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Establishing Prokaryotic Expression System of Angiotensin-Converting Enzyme 2 (ACE2) gene in pigs

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10 ## Abstract

11 In this paper, ACE2 gene of pigs was cloned and the purified protein was obtained via
12 the prokaryotic expression system. Polyclonal antibody of high titer and sensitivity
13 was obtained using Wastar rats immunization method and is then used to determine of
14 the expression of ACE2 using immunohistochemistry. The sequence of ACE2 in pigs
15 covered 2418 nucleotides and coded 805 amino acid (aa) residues. Sequence
16 homology analysis showed that the ACE2 sequence in pigs is highly conserved
17 among species at the nucleotide and amino acid levels. Genetic evolution analysis
18 revealed that ACE2 gene in pigs has the shortest genetic distance with that in goats
19 while residing in a totally different branch from that in zebra fishes. Analysis of
20 protein structure predicted that ACE2 protein is a transmembrane secreted protein
21 with high hydrophilicity, containing a signal peptide sequences locating between 1aa
22 to 17aa. The ACE2 fusion protein expressed (under the induction with 1.0 mmol/L
23 IPTG for 10 h) was of approximately 100 kDa and mainly existed in inclusion body.
24 Wastar rats immunization showed that the titer of the anti-ACE2 antiserum in rats was
25 1: 3200. Western blot showed that the antibody binds specifically.

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26 Immunohistochemistry showed that the ACE2 protein was expressed in all major
27 tissues of pigs. It is the first time that polyclonal antibody of ACE2 in pigs was
28 obtained and the expression of ACE2 was confirmed. These results will provide a
29 basis for investigating on ACE2's biological activity in pigs.

30 **Key words:** Angiotensin Converting Enzyme 2, Pig, Gene cloning, Bioinformatics
31 analysis, Expression

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32 # 1 INTRODUCTION

33 The renin–angiotensin system (RAS) is an importance regulator of
34 cardiovascular and renal function, both under physiological and pathological
35 conditions(Tipnis et al., 2000). Angiotensin converting enzyme 2 (ACE2) was
36 discovered as a new member of the RAS system in 2000. Earlier studies showed that
37 ACE2 in rats or humans was highly expressed in the kidney, heart and testicle tissues,
38 and was later confirmed existence in the lungs, brain, and the intestines and other
39 organs(Lambert et al., 2010; Pei et al., 2010; Briones et al., 2010; Imai et al., 2005)
40 The ACE2 genes in human, rat and mouse have been located on the X chromosome.
41 The ACE2 protein main structure consists of an extracellular N terminal catalytic
42 domain, a transmembrane region and a C terminal anchored on the cell
43 membrane(Danilczyk et al., 2006). The metalloproteinases, such as ADAM metal
44 protease domain 17 (ADAM17), can hydrolyze the extracellular domain (which is of
45 enzyme activity) to produce active soluble ACE2 form, which has carboxypeptidase
46 activity and participates in the hydrolysis of AngI, AngII and other peptides. Among
47 all, the main function of it is to hydrolyze the phenylalanine at the C-end of the AngII
48 to produce Ang (1-7). Ang (1-7) is later combined with the Mas receptor and plays
49 various protective functions, which antihypertensive activities and exhibits some
50 biological actions that are quite distinct from Ang II(Hashimoto et al,. 2012; Wang et
51 al,. 2016).

52 So far, the research on ACE2 has been focused on rodents and human beings.
53 The research gaps in other animals, especially production animals, such as cattle,
54 sheep, pigs still need to be filled. Particularly, GenBank still lacks the information of
55 ACE2 gene in pigs. Recently, Zhang has reported a segment of the ACE2 gene
56 sequence in Chinese Jiangsu local Hybrid Pigs(Zhang et al,. 2012). However the size
57 of the gene fragment is only 641 bp.

58 Therefore, this study was aimed at determining the whole cDNA sequence of
59 porcine ACE2 gene using gene cloning and other molecular biology techniques and
60 establishing a prokaryotic expression system of ACE2 in pigs. The porcine ACE2

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61 polyclonal antibody was obtained and the expression distribution of ACE2 was
62 determined. We hope to be able to provide the whole sequence of ACE2 gene and other
63 related information for Genbank.

64 2 MATERIALS AND METHODS

65 2.1 Ethics Statement

66 All animal procedures were approved by the Institutional Animal Care and Use
67 Committee of Nanjing Agricultural University. The protocols were reviewed and
68 approved, and the project number 2011CB100802 was assigned. The slaughter and
69 sampling procedures strictly followed the 'Guidelines on Ethical Treatment of
70 Experimental Animals' (2006) no. 398 established by the Ministry of Science and
71 Technology, China and the 'Regulation regarding the Management and Treatment of
72 Experimental Animals' (2008) no. 45 set by the Jiangsu Provincial People's
73 Government.

74 2.2 Samples collection

75 The Healthy newborn piglets were terminally anesthetized via an intraperitoneal
76 (ip.) injection of 20% urethane (5 mL/kg body weight), were decapitated within 15
77 min after anesthesia took effect (i.e. animals lost conciseness and whole body muscle
78 relaxation was observed), and all tissues including heart, liver, hung, kindey, stomach
79 and soon were collected and frozen immediately in liquid nitrogen and then stored at
80 -80°C , Part of tissues were fixed immediately with 4% buffered paraformaldehyde
81 for 24 hours, then transferred to 20% sucrose solution for 3 h.

82 2.3 Primer design

83 Primers for the ACE2 and β-actin genes were designed with Primer 5.0 software
84 and were synthesized by Dingguo, Beijing, China based on their respective porcine
85 sequences to produce an amplification product. which the primers for β-actin (an I
86 nternal control) were forward 5'-GATCTGGCACCACACCTTCT-3', and reverse
87 5'-CCAGAGGCATACAGGGACAG3'; and the primers for ACE2 were forward 5'
88 GGATCCATGTCAGGCTCTTTCTGGCT-3', and reverse
89 5'-GAGCTCCTAAAACGAAGTCTGAATGTCATCGC-3'; The expected size of the

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90 porcine ACE2 gene product was 2418 bp (GenBank accession no. NM_001123070).

91 **2.4 RNA extraction and RT-PCR**

92 Total RNA was extracted by using the RNeasy mini kit (Qiagen, Valencia CA),
93 and RNA concentrations were determined by a ultraviolet colorimetry
94 (ThermoScientific, Wilmington, DE). One microgram of total RNA was reverse
95 transcribed to cDNA using two step reverse transcription inversion following the
96 manufacturer's protocol. ACE2 mRNA expression were amplified by PCR with
97 β-actin as an internal control (Bt03279175); The amplification conditions of PCR
98 reaction were 95°C for 5 min, 32 cycles of denaturation at 98 °C for 10 sec, annealing
99 at 57°C for 15 sec, extension at 72°C for 30 sec, and finally an additional extension
100 step at 72°C for 10 min. The amplified products were identified by 1% agarose gel
101 electrophoresis.

102 **2.5 Purification, cloning and sequence analysis of PCR products**

103 **2.5.1 Purification of PCR products**

104 The target gene from PCR was recovered by a gel Recovery Kit (Axygen, USA) ,
105 and the PCR products recovered were connected with pMD-19T carrier（Takara,
106 Janpan）, then converted to DH5 alpha receptive cells (Vazyme, China), and evenly
107 coated into the LB agar plate containing 0.1 mM ampilin for single monoclonal
108 colony culture. After 18 h，positive colony was collected. and the bacteria solution
109 was verified by PCR. The PCR reaction and conditions were the same as described
110 previously.

111 **2.5.2 Cloning of PCR products**

112 Omega Plasmid Extraction Kit was used to extract recombinant plasmid vector.
113 The extracted plasmid was identified by single enzyme digestion (HindIII) and double
114 enzyme digestion (BamHI and SacI) according to the Takara enzyme product
115 instructions, and the enzyme digestion products were identified by 1% agarose gel
116 electrophoresis.

117 The amino acid sequence was deduced from the Editseq program in DNASTAR
118 software and MegAlign software plotted the genetic evolution tree for affinity
119 analysis. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) predicted the location

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120 and sequence of the protein amino acid as well as the sequence signal peptide of the
121 ACE2 gene encoding. TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/)
122 predict the transmembrane and transmembrane directions of ACE2 encoded protein
123 and Kyte-Doolittle method predicts the hydrophilic region by DNASTAR software.

124 **2.6 Construction and identification of recombinant expression vector**

125 The plasmid of pMD-19T-ACE2 and pET-32a was digested by BamHI, SacI
126 enzyme, the target fragment was recovered and the recombinant protein expression
127 vector pET-32a-ACE2 was constructed used T4 DNA ligase, and transformed into
128 Escherichia coli DH5 alpha. The positive recombinant plasmid were screened for the
129 monoclonal bacterial liquid PCR and the double enzyme digestion.

130 **2.7 Induction and expression of fusion protein**

131 The Escherichia coli BL21 (DE3) containing the recombinant plasmid containing
132 pET-32a-ACE2 was inoculated into the liquid medium LB containing Amp (100
133 μg/mL), 37°C, 180 rpm/min until OD_{600} 0.6~0.8. then The 1.0 mmol/L of IPTG was
134 added to the Escherichia coli BL21 (DE3), and collecting 1 mL bacteria solution after
135 inducting 2, 4, 6, 8, 10 h and overnight respectively. After centrifuging in 8000
136 rpm/min for 15 min, these bacteria solution were detected by SDS-PAGE.

