows comes from the observation that islet zinc content is low in sheep (Galabova et al. 1971) and cows (Petkov et al. 1970), consistent with the observation that deletion of *Slc30a8* markedly decreases zinc content (Davidson et al. 2014; Rutter and Chimienti 2015).

A very different outcome was observed when these genome analyses were repeated in alpacas. In this herbivore a BLAST search of the NCBI nr database using the human *SLC30A8* cDNA sequence (NM_173851) identified a *SLC30A8* homolog (XM_006210943) that was derived by computer prediction from a genomic scaffolds that contain the entire putative alpaca (NW_005882820) *SLC30A8* gene as a contiguous sequence. The cDNA contains no in frame stop codons (Supplemental Figure 2) and the gene contains 8 exons whose sizes precisely match those in humans (data not shown) suggesting that the *SLC30A8* gene is intact in alpacas. This result suggests that our hypothesis is incorrect and the nature of the diet does not negate the need for ZnT8 in all herbivores.

Another species in the same evolutionary branch as sheep, bison, cows and alpaca are pigs (*Sus scrofa*). Pigs are omnivores and have high islet zinc content (Petkov and Galabova 1969). The *SLC30A8* gene appears to be intact in this species (Supplemental Figure 2), consistent with a previous report (Schweiger et al. 2013). These data suggest that in this evolutionary branch inactivation of the *SLC30A8* gene is restricted to a sub-group of species, namely sheep, bison and cows.

A similar outcome was observed when these genome analyses were repeated in rabbits and pika. In these herbivores a BLAST search of the NCBI nr database using the human *SLC30A8* cDNA sequence (NM_173851) identified *SLC30A8* homologs of rabbits (XM_008255843) and pika (XM_004580693) that were derived by computer prediction from genomic scaffolds that contain the entire putative rabbit (XM_008255843) and pika (XM_004580693) *SLC30A8* genes as contiguous sequences. In both cases the predicted cDNAs contain no in frame stop codons (Supplemental Figure 2) and the genes contain 8 exons whose sizes precisely match those in humans (data not shown) suggesting that the *SLC30A8* gene is intact in these species. Supporting evidence for the conclusion that the

SLC30A8 gene is active in rabbits comes from the observation that rabbit islet zinc content is very high (Ynsa et al. 2009). This result confirms that our hypothesis was incorrect and the nature of the diet does not negate the need for ZnT8 in all herbivores.

Other species in the same evolutionary branch as Guinea pigs, chinchillas, naked mole rats, rabbits and pika include rats (*Rattus norvegicus*) and Chinese hamsters (*Cricetulus griseus*). Both are omnivores and both have high islet zinc content (Havu et al. 1977). The *SLC30A8* gene appears to be intact in both species (Supplemental Figure 2). These data suggest that in this evolutionary branch inactivation of the *SLC30A8* gene is restricted to a sub-group of species, namely Guinea pigs, chinchillas and naked mole rats.

Expanding this analysis more broadly we found that the *SLC30A8* gene appears to be intact in hedgehogs (*Erinaceus europaeus*), horses (*Equus caballus*), dogs (*Canis lupus familiaris*), pumas (*Puma concolor*), elephants (*Loxodonta africana*), chickens (*Gallus gallus*) and frogs (*Xenopus tropicalis*) (Supplemental Figure 2). These species represent a mix of herbivores, omnivores and carnivores. Overall, these data suggest that the *SLC30A8* gene has been retained broadly across different species and there appear to be no clear patterns or transitions in *SLC30A8* evolution across the mammalian phylogeny.

The chinchilla, naked mole rat, sheep, bison and cow pseudogenes are flanked on one side by a homolog of *RAD21* and on the other by a homolog of *MED30*. This relationship and the orientation of these *SLC30A8* pseudogenes relative to the *RAD21* and *MED30* genes is consistent with intact *SLC30A8* genes including those in mice, humans, horses, chickens and frogs. Since sequencing of the genomes for chinchillas, naked mole rats, sheep, bison and cows is nearly complete, the possibility that the *SLC30A8* gene underwent duplication in these species and that we have failed to identify an intact *SLC30A8* variant appears low. These data strongly suggest that the *SLC30A8* gene is a pseudogene in all five species.

