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Postnatal lethality and abnormal development of foregut and spleen in *Ndrg4* mutant mice

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ABSTRACT

NDRG4 is a member of the *NDRG* family (N-myc downstream-regulated gene), which is highly expressed in brain and heart. Previous studies showed that *Ndrg1*-deficient mice exhibited a progressive demyelinating disorder of peripheral nerves and *Ndrg4*-deficient mice had spatial learning deficits and vulnerabilities to cerebral ischemia. Here, we report generation of *Ndrg4* mutant alleles that exhibit several development defects different from those previously reported. Our homozygous mice showed growth retardation and postnatal lethality. Spleen and thymuses of $Ndrg4^{-/-}$ mice are considerably reduced in size from 3 weeks of age. Histological analysis revealed abnormal hyperkeratosis in the squamous foregut and abnormal loss of erythrocytes in the spleen of $Ndrg4^{-/-}$ mice. In addition, we observed an abnormal hind limb clasping phenotype upon tail suspension suggesting neurological abnormalities. Consistent to these abnormalities, Ndrg4 is expressed in smooth muscle cells of the stomach, macrophages of the spleen and neurons. Availability of the conditional allele for *Ndrg4* should facilitate further detailed analyses of the potential roles of Ndrg4 in gut development, nervous system and immune system.

1. Introduction

Cytoplasmic protein Ndrg4 belongs to the N-myc downstream regulated gene (NDRG) family, which consists of four related members, NDRG1-NDRG4, in mammals and are well conserved through evolution [1,2]. The proteins consist of 325–394 amino acid residues, and share 53–65% sequence identity with each other [3]. Although the precise molecular and cellular function of these NDRG members has not been fully elucidated, emerging evidence implicates their roles in development, cancer metastasis, and the immune system [4–8]. The first described mouse mutant model for this family was *NDRG1*-deficient mice, which were unable to maintain myelin sheaths in peripheral nerves [9]. This phenotype was consistent with human hereditary motor and sensory neuropathy, Charcot-Marie-Tooth disease type 4D, caused by a nonsense mutation of *NDRG1* [10]. *NDRG2*-deficient ($NDRG2^{-/-}$) mice were recently reported to be viable and fertile without apparent physical abnormalities, but had a markedly shorter lifespan than the wild-type (wt) or $NDRG2^{+/-}$ mice, and they are susceptible to tumor formation, including lymphoma, hepatocellular carcinoma and bronchoalveolar carcinoma, suggesting that NDRG2 is a possible tumour suppressor in various types of cancer [11]. In addition, loss of *Ndrg2* in mice resulted in vertebral homeotic transformations in thoracic/lumbar and lumbar/sacral transitional regions in a dose-dependent manner [12].

An *NDRG4*-deficient mouse model was previously described [13]. Although the homozygous mutant mice were born at normal Mendelian ratios, they showed impaired phenotypes in spatial learning and memory, and neuroprotection with decreased levels of brain-derived neurotrophic factor. Here, we have generated mice with a conditional allele for the *Ndrg4* gene. When a global null mutation for the *Ndrg4* gene was generated from the floxed mice, to our surprise, we observed several more severe phenotypes different from those previously reported: an abnormal hind limb clasping phenotype and growth retardation with disproportional small spleens and thymuses from the age of about 3 weeks. In addition, our homozygous mutant $Ndrg4^{-/-}$ mice also exhibited partial postnatal lethality. In agreement with reduced size in the forestomach and the spleen of *Ndrg4* mutant mice, we observed

Abbreviations: NDRG, N-myc downstream regulated gene; ES, embryonic stem; PP, Peyer's patches; wt, wild-type; wp, white pulp; rp, red pulp.

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abnormal hyperkeratosis in the squamous foregut and abnormal loss of erythrocytes in the spleen of *Ndrg4*$^{-/-}$ mice. We also detected a considerable Ndrg4 expression in stomach, spleen and neurons of wt mice.

2. Methods

2.1. Mouse breeding and generation of Ndrg4 mutant alleles

All strains were maintained on a mixed 129 and C57/BL6 background. The animals were handled in accordance with institutional guidelines with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine. The authors are currently exploring possibilities to deposit the newly developed mice to public repositories and are happy to share them upon request.

