EpCAM is essential for maintenance of the small intestinal epithelium architecture via regulation of the expression and localization of proteins that compose adherens junctions

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Abstract. Epithelial cell adhesion molecule (EpCAM) is highly expressed in mammalian intestines, and is essential for maintaining the homeostasis of the intestinal epithelium. EpCAM protein is localized at tight junctions and the basolateral membrane of the intestinal epithelium, where it interacts with many cell adhesion molecules. To explore the molecular functions of EpCAM in regulating adherens junctions in the intestinal epithelium, EpCAM knockout embryos and newborn pups were analyzed. Hematoxylin and eosin staining was used to assess the histology of the duodenum, jejunum, ileum and colon from wild-type and EpCAM$^{-/-}$ mice at E18.5, P0 and P3. The expression and localization of adherens junction-associated genes and genes that encode the proteins that participate in the assembly of adherens junctions were measured at the mRNA and protein levels using qPCR, western blot analysis and immunofluorescence staining. The results showed that although there was no significant damage to the intestines of EpCAM$^{-/-}$ mice at E18.5 and P0, they were significantly damaged at P3 in mutant mice. The expression of adherens junction-associated genes in EpCAM mutant mice was normal at the mRNA level from E18.5 to P3, but their protein levels were gradually reduced and mislocalized from E18.5 to P3. The expression of nectin 1, which can regulate the assembly and adhesion activity of E-cadherin, was also gradually reduced at both the mRNA and protein levels in the intestinal epithelium of EpCAM mutant mice from E18.5 to P3. In summary, the loss of EpCAM may cause the reduction and mislocalization of proteins that compose adherens junctions partly via the downregulation of nectin 1 in the intestines.

Introduction

EpCAM, the epithelial cell adhesion molecule, is a hemophilic Ca^{2+}-independent cell-cell adhesion molecule, and it is expressed in different kinds of epithelial tissues (1-3). In addition to its function in cell adhesion, EpCAM has been reported to contribute to various biological processes, such as signaling, migration and proliferation (2,4). EpCAM has been considered to be a cell surface marker for many kinds of stem cells (5,6). Moreover, EpCAM is also highly expressed in epithelial tumor tissues (7). However, the molecular mechanisms of these processes remain to be elucidated.

EpCAM mutations in humans can cause congenital tufting enteropathy (CTE), a rare diarrheal disease that can cause neonatal death (8). Several EpCAM knockout mouse models have been reported to generate a CTE phenotype (9-11). From these reports, it can be concluded that EpCAM has important physiological functions in the intestines of human and mammalian animals. EpCAM was determined to be highly expressed in developing adult intestinal epithelium (2,10). In addition, EpCAM is also highly expressed in many kinds of stem cells, neoplastic tissues, and other normal epithelial tissues, such as embryonic stem cells, hepatocyte progenitor cells, and labyrinthine layer of placentas (1,12-14). EpCAM recruits proteins of the claudin family to maintain functional tight junctions in the intestinal epithelium (10,15,16). Loss of functional tight junctions in the intestinal epithelium may be one of the important

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causes of diarrheal disease in humans and mice with mutated EpCAM. EpCAM is not only localized to tight junctions but is also enriched at the basolateral membrane of the intestinal epithelium (2). This suggests that EpCAM may have some interactions with other types of cell junctions. However, the conclusions regarding the function of EpCAM in regulating adherens junctions are contradictory. Previous findings have shown that EpCAM may suppress E-cadherin-mediated cell aggregation by disrupting the association of E-cadherin with the cytoskeleton in cultured murine fibroblast L cells (17,18). It has also been reported that E-cadherin could exert opposing effects on EpCAM, especially in tumor tissues (2). In addition, E-cadherin protein was decreased (19) or mislocalized (20) in EpCAM-depleted human colorectal adenocarcinoma Caco-2 cell line. However, Wu *et al* found that the expression and localization of E-cadherin and β-catenin were still normal after EpCAM knockdown in human colorectal adenocarcinoma T84 cell line (16). Results from *in vivo* studies were also different. In biopsy specimens from children with CTE, the expression of E-cadherin was normal (21). Lei *et al* also found that the expression level and localization of E-cadherin and β-catenin were normal in the embryonic intestines of EpCAM knockout mice (10). However, it has been reported that E-cadherin and β-catenin proteins progressively lose cell membrane localization and accumulate in the cytoplasm in the intestinal epithelium of EpCAM knockout mice after birth (9). In EpCAM mutant zebrafish, the expression of E-cadherin, β-catenin and α-catenin was reduced in the enveloping layer, but the localization of these proteins was still normal (4). Moreover, global depletion of EpCAM in Xenopus embryos caused a significant decrease in C-cadherin at the protein level (19). The molecular roles of EpCAM in regulating adherens junctions, especially in mammalian models, are still unclear.

In the present study, to investigate the functions of EpCAM in regulating adherens junctions in the mammalian intestinal epithelium, the expression of proteins that compose adherens junctions was assessed at both the mRNA and protein levels in different sections of the intestinal epithelium from EpCAM$^{-/-}$ and wild-type (WT) mice at E18.5 to P3, providing new evidence for the effects of EpCAM on adherens junctions in the intestinal epithelium.

Materials and methods

Animal treatment. All mouse experiments were approved by the Committee on Laboratory Animal Care and Use of Guangdong Pharmaceutical University [Guangzhou, China (gdpu2016073)].

EpCAM$^{-/-}$ mice were previously generated by CRISPR/Cas9 technology (22). Mice were housed in the specific pathogen-free (SPF) animal facility at 25°C with a 12-h light/dark cycle and 50-55% humidity. The animals had free access to water and food. Heterozygous males and females were mated and a total of 47 pairs of WT and EpCAM$^{-/-}$ embryos and pups were used for the current study. E18.5 embryos were harvested from pregnant females. E18.5 embryos and P0 and P3 pups were sacrificed, after which their intestines were collected for experiments. Pregnant mice were sacrificed by cervical dislocation. E18.5 embryos were obtained by dissecting pregnant

females. E18.5 Embryos, P0 and P3 pups were sacrificed humanely by decapitation. The information of the number and genotype of mice used in the present work was summarized in Table SI.

Histological analysis. For histological analysis, duodenum, jejunum, ileum and colon tissues from WT and EpCAM$^{-/-}$ mice were fixed overnight with 4% paraformaldehyde in PBS at 4°C before being dehydrated and embedded in paraffin. Then, hematoxylin and eosin (H&E) staining was performed on 4-μm paraffin sections; the sections were incubated in hematoxylin for 2 min and eosin for 30 sec at room temperature. Images were captured using the PerkinElmer Automated Quantitative Pathology System.

