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Generation of a *Slc39a8* Hypomorph Mouse: Markedly Decreased ZIP8 $\text{Zn}^{2+}/(\text{HCO}_3^-)_2$ Transporter Expression

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Abstract

Previously this laboratory has identified the mouse *Slc39a8* gene encoding the ZIP8 transporter, important in cadmium uptake. ZIP8 functions endogenously as a electroneutral $\text{Zn}^{2+}/(\text{HCO}_3^-)_2$ symporter, moving both ions into the cell. The overall physiological importance of ZIP8 remains unclear. Herein we describe generation of a mouse line carrying the *Slc39a8(neo)* allele, containing the *Frt*-flanked neomycin-resistance (*neo*) mini-cassette in intron 3 and *loxP* sites in introns 3 and 6. Cre recombinase functions correctly in *E. coli* and in adeno-Cre-infected mouse fetal fibroblasts, but does not function in the intact mouse for reasons not clear. *Slc39a8(neo)* is a hypomorphic allele, because *Slc39a8(neo/neo)* homozygotes exhibit dramatically decreased ZIP8 expression in embryo, fetus, and visceral yolk sac—in comparison to their littermate wild-type controls. This ZIP8 hypomorph will be instrumental in studying developmental and in utero physiological functions of the ZIP8 transporter.

INTRODUCTION

Cadmium (**Cd**, Cd^{2+}) is classified by IARC as a “Category I” human lung carcinogen. Individuals at highest risk for Cd-induced lung cancer and chronic renal disease include cigarette smokers, those on a diet rich in high-fiber foods or contaminated shellfish, women having low body-iron stores, and malnourished populations [1-4]. It has long been known that Cd causes damage to the central nervous system, lung, bone, gastrointestinal tract, liver, ovary, testis, placenta, and developing embryo [5; 6]. Chronic Cd exposures [7] cause renal proximal tubular metabolic acidosis and osteomalacia (renal Fanconi syndrome).

Recent studies in the mouse showed a relationship between a specific genotype (*Slc39a8* allelic differences) and phenotype (susceptibility to Cd-induced testicular necrosis). Starting with genetically “sensitive” vs “resistant” mice [8], our lab confirmed that the major locus (*Cdm*) was responsible for this trait [9]. We identified by positional cloning the *Slc39a8* gene, which encodes the metal transporter ZIP8, as the most likely candidate for the *Cdm* locus; high ZIP8 mRNA expression occurs in endothelial cells of the testicular vasculature

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in two Cd-sensitive mouse lines, whereas ZIP8 expression is negligible in this cell type from two Cd-resistant mouse lines [10]. We proved that the *Slc39a8* gene is indeed the *Cdm* locus by creating a bacterial artificial chromosome (BAC)-transgenic mouse line, *BTZIP8-3*. A 168.7-kb BAC, containing only the *Slc39a8* gene from a 129S6/SvEvTac (cadmium-sensitive) BAC library, was inserted into the Cd-resistant C57BL/6J genome; Cd treatment caused testicular necrosis in BAC-transgenic *BTZIP8-3* mice but not in non-transgenic littermates [11].

ZIP8 functions endogenously as an electroneutral $\text{Zn}^{2+}/(\text{HCO}_3^-)_2$ as well as a $\text{Mn}^{2+}/(\text{HCO}_3^-)_2$ symporter—moving both ions into the cell [7; 12; 13]. Cd has a binding affinity for ZIP8 in the same range as zinc (Zn) [13]; thus, Cd is readily able to displace Zn and enter cells that express the functional ZIP8 transporter.

The next step in understanding ZIP8's endogenous functions was to generate a knockout mouse line. In building the *Slc39a8* knockout construct, we inserted the *Frt*-flanked neomycin-resistance gene (*neo*) into intron 3, along with *loxP* sites in introns 3 and 6. A hypomorphic phenotype was generated—in mice retaining the *neo* gene. Curiously, Cre recombinase-mediated *loxP* excision occurred in bacteria but not in the intact mouse; herein we examine possible reasons for this excision failure.

