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***Basic Study***

**Claudin-7 gene knockout causes the destruction of intestinal and death of mice**

Xu C *et al*. Cldn7 knockout causes intestinal structure destruction

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**Abstract**

***BACKGROUND***

Claudin-7, one of the important components of cellular tight junctions, is currently considered to express abnormally in colorectal inflammation and colorectal cancer. However, there is currently no effective animal model to study its specific mechanism. Therefore, we constructed three Claudin-7 knockout mouse models using the Cre/Loxp system.

***AIM***

To determine the function of tumor suppressor gene Claudin-7 by generating three Claudin-7 gene knockout mouse models.

***METHODS***

We crossed Claudin-7-flox mice with CMV-Cre, vil1-Cre and villin-CreERT2 transgenic mice, and the offspring were self-crossed to obtain Claudin-7 conventional knockout mice, Claudin-7 intestinal conditional knockout mice and Claudin-7 inducible conditional knockout mice. Intraperitoneal injection of tamoxifen into the latter mice can induce the knockout of claudin-7. PCR and agarose gel electrophoresis were used to identify mice genotypes, and Western blot was used to detect the knockdown efficiency of Claudin-7. The mental state, body length and survival time of these mice were observed. The dying mice were sacrificed, and hematoxylin-eosin (HE) staining and immunohistochemical staining were performed on the intestine to observe changes in intestinal structure and proliferation markers.

***RESULTS***

We generated Claudin-7-flox mice and three Claudin-7 gene knockout mouse models using the Cre/LoxP system successfully. Claudin-7 conventional knockout mice and Claudin-7 intestinal conditional knockout mice were stunted and died during the perinatal period, intestinal HE staining of these mice revealed mucosal gland structure disappearance and connective tissue hyperplasia with extensive inflammatory cell infiltration. Claudin-7 intestinal inducible conditional knockout mice had a normal phenotype at birth, but after the induction of tamoxifen, the mice depicting a dying state. Intestinal HE staining showed significant inflammatory cell infiltration, and atypical hyperplasia and adenoma were also observed. Intestinal immunohistochemistry analysis showed abnormal expression and distribution of Ki67, the normal intestinal proliferation balance was disrupted. By measuring the crypt diameter, the intestinal crypt size in Claudin-7 intestinal inducible conditional knockout mice was also increased compared with control mice (small intestine: 54.1 ± 2.96 *vs* 38.4 ± 1.63; large intestine: 44.7 ± 1.93 *vs* 27.4 ± 0.60; *P* < 0.001).

***CONCLUSION***

The knockout of Claudin-7 *in vivo* causes extensive inflammation, atypical hyperplasia and adenoma in intestinal and death in mice. Claudin-7 may act as a tumor suppressor gene in the development of colorectal cancer.

**Key words:** Claudin-7; Gene knockout; Inflammation; Adenomas; Colorectal carcinoma

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**Core tip:** The intestinal tract of Claudin-7 conventional knockout mice and Claudin-7 intestinal conditional knockout mice is characterized by extensive and severe inflammation. The construction of Claudin-7 intestinal inducible conditional knockout mice can control the knockout of Cldn7 site-specific or time-specific, prolong the survival time of mice, and exhibit atypical hyperplasia and adenoma in the intestine. This study reveals the inhibitory role played by Claudin-7 in colorectal inflammation and colorectal cancer.

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**INTRODUCTION**

Members of the claudin family serve as important components of cellular tight junctions (TJs), and they mainly function to maintain cell polarity, regulate intercellular small molecule flux and facilitate cell proliferation and differentiation[1-3].Claudin-7 (Cldn7), one of the 27 members of the claudin family, is mainly distributed in the stomach, lung, intestine, bladder and kidney. Cldn7 was originally found in an extracellular Cl- barrier and Na+ channel and shown to affect extracellular permeability[4]. However, recent studies have shown that Cldn7 is abnormally expressed in different cancer tissues, especially in colon cancer, suggesting that alterations in its expression may affect the normal structure and function of TJs and be related to the occurrence of intestinal tumors[5-8]. Cldn7 is currently considered to play an inhibitory role in colorectal inflammation and colorectal cancer by most scholars[9-11].