Immunofluorescence staining. For immunofluorescence staining, duodenum, jejunum, ileum and colon tissues from WT and EpCAM$^{-/-}$ mice were fixed overnight with 4% paraformaldehyde in PBS at 4°C before being dehydrated and embedded in optimal cutting temperature compound (OCT) (Sakura Finetek). Then, 7-μm frozen sections were boiled in 10 mM citric acid (Merck) at pH 6.0 for 5 min. After exposure to goat serum blocking buffer (ZSGB-BIO, ZLI-9056) at room temperature for 1 h, the sections were incubated overnight at 4°C with primary antibodies and then with secondary antibodies at room temperature for 1 h. Primary antibodies were used as follows: Rabbit anti-E-cadherin (Cell Signaling Technology, Inc.; cat. no. 14472, 1:200), rabbit anti-p120-catenin (Santa Cruz Biotechnology, Inc.; cat. no. 15D2, 1:200), and rabbit anti-β-catenin (BD Biosciences; cat. no. 610154, 1:200). Immunofluorescence analysis was performed with Alexa Fluor 488-labeled donkey anti-rabbit IgG (H+L) secondary antibodies (Thermo Fisher Scientific, Inc.; cat. no. A21206, 1:1,000), and DAPI (Sigma-Aldrich; Merck KGaA; cat. no. D9564, 1:10,000) was used to stain the nuclei of tissues. Immunofluorescence images were observed using a PerkinElmer Automated Quantitative Pathology System.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from mouse small intestines was extracted using an RNAiso Plus kit (Takara; cat. no. 9109), which was subjected to reverse transcription using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara; cat. no. RR047A) at 37°C for 15 min and 85°C for 5 sec. The sequences of primers for RT-qPCR are listed in Table SII and they were produced by Sangon Biotech Co., Ltd. RT-qPCR was conducted using a TB Green® Premix Ex Taq™ II Kit (Takara; cat. no. RR820A) and the PikoReal PCR system (Thermo Fisher Scientific, Inc.). The thermocycling program was 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 65°C for 15 sec. Mouse GAPDH was used as an internal reference gene.

Western blot analysis. The small and large intestines of mice were collected and washed in cold PBS (HyClone). Then, they were lysed in radioimmunoprecipitation assay lysis buffer containing 1% PMSF as well as 1% protease inhibitor cocktail (Dalian Meilun Biotechnology Co., Ltd.), and centrifuged at 13,680 x g at 4°C for 30 min, and the supernatant was frozen at -80°C. Protein concentration was determined by BCA assay (Beyotime), and equal amounts of protein (20 μg) were
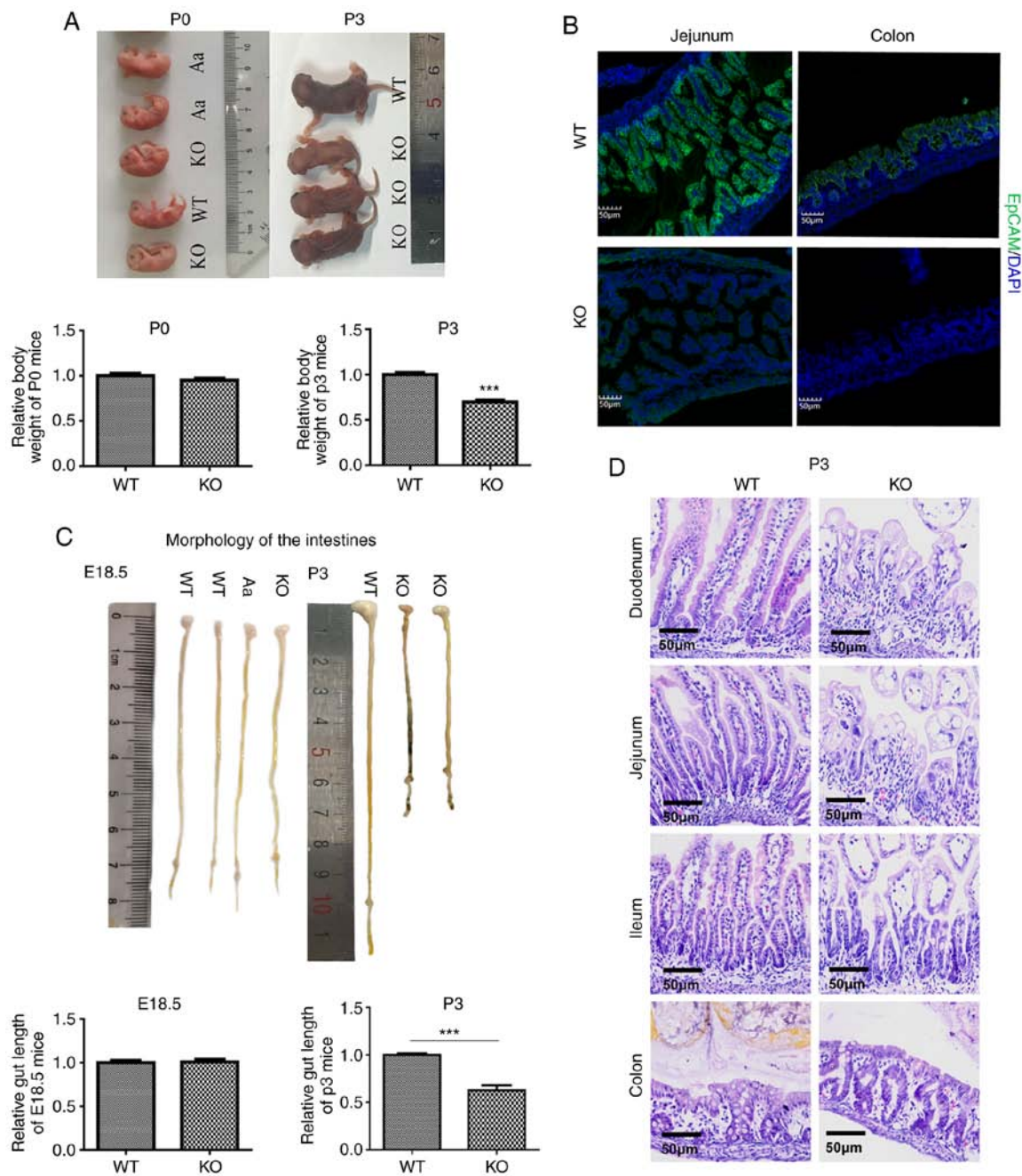
Figure 1. The phenotype and intestinal defects of EpCAM^{-/-} mice. (A) The body size and body weight of WT and EpCAM^{-/-} mice at P0 (n=20) and P3 (from 5 WT pups and 6 EpCAM^{-/-} pups) stages. (B) Images of immunofluorescent staining of EpCAM in the jejunum and colon of WT and EpCAM^{-/-} mice at E18.5 stage. Nuclei were also stained with DAPI. Bar, 50 μm. (C) The morphology and length of intestines from WT and EpCAM^{-/-} mice at E18.5 (n=8) and P3 (n=6) stages. (D) Images of H&E staining of the duodenum, jejunum, ileum and colon from WT and EpCAM^{-/-} mice at P3 stage. Scale bar, 20 μm. ***P<0.001. WT, wild-type; EpCAM, epithelial cell adhesion molecule; H&E, hematoxylin and eosin.