137 **2.8 Identification and purification of the expression of fusion protein**

138 The IPTG stimulation time of high expression was selected according to the
139 results of the former. The bacterial solution was centrifuged in 8000 rpm/min for 15
140 min, and the bacterial sedimentation was collected and suspended used PBS. The
141 bacterial supernatant and precipitate after ultrasonic breakage (ice bath conditions)
142 were purified, and precipitated by SDS-PAGE. Sure. The fusion protein was purified
143 by staining with reference to Gao Shen Yang(Gao et al,.2010), and the size and purity
144 of the purified protein were detected by SDS-PAGE.

145 **2.9 Preparation of anti ACE2 polyclonal antibody**

146 Male New Zealand White rabbits were immunized by subcutaneous injec-tion to
147 gain ACE2 antibody. Briefly, the ACE2 antigen mixed 1:1 with Freund's complete
148 adjuvant was injected into rab-bits, and booster injections were administered once
149 at10-days intervals with the same amount of antigen mixed 1:1 with incomplete

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150 Freund's adjuvant. Serum was collected from heart method and stored at −80°C.

151 **2.10 Extraction of total protein in pig and different species tissues**

152 The protein of heart, spleen, kidney, liver, duodenum, liver of pig, and also liver
153 and kidney tissue of chicken were extracted from Roe. The protein concentration of
154 each tissue was measured by BCA kit（Beyotime Biotechnology, China）.

155 **2.11 Indirect ELISA method for determination of polyclonal antibody titer of pig**
156 **ACE2**

157 The serum and pre immune serum of the rat were diluted by 1:50, 1:100, 1:200,
158 1:400, 1:800, 1:1600, 1:3200, 1:6400, and then incubated in 96 well plate. After
159 washing, Sheep anti rat marked by HRP were added. Finally, TMB was used as the
160 substrate. The OD_{450} value of each well was read on the enzyme standard analyzer
161 and pre immunized serum. As a negative control, the OD_{450} value was N, the serum
162 OD_{450} was P after immunization, and was positive by P/N>2.1.

163 **2.12 Westen-Blot detection of anti ACE2 antiserum specificity in rats**

164 After SDS-PAGE electrophoresis of the total tissue protein extracted from the
165 ACE2 fusion protein and the proteins from former, the corresponding strips on the gel
166 were transferred to the PVDF membrane, and the PVDF membrane was placed in the
167 50 g/mL skimmed milk powder in 2 h at room temperature, and then transferred to the
168 rat anti ACE2 blood albumin diluted with TBST 1:6400 times in overnight at 4°C
169 temperature, and the film was washed with a TBST liquid for 5 times. Then
170 transferred to the Goat anti mouse IgG labeled with 1:5000 diluted horseradish
171 enzyme in TBST solution, incubated at room temperature for 2 h and TBST washing
172 solution for 5 times. After color rendering, the images were observed in the automatic
173 chemiluminescence image analysis system.

174 **2.13 Immunofluorescent detection of anti ACE2 serum specificity**

175 The IPEC-J2 cells were cultured in 6 orifice plates with cell crawling slices.
176 After being placed in 37°C and 5%CO_2 incubators to 70% of fusion degree, After
177 washing with PBS for 2 times, then 4% polyformaldehyde was used to fix 15 min,
178 PBS was washed 5 times and permeated with 0.5%Triton 100 for 20 min, and then
179 washed again, placed in the 50g/mL skimmed milk powder in 2 h at room temperature

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180 and then transferred to rat anti ACE2 serum at 4°C incubated overnight. After
181 washing with PBS for 5 times, transferring into the Goat anti rat IgG which diluted by
182 PBS at 1:500 in 2 h at room temperature and washed for 5 times by PBS. The DAPI
183 staining 5 min for 100 mg/mL, and PBS washing for 3 times, observed in a confocal
184 microscope/ultrahigh resolution microscope STORM.

185 **2.14 Immunohistochemical identification and localization of murine anti ACE2**
186 **protein polyclonal antibody in pig tissues**

187 Immunohistochemical was performed on formalin-fixed tissue. Gut fragments,
188 3×1 cm (7~10 cm away from the stomach), were flushed with water, bound in
189 surgical tape and fixed in ice-cold 10% neutral buffered formalin for a maximum of
190 14 h at 4°C. All fixed samples were embedded in paraffin and sectioned to 5 μm on
191 poly L-lysine slides. Antigen retrieval was performed by boiling in citrate buffer
192 (LabVision, Fremont, California, USA) for 10 min at 100°C. The endogenous
193 peroxidase was blocked (only for immunohistochemical staining) by EnVision
194 blocking solution (DakoCytomation, Glostrup, Denmark) for 5 min. The non-specific
195 binding was blocked for 30 min with 5% rabbit serum (DakoCytomation) for the
196 primary mouse serum(1:600), and primary antibody incubation was performed at 4°C
197 overnight. The immunohistochemical detection was performed using secondary
198 antibody horseradish peroxidase labelled polymer and DAB reagent (Dako
199 Cytomation).

200 # 3 RESULTS

201 **3.1 Cloning and sequence analysis of the target gene of porcine ACE2**

202 4 reverse transcriptional products were randomly selected for PCR amplification.
203 A single band was found in the spleen, duodenum, jejunum and ileum by 1% agarose
204 gel electrophoresis (Line 1-4). The size of the strip was about 2400 bp, and was the
205 same as expected (2418 bp) (Figure 1-A).

206 **3.2 The recombinant vector of PMD-19T-ACE 2 was successfully constructed**

207 In order to verify whether the ACE2 gene fragment was successfully inserted
208 into the pMD19-T vector, 2 positive colonies were selected and the plasmid was

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209 extracted by plasmid DNA, and 1% agarose gel electrophoresis results were found in
210 figure 1-B. As can be seen from figure 1-B, 1 treaty 5110 bp bands were found after
211 the Hind III single enzyme was cut, and the size was consistent with the sum of the
212 pMD-19T vector (2692 bp) and the target gene (2418 bp). BamH I and Sac I double
213 digestion showed about two bands around 2700 bp and 2400 bp, which confirmed that
214 pMD-19T-ACE2 recombinant vector had been successfully constructed. The positive
215 plasmid was selected and sent to Shanghai Ying Jun Biotechnology.

216 **3.3 Homology analysis of ACE2**

217 Using DNAstar software, EditSeq template was used to analyze the coding
218 region sequence and homology analysis of wild boar and other genes in Genbank.
219 From Table 1, we can see that the nucleotide sequence obtained from this experiment
220 is compared with the ACE2 nucleotide sequence of 2 pig species found on Genbank,
221 and its homology is 99.5% and 99.1% respectively. Their homology with cattle, goats,
222 domestic cats, domestic dogs, rhesus monkeys, humans and Rattus norvegicus were
223 between 82.0%~90.0%, and the lowest homology with zebrafish was less than 50%.

224 According to the results of nucleotide sequencing, the corresponding amino acid
225 sequence was conjectured. The piglet encodes 805 amino acids, which is consistent
226 with the number of ACE2 amino acids in human and Rattus norvegicus, and an amino
227 acid difference with cattle and sheep (804 amino acids).

228 **3.4 Homology analysis of ACE2**

229 Using the MegAlign module in DNAstar software, the amino acid sequences
230 derived from the cloned pig ACE2 nucleotide sequence and the amino acid sequences
231 derived from wild boar and domestic pig ACE2 nucleotides were 99.3% and 98.5%,
232 respectively, compared with the other species ACE2 amino acid sequences, and the
233 results were compared with sheep, mountain cattle, domestic cats, domestic dogs,
234 macaques, brown, brown, and brown. Homology of rodents, humans and alpacas
235 reached over 80%, and the lowest homology with zebrafish was 46.8% (Fig. 2-A).
236 From the tree of genetic evolution (Figure 52-B), the fragment is in the same
237 evolutionary group with the ACE2 amino acid sequence of goats and cattle, and the
238 zebrafish is in a completely different 2 branch, and the genetic evolution is consistent

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239 with the evolution of amino acid.

240 **3.5 Structural Analysis of ACE2 protein**

241 The cross membrane region analysis of weaned piglets ACE2 protein was
242 analyzed by TMHMM2.0 software. The results showed that the porcine ACE2 protein
243 belonged to I type transmembrane protein, and the transmembrane types of ACE2
244 proteins, such as people and sheep, were the same. Among them, part 1~739aa is the
245 extracellular part, and there is a transmembrane helix between 740~762 aa, and the
246 763~805 AA is the intracellular part (Fig. 3-A).

247 The Signal P4.1 software predicts the amino acids encoded by the pig ACE2
248 gene. The results are as shown in figure 3-B: the C value at the frame is maximum,
249 the S value is steep, and the Y value is the highest. It is predicted here that there is a
250 potential cracking site between 17-18aa and the protein is secreted protein (this is
251 judged by the value of D, D is the average value of S-mean and Y-max. It is important
252 to distinguish whether the protein is secreted or not, the D value is 0.883, greater than
253 that of 0.450--, which is the criterion for the secretion of protein. Through online
254 SMART analysis (http://smart.embl-heidelberg.de/), the signal peptide region is
255 between 1-17aa, which is consistent with the results predicted by the transmembrane
256 region analysis.