The most effective way to study inhibitors *in vivo* is to knock out the gene in an animal and observe its overall phenotype. In recent years, Cre/Loxp recombinase system has been widely used in novel gene targeting[12,13]. LoxP was inserted at both ends of the Cldn7 sequence to obtain heterozygous flox mice. After hybridization with CMV-Cre and vil1-Cre mice, the sequence between the two LoxP sites was excised and inherited by daughter cells. Shimizu was the first to report time-specific gene knockout animal models in which the time of gene knockout could be artificially controlled by injection on an inducer[14].Therefore, we constructed Cldn7 gene conventional knockout (CKO) mice and conditional knockout (cKO) mice using Cre/Loxp system, we also generated Cldn7 inducible conditional knockout (ICKO) mice and induced Cre expression by injecting tamoxifen. Hematoxylin-eosin (HE) staining showed that the intestinal structures in the CKO and cKO mice were severely damaged, and numerous inflammatory cells were infiltrated. By injecting tamoxifen into the ICKO mice, we successfully established atypical hyperplasia and intestinal adenoma models. Immunohistochemistry analysis indicated that the expression and distribution of Ki67 in the intestinal tissues were dysregulated. The successful construction of mouse intestinal inflammation and intestinal adenoma models could provide quality animal carriers for further studying the role of Cldn7 in intestinal tumors.

**MATERIALS AND METHODS**

***Experimental animal species and animal care and use statement***

We inserted a LoxP site into the intronic sequence downstream of exon 4 of the Cldn7 gene and inserted the FRT-neo-FRT-LoxP element into the upstream intronic sequence of exon 2 to obtain Cldn7-flox mice. The CMV-Cre mice were purchased from the National Resource Center for Mutant Mice, the vil1-Cre mice were obtained from the Nanjing University of Chinese Medicine, and the villin-CreERT2 mice were donated by French professor Sylvie Robine. All mice were housed according to specific pathogen-free grade animal feeding standards at an indoor temperature of 20-26 °C and a 12-h day/night cycle. The mice were fed a standard diet after sterilization and had free access to food and water. All animals were euthanized for tissue collection. All animal assay protocols were reviewed and approved by the Medical Ethics Committee of the Capital Medical University Affiliated Beijing Shijitan Hospital Institutional Review Board.

***Construction of Claudin-7-flox mice***

We constructed a targeting vector as shown in Figure 1A. After the final vector was sequenced for validation, it was transfected into embryonic stem (ES) cells by electroporation. From the 8th-10th day, ES cell clones were picked, and genomic DNA was extracted, amplified, digested with the *Eco*RV enzyme overnight, and slowly electrophoresed for 36 h. The target clones were screened by long range PCR and Southern blot.

Approximately 4-wk-old C57BL/6N female rats were selected and injected with pregnant mare serum gonadotropin and human chorionic gonadotropin to promote ovulation. Embryos were harvested on the 2nd day after cohousing the female rats with the male rats, and 12-15 ES cells were injected into each blastocyst after culturing overnight. After the injection, the blastocysts were cultured for 3 h in an incubator, and those with a normal morphology and intact transparent bands were selected for transplantation. After 8-10 wk of sexual maturation, C57BL/6N female mice were selected for uterine blastocyst transplantation. The mice born after successful transplantation were identified by PCR, and those with the *fln/wt* genotype were deemed to be chimeric mice. Chimeric mice were crossed with Flper mice and then backcrossed with wild-type C57BL/6N mice to obtain Cldn7-flox mice (genotype: *fl/wt*), which were missing the entire Neo resistance gene.

***Construction of three knockout mouse models***

Cldn7-flox mice were crossed with CMV-Cre mice, vil1-Cre mice and villin-CreERT2 mice, and Cldn7 CKO mice, Cldn7 cKO mice and Cldn7 ICKO mice were then obtained by self-progeny. Next, 50 mg of tamoxifen was dissolved in 5 mL of sterilized sunflower oil and mixed for 30 min to obtain the tamoxifen dilution. Six- to eight-week-old ICKO mice were intraperitoneally injected with 100 μL of the tamoxifen dilution every 5 d to induce the Cldn7 knockout.

***Western blot***

Various Cldn7 knockout mouse tissues were minced on ice and mixed with appropriate grinding beads and total protein extraction reagents containing different protease inhibitors; a tissue homogenizer was then used to extract total protein. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with a diluted rabbit polyclonal anti-Cldn7 antibody (ab27487, 1:1000, Abcam, United States) at 4 °C overnight and then with a donkey anti-rabbit IgG antibody (1:10000, ab175780, Abcam, United States). After blotting, the signals were detected by a Western blot scanner. GAPDH was used as the internal reference.

***Hematoxylin-eosin staining***

The intestines of Cldn7 knockout mice and control mice were washed in PBS and then placed in 10% formalin/PBS at 4 °C. After dehydration and transparency, the intestines were immersed in wax and then cut into 5-8-micron-thick sections. The sections were then dewaxed and stained with hematoxylin-eosin (HE).