subjected to SDS-PAGE on a 10 or 12% gel. The separated proteins were transferred electrophoretically to a PVDF membrane, after which the membrane was blocked with 5% non-fat milk at room temperature for 1 h and then incubated with primary antibodies including E-cadherin (Cell Signaling Technology, Inc.; cat. no. 14472, 1:1,000), p120-catenin (Santa Cruz Biotechnology, Inc.; cat. no. 15D2, 1:1,000), β-catenin (BD Biosciences; cat. no. 610154, 1:1,000), nectin 1 (Abcam; cat. no. ab66985), and α-catenin (Sigma-Aldrich; Merck KGaA; cat. no. C2081) at 4˚C overnight. Subsequently, the membrane was incubated with horseradish peroxidase-labeled antibodies [goat anti-rabbit IgG H&L (HRP) (Abcam; cat. no. ab6721,

1:2,000) and goat anti-mouse IgG H&L (HRP) (Abcam; cat. no. ab6789, 1:5,000)] at room temperature for 1 h. The resultant signals were detected using enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.; cat. no. 170-5060). Western blotting bands were quantitatively analyzed using Lane 1d software (version 5.1.0.0; SageCreation).

Statistical analysis. Statistical differences were determined using SPSS software (23.0). Unpaired two sample t-test was used to determine the difference among groups. The data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.
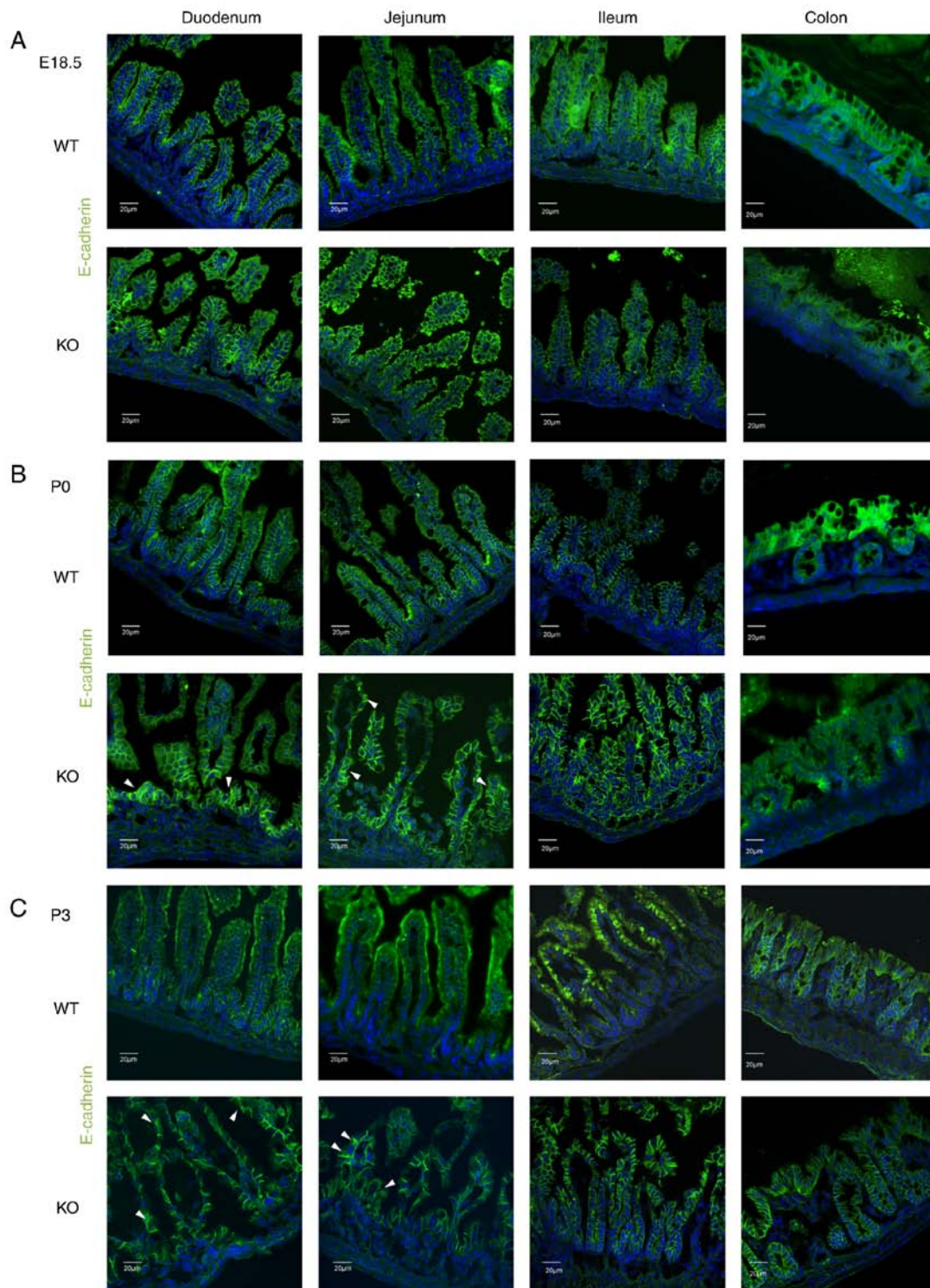
Figure 2. The E-cadherin protein gradually mislocalized in the small intestinal epithelial cells of postnatal EpCAM mutant mice during development. Images of immunofluorescent staining of E-cadherin in the duodenum, jejunum, ileum and colon from WT and EpCAM$^{-/-}$ mice at E18.5 (A), P0 (B), and P3 (C) stages. Scale bar, 20 μm. White arrowhead, the mislocalization of E-cadherin. WT, wild-type; EpCAM, epithelial cell adhesion molecule.

Results

The architecture of intestines from postnatal EpCAM mutant mice was gradually broken down during development. To study the functions of EpCAM *in vivo*, EpCAM$^{-/-}$ mice that we previously generated through CRISPR-Cas9 technology were used software used for densitometry (22). The phenotype of EpCAM$^{-/-}$ mice is similar to that of EpCAM mutant mice, which were

generated using traditional gene targeting methods (9,10,15). The newborn EpCAM$^{-/-}$ mice were indistinguishable from their WT and heterozygous littermates at P0. However, there was no increase in the body size of EpCAM$^{-/-}$ pups during development, and the body weight of P3 EpCAM$^{-/-}$ pups was significantly lower than that of their littermates (Fig. 1A).