MATERIALS and METHODS

Materials and Methods

Creation of the *Slc39a8* construct—This lab previously had characterized a 168.7-kb BAC, in the middle of which is located *Slc39a8* as the only protein-coding gene [11]; the longest transcript of the mouse *Slc39a8* gene spans 64.7 kb [11; 14]. From this BAC, we isolated a 9.7-kb subclone into pBluescript II SK(–) vector with a BAC subcloning kit (Gene Bridges; Dresden, Germany); this subclone contains *Slc39a8* exons 4–5–6 and portions of introns 3 and 6 (**Fig. 1A, top**). Following the protocol of the conditional-gene-knockout kit (Gene Bridges; Dresden, Germany), we first inserted the *loxP-neo-loxP* template into the distal site in intron 6 by ET-recombineering [15]. Cre excision in *E. coli* resulted in a single *loxP* site in intron 6, located 316 bp downstream of exon 6. Next, we inserted the *loxP-Frt-neo-Frt* template (Gene Bridges) into intron 3 by ET-recombineering (**Fig. 1A, 2nd line**); this template contains a phosphoglycerate kinase (PGK) promoter-derived *neo* mini-cassette flanked by two *Frt* sites plus one proximal *loxP* site [16]. The *neo* mini is needed for embryonic-stem (ES)-cell-positive selection for gene targeting. However, the *neo* cassette in many cases dampens gene expression; thus, it is common to include flanking *Frt* sites; FLP recombinase (expressed in FLP-transgenic mice) is then able to recombine with the two *Frt* sites and remove the *neo* mini-gene, as needed. If the *neo* gene is removed, this *loxP* site would be located 301 bp upstream of exon 4 (**Fig. 1A, 3rd line**). This “floxed” allele (containing two *loxP* sites) in most cases is regarded as equivalent to the wild-type allele, and should have a minimal interfering effect on the targeted gene.

ES cells (129S6/SvEvTac) were electroporated with the linearized knockout construct, using standard methods. Neomycin-resistance clones were picked, expanded, and probed via Southern blot to screen for the correctly targeted clones (**Fig. 1B**). The genomic DNA was digested with *ScaI*; the Southern blot probe is described in **Supplementary Data** online

The correctly targeted ES cell clone was microinjected into mouse blastocysts—with help from the University Cincinnati Gene Targeting Core Facility. Chimeric mice were generated and bred further with B6 mice. By genotyping agouti pups, germ-line transmission of the *Slc39a8(neo)* allele (**Fig. 1A, 2nd line**) was confirmed. Genotypes of the wild-type and

Slc39a8(neo) alleles were verified by PCR. All genotyping primers are listed in **Supplementary Data Table S1** online.

Animals—For this study, the *Slc39a8(neo)* allele was in the mixed C57BL/6J (**B6**) and 129S6/SvEvTac background. All mouse experiments were conducted in accordance with the National Institutes of Health standards for the care and use of experimental animals and the University Cincinnati Medical Center Institutional Animal Care and Use Committee.

For in utero experiments, the morning on which the vaginal plug was found is considered gestational day-0.5 (**GD0.5**). *Slc39a8(+/+)*, *Slc39a8(+/-)* and *Slc39a8(neo/ne)* embryos/fetuses, placentas and visceral yolk sacs were collected and genotyped at GD11.5, GD13.5 and GD16.5 for RNA isolation.

Total RNA preparation—Using Tri-Reagent (TR18, Molecular Research Center, Inc.; Cincinnati, OH), we isolated total RNA (combining those of the same genotype from one litter) from embryos, fetuses and yolk sacs; in each case, N = 3 or more pups.

Reverse transcription and quantitative real-time PCR analysis—Total RNA (2.5 µg) from embryos, fetuses, placenta or yolk sac—was used as a template for reverse transcription and primed with oligo(dT), using the SuperScript III first-strand kit, following the manufacturer's recommendations (Invitrogen). Total RNA (2.5 µg) was added to reactions containing 3.8 µM oligo(dT)₂₀ and 0.77 mM dNTP, to a final volume of 13 µL. Reactions were incubated at 65°C for 5 min, then 4°C for 2 min. Next, we added 7 µL of a solution containing 14 mM dithiothreitol and 40 units of RNaseOUT Recombinant RNase inhibitor™ (Verso cDNA kit, AB-1453/B, Thermo Scientific; Waltham, MA). After incubation at room temperature for 2 min, 1 µL of RT enzyme was added to each sample. Reaction tubes were incubated at 42°C for 30 min, followed by 75°C for 10 min (to inactivate reverse transcriptase) and placed immediately on ice. Diethylpyrocarbonate (**DEPC**)-treated distilled water (80 µL) was added to dilute the cDNA that had been generated, and the resultant mixture was stored at -80°C until use.