***Immunohistochemical staining***

All tissues were embedded in wax blocks and cut into paraffin sections. After dewaxing, hydration and antigen retrieval, the tissue sections were incubated for 10 min in 3% H2O2 and washed with 0.01 mol/l PBS. The sections were then incubated with a diluted rabbit polyclonal anti-Cldn7 antibody (ab27487, 1:200, Abcam, United States), a rabbit monoclonal anti-Ki67 antibody (ab16667, 1:1000, Abcam, United States), followed by incubation with the corresponding horseradish peroxidase-labeled secondary antibody. The proteins were then developed in 3,3′-diaminobenzidine for coloration and assessment.

***Statistical analysis***

Statistical analyses were performed using IBM SPSS version 17.0 and GraphPad Prism version 6.0. All data are expressed as the mean ± SD. Differences between two groups were analysed by Student’s *t*-test and considered significant at *P* < 0.05.

**RESULTS**

***Claudin-7-flox mice were constructed successfully***

We constructed the Cldn-7 gene knockout targeting vector as shown in Figure 1A, and sequenced the final vector using a unidirectional primer (LoxPtF: GTACGAGTTTGGACCTGCCA) to detect whether the 34 bp LoxP site was inserted correctly (Figure 1B). The LoxP site is shown in yellow, the 3’UTR is shown in green. FRT sequencing from the 5’ end was performed using a unidirectional primer (Cldn7-FRT-tF: CTGATCTGGGTGTCCCACGT), as the FRT site serves as a screening marker for Neo. By removing the Neo resistance gene, the FRT site was also removed. The second LoxP site is shown in green, the FRT site is shown in yellow, and Neo is shown in purple (Figure 1C). FRT 3’ sequencing was then performed (FRT-tR: CGATGAAACCGTTCCAGGTA), the presence of another FRT site is shown in pink font (Figure 1D). Therefore, the final gene targeting vector was correct.

The targeting vector was electroporated into B6/BLU ES cells for targeting, and some drug-resistant ES cell clones were obtained. Two methods were used to prevent false-positive results and detect target clones, long range PCR and Southern blot. First, we tested whether the 5' homologous arm strip was correct (Figure 2A). The 5496 bp product was a positive clone containing LoxP. The 3’ end was also detected (Figure 2B), and the 5204 bp product was a positive clone. The Southern blot identification results are shown in Figure 2C. Genomic DNA from the transfected ES cells was digested with *Spe* I restriction endonuclease. Gene fragments of 17.8 kb and 9.5 kb were obtained from the Cldn7 5’ end of the wild-type and target clones, respectively, and gene fragments of 17.8 kb and 7 kb were obtained from the Cldn7 3’ end. When using the *Eco*RV restriction enzyme (probe on Neo), an 11.7 kb gene fragment was obtained from the target clone. The Southern blot results showed that clones 8D, 8E, 4F, 11E, and 11F were the final positive clones.

The positive clone 8D was selected for blastocyst injection, and the newborn mice after blastocyst transfer were genotyped by PCR. The primer information is shown in Table 1. The mice numbered 50 and 77 were deemed chimeric mice with the genotype *fln/wt* (Figure 2D and E). The chimeric mice were crossed with Flper mice to remove the Neo resistance gene, and the resulting mice were mated with wild-type C57BL/6N mice to completely delete the Neo resistance gene, successfully yielding Cldn-7-flox mice.

***Claudin-7 conventional knockout mice die in*** ***the perinatal period and have severe intestinal damage***

Cldn7-flox mice were crossed with CMV-Cre mice, and the offspring were then self-crossed. Genomic DNA was isolated from the tails for genotyping. The primer information is shown in Table 2. The mouse with the Null/Null CreW genotype was considered the Cldn7 CKO mouse. We considered newborn mice from the same litter as an example (Figure 3). We first evaluated whether Neo was completely deleted. None of the samples showed a fln band at 515 bp, indicating that Neo had been deleted completely. Furthermore, none of the samples showed the Cre band at 481 bp, suggesting that the genotype of all samples was CreW. Next, the banding results showed that mice numbered 1, 5, 6, and 10 had a null band at 640 bp, suggesting that Cre was recombined. The final step was to identify whether the mice were homozygous, and mouse 6 was determined to be homozygous for the Null/Null genotype. Therefore, mouse 6 was deemed the Cldn7 CKO mouse with the Null/Null CreW genotype (Figure 3A).

Cldn7 CKO mice were born similar to heterozygous and wild-type mice. However, the lengths of the mice were measured daily, and the lengths of the Cldn7 CKO mice increased significantly slower than those of the control mice (*P* < 0.05).

Beginning on the third day, CKO mice were thin, lacked energy, showed signs of lethargy, exhibited decreased body temperature, activities were reduced, and the mice thus showing a state of dying (Figure 3B).