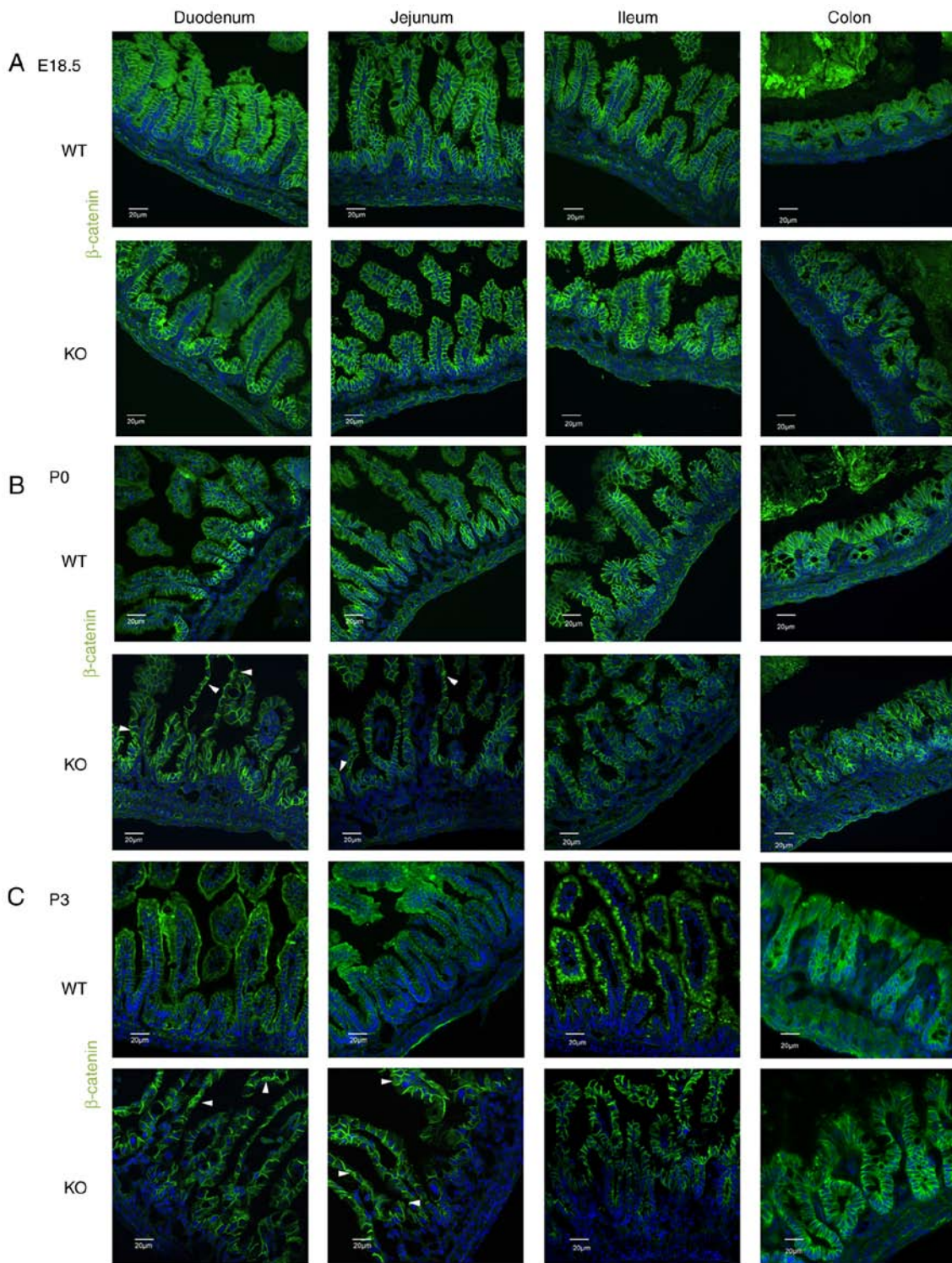Since a loss-of-function mutation in EpCAM can cause CTE in patients (8) and the intestines were seriously affected

Figure 3. The β-catenin protein gradually mislocalized in the small intestinal epithelial cells of postnatal EpCAM mutant mice during development. Images of immunofluorescent staining of β-catenin in the duodenum, jejunum, ileum and colon from WT and EpCAM^{-/-} mice at E18.5 (A), P0 (B), and P3 (C) stages. Scale bar, 20 μm. White arrowhead, the mislocalization of β-catenin. WT, wild-type; EpCAM, epithelial cell adhesion molecule.

in previously reported EpCAM mutant mouse models (9), the morphological and histological changes of intestines of EpCAM^{-/-} mice from E18.5 to P3 were investigated in this study. It was first confirmed that the EpCAM protein was completely lost in the intestines of EpCAM^{-/-} mice (Fig. 1B). The morphology of the intestines from EpCAM^{-/-} mice looked normal for both E18.5 (Fig. 1C) and P0 (data not shown). Then, it became abnormal at P3. The length of the intestines

of mutant mice at P3 was significantly shorter than that for WT pups at P3. The diameter of the ileum from the WT pups was thinner than that of the duodenum and jejunum, but in EpCAM^{-/-} pups, the diameter of the ileum became larger than that of the duodenum and jejunum. Blood could be observed in the intestinal lumen of P3 EpCAM^{-/-} mice (Fig. 1C). Tufts of villi could be detected in the small intestines of EpCAM^{-/-} mice from E18.5 to P3, as shown by H&E staining (Figs. 1D and S1).
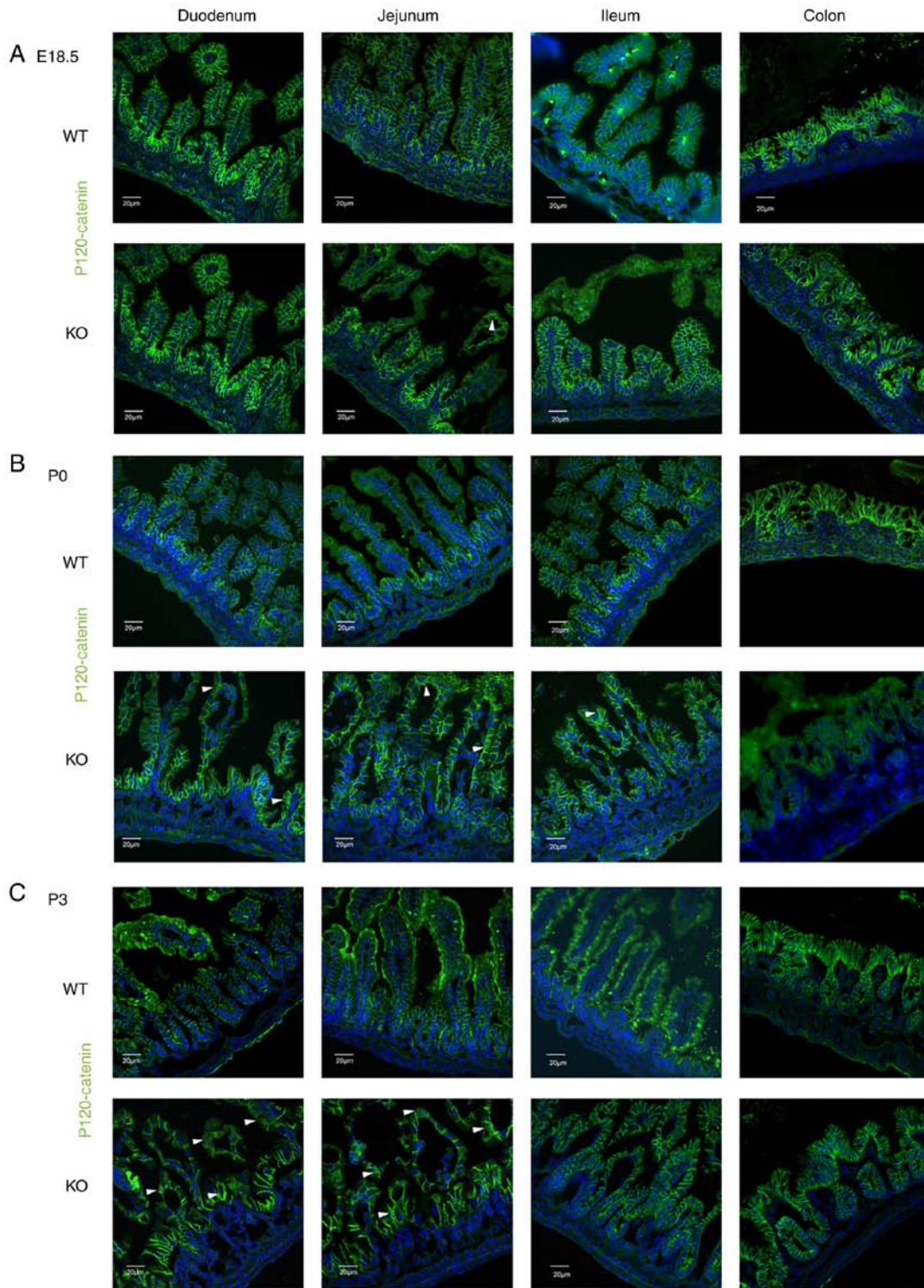
Figure 4. The p120 protein gradually mislocalized in the small intestinal epithelial cells of postnatal EpCAM mutant mice during development. Images of immunofluorescent staining of p120 on the duodenum, jejunum, ileum and colon from WT and EpCAM$^{-/-}$ mice at E18.5 (A), P0 (B), and P3 (C) stages. Scale bar, 20 μm. White arrowhead, the mislocalization of p120. WT, wild-type; EpCAM, epithelial cell adhesion molecule.