We performed qRT-PCR in the Bio-Rad DNA Engine Opticon 2™ (Bio-Rad Laboratories; Hercules, CA), using iQ SYBR Green Supermix™ (170-8882, Bio-Rad Laboratories). The housekeeping gene β -actin (**ACTB**) mRNA was employed as the internal control. The GD11.5 *Slc39a8(+/+)* wild-type ZIP8 mRNA/ACTB mRNA ratio was arbitrarily set as “1.0” and the “fold-induction” or relative levels of all other samples was compared with that value. A standard curve using serial dilutions of total RNA resulted in excellent linearity ($r = 0.99$), indicating the qRT-PCR results were valid. Primers used are listed in **Supplementary Data Table S1** online.

Cell culture—Mouse fetal fibroblasts (**MFFs**) were generated, using GD14.5 fetuses of *Slc39a8(+/+)*, *Slc39a8(+/-)* and *Slc39a8(neo/ne)* mice and cultured at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (**DMEM**) (Invitrogen; Carlsbad, CA) plus 10% fetal bovine serum (Hyclone; Logan, UT). Details of these procedures have previously been described [10; 12]. When MFF cells of the three genotypes reached log-phase growth, they were treated with adeno-Cre virus (University of Iowa; Iowa City, IA), added at a multiplicity of infection (**MPI**) of ~100. Cells were harvested 3 days later. Genomic DNA was extracted using standard procedures with Proteinase K and ethanol, and PCR-genotyping was carried out for the *Slc39a8(+)* wild-type allele, the targeting *Slc39a8(neo)* allele, and the recombined *Slc39a8(-)* null allele.

Statistical analysis—Statistical significance between groups was determined, using a 4-way Student's *t* test. Statistical analyses were performed with the use of SAS statistical

software (SAS Institute Inc., Cary, NC). P-values <0.05 are considered statistically significant.

RESULTS AND DISCUSSION

Generation of *Slc39a8* alleles in this study

From the *Slc39a8*(+) wild-type allele (**Fig. 1A, top line**), we built the plasmid construct harboring the *Slc39a8*(*neo*) allele (**Fig. 1A, 2nd line**). *Slc39a8*(+) and *Slc39a8*(*neo*) alleles were verified by PCR analysis (data not shown). Southern blot analysis (**Fig. 1B**) confirmed the clone had the correct insertion. The ES clone carrying the targeted *Slc39a8*(*neo*) was injected into mouse blastocysts; resulting chimeric mice were subjected to further breeding, and the allele was confirmed to have gone germ line.

By crossing *Slc39a8*(+/*neo*) mice with Flp recombinase mice, we obtained heterozygotes carrying the *Slc39a8*(*f*) allele with *neo* removed (**Fig. 1A, 3rd line**). Crossing *Slc39a8*(+/*f*) heterozygotes resulted in pups of all three genotypes in the expected Mendelian ratio and showing normal viability and fertility (data not shown), indicating that presence of the two *loxP* sites in introns 3 and 6 does not affect *Slc39a8* function.

Both CMV-Cre mice [17] and β -actin Cre mice [18] were bred with *Slc39a8*(+/*neo*) heterozygotes, attempting to generate the *Slc39a8*(-) allele. We also employed zygotic injection of both the CAGGS-Cre expression plasmid [19] and the IRES-Cre mRNA [20], trying to generate the *Slc39a8*(-) allele. None of these four different types of Cre recombinase was successful in generating the *Slc39a8*(-) null allele (**Fig. 1A, bottom line**). Interbreeding *Slc39a8*(+/*neo*) mice generated one-fourth *Slc39a8*(*neo/neo*) homozygotes in predicted Mendelian ratio; phenotypic characterization of the *Slc39a8*(*neo/neo*) homozygote, which lives to the neonatal period, will be described elsewhere (manuscript in preparation).