The dying mice were sacrificed, and their lungs, stomachs, bladders, kidneys, small intestines and large intestines were collected. Western blot analysis showed that Cldn7 was not expressed in any of the tissues analyzed in CKO mice, while the control mice expressed Cldn7 in all of these tissues (Figure 3C). Intestinal HE staining showed obvious atrophy, thinning or loss of intestinal mucosa, connective tissue hyperplasia with inflammatory cell infiltration, residual intestinal mucosal epithelial vacuolar degeneration, villus shortening, and lymphatic expansion. Intestinal HE staining of the control mice showed no obvious histopathological changes (Figure 3D). Therefore, Cldn7 CKO mice showed significantly slow growth and appeared to be dying on the third day. HE staining showed severe intestinal destruction, loss of intestinal mucosal structure, and infiltration of numerous inflammatory cells.

***Claudin-7 intestinal conditional gene knockout mice have longer survival*** ***times***

Cldn7-flox mice were crossed with vil1-Cre mice, and the offspring were then self-crossed. Genomic DNA was isolated for genotyping analysis. The mouse with the Cldn7fl/fl; villin-CreT (*fl*/*fl* CreT) genotype was deemed the Cldn7 cKO mouse. None of the samples showed a fln band at 515 bp or a null band at 640 bp, indicating that Neo had been deleted completely and that no Cre recombination occurred. We next detected whether flper recombination occurred by the detection of a *fl* band at 756 bp. The results showed that mouse no. 213 had the *fl*/*wt* genotype, while all other mice had the *fl*/*fl* genotype. Finally, Cre was detected, and a band at 481 bp, which corresponded to CreT, was observed in mice numbered 213, 215, and 217, while all other mice displayed a band corresponding to CreW. Therefore, the mice numbered 215 and 217 were deemed Cldn7 cKO mice.

Cldn7 cKO mice were normal at birth compared to control mice. But Cldn7 cKO mice were obviously thin, their body length increased slowly from the fifth day after birth, and their growth rate was significantly slower than that of control mice (*P* < 0.05). On the 9th day, Cldn7 cKO mice appeared to be languid, with reduced or even inactive activities, leaving only a slight breath. The body temperature of the mouse was reduced and it was in a state of dying (Figure 4B).

The dying mice were sacrificed, and we found the expression levels of Cldn7 in the lung, stomach, bladder and kidney tissues of cKO mice were normal, while Cldn7 expression was knocked out in the small and large intestines. All control mouse tissues expressed Cldn7 (Figure 4C). Intestinal HE staining showed obvious mucosal atrophy, mucosal gland structure disappearance, and connective tissue hyperplasia with extensive inflammatory cell infiltration. Inflammatory lesions were observed everywhere (Figure 4D), and mucosal epithelial vacuolar degeneration was observable after magnification. No obvious histopathological changes were observed in the mucosal glands, submucosa, or muscular layers of the intestines of *fl*/*fl* CreW mice. Cldn7 cKO mice excluded the influence of other organs lacking Cldn7 expression on mice and prolonged the survival time. However, because the cKO mice still showed intestinal inflammation, we generated Cldn7 ICKO mice on this basis to try to obtain an adenoma model.

***Claudin-7 inducible conditional gene knockout mice display atypical hyperplasia and adenoma and dysregulated proliferation in their intestines***

Cldn7-flox mice were crossed with villin-CreERT2 mice, and the offspring were then self-crossed. PCR and agarose gel electrophoresis results showed the mice numbered 135-137, 139-142, and 144 were Cldn7 ICKO mice with the genotype of Cldn7fl/fl; villin-CreERT2 (*fl*/*fl* CreERT2).

Cldn7 ICKO mice were normal at birth and developed smoothly, not unlike Cldn7fl/fl; villin-CreW (*fl*/*fl* CreW) mice (Figure 5B left). A tamoxifen solution (10 mg/mL, 100 μL) was intraperitoneally injected into 6 to 8-wk-old ICKO mice every 5 d. Beginning at the 7th injection, the ICKO mice were lethargic, lack of activity, appeared thin, and exhibited a dying state, while the control mice showed no abnormalities (Figure 5B left). All ICKO mice died within 75 d (15 tamoxifen injections, Figure 5B right).

Both the dying mice and control mice were sacrificed. The expression levels of Cldn7 in the lung, stomach, bladder and kidney tissues of ICKO mice were normal, but Cldn7 expression was knocked out in their small and large intestines. All control mouse tissues expressed Cldn7 (Figure 5C). Intestinal HE staining showed obvious inflammatory manifestations (Figure 5D, E, G, and H), numerous infiltrated inflammatory cells, abnormal or absent intestinal villi and intestinal gland structure, mucosal epithelial cell shedding, and disordered residual intestinal villus mucosal epithelial cells that lacked polarity. Atypical hyperplasia (Figure 5J and M) and intestinal adenoma (Figure 5K and N) were also observed. The intestinal structure was normal and intact in the control mice (Figure 5F, I, L, and O), no obvious pathological changes were observed.