257 **3.6 ACE2 protein has good hydrophilicity**

258 The hydrophilicity of ACE2 protein was analyzed according to the amino acid
259 hydrophilic standard of Kyke-Doolittle. Results are shown in Figure 4. The proportion
260 of hydrophilic region of ACE2 protein in piglets is larger, and the distribution is more
261 uniform (more than 0 lines in the map), indicating that the protein has good
262 hydrophilicity.

263 **3.7 Construction and identification of prokaryotic expression vectors**

264 After the recombinant plasmid and the expression vector were cut by BamH I
265 and Sac I, the T4 DNA ligase was linked and transformed into E.coli BL21 (DE3),
266 and the positive colony was picked up for PCR, and then the plasmid was identified
267 by BamH I and double enzyme digestion. 1% agarose gel was used to detect the strain
268 and about two bands. (Fig.5) the sequencing results were correct, indicating that

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269 pET-32a-ACE2 recombinant plasmid was successfully constructed.

270 **3.8 Induced expression, identification and purification of recombinant protein**

271 The recombinant expression bacteria were induced and expressed at the final
272 concentration of IPTG at 1 mmol/L. The expression products were collected at
273 different time points. After SDS-PAGE analysis, the target protein was obtained in the
274 induction of 2, 4, 6, 8, 10 h and overnight, and the expression of target protein was the
275 highest when 10 h was induced (Figure 6-A). After ultrasonic breakage centrifugation,
276 the supernatant and precipitation were taken respectively. After SDS-PAGE, the
277 recombinant protein of ACE2 was found mainly in the precipitate, that is, in the form
278 of inclusion body (Figure 6-B). The purified fusion protein (Fig 6-C) was purified and
279 purified by KCl staining, which could be used for subsequent polyclonal antibody
280 preparation.

281 **3.9 Determination of anti ACE2 polyclonal antibody titer by indirect ELISA**
282 **method**

283 The purified pET-32a-ACE2 fusion protein was immunized with 4 Wastar
284 female rats. After 4 immunizations, the antibody test results were all positive, the rats
285 were slaughtered, the isolated serum obtained the anti ACE2 antibody of the
286 polyclonal mice, and the antiserum titer was detected by indirect ELISA method.
287 Results as shown in Figure 7, the serum dilution ratio of the positive serum OD_{450} was
288 about 1, and the P/N value (P: the mean OD_{450} value of the positive hole, the mean
289 value of the negative pore, OD_{450}) was the best serum dilution degree, that was 3200.

290 **3.10 Detection of serum specificity of anti ACE2 in rats by Western blot**

291 The rat anti ACE2 serum was prepared with the best multiple dilution as a single
292 antibody, and the Sheep anti rat IgG-HRP was used as a two antibody. The purified
293 fusion protein, pig heart, spleen, kidney, liver, duodenum, sheep liver, rat liver, rat
294 liver and chicken liver and kidney tissue protein were analyzed by Western blot. The
295 results showed the fusion protein and pig heart. There was a specific binding protein
296 in the size of 100 kDa in the viscera, spleen, kidney, liver and duodenum (Fig. 8), and
297 the rest did not appear. The results show that the ACE2 polyclonal antibody can
298 identify the fusion protein and the ACE2 protein in the pig tissues, but the ACE2

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299 protein in other species can not be combined, that is, the ACE2 polyclonal antibody
300 against the pig's mouse has good immune response and specificity.

301 **3.11 Anti ACE2 serum specificity in mice by immunofluorescent**

302 The mouse anti ACE2 serum was diluted by multiple 1:600 as one antibody, the
303 fluorescent enzyme labeled Sheep anti mouse IgG-HRP was two resistance, and the
304 final concentration was 100 ng/mL DAPI for 5 minutes. The immunofluorescence
305 analysis of the pig intestinal epithelial cells, as shown in Figure 9, that the mouse anti
306 ACE2 could specifically identify the ACE on the membrane of the pig small intestinal
307 epithelial cells. The 2 protein, that is, the polyclonal antibody of pig anti mouse ACE2
308 prepared, has better immunoreactivity.

309 **3.12 Serum sensitivity of anti ACE2 in rats by Western blot**

310 As shown in Figure 10, the proteins extracted from pig spleen, kidney, liver, lung,
311 pancreas, lymph node, stomach, duodenum, jejunum, ileum, colon, rectum and cecum
312 were analyzed by Western blotting, and the rat anti porcine polyclonal antibody was
313 diluted with 1: 3200 as one resistance, and the IgG of sheep resistant to HRP was two.
314 Resistance, the results showed that the tissue protein extracted from pig kidney,
315 jejunum and rectum had obvious bands at 92 kDa, and the highest content in kidney,
316 but not in other tissues. Under the same condition, the Sheep anti rabbit polyclonal
317 antibody was diluted with 1:1000 multiple as a single antibody, and the HRP labeled
318 Goat anti rabbit IgG was two. The results showed that the tissue protein extracted
319 from pig spleen, kidney, lung, pancreas, lymph node, stomach, duodenum, jejunum,
320 ileum, colon, rectum, and cecum was marked at 92 kDa. The results showed that the
321 sensitivity of mouse against polyclonal antibody was poor.

322 **3.13 Immunohistochemistry was used to detect the expression of ACE2 in pig**
323 **tissues.**

324 The cellular localization of ACE2 in porcine tissues was analyzed after dilution
325 of 1:600 prepared by anti ACE2 serum. The criterion is that if Brown is positive, the
326 result is shown in Fig. 11. As can be seen from figure 3-21, ACE2 protein exists in
327 various tissues and organs of the pig, mainly expressed in the mucosa of the gastric
328 fundus (Fig. 11-A), the myometrium of the small intestine, serous layer, and villi

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329 epithelium (FIGS. 11-B, C, D). In the large intestine, it is mainly expressed in the
330 intestinal gland (FIGS. 11-E, F), and the result of this group on the pig WB Like. The
331 results further confirmed the specificity of the mouse anti ACE2 polyclonal antibody
332 prepared in this experiment.

333 # 4 DISCUSSION

334 In 2000, Tipnis amplified the genes encoding ACE2 from the cDNA Library of
335 human lymphoid cancer(Tipnis et al., 2000). It was named as ACEH and numbered as
336 AF241254 in the gene pool. Further study found that the open reading frame of cDNA
337 of the ACEH was composed of 2418 nucleotides, encoding 805 amino acids, and the
338 gene was located at the chromosome Xp22 site and contains 18 exons. In the same
339 year, Donoghue et.al constructed a full-length ACE2 expression vector from the
340 cDNA Library of heart failure patients through transfection of CHO cells(Donoghue
341 et al,. 2000). The gene sequence was recorded by GenBank and numbered as
342 AF291820. Later, it was confirmed that ACE2 and ACEH were the same. In 2003,
343 Moore confirmed that ACE2 was the main functional receptor of the SARS
344 coronavirus(Li et al,. 2003). In order to further understand the route of infection, Li
345 cloned the full-length ACE2 sequence of the beaver, and confirmed that the beaver
346 was infected mainly by the S protein region of the SARS coronavirus combined with
347 its ACE2(Ge et al,. 2013; Li et al,. 2005). Wang extracted the total RNA from the
348 lungs of 9 month old cats and amplified the ACE2 gene sequence of the home cat by
349 RT-PCR(Wang et al. 2005). The gene number in Genebank was AY957464 and its
350 sequence size was 2418 bp. It encodes 805 amino acids. It was suggested that cat
351 ACE2 might be more likely to mediate the invasion of SARS-CoV. In 2005, Xie
352 successfully amplified the full length cDNA sequence of the ACE2 gene from the
353 mouse kidney tissue, and the sequence size was 2418 bp(Xie et al,. 2005). Then Xu
354 uploaded the partial ACE2 gene sequence of kidney of Trionyx sinensis（Genbank：
355 HM107424）(Xu et al,. 2011). In 2018 Chen cloned the whole ACE2 gene sequence
356 from the kidney and lung of monkeys. Compared with the human ACE2 sequence,
357 there were 38 NS mutations(Chen et al,. 2008). The specific fragment of the ACE2

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358 gene coding region of the goat kidney tissue was amplified by Yang in the laboratory
359 for the first time. The length of the nucleotide sequence was 2415 bp(Yang et al,.
360 2016).

361 In this study, the pig ACE2 gene was cloned, analyzed and prokaryotic expressed
362 successfully, and the polyclonal antibody of mouse ACE2 was prepared. The whole
363 gene sequence of ACE2 in pig was 2418 bp and 805 amino acids were encoded.
364 Homology analysis on GenBank found that the cloned ACE2 gene were above 99%
365 similar to other porcine ACE2 genes, which shows that the pig ACE2 gene cloning
366 was successful. The full-length gene sequence of pig ACE2 gene was provided to
367 GenBank database., It is deduced that the pig ACE2 protein belongs to the type I
368 secretory transmembrane protein and its signal peptide sequence is located between
369 1~17 AA, which is similar to that of goats (1~18 AA) (Yang et al,. 2016).

