Identification of the Paneth cells in chicken small intestine

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ABSTRACT The Paneth cells are highly specialized cells in the epithelium of the small intestine of many vertebrate species. These cells reside at the base of crypts of the Lieberkühn and contain abundant secretory granules. Previous studies suggesting the existence of Paneth cells in the chicken (*Gallus gallus)* remained controversial. Here we seek to identify the Paneth cells in the chicken small intestine through morphological examination and specific gene expression. Histological staining and transmission electron microscope confirmed the presence of granulated secretory cells at the base of the crypts in the chicken small intestine. Western blotting experiment also manifested the expression of lysozyme protein, which is specifically secreted by the Paneth cells in the small intestine. Moreover, lysozyme *c* and lysozyme *g* mRNAs were expressed in the small intestine of chickens at different ages. Lysozyme *c* mRNA, in particular, was located at the base of the small intestinal crypts as displayed by in situ hybridization. Collectively, we provide evidences that the Paneth cells indeed exist in the small intestine of the chicken.

Key words: paneth cell, chicken, crypt, lysozyme

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INTRODUCTION

In mammals, the Paneth cells play important immune defense roles in the intestines, particularly relating to intestinal epithelium renewal and early stages of intestinal inflammation. The Paneth cells are restricted to the crypts of the small intestine, along with goblet cells, enterocytes, tuft cells, and enteroendocrine cells. These cells represent the principal cell types of the epithelium of the small intestine (van Es and Clevers, 2014). The Paneth cells were first described by J. Paneth in 1888 as granulated cytoplasmic cells located at the base of small intestinal crypts, the "crypts of Lieberkühn". Classical Paneth cells are pyramidal shaped secretory cells with basally situated nuclei, extensive endoplasmic reticulum and Golgi network, and prominent, large apical granules that occupy most of their cytoplasm. The large secretory granules in the Paneth cells are rich in antimicrobial peptides (such as lysozyme, α-defensins/cryptdins, and secretory phospholipase A2), immune modulators (such as IgA), and trophic factors (such as carboxylic ester hydrolase) (Porter et al., 2002; Elphick and Mahida, 2005; Ouellette, 2010; Bevins and Salzman, 2011; Clevers and Bevins, 2013). Antimicrobial peptides or immune modulators released from the Paneth cells protect the host from enteric pathogens (van Es and Clevers, 2014). In addition, the secretions of the Paneth cells constitute the niche for Lgr5 stem cells in the intestinal crypts (Sato et al., 2011). The Paneth cell can also act as the site of origin for intestinal inflammation (Adolph et al., 2013).

In previous studies, by immunostaining with lysozymes (Erlandsen et al., 1974), defensins (Cunliffe et al., 2001), and with secretory phospholipase A2 (Nevalainen et al., 1995), a wide variety of species including primates, rodents and horse have been demonstrated to be abundantly populated with the Paneth cells. Conversely, the absence of the Paneth cell in other species' intestines, including those of the cow, sheep, ostrich, sloth, seal, crocodile, and colubrid snake, has also been noted. For some other species, including domestic cats and the dogs, the presence of the Paneth cell remains controversial and unconfirmed (Porter et al., 2002). Whether the Paneth cells are present in the avian gastrointestinal tract was debatable. Humphrey and Turk (1974) reported the presence of the Paneth cells in the crypts of the chick intestine epithelium by using low-power light microscopy (Humphrey and Turk, 1974). However, there is still doubt about the existence of the Paneth cells in the chicken small intestine, based on the observation that the continuous expression of lysozyme *c* gene remained undetected (Nile et al., 2004). In the present study, we attempted to provide clearer evidence to demonstrate the existence of the Paneth cells in the chicken small

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Table 1. Primers for PCR analysis.