Immunohistochemistry analysis was also used to detect the expression of intestinal Cldn7 and the nuclear proliferation marker Ki67 in ICKO and control mice, revealing that Cldn7 was strongly expressed in the intestinal epithelial junction of control mice, while its expression was significantly weakened in the intestines of ICKO mice (Figure 6A-D). In addition, the expression of Ki67 was also altered. In control mice, Ki67 was mainly distributed in portions of the crypt, while Ki67-positive cells occupied the entire intestinal crypt in ICKO mice (Figure 6E-6H). By measuring the crypt diameter, the intestinal crypt size in ICKO mice was increased compared with that in control mice (Figure 6I and J). Furthermore, Ki67 was mostly expressed in the crypt area in control mice, while Ki67 in the intestinal tract of the ICKO mice was no longer confined to the crypt area. Cells expressing Ki67 were observed throughout the entire intestinal villi, and this manifestation was more pronounced in the large intestine.

**DISCUSSION**

Abnormal expression of Cldn7 can lead to the destruction of TJ structure and function as well as cell proliferation and migration abnormalities[15],which are closely related to the occurrence and development of malignant tumors, such as lung cancer, ovarian cancer and gastric cancer[16-18].However, the specific mechanisms underlying these phenomena remain elucidated.

Cldn7 is widely considered to be a tumor-suppressor gene[19-21], and one of the best methods for researching tumor-suppressor genes is to knock out the gene *in vivo* and then observe the phenotype of the entire animal[22]. Tamura *et al*[23] generated Cldn15-/- mice using the conventional gene targeting strategy. Cldn15 is similar to Cldn7 and strongly expressed in the duodenum, jejunum, ileum, and colon, while other claudin family proteins, such as Cldn6, 9, 10, 11, and 14, are not expressed in the intestine[20]. Cldn15-deficient mice formed mega intestines, in which the upper small intestine was two times larger than the normal intestine. Moreover, because Cldn15 deletion is not lethal, researchers can observe intestinal development at different time points, such as at 1 wk, 4 wk, and 10 wk after birth[22]. Similarly, we constructed Cldn7 CKO mice using the same strategy, and this mouse model showed severe intestinal defects that included mucosal ulcerations, epithelial cell sloughing and inflammation. However, Cldn7 CKO mice died beginning on the third day, which was not conducive to long-term observation or subsequent experiments. Additionally, the effects of Cldn7 deletion in other organs on survival time and morphological changes could not be excluded. Therefore, prolonging the survival time of Cldn7 knockout mice is necessary to further study the function of Cldn7.

Cre/LoxP technology makes it possible to knock out a gene in a site-specific or time-specific manner[22]. Cre recombinase can be expressed in specific cell types, and the hybridization between flox mice and Cre mice can then be used to remove genes between two LoxP sites in specific tissues. When Cre recombinase is fused to a ligand-binding domain of a mutant human estrogen receptor (ER), it becomes a tamoxifen-dependent Cre recombinase (Cre-ERT)[24]. Time-specific gene knockout can be achieved by injecting tamoxifen into transgenic mice at different growth stages. Using this method, we constructed Cldn7 intestinal cKO mice. A Western blot assay confirmed that only intestinal Cldn7 was knocked out. While Cldn7 cKO mice had a normal phenotype after birth, by the fifth day, the mice grew slowly and lacked energy. On the 9th day, the dying mice were sacrificed; their intestinal tracts showed severe inflammation, and their mucosal or glands appeared abnormal. Tanaka H et al. also constructed Cldn7 intestinal cKO mice[25],which had a longer survival period of 28 d due to the use of a different Cre enzyme mouse model. Tanaka H found that intestinal Cldn7 knockout changed only the paracellular flux of small molecule solutes and did not completely destroy the TJ structure. However, both knockout mice are intestinal inflammation models not yet showing adenomas or tumors. We further constructed Cldn7 ICKO mice and controlled their survival time and intestinal morphological changes by changing the dose and frequency of tamoxifen. When the mice received 1 mg of tamoxifen every day, they began dying after the 5th injection, and severe inflammation was observed in the intestine (data not shown). When the mice received 1 mg of tamoxifen every 5 d, they began to show signs of dying after the 7th injection, and all mice died within 15 tamoxifen injections. Approximately 71.4% of the mice developed adenomas in different regions, including the duodenum, jejunum, ileum, and colon.