There was no significant damage to villi from EpCAM$^{-/-}$ mice at E18.5 and P0 (Fig. S1). However, the intestinal epithelium was gradually broken down during development of the mutant mice after birth, and the size of intestinal epithelial cells in mutant mice at P3 became very large (Fig. 1D).

Proteins that compose adherens junctions gradually mislocalize in the epithelial cells of the small intestine in postnatal

EpCAM mutant mice during development. To investigate whether the composition and functions of adherens junctions (AJs) in the intestinal epithelium were affected by the loss of EpCAM, the proteins that compose the AJs were analyzed via immunofluorescence (IF) staining. E-cadherin, β-catenin and p120 were only found to be localized to the membrane between epithelial cells in the intestines of WT mice from E18.5 to P3 (Figs. 2-4). The localization of these proteins was normal in
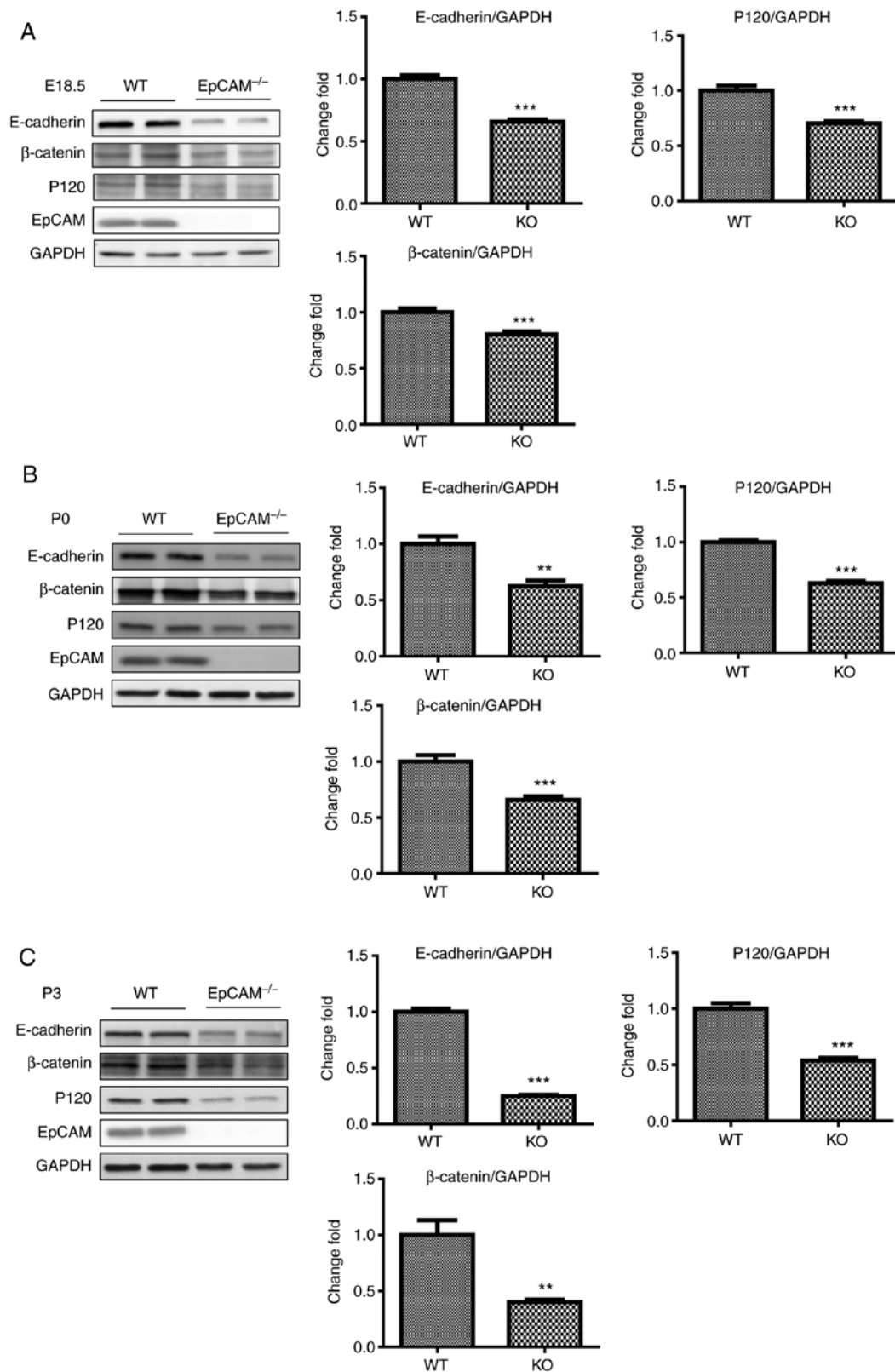
Figure 5. Proteins that compose adherens junctions were gradually reduced in the small intestinal epithelial cells of EpCAM mutant mice during development. Western blot results of E-cadherin, β-catenin, and p120 in the small intestines of WT and EpCAM^{-/-} mice at E18.5 (n=4) (A), P0 (n=4) (B) and P3 (n=4) (C) stages respectively. [**]P<0.01, and [***]P<0.001. WT, wild-type; EpCAM, epithelial cell adhesion molecule.