Gene	Accession no.	Primer sequence (5′–3′)	Product length (bp)
Lysozyme *c*	NM_205281	GACGATGTGAGCTGGCAG GGATGTTGCACAGGTTCC	225
Lysozyme *g*	X61002	CACGCTGGCAAAATACTGAAG TTCCCAACACCAGCATTGTAG	236
Lysozyme *g*2	XM_416896	CATTCCATCTTTGGTTGC CCACCTTTGAGCTGCTGTTC	246
β-actin	NM_205518	ACACCCACACCCCTGTGATGAA TGCTGCTGACACCTTCACCATTC	136

intestine based upon the examination of morphological characteristics and the expression of the marker genes or proteins.

Lysozyme, a product secreted by the Paneth cells, is a glycosidase that specifically hydrolyses peptidoglycan, a major component of the bacterial cell wall (Ghoos and Vantrappen, 1971; Callewaert and Michiels, 2010). Lysozyme is also widely found in the surface fluid of tears, and in breast milk, saliva, gastric and small intestinal secretions, as well as in the granules of macrophages and neutrophils (Erlandsen et al., 1974; Spicer et al., 1977). There are three types of lysozyme that have been identified: the c-type (*c*hicken or conventional type), which is highly expressed in the oviduct, macrophages, and small intestine (Hindenburg et al., 1974; Nile et al., 2004); the g-type (goose type), which is expressed in the bone marrow and the lung (Nakano and Graf, 1991); and the g2-type, which is identified in the small intestine, liver, and kidney (Nile et al., 2004). In the gastrointestinal tract, lysozyme c is expressed in the gastric and pyloric glands, duodenal Brunner's glands, and in the Paneth cells (Klockars and Reitamo, 1975). In addition, phloxine-tartrazine staining is a simple and reliable technique that stains Paneth granules in bright red color (Bevins and Salzman, 2011). In this study we focus on the detection of the expression and location of lysozyme *c* in the chicken intestine. Through studies of histology, protein and gene expression, we are able to provide evidence of the existence of the Paneth cells in the chicken intestine.

MATERIALS AND METHODS

Animals and Sample Preparation

Hyline chickens (*Gallus gallus*) were obtained from a commercial company. C57BL/6(B6) mice were purchased from the Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China). The duodenum, jejunum, and ileum were removed and washed with ice-cold PBS immediately after these animals were sacrificed. All procedures related to the animals were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals of Zhejiang University.*

RNA Extraction and RT-PCR Analysis

Total RNA was extracted using a TRIzol reagent (Invitrogen Co., Carlsbad, CA) and the purity and concentration were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, Waltham City, MA) to be 260/280 nm in the range of 1.8 to 2.0. The cDNA was generated from 2 μg total RNA by using the SuperScript First-Strand Synthesis System (Fermantas, Glen Burnie, MD). PCR amplification was performed on a 20 μL volume containing 2 μL cDNA. The PCR products were analyzed by electrophoresis in 1.5% agarose gel and the pictures were taken with a Tanon Gel Imaging system (Tanon, Shanghai, China). The sequences of the primers were listed in Table 1.

Histological Staining and Transmission Electron Microscopy

Paraffin sections (5 μm thickness) were routinely prepared for phloxine-tartrazine staining. For transmission electron microscopy (TEM), the specimen was fixed with 2.5% glutaraldehyde in a phosphate buffer (0.1 M, pH7.0) overnight and postfixed with 1% OsO_4 in a phosphate buffer (0.1 M, pH7.0) for 1 to 2 h. The specimen was then embedded in Spurr resin and sliced using a ultratome (LEICA EM UC7). It was then stained by uranyl acetate and alkaline lead citrate (each for 5 to 10 min) and observed in a Hitachi Model H-7650 TEM.