Immunohistochemical staining showed that compared with that in control mice, the intestinal crypt size in ICKO mice was increased, and cells positively expressing Ki67 covered the entire crypt. Ki67 expression was no longer limited to the crypt but rather to the crypt-villus axis. These phenomena suggested that the loss of Cldn7 led to both expansion of the intestinal crypt and proliferation of cells at the crypt, and the normal proliferation-differentiation balance of intestinal cells along the crypt-villus axis was disrupted.

In the intestine, proliferating epithelial cells are specifically confined to the crypts[26]. Recent studies have shown that the crypt microenvironment is important for the generation and maintenance of proliferating cells[27]. Because TJs play a critical role in maintaining inner homeostasis, they may be important for formation of the niche, the microenvironment of the crypt for stem and transit-amplifying cells[24]. Therefore, the construction of Cldn7 ICKO mice is of great significance for studying the relationship among intestinal barrier destruction, dysregulation of intestinal stem cell proliferation and differentiation along the crypt-villus axis, changes in the intestinal crypt microenvironment and intestinal tumor formation.

In conclusion, we report the novel findings that Cldn7 knockout cause extensive inflammation, atypical hyperplasia and adenoma in intestinal and death in three mouse models. Therefore, Cldn7 may act as a tumor suppressor gene in the development of colorectal cancer, the specific mechanism of Cldn7 can be elucidated by performing further research on the ICKO mouse model.

**ARTICLE HIGHLIGHTS**

***Research background***

Claudin-7, one of the important components of cellular tight junctions, is currently considered to express abnormally in colorectal inflammation and colorectal cancer. However, there is currently no effective animal model to study its specific mechanisms. Therefore, we constructed three Claudin-7 knockout mouse models using the Cre/Loxp system to provide animal carriers for further studying the role of Claudin-7 in intestinal tumors.

***Research motivation***

Claudin-7 is currently considered to play an inhibitory role in colorectal inflammation and colorectal cancer. The most effective way to study inhibitors *in vivo* is to knock out the gene in an animal and observe its overall phenotype. Therefore, we constructed Claudin-7 gene conventional knockout (CKO) mice and conditional knockout (cKO) mice using Cre/Loxp system, we also generated Claudin-7 inducible conditional knockout (ICKO) mice and induced Cre expression by injecting tamoxifen. The successful construction of these mouse models and intestinal inflammation and intestinal adenoma models could provide quality animal carriers for further studying the role of Claudin-7 in intestinal tumors.

***Research objectives***

The main objective is to construct three Claudin-7 knockout mouse models to achieve space-specific and time-specific knockout of Claudin-7, precise the site of gene knockout, and prolong the survival time of mice. Due to the prolonged growth time of mice, the organs were differentiated and matured when claudin-7 was knocked out. So this animal model can provide a good vector for further study of Claudin-7.

***Research methods***

We constructed three Claudin-7 knockout mouse models using the Cre/Loxp system. Firstly, chimeric mice were constructed by transfecting the target vector into embryonic stem cells, screening the hybrid clones into the female mouse blastocysts, and transplanting the blastocysts into the female mouse uterus. Chimeric mice were then purified to obtain Claudin-7-flox mice. Secondly, Claudin-7-flox mice were crossed with CMV-Cre mice, vil1-Cre mice and villin-CreERT2 mice, and Claudin-7 CKO mice, Claudin-7 cKO mice and Claudin-7 ICKO mice were obtained by self-progeny. ICKO mice were induced by intraperitoneal injection of tamoxifen to knockout Claudin-7 in intestinal. Finally, western blot was used to verify the knockout efficiency of Claudin-7. Hematoxylin-eosin (HE) staining confirmed the structural changes and pathological changes of intestinal tract in Claudin-7 knockout mice. Immunohistochemical staining was used to observe the proliferation markers. The construction of cKO mice prolonged the lifespan of CKO mice, and the ICKO mouse was the first animal model to specifically knock out Claudin-7 in space and time.

***Research results***

We generated Claudin-7-flox mice and three Claudin-7 gene knockout mouse models successfully. Claudin-7 CKO mice and Claudin-7 cKO mice were stunted and died during the perinatal period, intestinal HE staining of these mice revealed mucosal gland structure disappearance and connective tissue hyperplasia with extensive inflammatory cell infiltration. Claudin-7 ICKO mice had a normal phenotype at birth, but after the induction of tamoxifen, the mice depicting a dying state. Intestinal HE staining showed significant inflammatory cell infiltration, and atypical hyperplasia and adenoma were also observed. Intestinal immunohistochemistry analysis showed abnormal expression and distribution of Ki67, the normal intestinal proliferation balance was disrupted.