the intestines of EpCAM^{-/-} mice at E18.5, and the IF signal of these proteins was even higher in the duodenum and jejunum of EpCAM^{-/-} mice than that in the WT mice at this stage (Figs. 2A, 3A, and 4A, and S2A, S3A, and S4A). Since the intestinal

tight junctions of EpCAM^{-/-} mice were impaired at the E18.5 stage (10), the intestinal epithelium was affected, although it was still not clear at the morphology level. The higher IF signal was induced by the affected tissues of the EpCAM^{-/-} mice. However,
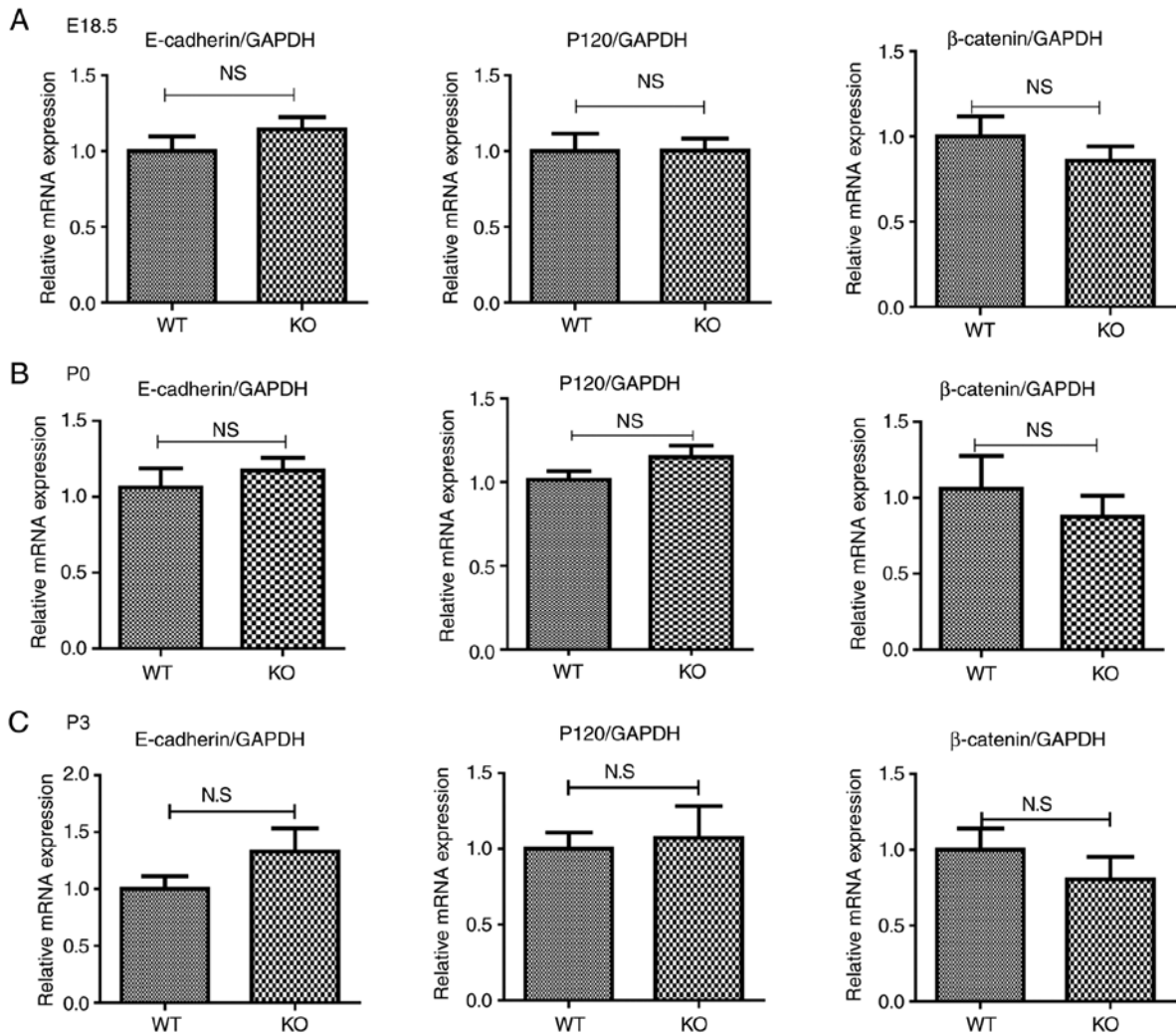
Figure 6. The mRNA expression of proteins that compose adherens junctions was normal in the small intestines of EpCAM mutant mice during development. qPCR results of E-cadherin, β-catenin, and p120 in the small intestines of WT and EpCAM$^{-/-}$ mice at E18.5 (A), P0 (B) and P3 (C) stages respectively. Eight samples from each group were used at E18.5 and P3 stages. At P0 stage, 4 samples from each group were used to test the mRNA levels of E-cadherin and β-catenin, and 7 samples from each group were used to test the expression level of p120. WT, wild-type; EpCAM, epithelial cell adhesion molecule.

some of these proteins began to be detected in the cytoplasm of epithelial cells in the duodenum and jejunum of EpCAM$^{-/-}$ pups at P0 (Figs. 2B, 3B, and 4B, and S2B, S3B, and S4B). At P3, the signals of these proteins clearly localized to the cytoplasm of epithelial cells in the duodenum and jejunum of EpCAM$^{-/-}$ pups. The localization of E-cadherin, β-catenin and p120 in the epithelium of the ileum and colon in EpCAM$^{-/-}$ mice was still normal from E18.5 to P3. The signal of these proteins was not detected in the nucleus of the epithelial cells of the intestines of either WT or EpCAM$^{-/-}$ mice from E18.5 to P3 (Figs. 2C, 3C, and 4C, and S2C, S3C, and S4C).

Proteins that compose adherens junctions are gradually reduced in the intestinal epithelial cells of postnatal EpCAM mutant mice during development. The expression levels of proteins that compose adherens junctions in the small intestines were further analyzed by western blot analysis. The protein levels of E-cadherin, β-catenin and p120 in the small intestines of EpCAM$^{-/-}$ mice at E18.5 was slightly lower than that of WT mice, but their reduction was significant (Fig. 5A). At P0, the reduction in the protein levels of E-cadherin,

β-catenin and p120 in the small intestines of EpCAM$^{-/-}$ mice was more apparent (Fig. 5B). At P3, only approximately half as much p120 protein was observed in the small intestines of EpCAM$^{-/-}$ pups, and the protein levels of E-cadherin and β-catenin in the small intestines of EpCAM$^{-/-}$ pups were reduced by more than half compared with the levels in WT pups (Fig. 5C).

