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Zic2 is required for enteric nervous system development and neurite outgrowth: a mouse model of enteric hyperplasia and dysplasia

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Abstract

From a forward genetic screen in mice we isolated a novel *Zic2* allele with abnormal aggregation of meconium in the gastrointestinal tract. *Zic2*^{m1Nisw} mutant embryos show an increase in the number of enteric neurons in vivo and disorganization of the neurite network. Explant culture of *Zic2*^{m1Nisw} gastrointestinal tract show extensive neurite outgrowth, suggesting that *Zic2* is a negative regulator of nerve fiber growth. These studies demonstrate a previously unknown function of *Zic2* and provide a novel animal model of enteric nervous system dysplasia and hyperplasia.

Keywords

dysplasia; enteric neural crest cell; hyperplasia; neurite outgrowth; *Zic2*

INTRODUCTION

The enteric nervous system (ENS) is composed of neurons and glial cells that cluster to form the ganglia of the mature ENS, which acts to control intestinal peristalsis and secretion. To identify novel molecules required for ENS development, we performed *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screens for recessive mutations in mice which cause defects in the gastrointestinal tract.¹ Normally, intestines of wild-type embryos at E18.5 were yellow color. From this screen, we isolated the mouse Line 7–12 in which the intestines of mutant embryos were green and/or dark red and there was an abnormal accumulation of material in the gut (Fig. 1A–B). We initially mapped the ENU-induced mutation associated with the ENS phenotype to a ~7 Mb region on mouse chromosome 14 and this interval was refined to ~1 Mb using strain-specific polymorphisms and meiotic recombination mapping (Fig. S1A). Analysis of this 1 Mb region for genes previously associated with embryonic development revealed two strong candidate genes, *Zic2* and *Zic5* (Fig. S1A), zinc-finger transcription factors that function during central nervous system development.² Thus, using

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DISCLOSURES The authors have no competing interests.

AUTHOR CONTRIBUTIONS YZ performed the research; YZ and LN designed the research study, analyzed the data and wrote the manuscript.

SUPPORTING INFORMATION Additional Supporting Information may be found in the online version of this article:

Figure S1. Line 7–12 carries a mutation in the *Zic2* gene.

Figure S2. Differentiation appears normal in *Zic2*^{m1Nisw/m1Nisw} embryonic gut.

genomic DNA, we sequenced the coding regions of *Zic2* and *Zic5* from wildtype and mutant. This identified a single missense A to T mutation in the *Zic2* gene that changes glutamic acid (Glu) to valine (Val) within the zinc-finger domain. This strongly suggests that Line 7–12 represents a new allele of *Zic2* (renamed *Zic2^{m1Nisw}*). Moreover, the *Zic2^{m1Nisw}* mutation appears to create a hypomorphic allele as *Zic2* protein level is not affected in the mutant (Fig. S1B). Hypomorphic alleles have the advantage that they may reveal new gene functions in developmental processes not previously recognized to be controlled by that gene. This new hypomorphic *Zic2* mutation reveals a previously unknown function for *Zic2* in ENS development.

To characterize enteric neural crest cells (ENCC) in the gut, we analyzed the expression of nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, which highlights the differentiated nitric oxide synthase (NOS)-expressing population in myenteric plexus at E18.5 (Fig. 1C–H). This showed a marked ENS dysplasia and a reduced distance between internodal strands (neurite bundles connecting enteric ganglia) in the *Zic2^{m1Nisw/m1Nisw}* gut compared with wild type. This phenotype was confirmed by examining the expression of pan-neuronal markers PGP9.5 and Ret (Fig. 1J–K’). Quantification of NADPH diaphorase-positive cells along various regions of the gut showed a significant increase of ENCCs in *Zic2^{m1Nisw/m1Nisw}* (Fig. 1I). The motor function of the gastrointestinal tract is regulated by the equilibrated activities from excitatory and inhibitory neurons which are synaptically linked to each other in a reflex circuit.³ Neurite outgrowth is a key component in establishing this highly precise configuration of connections between various kinds of neurons. Moreover, the coordination of the outer myenteric plexus and inner submucosal plexus is necessary to control normal function of gastrointestinal tract.³ To explore whether the organization of ganglion strands was disturbed in both plexus layers, we examined the expression of p75^{NTR}, a nerve growth factor receptor (Fig. 1L–O). Wild-type ENCCs tend to form large ganglia and each ganglion is well interconnected (Fig. 1L, N). In contrast, the distance between the internodal strands in the mutant was decreased in both plexus layers (Fig. 1M, O). Quantification of distance between internodal strands showed significantly closer spacing in the mutant (Fig. 1P), although the length of the gut was not significantly affected (measured at E14.5 and E18.5), further suggesting that the neurite network is disturbed.

To examine possible morphological changes of the mutant neurites, the midgut was dissected from E13.5 wild type or *Zic2^{m1Nisw/m1Nisw}* embryos and cultured with GDNF. After 3 days of culture, wild-type ENCCs showed extensive migration out of the explant with limited neurite outgrowth (Fig. 1Q). The mutant ENCCs migrated out of the explant to a similar extent as wild type, however, the mutant cells showed a statistically significant increase in neurite length (Fig. 1R, S). To determine whether increased myenteric neurite outgrowth resulted from altered differentiation of mutant ENCCs, neuronal (neurofilament 100, PGP9.5, Tuj1) and glia markers (GFAP, S100) were examined and the ratio of neuron : glia was quantitated. No difference in expression pattern or neuron : glia ratio was detected between wild type and mutant (Fig. S2). Taken together, these results indicate that abnormal neurite outgrowth in the *Zic2* mutants is not due to an alteration in differentiation.