370 In conclusion, In this paper, the basic information of porcine ACE2 protein was
371 provided and the specific anti-porcine polyclonal antibody was successfully, which
372 could lay the foundation for further research on the biological activity of ACE2 in
373 pigs.

374 ACKNOWLEDGMENTS

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376 (31972640).

377 CONFLICT OF INTEREST

378 The authors declare no conflflict of interest.

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440 # FIGURE LEGENDS

441 **Figure 1.** ACE2 gene sequencing.

442 A: The product of PCR amplification of ACE2 gene; M: DL 5000, Line 0: negatively control, Line 1: ACE2 in
443 spleen; Line 2: ACE2 in duodenum; Line 3:ACE2 in jejunum; Line 4: ACE2 in ileum; B: Single and double
444 enzyme digestion of recombinant plasmid. Line 0: negative control; Line 1: Recombinant plasmid
445 ACE2-pMD19T; Line 2: Recombinant plasmid was digested by Hind III; Line 3: Recombinant plasmid was
446 digested by BamHI and SacI.

447 **Figure 2.** Homology / phylogenetic tree analysis of ACE2 protein.

448 A: Comparison of the deduced amino acid sequences betweendifferent species of ACE2; B: Phylogenetic tree of
449 the deduced amino acid sequence of ACE2 nucleotide sequences between different species.

450 **Figure 3.** Prediction of transmembrane domain /signal peptide analysis of ACE2 protein from
451 piglet.

452 A: Prediction of transmembrane domain; B: signal peptide analysis.

453 **Figure 4.** Hydrophilic analysis of ACE2 protein in piglet.

454 **Figure 5.** Development of prokaryotic expression construction of ACE2.

455 M_1: DL10000; Line 1: Recombinant plasmid; Line 2: Recombinant plasmid was digested by Hind III; Line 3:
456 Recombinant plasmid was digested by BamHI and SacI.

457 **Figure 6.** Expression Purification of pET-32a-ACE2 fusion protein.

458 A: Induced expression of pET-32a-ACE2 in E:coli BL21(DE3); M: Protein molecular weight Marker; Line 1~7:
459 Recombinant pET-32a-ACE2 expression products in E:coli BL21(DE3) induced with IPTG at 0、2、4、6、8、10
460 and overnight; B: Solubility analysis of pET-32a-ACE2 fusion protein; M: Protein molecular weight Marker; Line
461 1:Recombinant bacterium; Line 2: The upernatant; Line 3: The deposit; C: Purification of pET-32a-ACE2 fusion
462 protein; M: Protein molecular weight Marker; Line 1 : Recombinant bacterium; Line 2~3: Purificated fusion
463 protein.

464 **Figure 7.** ELISA detection of rats anti-ACE2 titer.

465 **Figure 8.** Western blotting analysis of specificity of polyclonal antibody.

466 A: Western blotting analysis of purified protein; M: Protein molecular weight Marker; Line 1~2: purified protein;
467 B: Western blotting analysis of purified protein of different tissue proteins; M: Protein molecular weight Marker;
468 Line 1: Cardiac tissue protein of porcine; Line 2: Spleen tissue protein of porcine; Line 3: Duodenum tissue
469 protein of porcine; Line 4: Kidney tissue protein of porcine; Line 5: Liver tissue protein of porcine; Line 6: Liver
470 tissue protein of goat; Line 7: Liver tissue protein of rat; Line 8: Liver tissue protein of chicken; Line 9: Kidney

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471 tissue protein of chicken.

472 **Figure 9.** Immunofluorescence analysis of specificity of polyclonal antibody.

473 **Figure 10.** Western blotting analysis of differert tissues from porcine.

474 Line 1: Live tissue protein of porcine; Line 2: Spleen tissue protein of porcine; Line 3: lymph tissue protein of
475 porcine; Line 4: stomach tissue protein of porcine; Line 5: duodenum tissue protein of porcine; Line 6: jejunum
476 tissue protein of porcine; Line 7: ileum tissue protein of porcine; Line 8: colon tissue protein of porcine; Line 9:
477 rectum tissue protein of porcine; Line 10: caecum tissue protein of porcine; Line 11: lung tissue protein of porcine;
478 Line 12: kindey tissue protein of porcine; Line 13: pancreas tissue protein of porcine.

479 **Figure 11.** ACE2 immunohistochemical map of differert tissues from pigs.

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Table

Table 1 Comparison of nucleic acid homology between the piglet and other organisms

Species	GenBank accession No.	Homology (%)
Sus scrofa	NM_001123070.1	99.5
Sus scrofa domestica	GQ262781.1	99.1
Bos taurus	NM_001024502.4	88.8
Felis catus	NM_001039456.1	87.4
Canis lupus familiaris	NM_001165260.1	86.6
Rattus norvegicus	NM_001012006.1	82.0
Macaca mulatta	FJ170098.1	84.8
Capra hircus	NM_001290107.1	89.2
Danio rerio	NM_001007297.1	49.7
Homo sapiens	NM_021804.2	84.9