ZIP8 mRNA expression

In the whole embryo/fetus, as well as placenta and visceral yolk sac (**Fig. 2**), ZIP8 mRNA levels in *Slc39a8*(*neo/neo*) homozygotes were dramatically lower than that in the wild-type—at GD11.5, GD13.5 and GD16.5. ZIP8 protein levels on Western immunoblots were consistent with mRNA levels (data not shown). We conclude that, in the developing embryo/fetus and placenta and yolk sac, the *Slc39a8*(*neo*) allele is associated with strikingly decreased levels of ZIP8 expression.

In some cases, ZIP8 mRNA in *Slc39a8*(+/*neo*) heterozygotes was not different from that in *Slc39a8*(+/+) wild-type, whereas in other cases ZIP8 mRNA in *Slc39a8*(+/*neo*) heterozygotes was not different from that in *Slc39a8*(*neo/neo*) homozygotes. Transvection might explain why loss of one copy of the *Slc39a8*(*neo*) allele can cause decreases in ZIP8 expression similar to that found in *Slc39a8*(*neo/neo*) pups. Transvection is an epigenetic phenomenon of activation or repression resulting from the interaction between an allele on one chromosome and the corresponding allele on the homologous chromosome [22-26]. Although transvection is somewhat rare, it has been reported in *Slc39a4*(+/-) mice [27].

Adenovirus-mediated Cre excision in MFF cultures

Cre recombinase packaged by adenovirus can achieve high viral titers, high infection efficiency and, therefore, high Cre activity. We therefore studied Cre recombinase function in adeno-Cre-infected MFF cultures. The *Slc39a8*(+) wild-type allele (276-bp band) was found (**Fig. 3A, top**) in *Slc39a8*(+/*neo*) and *Slc39a8*(+/+) cells but not in *Slc39a8*(*neo/neo*) cells. The *Slc39a8*(*neo*) allele (876-bp band) was observed in *Slc39a8*(*neo/neo*) and

Slc39a8(+/neo) cells but not in *Slc39a8*(+/+) cells (**Fig. 3A, bottom**). Without adeno-Cre activity, no *Slc39a8*(-) null allele was seen (**Fig. 3B, top**). Following adeno-Cre action, the *Slc39a8*(-) allele (425-bp band) occurred in *Slc39a8*(neo/neo) and *Slc39a8*(+/neo) cells but not in *Slc39a8*(+/+) cells (**Fig. 3B, bottom**). Hence, just like in *E. coli*, Cre recombinase activity is able to recombine with the *Slc39a8* gene at the two *loxP* sites in adenovirus-infected MFF cultures.

Why does adenovirus-driven Cre recombinase excise the floxed allele in MFF cultures, whereas none of four types of Cre recombinase functions in the intact mouse? It is estimated that at least four Cre recombinase molecules per cell are necessary to achieve efficient Cre recombinase activity (Jon Neumann, University Cincinnati Transgenesis Core, *personal communication*). One possibility for the failure of these four types of Cre recombinase is that sufficient numbers of Cre recombinase were not reached; on the other hand, with adeno-Cre infections in MFF cultures, these levels of Cre recombinase were achieved.

Sequence analysis surrounding both *loxP* sites

When we chose the regions in introns 3 and 6 to insert the *loxP* sites more than 5 years ago, accurate comparative sequences of these regions in other mammalian *SLC39A8* genes were unavailable. We therefore repeated the comparative alignments of these regions. Whereas no significant regions of homology were found anywhere near the proximal *loxP* site in intron 3 (not shown), we did find substantially conserved segments (in the mouse, rat, human, orangutan, dog and horse) surrounding the distal *loxP* site in intron 6. Regions most highly conserved (**Fig. 4A**) are within exon 6 among all six mammals (true for internal exons in virtually any gene). In the region where the distal *loxP* site was inserted, however, there are significant regions of homology—especially within 10-25 bp 3'-ward.