Neurite formation starts when membrane bound receptors are activated by extracellular cues. Activated receptors trigger intracellular signaling cascades that act to control expression of genes essential for cyto-skeleton remodeling and neurite extension. During ENS development, RET signaling has been implicated in promoting ENCC proliferation and differentiation.^{4,5} Negative regulators of Ret signaling, such as Sprouty2 and Kif26a, have been shown to be crucial in regulating ENS proliferation and neurite growth.^{6,7} Mice that are homozygous mutant for Sprouty2 or Kif26a display intestinal hyperplasia as well as disorganized neurite growth. The similar phenotype in *Zic2* mutants suggests that *Zic2* may

act as a negative regulator of Ret signaling to control enteric neurite growth, an area of future study.

The studies reported here reveal a new role for *Zic2* in repressing neurite outgrowth during ENS development. ENU-generated *Zic2^{m1Nisw/m1Nisw}* mutant embryos exhibit neuronal dysplasia and abnormal neurite outgrowth, leading to defective function of the ENS and retention of meconium in the embryonic gut. It has been shown that enteric hyperganglionosis and hyperinnervation can contribute to megacolon in human patients.⁸ Homozygous *Zic2^{m1Nisw}* embryos do not survive beyond birth due to neural tube defects, and thus it was not possible to examine whether *Zic2* mutant animals would develop megacolon, similar to that observed in human patients, although this would be expected based on the embryonic hyper-ganglionic phenotype and retention of meconium. *Zic2^{m1Nisw/m1Nisw}* can be therefore considered a useful model for studying ENS dysfunction due to neuronal hyperplasia and defective neurite patterning.

MATERIALS AND METHODS

ENU mutagenesis

The methods of ENU treatment and genetic crosses to obtain embryos carrying homozygous mutations have been described.¹ Following timed matings of G2 daughters with their F1 father, embryos were dissected and analyzed at E10.5, E12.5, E14.5, or E18.5.

Genotyping

Zic2^{m1Nisw} mutant embryos were genotyped with SSLP markers D14mit107 (Left: 5' AAATGGTCATCCCTGAAAAGA 3' Right: 5'CAGGCCTCTCCAAAGTACCA 3') and D14mit177 (Left: 5' CCAGTACTAAGAAGGAAACGTGC 3' Right: 5'CACACACATACAAACAACATGTCC3'). The specific point mutation in *Zic2* was always concordant with the ENS phenotype in animals that had been backcrossed for over 10 generations to a wild-type 129/SvJ strain.

Immunostaining

For whole-mount immunostaining, embryonic guts were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight, and then treated with 0.1% triton and 1% goat serum for 2 h at room temperature. Primary antibody was added and incubated at 4 °C for 2 days. After washing, secondary antibody was added and incubated at RT for 2 h. To analyze the submucosal plexus, the circular and longitudinal muscles were removed before performing IHC.

Nicotinamide adenine dinucleotide phosphate diaphorase staining were performed by incubating fixed tissues in diaphorase solution [0.1 mol L⁻¹ PBS (pH7.4), 1 mg mL⁻¹ β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (Sigma), 0.1 mg mL⁻¹ nitro blue tetrazolium (NBT; Sigma, St. Louis, MO, USA), 0.3% Triton X-100] for 1.5 h at 37 °C.

Gut explant culture

For collagen gel gut explant cultures, E13.5 proximal hindgut segments were placed on a glass bottom dish (MatTek, Ashland, MA, USA) coated with 1 mg mL⁻¹ 3D collagen gel (R&D, Minneapolis, MN, USA) with 10 ng mL⁻¹ GDNF (USBiological, Marblehead, MA, USA) and cultured for 3 days.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Abnormal ENS development and neurite outgrowth in *Zic2^{m1Nisw/m1Nisw}* embryos (A, B) Material is retained in the intestines of E18.5 mutants (B) presenting as green intestines vs the normal yellow intestines of wild-type embryos (A); $N > 50$. (C–H) Whole-mount NADPH diaphorase staining of E18.5 wild type (C, E, G) and *Zic2^{m1Nisw/m1Nisw}* (D, F, H) gut regions; $N = 5$. (I) Statistically significant increase in NADPH diaphorase-stained cells in the mutant gut compared with wild type (E18.5; $N = 4$). (J–O) Whole-mount immunostaining for the indicated markers of E18.5 wild type (J–J'', L, N) or *Zic2^{m1Nisw/m1Nisw}* (K–K'', M, O) midguts showing abnormal neurite pattern; $N = 5$. (P) Statistically significant decrease in the distance between internodal strands in the mutant gut compared with wild type (E18.5). (Q–R) Segments of E13.5 proximal midgut were cultured in 3D collagen matrix with GDNF for 3 days and stained with p75^{NTR} antibody (green) to detect ENCC. *Zic2^{m1Nisw/m1Nisw}* mutant ENCCs (R) showed extensive outgrowth of neurites compared with wild type (Q); $N = 4$. (S) Statistically significant increase in neurite outgrowth in the mutant gut compared with wild type. Neurite length was measured from the confluent edge of the migrating ENCC to the distal tip of the neurite (highlighted by an arrow). Data expressed as mean $\pm$ standard deviation (SD) ($*P < 0.05$, $***P < 0.001$, Student's *t*-test).