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Figure 1

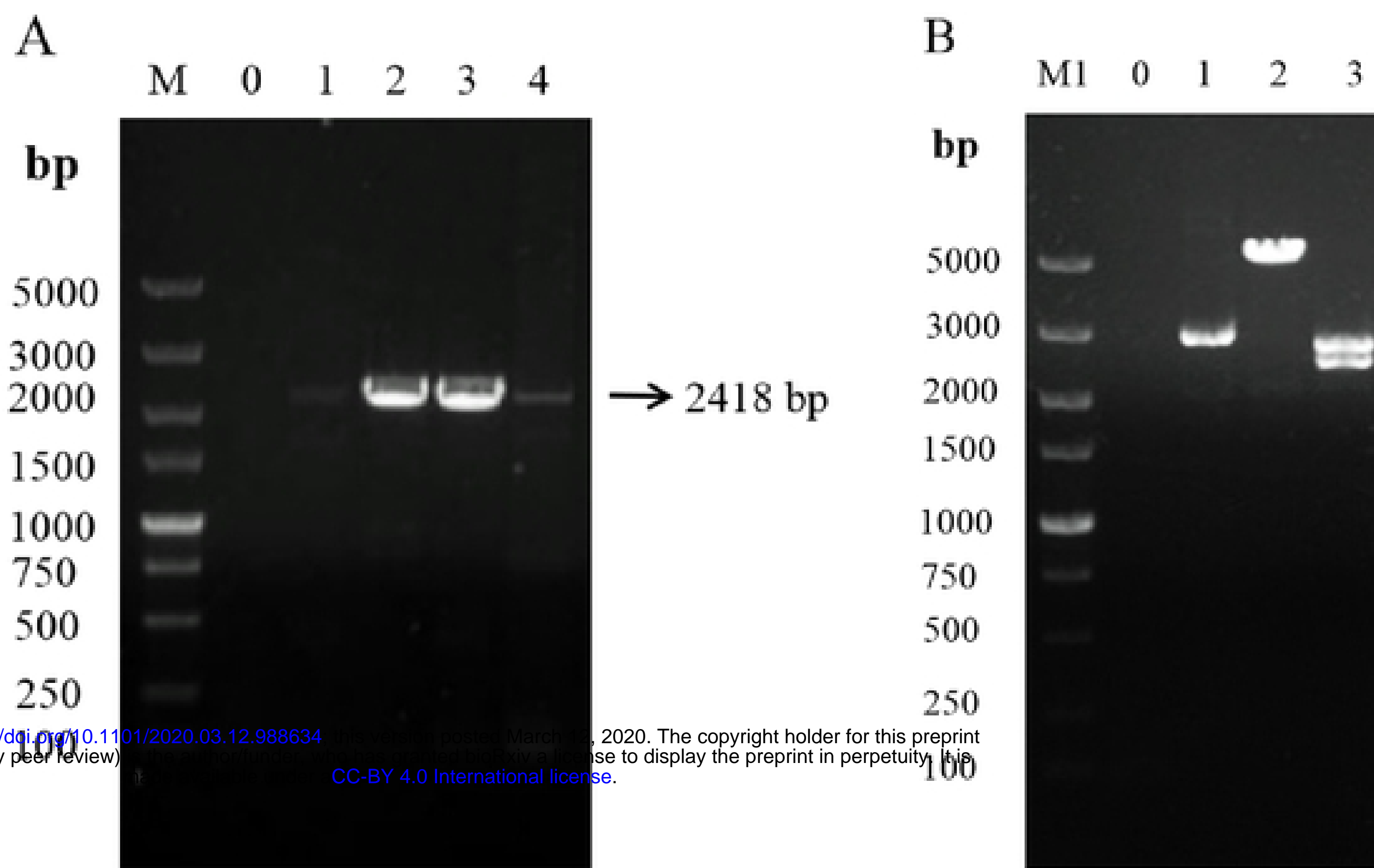

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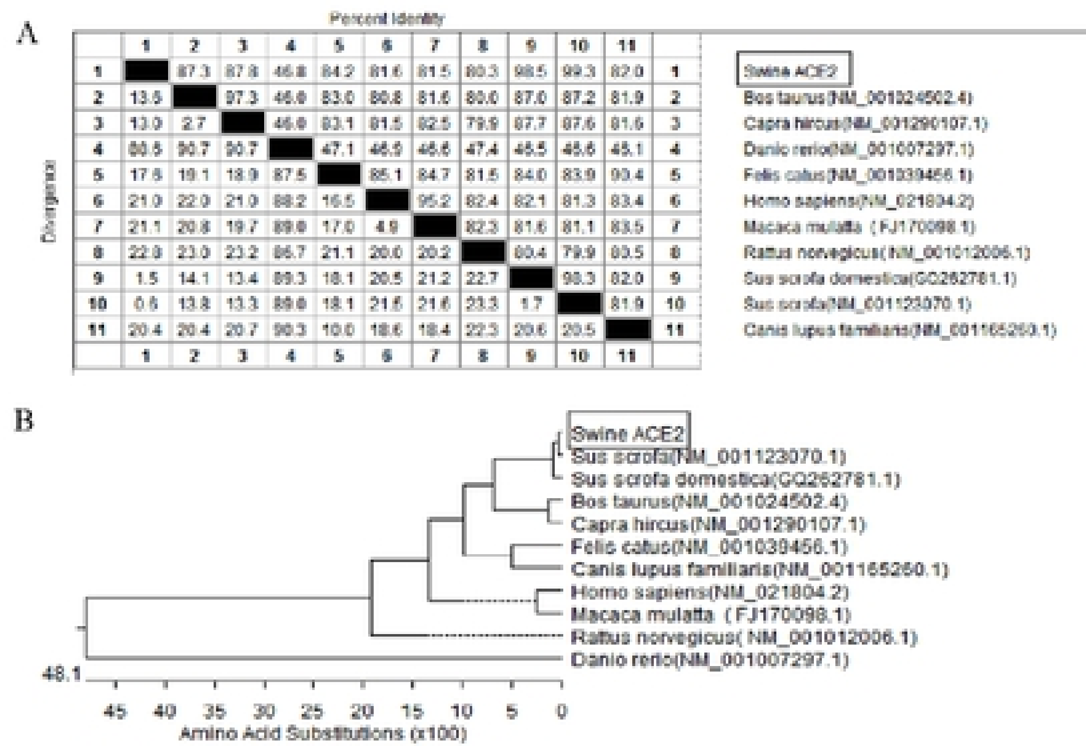
Figure 2



A

Percent Identity (upper right) / Divergence (lower left)

	1	2	3	4	5	6	7	8	9	10	11	
1		87.3	87.8	46.8	84.2	81.6	81.5	80.3	98.5	99.3	82.0	1
2	13.5		97.3	46.0	83.0	80.8	81.6	80.0	87.0	87.2	81.9	2
3	13.0	2.7		46.0	83.1	81.5	82.5	79.9	87.7	87.6	81.6	3
4	80.5	90.7	90.7		47.1	46.5	46.6	47.4	46.5	46.6	45.1	4
5	17.6	19.1	18.9	87.5		85.1	84.7	81.5	84.0	83.9	90.4	5
6	21.0	22.0	21.0	88.2	16.5		95.2	82.4	82.1	81.3	83.4	6
7	21.1	20.8	19.7	89.0	17.0	4.9		82.3	81.6	81.1	83.5	7
8	22.8	23.0	23.2	86.7	21.1	20.0	20.2		80.4	79.9	80.5	8
9	1.5	14.1	13.4	89.3	18.1	20.5	21.2	22.7		98.3	82.0	9
10	0.6	13.8	13.3	89.0	18.1	21.5	21.6	23.3	1.7		81.9	10
11	20.4	20.4	20.7	90.3	10.0	18.6	18.4	22.3	20.6	20.5		11
	1	2	3	4	5	6	7	8	9	10	11	

1. Swine ACE2
2. Bos taurus(NM_001024502.4)
3. Capra hircus(NM_001290107.1)
4. Danio rerio(NM_001007297.1)
5. Felis catus(NM_001039456.1)
6. Homo sapiens(NM_021804.2)
7. Macaca mulatta (FJ170098.1)
8. Rattus norvegicus(NM_001012006.1)
9. Sus scrofa domestica(GQ262781.1)
10. Sus scrofa(NM_001123070.1)
11. Canis lupus familiaris(NM_001165260.1)