***Research conclusions***

This study innovatively constructed three Claudin-7 knockout mouse models, which clarified that Claudin-7 plays an inhibitory role in colon inflammation and colon adenoma, and initially found that Claudin-7 may promote the development of colon adenomas by affecting proliferation. This study successfully simulated two phenomena of intestinal inflammation and intestinal adenoma, and proposed a new animal model research method. This study is important to clarify the role of Claudin-7 in colonic inflammation and tumors, laying the groundwork for finding early clinical diagnosis and potential therapeutic targets.

***Research perspectives***

This article details described the construction of Claudin-7-flox mice and the process of hybridization with three Cre mice. Based on this experience, we can construct ICKO mice that knock out Claudin-7 in kidney and skin and some other organs, and then observe changes in mice before and after Claudin-7 deletion. In the next step of the study, the dose of tamoxifen should be clarified, and the tumor should be induced in the intestinal tract of ICKO mice. The direction of the future research is to clarify the specific mechanism of Claudin-7 in inflammatory bowel disease and intestinal tumorigenesis, invasion and metastasis; and to explore the relationship between Claudin-7 and stem cells, and the role played in intestinal development.

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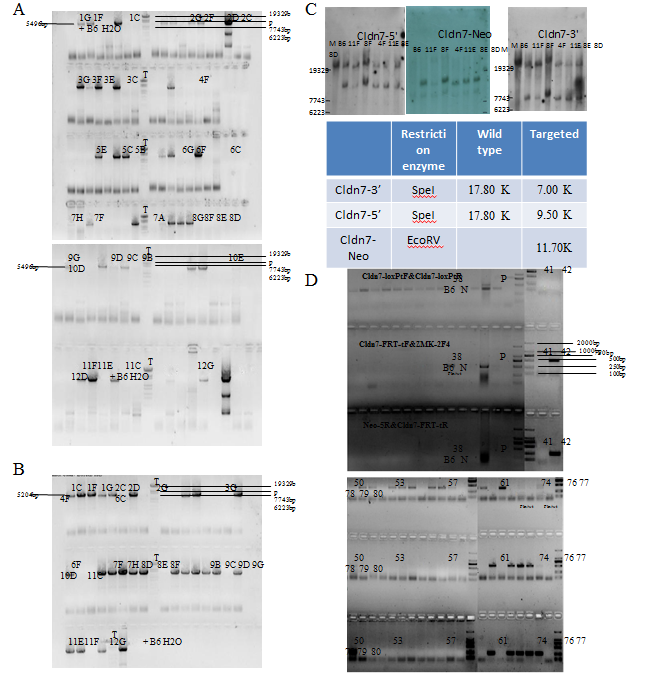
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**Figure 1** **Strategic design and final vector sequencing results.** A: Schematic diagram of the Cldn7 gene knockout targeting vector; B-D: The first and second regions in red show the sequencing results at LoxP and FRT 5’, while the grey region shows the FRT 3’ sequencing results; B-D indicate that the Cldn7 terminal vector was correct. Cldn7: Claudin-7.



**Figure 2 Long range PCR and Southern blot were used to detect target clones.** A: The 5’ homologous arm band was first detected, and the product with a length of 5496 bp was a positive clone containing LoxP; B: The 3’ end was then detected, and the product with a length of 5204 bp was a positive clone; C: The Southern results showed that clones 8D, 8E, 4F, 11E, and 11F were the final targeted clones; D-E: After blastocyst transfer, newborn mice were genotyped by PCR. The mice numbered 50 and 77 were deemed chimeric mice with the genotype *fln*/*wt*.



**Figure 3** **Phenotypic and intestinal pathological changes in Cldn7 CKO mice.** A: Genotype analysis showed that mouse no. 6 was a Cldn7 CKO mouse with the genotype *Null*/*Null* CreW, marker: 8000/5000/3000/2000/1000/750/500/250/100bp; B: Cldn7 CKO mice were similar to control mice at birth but grew slower than control mice (*P* < 0.05); from the third day, CKO mice appeared in a dying state; C: CKO mice expressed no Cldn7 in any tissues, while all tissues of control mice expressed Cldn7; D: Intestinal HE staining of Cldn7 CKO mice showed obvious atrophy of intestinal mucosa with inflammatory cell infiltration (as indicated by the arrow), villus shortening and lymphatic expansion (as indicated by the arrowhead); the control mice showed no obvious histopathological changes. Cldn7: Claudin-7; CKO: Conventional knockout.