If the first 1.2 kb of intron 6 (comparing mouse *Slc39a8* with human *SLC39A8*) is aligned (**Fig. 4B**), the 431-bp segment from +190 through +620 bp has 72% identity; moreover, the 61-bp segment from +295 through +355 bp shows 86% identity. The asterisk (**Fig. 4B**) denotes the precise *loxP* location (base +316; between adenines on either side).

Such conserved intronic segments strongly suggest that transcription-factor regulatory proteins might be binding to this DNA region. This would provide another plausible explanation as to why Cre recombinase activity does not function in the intact mouse, whereas it functions perfectly well in *E. coli* and MFF cultures. In MFF cultures, and certainly in *E. coli*, many components of genetic architecture can be very different from that in the intact animal. Thus, perhaps transcription factors and other regulatory proteins do not bind to the distal *loxP* site region in MFFs, allowing Cre recombinase to recognize *loxP* and excise the *Slc39a8*(neo) allele successfully (**Fig. 3**).

Location of *neo* inside or near a gene can decrease gene expression

There are several known examples in which placement of the *neo* cassette inside an intron, or outside the transcribed gene, severely dampens expression of that gene and/or neighboring genes: targeting the granzyme B (*Gzmb*) gene (the most 5'-ward gene in the *Gzm* seven-gene cluster) decreases expression also of downstream *Gzm* genes; targeting the β -globin locus control region (**LCR**) down-regulates expression of multiple downstream globin genes [28]; and targeting of *Hox13* gene [29] and *Hox11* gene [30] expression. In contrast, targeting the cathepsin G (*Ctsg*) gene, lying 3'-ward to the four-gene *Cts* cluster, has minimal effects on upstream *Cts* gene expression [28].

Concluding remarks

We have generated a hypomorphic *Slc39a8(neo)* allele, associated with striking down-regulation of ZIP8 expression. This hypomorph mouse model provides us with a unique opportunity to study developmental and in utero physiological functions of ZIP8, whereas we predict the global *Slc39a8(−/−)* knockout would be early-embryolethal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The mouse *Slc39a8* gene encodes the ZIP8 transporter
- ZIP8 functions endogenously as a electroneutral $\text{Zn}^{2+}/(\text{HCO}_3^-)_2$ symporter
- A *Slc39a8(neo/neo)* hypomorph mouse, due to retention of the *neo* mini-gene, has been created
- ZIP8 expression in utero is ~90% decreased in all tissues examined
- This mouse model will be useful for studying developmental and in utero physiological functions of ZIP8

A***Slc39a8*(+) wild-type allele** ***Slc39a8*(neo) allele** ***Slc39a8*(f) allele without neo** ***Slc39a8*(-) null allele** **B** **FIG. 1.**

Characterization of the *Slc39a8* alleles. (A) Diagram of the four *Slc39a8* alleles discussed. The mouse *Slc39a8* transcript (nine exons, eight introns) spans ~64.7 kb; total lengths of introns 3 and 6 are 6,725 and 25,955 bp, respectively [11; 14]. *Top*, *Slc39a8*(+) wild-type allele, showing exons 4 (170 bp), 5 (123 bp) and 6 (171 bp), introns 4 (1,409 bp) and 5 (465 bp), and portions of introns 3 and 6. *Second line*, *Slc39a8*(neo) allele—proximal *loxP* site was inserted into intron 3, 301 bp upstream from start of exon 4, using the *Frt-neo-Frt-loxP* template [15]; distal *loxP* site in intron 6 is located 316 bp downstream from end of exon 6. *Third line*, *Slc39a8*(f) floxed allele in which *neo* was removed by FLP recombinase in mouse via breeding with FLP-transgenic mice. *Bottom*, *Slc39a8*(-) knockout allele following Cre recombinase-mediated excision, detected in MFF cultures (*see text*). (B)

Southern blot, digestion by *ScaI*, confirming the *Slc39a8(t)* targeted allele (3,017 bp) as distinct from the *Slc39a8(+)* wild-type allele (5,072 bp).

FIG. 2.