Figure 3

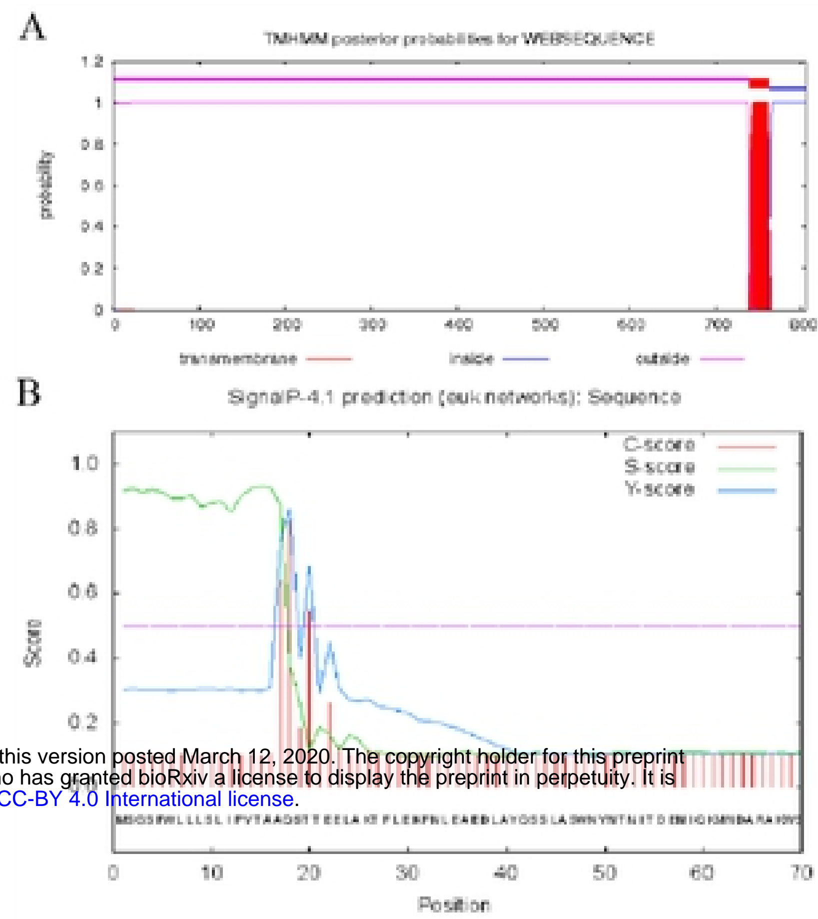


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Figure 4

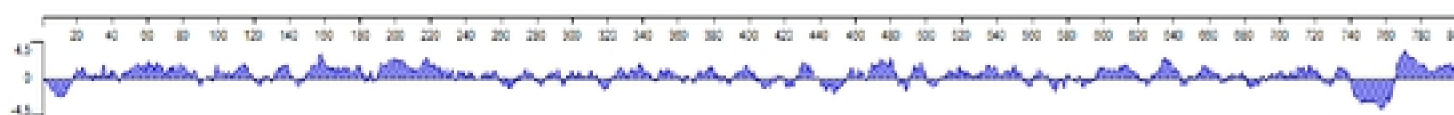

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Figure 5

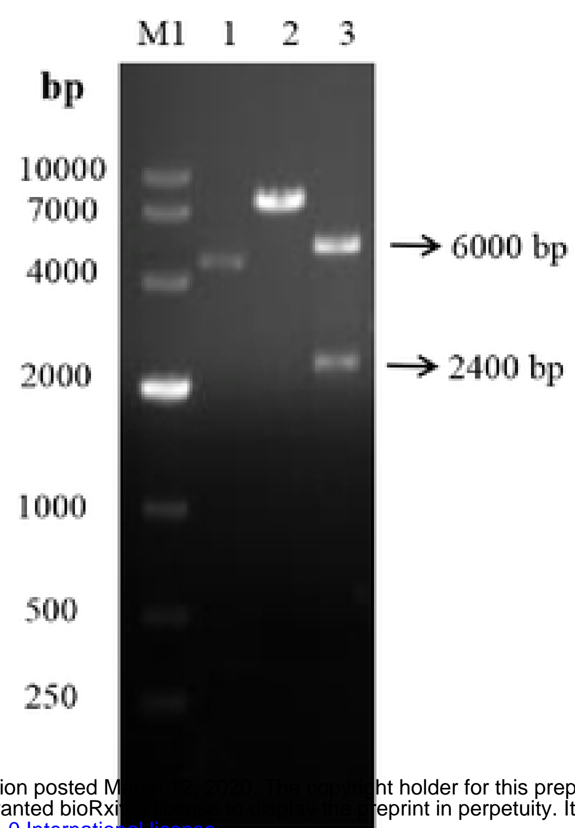

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Figure 6

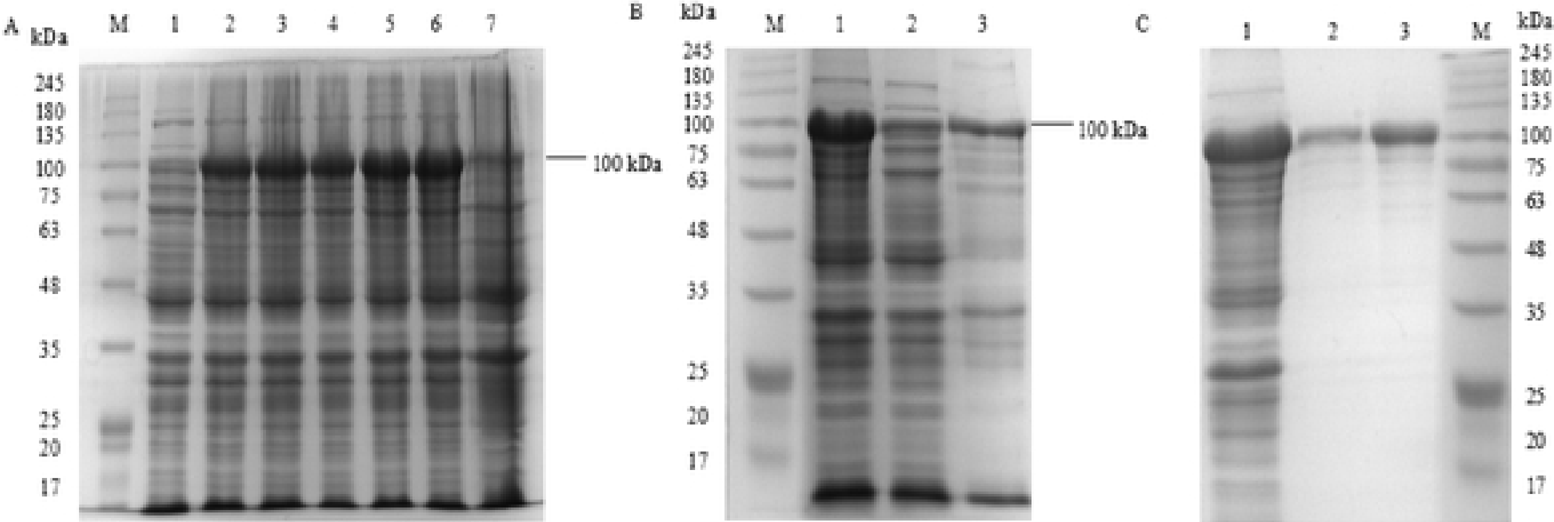

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Figure 7

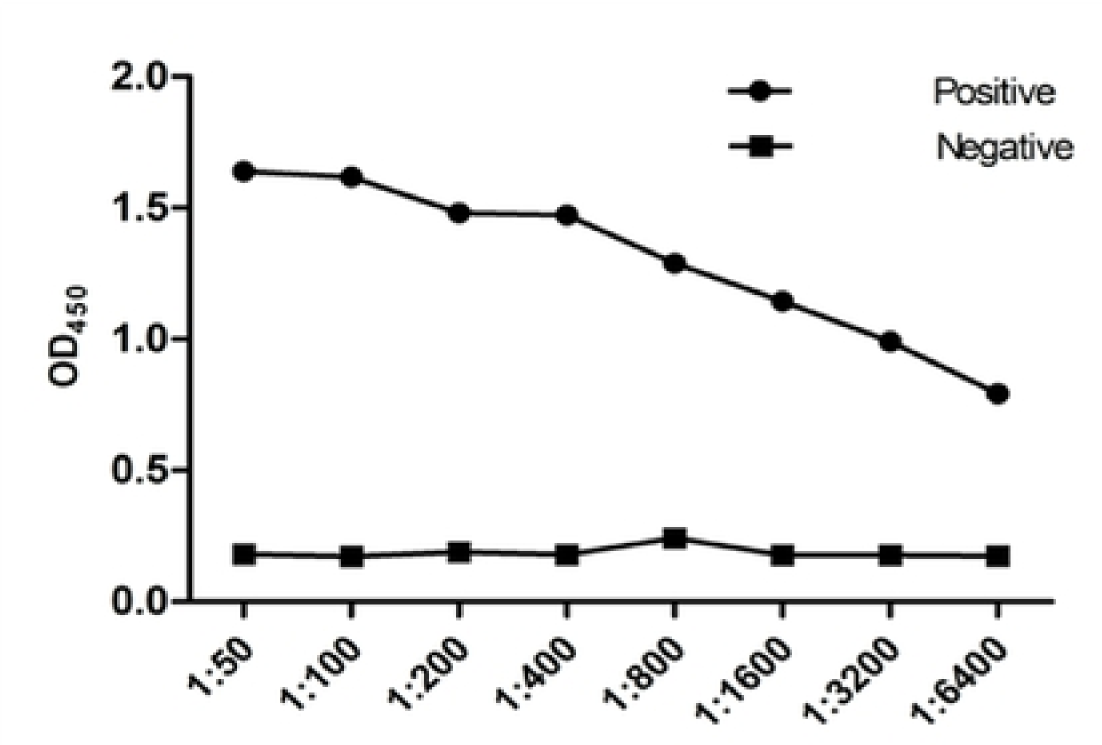