mRNA expression of proteins that compose adherens junctions was normal in the intestines of postnatal EpCAM mutant mice during development. To examine how EpCAM regulated the localization and expression of proteins that compose adherens junctions, their gene expression was assessed at the mRNA level by qPCR. At E18.5 and P3, the expression of E-cadherin, β-catenin and p120 mRNA in the small intestines was similar between WT and EpCAM$^{-/-}$ mice (Fig. 6). The expression of E-cadherin mRNA in the small intestines of EpCAM$^{-/-}$ pups was increased compared with that of WT pups at the same stage, and the expression of β-catenin mRNA in the small intestines of EpCAM$^{-/-}$ mice was reduced, but these changes were not significant (Fig. 6).
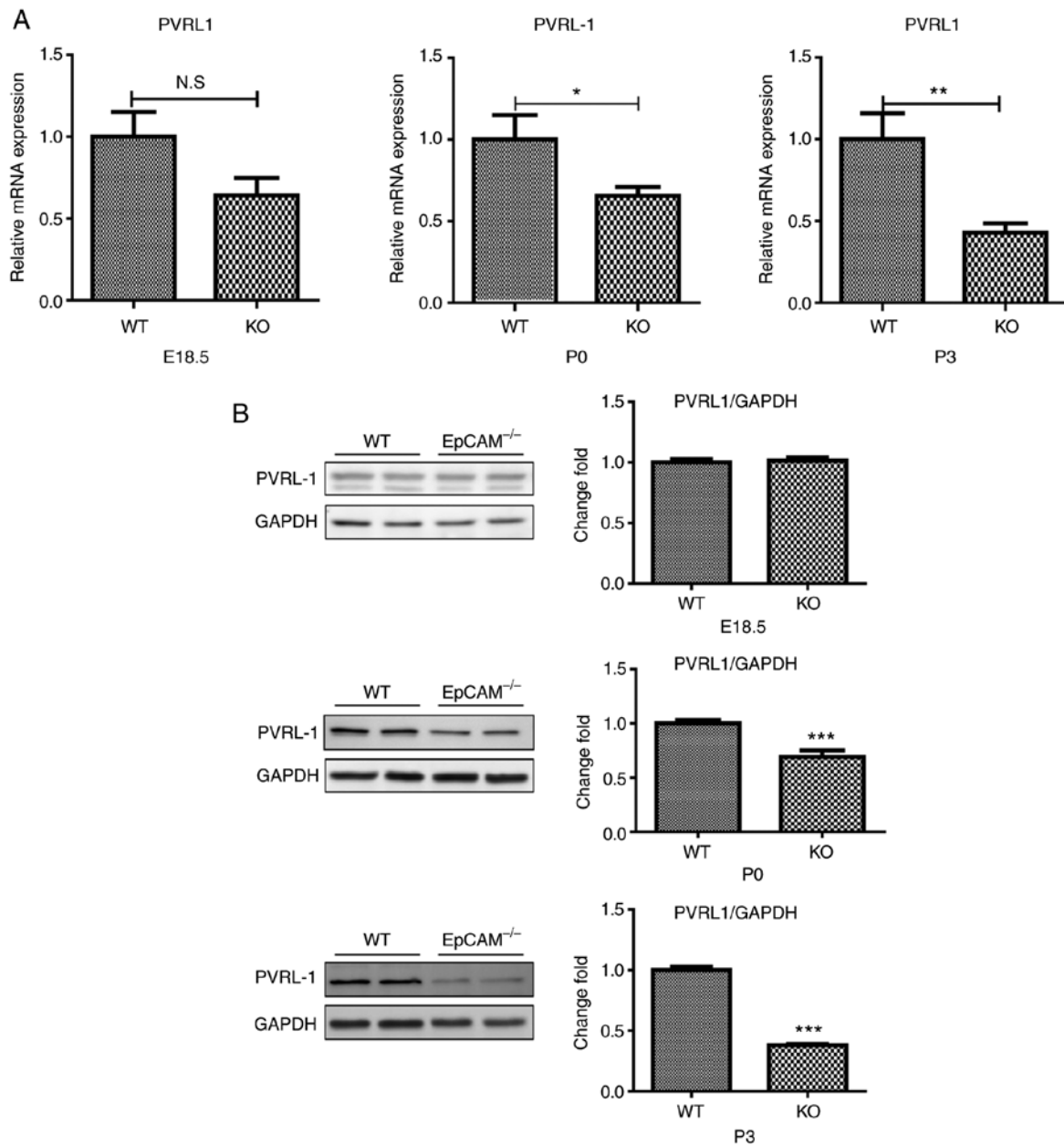
Figure 7. The expression of nectin 1 gradually decreased in the intestines of postnatal EpCAM mutant mice during development. (A) qPCR results of nectin 1 in the small intestines of WT and EpCAM^{-/-} mice at E18.5 (n=6), P0 (n=7) and P3 (n=8) stages. (B) Western blot results of nectin 1 in the small intestines of WT and EpCAM^{-/-} mice at E18.5, P0 and P3 (n=4). *P<0.05, **P<0.01, and ***P<0.001. WT, wild-type; EpCAM, epithelial cell adhesion molecule.

Expression of nectin 1 became gradually reduced in the intestines of postnatal EpCAM mutant mice during development. The expression of genes that encoded proteins that compose adherens junctions was further assessed. Nectin 1, also known as PVRL1, which can regulate the assembly and adhesion activity of E-cadherin (23), was found to be significantly changed in the intestines of EpCAM^{-/-} mice (Fig. 7A and B). The qPCR results showed that the expression of nectin 1 mRNA in the small intestines of EpCAM^{-/-} mice was significantly reduced at P0 and P3, although it was still normal at E18.5 (Fig. 7A). The expression of nectin 1 protein in the small intestines of EpCAM^{-/-} mice was normal at E18.5 and P0, but compared to control mice, there was only half as much nectin 1 protein in the intestines of P3 EpCAM^{-/-} mice (Fig. 7B).

Discussion

In the present study, we confirmed that the adherens junctions in the upper part of the small intestinal epithelium of postnatal EpCAM^{-/-} mice were gradually affected, but they were still normal in the lower part of the intestinal epithelium in EpCAM^{-/-} mice. The results of H&E staining showed that the architecture of the duodenum and jejunum of EpCAM^{-/-} mice at P3 was broken down, but the architecture of the ileum and colon was not affected even at P3. Moreover, the expression level and localization of E-cadherin, β-catenin and p120 were also gradually affected in the duodenum and jejunum of EpCAM^{-/-} mice from E18.5 to P3 stages, but they were not affected in the ileum and colon of EpCAM^{-/-} mice even at P3 stage. Finally, the expression of nectin 1 was

reduced at both the mRNA and protein levels, which may be one of the important reasons for the breakdown of adherens junctions in the intestinal epithelium of postnatal EpCAM^{-/-} mice. These results first showed the detailed process and mechanism of the adherens junctions breakdown in the intestinal epithelium of EpCAM^{-/-} mice.