In situ Hybridization

The triphase oligonucleotide probes (1) 5′-ATGAA GCGTC ACGGA CTTGA TAACT ATCGG GGATA-3′, (2) 5′-GACTA CGGAA TCCTA CAGAT CAACA GCCGC TGGTG-3′, and (3) 5′-TGGGT CGCCT GGCGC AACCG CTGCA AGGGT ACCGA-3′ were labeled with digoxigenin as probes to detect lysozyme *c* mRNA. Paraffin sections (5 μm thickness) were deparaffinized and incubated in 3% H_2O_2 for 5 min at room temperature. After washing in distilled water, sections were incubated in pepsin (3% sodium citrate) for 3 to 30 min at 37°C followed by fixation in 1% paraformaldehyde for 10 min at room temperature. The sections were then acetylated in a pre-hybridization buffer for 2 to 4 h at 38°C to 42°C and incubated overnight at 38°C to 42°C with a hybridization buffer containing Digoxigenin-labeled probes. Hybridized

sections were washed in succession with 2 × **SSC** (saline sodium citrate), 0.5 × SSC, and 0.2 × SSC, blocked with 5% BSA in buffered saline for 30 min, and incubated with biotin-labeled mouse anti-Digoxigenin for 60 min at 37°C. After a brief wash in PBS, the sections were detected using the 3,3′-diaminobenzidine tetrahydrochloride (**DAB**) system according to the manufacture's protocol (MK 10152, Boster Bioengineering Co., Ltd., Wuhan, China) and then counterstained with hematoxylin.

Western Blot

Protein was extracted from 0.1 g tissues using 1 mL RIPA Lysis Buffer (P0013B, Beyotime) containing 10 μL 100 mM PMSF (ST506, Beyotime). Electrophoresis was carried out by 12% sodium dodecyl sulfate polyacrylamide gel electropheresis (**SDS-PAGE**), followed by electrotransfer to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was incubated in 5% skimmed milk/Tris-buffered saline and Tween 20 at room temperature for 1 h and subsequently incubated with polyclonal rabbit anti-human lysozyme EC 3.2.1.17 antibody (1:200, A0099, Dako) and monoclonal mouse anti-avian β-actin antibody (1:200, sc-47778, Santa Cruz) at 4°C overnight. The rabbit anti-human lysozyme antibody was initially checked for the feasibility in chicken studies through the immunohistological staining and Western blot. After washing in Tris-buffered saline and Tween 20, the membranes were exposed to horseradish peroxidase labeled secondary antibody (goat anti-rabbit or goat anti-mouse IgG, dilution 1:1000) for 1 h at 37°C. The membranes were washed three times and visualized using an electrochemiluminescence (**ECL**) system (170-5056, BIO-RAD Laboratones. Inc., Shanghai, China). The β-actin band was adopted as the internal control. The bands obtained in the Western blot were scanned and analyzed by image analysis software (Gel-Pro Analyzer 4.5, Media Cybernetics). The data were expressed as the IOD of the bands, normalized to the IOD of the corresponding β-actin bands. The result was obtained from three replicates.

Data Analysis

The data were expressed as the means ± SD. The difference of lysozyme expression in the same intestine segment between mouse and chicken was analyzed by Independent-Samples t tests with SPSS11.5 (SPSS Inc., Chicago, IL). Level for determination of significance was 0.05.

RESULTS

Secretory Granules Exist in Rodlike Cells at the Base of Small Intestinal Crypts

Granules in Paneth cells can be recognized by phloxine-tartrazine staining. The staining result showed that some purplish red granules could only be observed in the small intestinal crypts but not in the villus (Figure 1A, B) of 6-month-old chickens. This result implied that Paneth cells might exist in the small intestinal crypts of the chicken intestine. Furthermore, TEM results showed that a large amount of secretory granules existed in the rodlike cells at the bottom of the chicken's intestinal crypts (Figure 1D). However, the granules in the goblet cells at villus showed a low electron density and are arranged in a specific shape (Figure 1C) while granules in the crypts presented a higher electron density (Figure 1D), these different characteristics indicated that the secretory cells in villus and crypt were of different types. It seemed likely that such cells, located at the base of the chicken's small intestinal crypt and confirmed as containing secretory granules, were Paneth cells.