**Figure 4 Phenotypic and intestinal pathological changes in Cldn7 cKO mice.** A: Genotype analysis showed the mice numbered 215 and 217 were Cldn7 intestinal cKO mice with the genotype Cldn7fl/fl; villin-CreT; B: Cldn7 cKO mice were normal at birth, but their body lengths increased more slowly from the fifth day after birth than those of control mice (*P* < 0.05); on the 9th day, cKO mice were in poor spirits and died thereafter; C: Cldn7 expression was normal in the lung, stomach, bladder and kidney tissues of cKO mice but knocked out in their small and large intestines; all tissues of the control mice expressed Cldn7; D: Intestinal hematoxylin-eosin staining of Cldn7 cKO mice showed connective tissue hyperplasia with extensive inflammatory cell infiltration in the submucosa (as indicated by the arrow); mucosal epithelial vacuolar degeneration can be observed after magnification (as indicated by the arrowhead); there were no obvious histopathological changes in *fl*/*fl* CreW mice. Cldn7: Claudin-7; Cko: Conditional knockout.



**Figure 5 Phenotypic and intestinal pathological changes in Cldn7 ICKO mice.** A: Genotype analysis showed that mice numbered 135-137, 139-142, and 144 were Cldn7 ICKO mice with the genotype Cldn7fl/fl; villin-CreERT2; B: Cldn7 cKO mice were normal at birth and developed smoothly, but beginning at the 7th injection, ICKO mice were in poor health and appeared to be dying; all ICKO mice died within 15 tamoxifen injections; C: Cldn7 was expressed at normal levels in the lung, stomach, bladder and kidney tissues of ICKO mice but knocked out in their small and large intestines; all tissues of the control mice expressed Cldn7; D-O: Intestinal hematoxylin-eosin staining of ICKO mice showed obvious inflammatory manifestations; atypical hyperplasia and intestinal adenoma were also observed; the intestinal structure of the control mice was normal. Cldn7: Claudin-7; cKO: Conditional knockout; ICKO: Inducible conditional knockout.



**Figure 6 Increased intestinal proliferation in Cldn7 ICKO mice.** A-D: Immunohistochemistry staining showed that Cldn7 was strongly expressed in the intestinal epithelial junctions of control mice but significantly weakened in ICKO mice; E-H: In control mice, Ki67 was mainly distributed in parts of the crypt, while Ki67-positive cells occupied the entire intestinal crypt and could be observed throughout the intestinal villi in ICKO mice; I-J: The size of the intestinal crypt in ICKO mice was increased compared with that in control mice (*P* < 0.001). Cldn7: Claudin-7; ICKO: Inducible conditional knockout.

**Table 1 PCR primers for chimeric mice genotype identification and corresponding band results**

|  |  |
| --- | --- |
| **Sequence(5’-3’)** | **Results** |
| Cldn7 loxptF primer : GTACGAGTTTGGACCT GCCA | Fln/Fln = 493 bp  Fln/wt = 493/375 bp  wt/wt = 375 bp |
| Cldn7-LoxP-tR primer : TGTGCAA GGATCTGGGTCTG |
| Cldn7-FRT-tF primer : CTGATCTGGGTGTCCCACGT | Fln/Fln = 507 bp  Fln/wt = 507/0 bp  wt/wt = 0bp |
| ZMK-2F4 primer: GCATCGCATTGTCTGAG TAGGTG |
| Neo-5R primer : GGCTGG ACGTAAACTCCTC | Fln/Fln = 259 bp  Fln/wt = 259/0bp  wt/wt = 0 bp |
| Cldn7-FRT-tR primer: CGATGAAACC GTTCCAGGTA |

**Table 2 PCR primers for gene knockout mice genotype identification and corresponding explanations**

|  |  |  |
| --- | --- | --- |
| **Primer sequence(5’-3’)** | **Results** | **Description** |
| Cldn7-FRTtF2: CCTGGGATCTGATCTGGGTG | wt = 600 bp, fln = none  Fl = 756 bp, Null = none | Check if flper is reorganized |
| Cldn7-FRTtR2: GGCAGGTAGCCTTAGGATGG |
| ZMK2F4: GCATCGCATTGTCTGAGTAGGTG | wt = none, fln = 515 bp fl = none, Null = none | Check if Neo is deleted completely |
| Cldn7-FRTtF2: CCTGGGATCTGATCTGGGTG |
| Cldn7-loxPtF2: CTTGGGAGACATCAGGTCCG | wt = 512 bp, fln = 630 bp fl = 630 bp, Null = none | Homozygous identification |
| Cldn7-loxPtR2: GAGGCAATAGGCCCAAGGAG |
| Cldn7-FRTtF2: CCTGGGATCTGATCTGGGTG | wt = none, fln = none fl = none, Null = 640 bp | Check if Cre is reorganized |
| Cldn7-loxPtR2: GAGGCAATAGGCCCAAGGAG |
| Cre-up: GCCTGCATTACCGGTCGATGC | T: 481 bp W: none | Detect Cre |
| Cre-low: CAGGGTGTTATAAGCAATCCC |