The intestine was the most severely damaged organ in the EpCAM mutant mice, and the mutation of EpCAM could also cause CTE in human. Therefore, we only assessed the AJs in the intestines at the current study. The assumption was that the AJs of some other organs in the EpCAM mutant mice may also be affected slightly, and we aim to test AJs in other tissues of EpCAM^{-/-} mice in future studies. The function of EpCAM in maintaining the architecture of the intestinal epithelium has been confirmed by several studies, since it was found that the mutation of EpCAM could cause CTE (8,9-11,24). Total or partial villus atrophy and crypt hyperplasia could be found in the epithelium of small intestines from CTE patients (8,25). Lei *et al* found that the intestines of EpCAM knockout mice were relatively normal at the neonatal stage, as revealed by both macroscopic and histological data, but the disruption of mucosal architecture and sloughing of epithelial cells could be detected in EpCAM knockout mice at P5, especially in the duodenum and jejunum (10). Guerra *et al* also reported the increasing severity of villous atrophy and an increase in epithelial tufts in EpCAM mutant intestines (9). In the present study, significant changes in the intestinal epithelium of EpCAM^{-/-} mice also occurred after birth. The upper part of the intestinal epithelium was broken down, but the lower part was normal even at P3. These results demonstrated that the intestinal architecture breakdown occurred earlier in the upper parts of the intestines than it did in the lower parts. Since the EpCAM mutation could impair tight junction formation in the intestinal epithelium, Lei *et al* suggested that the abundant proteases in the upper parts of the small intestines might be the cause of this phenotype (10). In the present study, we hypothesize that these proteases could easily penetrate the upper parts of the intestinal epithelium in the EpCAM mutant and cause damage. Therefore, many swollen cells appeared in the upper parts of the small intestines of EpCAM^{-/-} mice, which could exacerbate the architectural breakdown of the upper parts of the small intestines in the EpCAM mutant. Therefore, the compositions of adherens junctions in the duodenum and jejunum of EpCAM mutant mice may be affected by proteases.

Several proteins that compose adherens junctions have been demonstrated to be essential for maintaining the homeostasis and morphogenesis of intestines. Specific knocking out E-cadherin in the intestinal epithelium of adult mice can cause hemorrhagic diarrhea because of the abnormal intestinal epithelial architecture (26), and deletion of E-cadherin from the developing mouse intestinal epithelium causes death shortly after birth because of the impairment of intestinal morphogenesis (27,28). Transgenic expression of a dominant-negative N-cadherin (DN-cadherin) in the mouse small intestine can cause loss of endogenous E-cadherin protein and induce Crohn-like inflammatory bowel disease at the age of 3 months, with most of these mice developing

adenomas within 6 months (29). Hermiston *et al* also found that the forced expression of E-cadherin in the mouse intestinal epithelium resulted in slower cellular migration from crypts to villi (30). Knocking out p120 in the mouse intestinal epithelium resulted in mucosal damage and inflammation, leading to bleeding and death within 3 weeks of birth (31). Smalley-Freed *et al* found that limited ablation of p120-catenin in the adult mouse intestinal epithelium could promote adenoma formation by an indirect non-cell autonomous mechanism (32), and Short *et al* confirmed that p120 was an obligate haploinsufficient tumor suppressor in intestinal neoplasia, as shown by a conditional p120 knockout in Apc-sensitized mouse models (33). β-catenin is the key molecule in the Wnt signaling pathway (34), and it is essential for many biological processes in the intestines, such as maintenance of intestinal stem cells, inflammation and carcinogenesis (15,35-38). Therefore, any factor that could affect the expression or localization of proteins that compose adherens junctions in the intestines would affect the homeostasis of the intestinal epithelium. Since EpCAM is essential for maintaining the homeostasis of the intestinal epithelium, previous studies have tried to elucidate the changes in proteins that compose adherens junctions in the EpCAM mutant intestines (9,10,21), but their conclusions were based on different developmental stages and different parts of the intestines. Furthermore, intestinal tissues from CTE patients are very difficult to obtain. In the present study, we compared the whole intestines of WT and EpCAM^{-/-} mice at E18.5, P0 and P3, and we found that the E-cadherin, p120 and β-catenin proteins in the duodenum and jejunum of P0 EpCAM^{-/-} mice started to decrease and mislocalize at P0, and they were seriously affected at P3, but these proteins in the ileum and colon were not affected even at P3. These results demonstrated that EpCAM could protect the architecture of the duodenum and jejunum by maintaining the expression and localization of proteins that compose adherens junctions in these areas. The mRNA levels of these adherens junction-associated genes were not decreased, so we concluded that EpCAM may regulate the expression of these genes at the post-transcriptional level.

E-cadherin, β-catenin, and p120 are three important proteins which composed adherens junctions, and the interactions between them have been clearly studied. There is a multiple complex at adherens junctions, and the binding sides of β-catenin and p120 on E-cadherin are very clear now (39). If EpCAM could directly interact with one of these proteins in the multiple complex of the adherens junctions, it could be detected by Co-IP experiments. However, Wu *et al* have found that EpCAM and E-cadherin were not tightly associated in T84 cells through Co-IP experiments (16). It means that EpCAM is not directly associated with one of the proteins in the multiple complex of the adherens junctions.

Nectins are a type of immunogroblin (Ig)-like cell adhesion molecule, and they can regulate the formation of adherens junctions and tight junctions (40,41). We found that the mRNA level of nectin 1 was significantly reduced at the P0 and P3 stages, while its protein level was also significantly reduced at the P3 stage in EpCAM^{-/-} mice.

Sato *et al* reported that nectin 1 could regulate the assembly and adhesion activity of E-cadherin in MDCK cells (23). Martinez-Rico *et al* found that nectin 1 had important roles in the regulation of E-cadherin-based adhesion (42). We hypothesize that the reduction of nectin 1 may be one of the important reasons for the decrease and mislocalization of proteins that compose adherens junctions. It has been reported that EpCAM could control at least four independent signaling pathways (1-3), and the expression of nectin 1 may control one or more of these signaling pathways. Furthermore, the impaired tight junctions of the intestinal epithelium from EpCAM$^{-/-}$ mice may disrupt intracellular signaling pathways which are essential to regulate the nectin 1 expression. Our previous study had already shown that claudin proteins were downregulated in the intestines of EpCAM mutant mice (10).

In summary, we examined the expression of claudin-7 at both mRNA and protein levels after obtaining the current EpCAM$^{-/-}$ model through CRISPR/Cas9 technology. The results yielded were similar to those of our previous studies. Since the current study focused on adherens junctions of the intestinal epithelium, the claudin-7 results were not included. Nevertheless, further investigations are needed to examine the manner in which EpCAM regulates the expression of nectin 1.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JG and ZL designed the study and conceived its execution. ZL and YY analyzed and interpreted the results, and wrote and revised the manuscript. GC, WL and LH maintained the mouse model and performed all qPCR and immunostaining experiments. YY, LY, YL, and HW performed the H&E staining and western blots. All authors read the final manuscript and approved it.

Ethics approval and consent to participate

All mouse experiments were approved by the Committee on Laboratory Animal Care and Use of Guangdong Pharmaceutical University [Guangzhou, China (gdpu2016073)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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