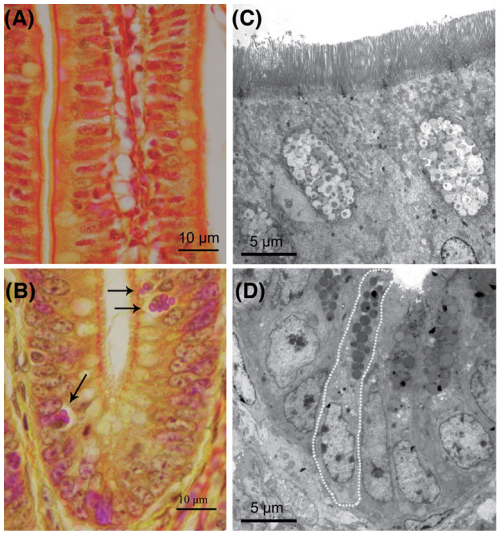

Figure 1. Secretory granules were observed in rodlike cells located at the base of chicken intestinal crypts at 6 months. (**A**) Phloxine-tartrazine staining of the small intestine villus in chicken at 6 months. Scale bar: 10 μm. (**B**) Phloxine-tartrazine staining of the small intestine crypt in chicken at 6 months. The arrow shows the suspected Paneth cells. Scale bar: 10 μm. (**C**) TEM image of the villus showing the ultrastructure of the granules in the goblet cells. Scale bar: 5 μm. (**D**) TEM image of the crypt showing the ultrastructure of the granules in the rodlike cells (indicated in white dotted lines). Scale bar: 5 μm.

Expression of Lysozyme in the Chicken Small Intestine

To investigate the Paneth cells in the chicken small intestine, we detected the expression of lysozyme by Western blot. As shown in Figure 2, lysozyme protein was expressed in the small intestine of chicken at 6 months old and mouse (positive control) without statistical significance. However, no positive band was detected in skin and muscle of chicken (negative control).

RT-PCR analysis of mRNA showed lysozyme c and lysozyme g mRNA expression in the intestine from 1 wk, 2 wk, 3 wk, 8 wk, and 6-month-old chickens (Figure 3A). Whilst the expression of lyszyme $g2$ in the jejunum and ileum remained uniform from 1 wk to

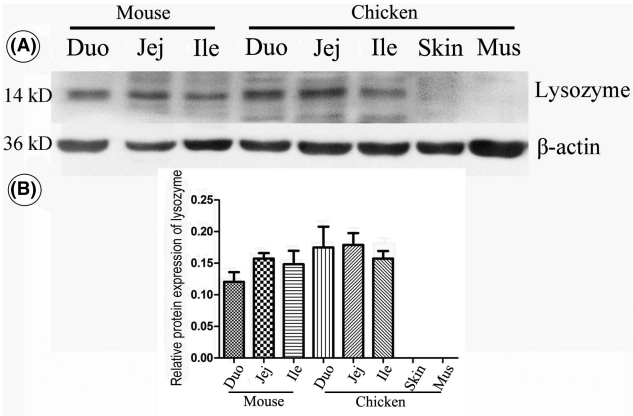

Figure 2. Western blot analysis of lysozyme protein in the chicken of 6 months old and mouse (A), and the statistical analysis result of three replicates (B). Duo, duodenum; Jej, jejunum; Ile, ileum; Mus, muscle. β-actin was adopted as the internal control. There was no statistical significance ($P < 0.05$) in the same intestine segment between the mouse and chicken.

