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The lncRNA, Cardiac Mesoderm Enhancer-Associated Noncoding RNA Is Indispensable for Intestinal Smooth Muscle Homeostasis in Female Mice as Revealed by a Novel Endogenous *Myh11*-Encoded Inducible Cre Model

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Next-generation sequencing has revealed an unexpectedly high number of noncoding RNA transcripts. Long noncoding RNAs (lncRNAs), more than 200 nucleotides in length and without protein-coding potential, have been shown to play emerging roles in a variety of cell types and settings.[1] However, the functional roles of many lncRNAs in vivo remain largely unknown due to a lack of effective genetic tools.

Our recent study demonstrated an essential role of the conserved smooth muscle cell (SMC)-specific lncRNA, cardiac mesoderm enhancer-associated noncoding RNA (*CARMN*), in maintaining the SMC contractile phenotype by using *Myh11-CreER*^{T2} transgene.[2–4] The location of the *Myh11-CreER*^{T2} allele is in Y chromosome,[2] however, prevented us from assessing the specific functional role of *Carmn* in postnatal SMCs in adult female mice. Given that numerous SMC-driven diseases show sex-dependent differences, we generated a new inducible SMC-specific Cre mouse model in which a CreER^{T2} cassette is knocked

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Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2023.12.012.

Conflicts of Interest:

The authors disclose no conflicts.

Ethical Statement:

The use of experimental animals is approved by the Institutional Animal Care and Use Committee and Biosafety Committee at Augusta University (2012–0502) in accordance with NIH guidelines.

Data Transparency Statement:

The *Myh11*^{CreERT2} mice generated in this study are available from Drs Zhou's and Cai's laboratories upon request.

Reporting Guidelines:

ARRIVE/Care and Use of Laboratory Animals.

in immediately after the initiation codon of the most highly SMC-restricted gene, *Myh11*, through homologous recombination[5,6] (Figure A and Figure A1A). Because the *Myh11* gene is on autosomal chromosome 16 in mice, this model is expected to circumvent the sex-specific limitations of the Y chromosome-linked *Myh11-CreER^{T2}* transgene and therefore allow us to study the function of the lncRNA *Carmn* in both sexes of mice. Data from polymerase chain reaction genotyping and sequencing of the genotyping polymerase chain reaction product confirmed that the CreER^{T2} cassette was successfully inserted after the start codon of the *Myh11* gene in both sexes (Figure A1A–C) and that *Myh11$^{CreERT2/+}$* heterozygous knock-in (KI) mice are viable and fertile. Data from Western blotting revealed that the CreER^{T2} protein in *Myh11-CreER$^{T2/+}$* heterozygous mice can be specifically detected in the colon muscularis and aorta in both sexes of mice without significantly affecting the expression level of endogenous MYH11 protein compared to the wild type (WT) mice, although the qRT-PCR results showed that the mRNA level of *Myh11* is reduced ~50% in the aorta but remains comparable level in the colon, compared to WT controls (Figure B and Figure A1D–J), suggesting that a single WT allele can largely compensate for the loss of the other allele due to CreER^{T2} KI.

We next sought to validate the utility of *Myh11*-driven Cre activity through crossing *Myh11$^{CreERT2/+}$* male with *mTmG* reporter female mice[7] to obtain *Myh11$^{CreERT2/+}$;mTmG$^{F/W}$* mice and *Myh11$^{+/+}$;mTmG$^{F/W}$* control mice (Figure A2A–B). The Cre-dependent recombination of mTmG in SMCs of the adult mice was subsequently analyzed by analysis of GFP fluorescence 24 days after tamoxifen injection (Figure A2C). Direct visualization of Cre-dependent green fluorescent protein expression revealed that tamoxifen-dependent Cre activity is specific for gastrointestinal organs such as the colon and ileum, the female reproductive and urinary system such as the uterus and bladder, and vascular tissues such as the thoracic aorta and coronary arteries (Figure A2D–F). Co-immunostaining revealed that the GFP fluorescence is colocalized with the SMC marker, ACTA2 in SMCs of the colon and aorta (Figure A2G). These observations collectively demonstrate that the endogenous *Myh11* promoter can, with a high degree of specificity, drive Cre expression and recombination of a floxed reporter gene in SMCs of adult female mice.