To identify tissue-specific functions of Ndrg4, we have created a conditional allele of the mouse *Ndrg4* gene by introducing Cre recombinase recognition sites (*loxP*) into the *Ndrg4* locus. This strategy involved introducing a *loxP* site and a neomycin resistance cassette within the intron 5, and another *loxP* site into the intron 7 (Fig. 1a), such that Cre recombination excises the *Ndrg4* exon 5–7 leading reading frame shift. A *Ndrg4* floxed targeting vector was constructed based on the 129-Sv mouse genomic fragment. A 744bp *Hpa*I-*Ase*I fragment containing *Ndrg4* exons 6–8 was inserted into the Floxed *Kpn*I-*Cla*I sites of the pDELBOY plasmid [14], which contains an *Frt*-site-flanked neomycin gene expressed from the phosphoglycerate kinase promoter (*PGK-neo*), two *loxP* sites and a cassette containing the herpes simplex virus thymidine kinase gene expressed from the phosphoglycerate kinase promoter (*PGK-HSV-tk*). Also, a 2.9-kb *Ase*I-*Bam*HI fragment (3′ short arm) and a 3.7-kb *Sal*I-*Hpa*I fragment (5′ long arm) were respectively inserted into the *Xho*I site and the *Sal*I site of this construct. The targeting vector was linearized with *Not*I and electroporated into G4 hybrid mouse embryonic stem (ES) cell line, which was established from male blastocyst derived from the natural mating of 129S6/SvEvTac female with C57BL/6Ncr male in the Nagy lab [15]. The correct gene targeting was confirmed by Southern blot with both 5′ and 3′ external probes (Fig. 1b). Presence of the 5′ loxP site was confirmed by genomic PCR using primers P1 (5′-TAGGCAGGGGCAGGTGGGTTTGT-3′) and P2 (5′-GGCGTCTCGATGTCATGTTCCTGT-3′). Targeted cells were then injected into C57/BL6 blastocysts and two of the resulting chimeras were found to transmit the targeted allele through the germline. Both lines were maintained on a 129 and C57/BL6 background and showed the same phenotype. Then, the neo cassette was removed by crossing with *ROSA26-Flp* transgenic mice to generate heterozygous mice for the floxed allele without neo (*+/fx*) (Farley et al., 2000). The resulting heterozygous floxed *Ndrg4* mutant mice (*Ndrg4*$^{+/fx}$) were interbred to make homozygous for the conditional allele (*fx/fx*). To make global *Ndrg4* knockout mice, the *Ndrg4*$^{+/fx}$ mice were crossed with *E2A-cre* transgenic mice, which express the Cre recombinase in germ cells [16]. Cre-dependent deletion was detected with primers P1, P2 and P3 (5′-GCTCCCACTCCAATGCCAATC-3′) (Fig. 1a, c). The resulting *Ndrg4*$^{+/-}$ mice were intercrossed to generate homozygous *Ndrg4* mutant mice.

2.2. Histological and western blot analysis

Stomach and spleen were fixed in 4% paraformaldehyde (PFA), embedded in paraffin and stained with hematoxylin and eosin (H&E) according to a standard procedure. For Western blotting, brains and hearts were homogenized in a dounce homogenizer using modified radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% NP-40, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with Complete Protease Inhibitors (Roche Diagnostics) and centrifuged at 12,000 g for 12 min. Lysates were subjected to Western blot analysis using standard technologies. The western blots were probed with the primary antibody (mouse monoclonal anti-NDRG4, clone 2G3, Novus Biologicals, T5168), followed by treatment with HRP-conjugated goat anti-mouse IgG (Promega, W4021). Positive signals were visualized using a Pierce ECL western blotting substrate detection Kit (Pierce, 32132). For normalization of signals, mouse anti-α-Tublin (clone B-5-1-2, Sigma, H00065009-M01) monoclonal antibody was used as a loading control.

2.3. Immunohistochemistry and antibodies

Immunostaining was performed on frozen sections as previously described [14]. Briefly, spleen and stomach were dissected, fixed in 4% PFA in phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C, washed with PBS, and cryoprotected with 30% sucrose in PBS overnight; embedded in OCT and then cryosectioned at 10 μm. The frozen sections were stained with the following primary antibodies and conjugates: rat monoclonal (RA3-6B2) anti-B220 (eBioscience, 14-0452), Fluorescein isothiocyanate (FITC) rat monoclonal (4E3) anti-CD21/CD35 (eBioscience, 11-0212), Alex Fluor 647 anti-mouse CD4 (eBioscience, 51-0041), rat monoclonal (ER-TR9) anti-SIGN Related 1 (SIGN R1) (eBioscience, ab37220), FITC anti-Mucosal vascular addressin-1 (MAdCAM1) (eBioscience, 11-5997), and Allophycocyanin (APC) anti-mouse TER-119 (eBioscience, 17-5921), rat monoclonal anti-ER-TR-7 (Santa Cruz, sc-73355), rat monoclonal (CL:A3-1) anti-F4/80 (abcam, ab6640), rabbit anti-NDRG4 antibody (N13) [17] and Cy3 conjugated anti-α-Smooth Muscle Actin (SMA) (Sigma, C-6198). Hippocampus neurons were isolated from E17.5 wt embryos, cultured for 35 days and then stained with antibodies for Ndrg4 and α-Tublin. For the non-conjugated primary antibodies, Alexa Fluor 488 and 594 fluorochrome-conjugated secondary antibodies (Invitrogen) were used for signal detection. Images were acquired on an Olympus fluorescent microscope or with a Leica TCS SP2 confocal system (Leica Microsystems), and processed in Adobe Photoshop.

2.4. Measurements of fasted blood glucose concentrations in mice

After overnight fasting, blood was obtained from tails (5 mice/genotype), and glucose concentrations were measured using a glucometer (One Touch Ultra; Johnson & Johnson).

2.5. Statistical analysis

Data are expressed as the means ± the standard error of the mean. Statistical analysis was conducted using the 2-tailed Student's t-test. A P value < 0.05 was considered significant.

3. Results and discussion

We and other labs originally documented that *Ndrg4* is abundantly expressed in the murine brain and heart [1,3,18] and that *Ndrg4* is essential for normal cardiac development in zebrafish [4]. To elucidate the effects of *Ndrg4* deficiency *in vivo* and allow temporal and tissue-specific gene inactivation, we have created a conditional allele of the mouse *Ndrg4* gene. We flanked the *Ndrg4* exon 5-7 with two *loxP* sites (Fig. 1a), such that Cre recombination excises the *Ndrg4* exon 5-7 leading reading frame shift. We verified homologous recombination at the gene-targeted locus in G4 ES cells [15] and mutant mice by Southern blot hybridization (Fig. 1b) and genomic PCR (Fig. 1c). Then, mice heterozygous for the *Ndrg4*$^{fx-}$

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