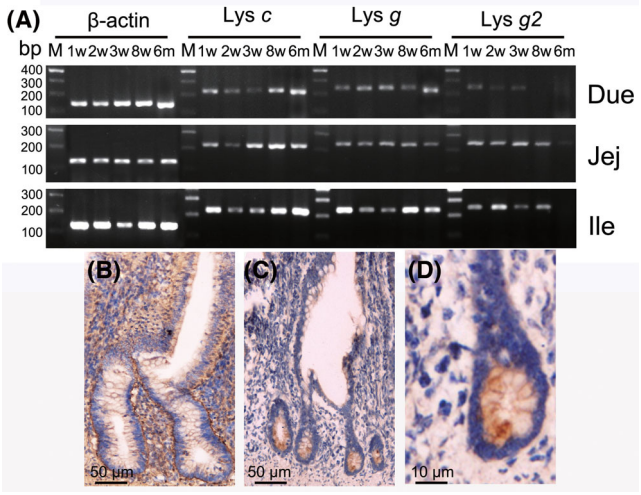

Figure 3. Expression of chicken lysozyme mRNA. (**A**) Expression of lysozyme *c* (lys *c*), lysozyme *g* (lys *g*), and lysozyme *g2* (lys *g2*) mRNAs on Due (duodenum), Jej (jejunum) and Ile (ileum) at the age of 1 wk, 2 wk, 3 wk, 8 wk and 6 months. (**B**) Negative control of in situ hybridization. (**C**) The lysozyme *c* gene was detected at the base of the crypts of the 6-month-old chicken intestine. (**D**) Magnified images from C. Scale bar: 50 μm for B and C, and 10 μm for D.

8 wk, in the duodenum it could only be detected from 1 wk to 3 wk.

To further confirm the cells at the base of small intestinal crypt to be Paneth cells, in situ hybridization analysis was performed on the small intestinal sections of chickens. The result showed that lysozyme *c* mRNA was expressed at the base of small intestinal crypts, at just the location that was presumed to be that of the Paneth cells (Figure 3C, D).

DISCUSSION

Paneth cells are specialized epithelial cells of the small intestine that are located at the base of the intestinal crypts of many vertebrate species. In Paneth cells, abundant secretory granules occur which contain lysozyme, secretory phospholipase A2, enteric α-defensins, cryptdin related sequence peptidesins, and angiogenin 4 (Bevins and Salzman, 2011). Among them, lysozyme is widely considered to be a marker of Paneth cells (Cadwell et al., 2008; Sato et al., 2009; Yin et al., 2014). To date, the presence of Paneth cells in the small intestine of the chicken has remained controversial. In 1974, Paneth cells in the avian intestine were reported based on the light-microscopic methods (Humphrey and Turk, 1974). However, a study by Nile et al (2004) showed that there were no Paneth cells in chicken intestine since the expression of lysozyme *c* gene at different ages could not be detected. In this study, by using phloxine-tartrazine staining and TEM, abundant secretory granules were observed at the rod-like cells which were located at the bottom of small intestinal crypts of the chicken (Figure 1B, D). Unlike the Paneth cells in mice and humans, these secretory cells in the chicken small intestinal crypts were thin and elongated into rod shapes. Species diversity may the cause of this difference in morphological characteristics in such cells between the chicken and mouse. However, these results did imply the existence of Paneth cells in the chicken small intestine.

Lysozyme is one of the most widely used markers for the Paneth cells based on its specific expression in the intestinal crypts. Our RT-PCR result revealed that lysozyme *c* and lysozyme *g* mRNAs is expressed in the chicken in different small intestine segments at various ages. Additionally, the lysozyme *c* gene was specifically located at the base of intestinal crypts which is the predicted location of Paneth cells as indicated by the preliminary morphological analysis above. Moreover, the location of lysozyme *c* mRNA expression in the chicken small intestine was consistent with that in humans (Erlandsen et al., 1974). Therefore, the Paneth cells were considered to exist in chicken small intestine.

In conclusion, we detected the Paneth cells in chicken small intestinal crypts by phloxine-tartrazine staining and TEM observation. We also detected the expression of lysozyme mRNA and protein expression in the chicken small intestine. Lysozyme *c* gene was also specifically expressed at the base of the intestinal crypts. Based on these results, we assume the presence of the Paneth cells in the chicken small intestine. However, the exact roles of the chicken Paneth cells involved in the innate immunity and maintenance of intestinal homeostasis requires further studies.

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