An analysis of *INS* mRNA in chinchillas (XM_005384189), naked mole rats (XM_004852092), sheep (XM_012167536), and cows (NM_173926) shows that in every case amino acid 10 in the B chain of the insulin molecule is a histidine, in contrast to Guinea pigs where this amino acid is an asparagine (Beintema and Campagne 1987; Blundell et al. 1972; Smith 1966; Zimmerman and Yip 1974). Both chinchilla and cow insulin have been shown to form crystals with zinc (Wood et al. 1975). The sequence of *INS* mRNA has not been reported in bison. These data support the hypothesis that evolution of *SLC30A8* rather than *INS* has driven the low pancreatic islet zinc content in these species. Given this low islet zinc content it is unclear why the insulin molecule in these species appears to have retained the ability to bind zinc in contrast with Guinea pigs.

A key unanswered question is whether the *SLC30A8* pseudogenes in Guinea pigs, chinchillas, naked mole rats, sheep, bison and cows are expressed and, if so, whether the predicted truncated proteins are generated (Supplemental Figure 2). Unfortunately, relatively few *SLC30A8* mRNAs have been identified in any of the species described above, even those with intact *SLC30A8* genes (Supplemental Figure 3). This may be because, based on data from mice and humans, *SLC30A8* is only highly expressed in pancreatic islets. Partial cDNAs have been reported for chinchillas and cows but in both cases these cDNAs represent

the products of alternate splicing events (see Supplemental Figure 3 for details). Importantly, sequence analysis of the cow cDNA reveals the presence of the same single base pair deletion in exon 4 as seen in cow genomic DNA sequence (see Supplemental Figure 3).

Future studies will explore whether mutation of the key B chain histidine in the presence of an intact *SLC30A8* gene is sufficient to lower islet zinc content. Thus, in Guinea pigs we cannot discount the possibility that inactivation of *SLC30A8* and the inability of Guinea pig insulin to bind zinc both contribute to low islet zinc content.

From an evolutionary perspective the key unanswered issue is the nature of the benefit conferred to Guinea pigs, chinchillas, naked mole rats, sheep, bison and cows by the loss of the *SLC30A8* gene and high islet zinc content. Or put another way, the benefit conferred to humans, mice, rabbits and pika by retaining high islet zinc content.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.
Comparison of the Exon/Intron Boundaries of *SLC30A8* Genes from Multiple Species

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 values given in the exon size column refer to the exon sizes either side of the splice junction. 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 other species are shown in blue. NMR, naked mole rat.