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Figure 8

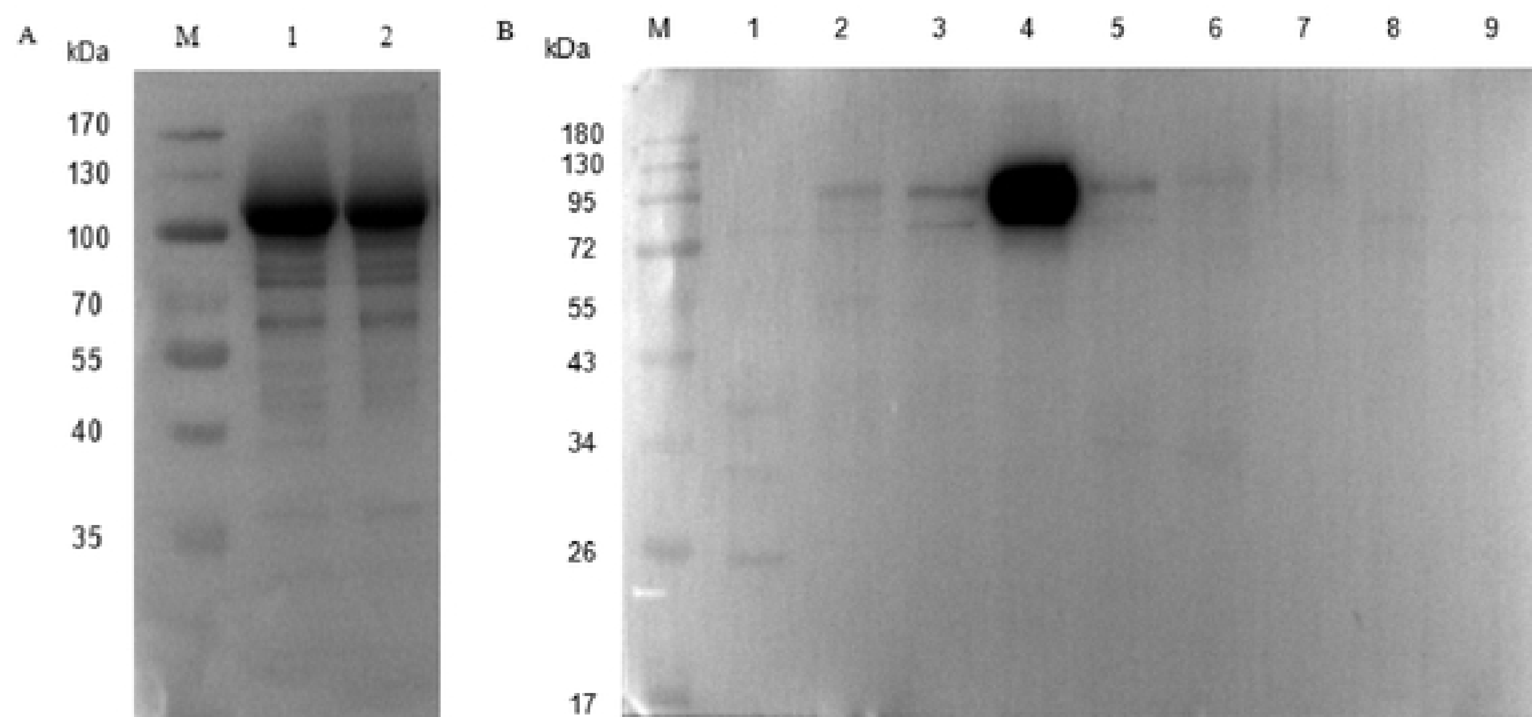

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Figure 9

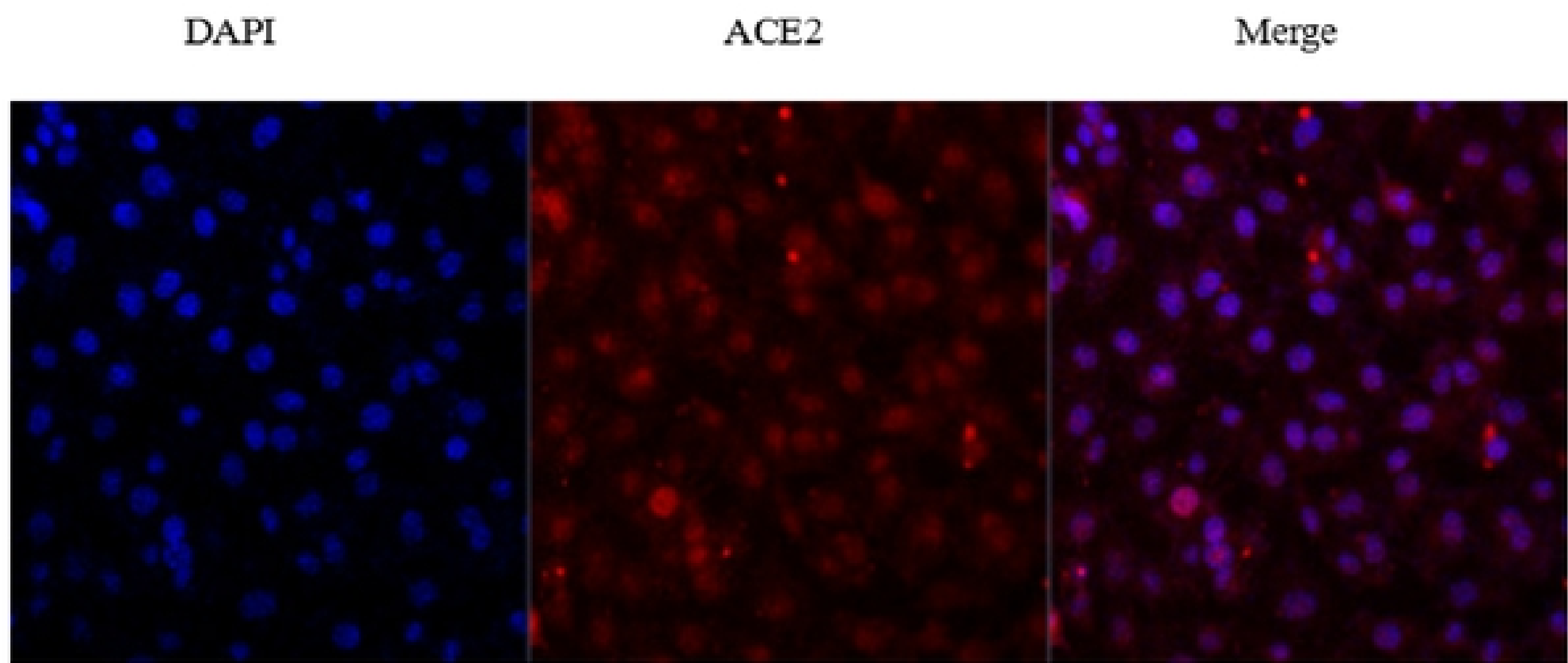

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Figure 10

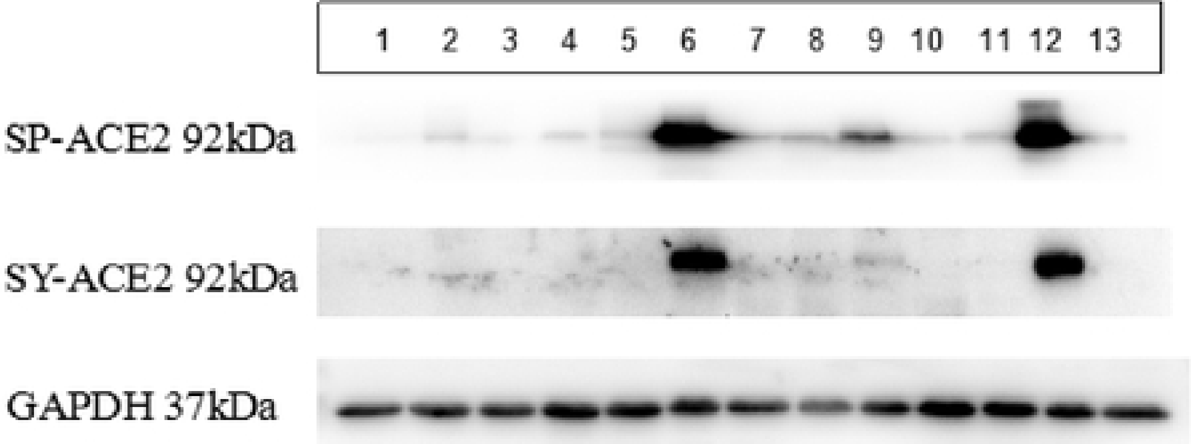

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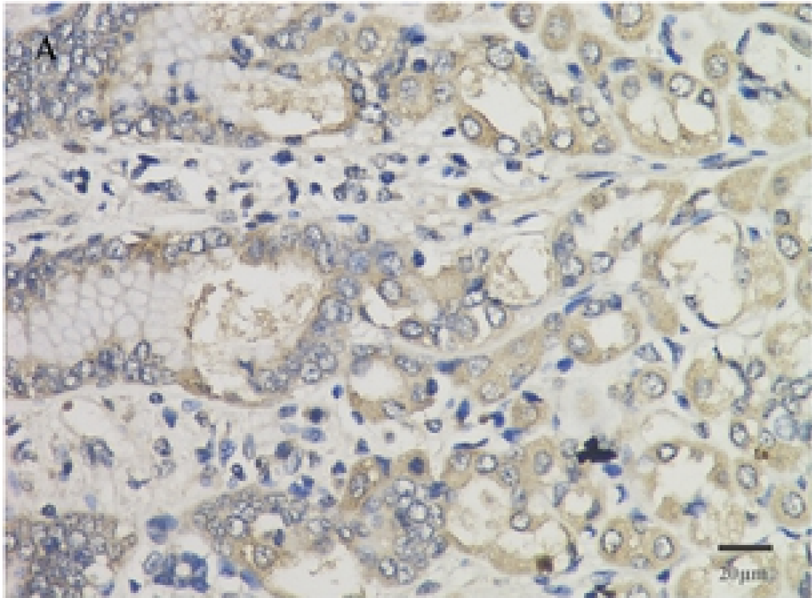
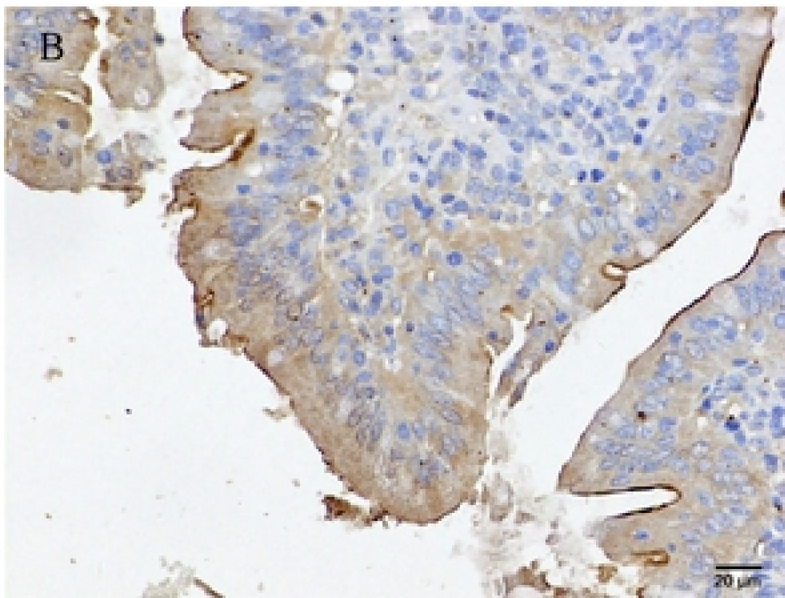
Figure 11