To investigate the functional role of *Carmn* in the postnatal SMCs of female mice, we crossed the conditional *Carmn$^{PFG/PFG}$* mouse[3] with the *Myh11$^{CreERT2/+}$* KI mouse (Figure C). A single *Carmn* allele deletion was designated as a control (referred to as iHet). Tamoxifen was administered to 8-week-old female mice, as illustrated in Figure D. GFP expression, due to tamoxifen-dependent Cre-mediated inversion of a reversed GFP trap cassette in the *Carmn* gene locus, reveals the endogenous *Carmn* expression pattern in vivo while simultaneously disrupting *Carmn* expression.[3] Compared to iHet control mice, female *Carmn* iKO mice exhibited premature lethality starting ~3 weeks after the first tamoxifen injection, with no mice surviving beyond 8 weeks (Figure D). Analysis of female iKO mice revealed a severe intestinal pseudo-obstructive phenotype consistent with the pathology observed using the Y-chromosome-restricted *Myh11-CreER^{T2}* transgene to delete *Carmn* in male mice.[4] This phenotype includes dramatic gastrointestinal tract distention with excess gas and feces accumulating predominantly in the cecum and colon (Figure E and F). Analysis of colon morphology via direct visualization of GFP signal from the *Carmn* KO

gene locus in female iKO mice showed drastic distention and significant thinning of the colonic muscular layer as compared to iHet mice (Figure G and H). A robust GFP signal can also be seen in both iHet and iKO female mouse aorta (Figure A2H). In conclusion, our development of a novel endogenous *Myh11*-driven inducible Cre mouse model enables, for the first time, analysis of the function of SMC genes in both sexes in vivo. We found that the lncRNA *Carmn* is essential for intestinal contractility in postnatal female mice and that *Carmn* deletion in adult female mice results in premature lethality due to a severe intestinal pseudo-obstructive phenotype. The novel $Myh11^{CreERT2/+}$ KI mouse provides a highly effective and specific genetic tool to investigate the function of the gene of interest in visceral and vascular SMCs in both female and male mice. It is recommended to include $Myh11^{CreERT2/+}$ positive mice lacking the floxed allele in the cohort for controlling potential confounding effects from the downregulated *Myh11* expression resulting from the $CreER^{T2}$ KI in *Myh11* gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

CARMN	cardiac mesoderm enhancer-associated noncoding
RNA	lncRNA, long noncoding RNA
KI	knock-in
SMC	smooth muscle cell
WT	wild type

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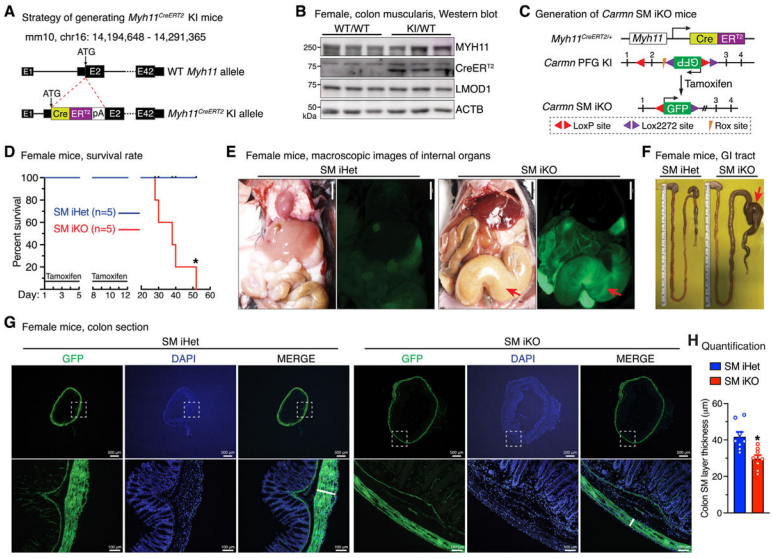

Figure.

SMC-specific, inducible deletion of *Carmn* in adult female mice results in premature lethality and drastic distension of colon and cecum. (A) Schematic diagram depicting the strategy to generate $Myh11^{CreERT2/+}$ knock-in (KI) mice. (B) Colon muscularis were isolated from WT or $Myh11^{CreERT2/+}$ female mice, and total proteins were extracted for Western blot analysis. (C) Strategy used to generate smooth muscle (SM)-specific *Carmn*-inducible knock-out (iKO) mice. (D) Kaplan-Meier survival curve showing respective longevity of iHet and iKO female mice after tamoxifen administration (n = 5). $*P < .05$ by log-rank (Mantel-Cox) test. (E) Macroscopic images acquired under bright field and GFP epifluorescence show a dramatically distended cecum (red arrows) in *Carmn* iKO female mice. Scale bar: 5 mm. (F) Representative macroscopic images of gastrointestinal tracts isolated from iHet and iKO female mice. Note the dramatically distended cecum and proximal colon (arrow) in female iKO mice. (G) Direct visualization of green fluorescent protein (GFP; green) in iHet and *Carmn* iKO mice. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). The boxed area is magnified under each respective panel. (H) The thickness of muscular wall of proximal colon from iHet and iKO mice (white bar in "G") was quantified and plotted. N = 8; $*P < .05$; unpaired student t test.