Intron	Species	5' Exon/Intron Junction	3' Intron/Exon Junction	Exon	Exon Size (bp)
A	Human	CACTAGAAAG/gtaataagatg	tcatccatag/TGTGGAAGCTC	1/2	71/200
	Guinea Pig	CCCTGGAGAG/gtaagagaca	tcatccacag/TATGGAAGCTC		71/197
	Chinchilla	CATTAGACAG/gtaagagaca	tcatccacag/TATCGAAGCTC		71/200
	NMR	CACTAGGCAG/gtaacagatg	gcatccacag/TATGGAAGCTT		71/296
	Sheep	CACTAGACAG/gtaacagaca	tcctccacag/CGTGGAAGCTC		71/200
	Bison	CACTAGACAG/gtaacagaca	tcctccacag/CCTGGAAGCTC		71/200
	Cow	CACTAGACAG/gtaacagaca	tcctccacag/CCTGGAAGCTC		71/200
B	Human	GAGGTCGTGG/gtgagtcttt	cattctctag/GTGGGCACAT	2/3	200/147
	Guinea Pig	GAGTCGTGG/gtaagtacgg	acccctgtag/TTGGGCACAT		197/147
	Chinchilla	GAGGTCGTGG/gtaagtactg	ctccccatag/GTGGGCACAT		200/147
	NMR	GAGGTGTGG/atatgacccc	acaccccttag/GTGGGCACAT		296/147
	Sheep	GAGGTCGTAG/gtgagtattt	atcccccttag/GTGGGCACAT		200/147
	Bison	GAGGTCGTGG/gtgagtactt	-----/-----		200/---
	Cow	GAGGTCGTGG/gtgagtactt	atcccccttag/GTGGGCACAT		200/147
C	Human	CACCGAGCAG/gtacggttca	gaattcctag/AGATCCTTGG	3/4	147/154
	Guinea Pig	TACCAAGCAG/gtacggttct	gaattctcac/AGATTTTGG		147/154
	Chinchilla	CACCGAGCAG/gtacggttct	gaaactacag/AGATCCTTGG		147/154
	NMR	CACTAAGCAG/gttcactgat	gaatccacag/AGTCCTCGG		147/149
	Sheep	CGCTGAGCAG/gtatagttca	gtatccctag/AGATCCTGGG		147/153
	Bison	-----/-----	atatacctag/AGATCCTTGG		---/153
	Cow	CGCCGAGCAG/gtatagttca	atatacctag/AGATCCTTGG		147/153
D	Human	CCAACATTGT/gtaagtcac	tctctttcag/ACTAAGTGTG	4/5	154/151
	Guinea Pig	CCGACGTTGT/tgtgtaagta	tctttttcag/AATAAAGTGTG		154/151
	Chinchilla	CCAGTGCTGT/gtaagtcacc	tctctttcag/AATAAGTGTG		154/151
	NMR	GCAATGTTGT/gtaagtcacc	tctctttcag/AATAAGTGTG		149/151
	Sheep	CC-ACATTAT/gtaagcgccc	tttctttcag/ACTAAGTGTG		153/151
	Bison	CCAACAATAT/gtaagtagcc	tttctttcag/ACTAAGTGTG		153/151
	Cow	CCAACAATAT/gtaagtagcc	tttctttcag/ACTAAGTGTG		153/151
E	Human	CTACTTTAAG/gtgagtttga	ttttttctag/CCAGAGTATA	5/6	151/106
	Guinea Pig	CTACTTTAAG/gtaaatgtga	tgttttccag/ACAGATTACA		151/106
	Chinchilla	CCACTTTAAG/gtgagtggat	tgttttccag/CCAGACTACA		151/105
	NMR	CTCCTTTAAG/atgagtgtgt	tattttccag/CTTGACTACA		151/106
	Sheep	TTACTTTAAG/gtgagtatat	ttttttctag/CCAGAGTATA		151/107
	Bison	CTACTTTAAG/gtgagtatat	ttttttctag/CCAGAGTATA		151/106
	Cow	CTACTTTAAG/gtgagtatat	ttttttctag/CCAGAGTATA		151/106
F	Human	CTCATGGAAG/gtaggagtga	cttttgcag/GTGTGCCAAA	6/7	106/135
	Guinea Pig	CTCATGGAAG/ataggaggga	ccttcatcag/GTGTAGCCAA		106/124
	Chinchilla	CTCATGGAAG/gtaggagaga	cctgctcag/GGGACCCAA		105/135
	NMR	CTCATGCAAG/gtaggaggga	tttttgcag/GTGTACCCAA		106/120
	Sheep	TTCATGGAAG/gtaggagtga	cttttgcag/GTGTGCCAAA		107/135
	Bison	CTCATGGAAG/gtaggagtga	cttttagtgg/GTGTGCCAAA		106/133
	Cow	CTCATGGAAG/gtaggagtga	ctttcgagg/GTGTGCCAAC		106/133
G	Human	GTTGCTACAG/gtcagtgaat	ttatcaacag/CAGCCAGCCG	7/8	135/146
	Guinea Pig	GTCACTGCAG/gtcactgagc	ctaccatcag/TGGCCAGCTG		124/146
	Chinchilla	GTTGCTACAG/gtcaggagac	ttaccaacag/TGGCCGGCTG		135/146
	NMR	GTTGCCACAG/gtcagtgaat	ttaccaacag/CGACAGCCCA		120/???
	Sheep	ATTGCTGCAG/gtcagtgaat	ttatcaacag/CCGCCAGCCG		135/146
	Bison	GCTGCTGCAG/gtcagtgaat	ttatcaacag/CAGCCAGCCG		133/146
	Cow	GCTGCTGCAG/gtcagtgaat	ttatcaacag/CAGCCAGCCG		133/145
Consensus		(A or T)G/gtaa	cag/G		