Fundic（400×）



Duodenum（400×）

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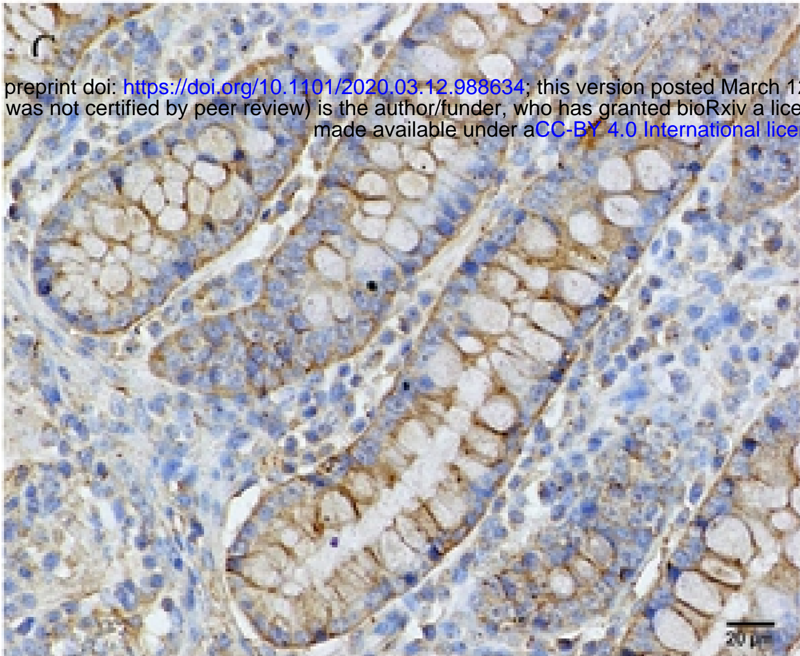
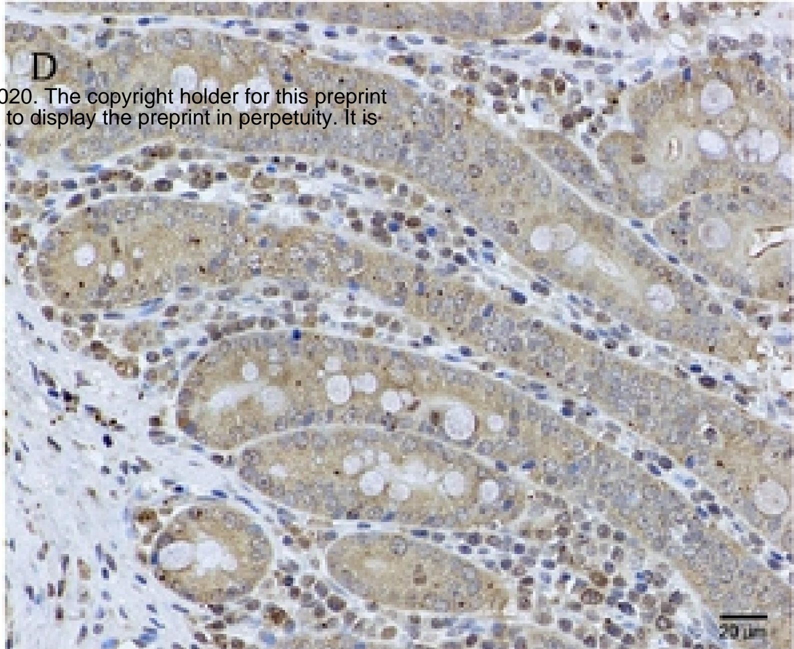
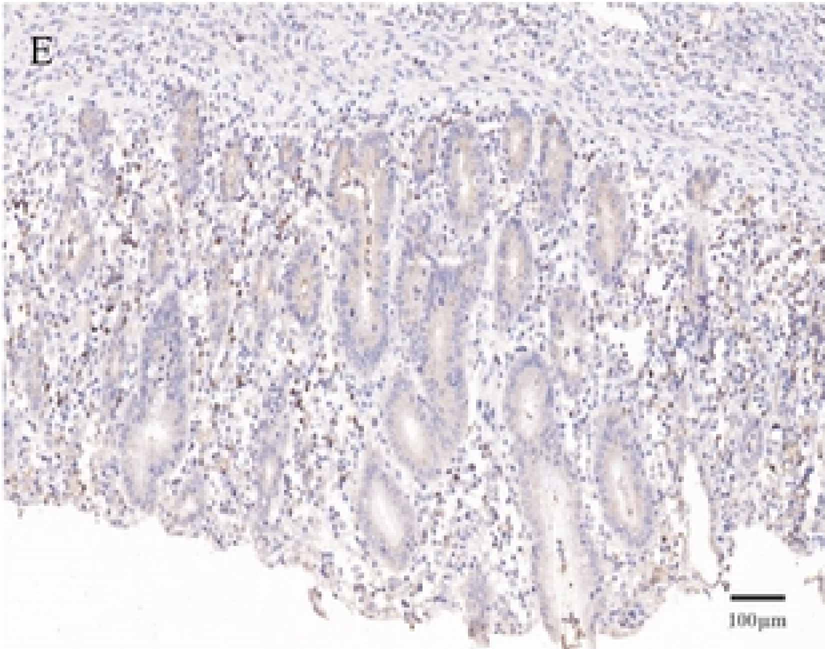


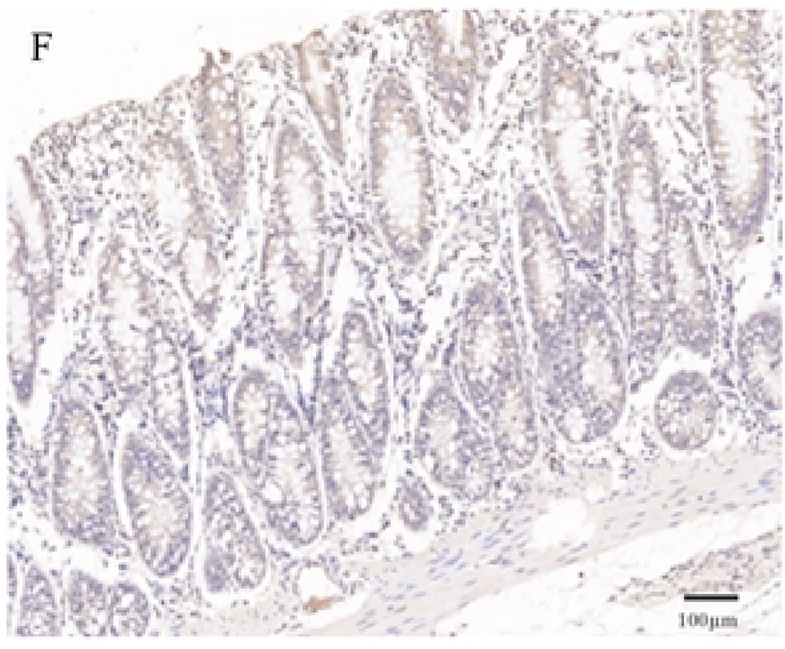
Jejunum（400×）



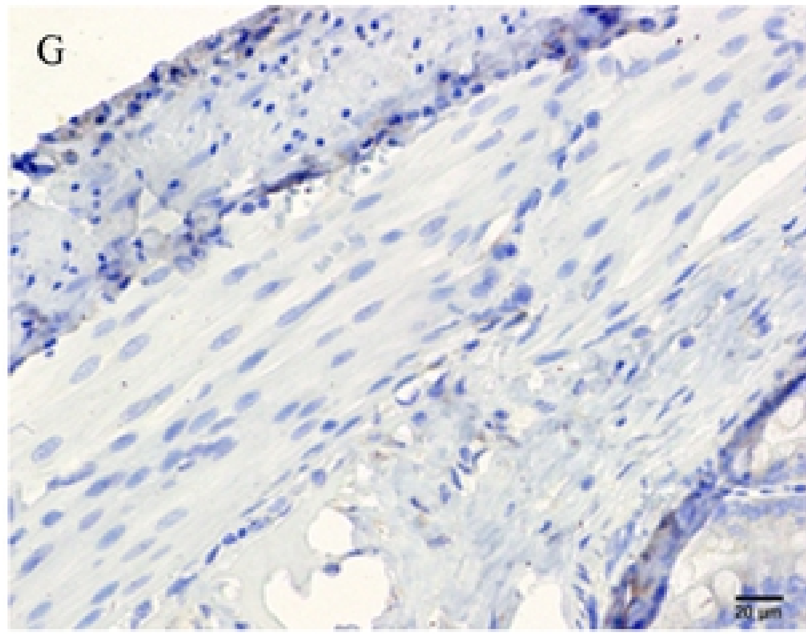
Ileum（400×）



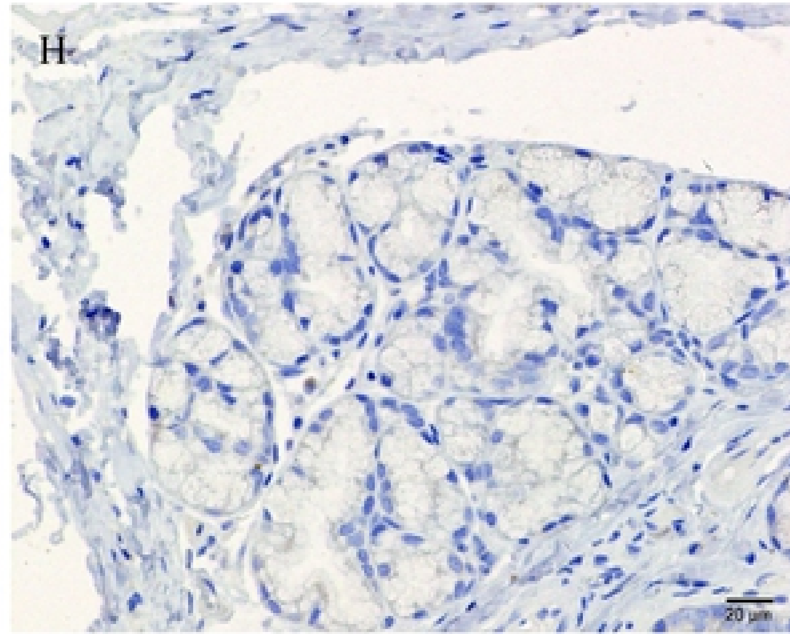
Colon（100×）



Rectum（100×）



Negative control (400×)



Negative control (400×)

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