Quantification of ZIP8 mRNA levels via qRT-PCR in three genotypes. At three gestational ages, mRNA levels were determined in whole embryo/fetus (*left*), placenta (*center*), and visceral yolk sac (*right*). It is well known that the hematopoiesis function switches from yolk sac to fetal liver well before GD16.5 [21]. N = individual samples from three litters pooled per developmental-age-point. Mouse β -actin (*ACTB*) mRNA was employed as the normalization control. The GD11.5 *Slc39a8*(+/+) wild-type ZIP8 mRNA/*ACTB* mRNA ratio was arbitrarily set at "1.0". For **total embryo: GD11.5**, (+/+) vs (*neo/neo*) $P = 0.009$ and (+/*neo*) vs (*neo/neo*) $P = 0.048$; **GD13.5**, (+/+) vs (*neo/neo*) $P < 0.001$ and (+/*neo*) vs (*neo/neo*) $P = 0.003$, and (+/+) vs (+/*neo*) $P = 0.043$. For **total fetus: GD16.5**, (+/+) vs (*neo/neo*) $P = 0.002$ and (+/*neo*) vs (*neo/neo*) $P = 0.004$. For **placenta: GD11.5**, (+/+) vs (*neo/neo*) $P = 0.001$, and (+/*neo*) vs (*neo/neo*) $P = 0.004$, and (+/+) vs (+/*neo*) $P = 0.043$; **GD13.5**, (+/+) vs (*neo/neo*) $P = 0.019$, and (+/+) vs (+/*neo*) $P = 0.032$; **GD16.5**, (+/+) vs (*neo/neo*) $P = 0.001$, and (+/*neo*) vs (*neo/neo*) $P = 0.003$. For **yolk sac: GD11.5**, (+/+) vs (*neo/neo*) $P = 0.001$, and (+/*neo*) vs (*neo/neo*) $P = 0.003$, and (+/+) vs (+/*neo*) $P = 0.028$; **GD13.5**, (+/+) vs (*neo/neo*) $P < 0.001$, and (+/*neo*) vs (*neo/neo*) $P < 0.001$; **GD16.5**, (+/+) vs (*neo/neo*) $P < 0.001$, and (+/*neo*) vs (*neo/neo*) $P < 0.001$.

FIG. 3.

Demonstration that adeno-Cre recombinase action was successful in MFFs. (A) Wild-type *Slc39a8*(+) PCR fragment (276 bp) is present in *Slc39a8*(+/neo) and *Slc39a8*(+/+) MFFs, but not in *Slc39a8*(neo/neo) MFFs; in contrast, the *Slc39a8*(neo) PCR fragment (876 bp) is present in *Slc39a8*(neo/neo) and in *Slc39a8*(+/neo) MFFs, but not in *Slc39a8*(+/+) MFFs. (B) Following adeno-Cre recombinase action, the *Slc39a8*(-) null allele (PCR fragment 425 bp) was found in *Slc39a8*(neo/neo) and *Slc39a8*(+/neo) MFFs, but not in *Slc39a8*(+/+) MFFs (lower gel). In contrast, no bands are seen in absence of adeno-Cre recombinase (upper gel).

FIG. 4.

DNA sequence of mammalian *SLC39A8* exon 6 and proximal intron 6. (A) Alignment across this region in mouse plus five other mammals. Vertical arrows (top) designate 200-bp intervals, and nucleotide numbers along mouse chromosome (**Chr 3**) are noted. Solid rectangle (upper left) depicts Exon 6 and the line represents proximal Intron 6. Red arrow indicates exact integration site of distal *loxP*. X-axis represents nucleotide identities across the region, comparing mouse genome with that for each of the other five mammals (lower portion in blue). Y-axis denotes relative degree of conservation at each nucleotide, among mouse and the other five species, calculated by the algorithm “30-Way Multiz Alignment & Conservation”. (B) Conserved sequences of mouse vs human and rat *SLC39A8* genes—in region of the intron 6 distal *loxP* site. Asterisk denotes precise location (base +316) at which distal *loxP* site was inserted. Numbering system starts with +1 as first nucleotide of intron 6. Cytosines are shown in blue, adenines in red, guanines in black, and thymine in green.