Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Characterization of Invariant Chain Distribution in Tissues of the Digestive Tract in the Muscovy Duck (*Cairina moschata*)

Sheng-Jie Liu[1,2], Chao Wu[2], Qing-Sheng Ni[2] and Wei-Yi Yu[2]*

[1]Biology and Food Engineering School, Fuyang Teachers' College of Anhui Province, Fuyang 236041, China
[2]Key Laboratory of Zoonoses of Anhui Province, Anhui Agricultural University, Hefei, Anhui 230036, China
*Corresponding author: yuweiyi@ahau.edu.cn

ARTICLE HISTORY (14-612)

Received: December 11, 2014
Revised: July 29, 2015
Accepted: July 31, 2015

Key words:
Digestive tract
Invariant chain
Muscovy duck (*Cairina moschata*)
Tissue distribution

ABSTRACT

The invariant chain (Ii) is a molecular chaperone for major histocompatibility complex class II (MHC II) and is therefore intimately involved in the process of antigen presentation. However, it also acts as a receptor for various molecules including macrophage migration inhibitory factor. To determine the localization of Ii in the digestive system of the Muscovy duck (*Cairina moschata*) (MDIi), frozen tissue sections were prepared and immunofluorescence performed using a mouse anti-MDIi antibody. The results show that MDIi was highly expressed by a large number of suspected macrophages and dendritic cells in connective tissues of the mucosa lamina propria in the digestive tract. In comparison, MDIi was only weakly expressed, but polarly distributed, in epithelial cells of the mucosa and glands of the digestive tract. Hepatocytes and endothelial cells around the sinus of the liver also weakly expressed MDIi, while suspected dendritic cells and Kupffer cells strongly expressed it. The findings presented in this study provide initial data for future research on the function of Ii in different organs of the digestive tract of the Muscovy duck, especially in relation to mucosal immunity and the treatment of liver disease.

To Cite This Article: Liu SJ, C Wu, QS Ni and WY Yu, 2015. Characterization of invariant chain distribution in tissues of the digestive tract in the Muscovy duck (*Cairina moschata*). Pak Vet J, 35(4): 510-515.

INTRODUCTION

In addition to being co-expressed with major histocompatibility complex class II (MHC II) by professional antigen-presenting cells (APCs), the invariant chain (Ii, CD74) has also been found co-expressed with MHC II in mucosal epithelial cells of the digestive tract in humans (Barrera *et al*., 2005; Beswick and Reyes, 2009; Metodieva *et al*., 2013). As the class II-associated invariant chain peptide is expressed in complex with MHC II (Beers *et al*., 2005), mucosal epithelial cells are also endowed with antigen-presenting ability. Furthermore, the expression levels of the MHC II/Ii complex were higher in mucosal epithelial cells of the stomach, small intestine, and colon under conditions of infection and cancer than in the corresponding normal tissue (Maharshak *et al*., 2010; Gold *et al*., 2010). Ii also plays an important role as a receptor for various molecules in humans (Metodieva *et al*., 2013; Otterstrom *et al*., 2014). For example, CD74 also is a receptor for macrophage migration inhibitory factor (MIF), which plays important roles in inflammation, proliferation and differentiation of cells, in both normal colonic epithelial cells and cancerous colon cells (Maharshak *et al*., 2010; Grieb *et al*., 2014). Therefore, CD74 and MIF have been used as therapeutic targets in gastric diseases (Zheng *et al*., 2012; Grieb *et al*., 2014). CD74 of gastric mucosa epithelial cells (GEC) was also reported to be the adhesion receptor for urease of *Helicobacter pylori* (Beswick and Reyes, 2009; Alzahrani *et al*., 2014). In the liver of humans and mice, Ii was detected in dendritic cells (DCs), Kupffer cells (KCs) and hepatic stellate cells (Heinrichs *et al*., 2011; Assis *et al*., 2014). Ii can also be ectopically expressed by hepatocytes of Ikkβ knockout of mice to promote antigen presentation and act as a receptor for MIF (Koch and Leffert, 2011).

The above findings on Ii have been obtained in the human digestive system, while in comparison studies on Ii in the digestive system of poultry are rare. In our previous study (Liu *et al*., 2011), the expression levels of *MDIi* in the small intestine were determined to be only slightly less than that in spleen by real-time relative quantitative PCR. *MDIi* was also highly expressed in the liver. However, which cells expressed MDIi and its function in the

digestive system remained unclear. Therefore, in this study, frozen sections of tissues from the digestive system of the Muscovy duck were prepared and MDIi expression localized using immunofluorescence.

MATERIALS AND METHODS

Tissue sampling and preparation of frozen sections: Five healthy adult Muscovy ducks of approximately 5 months of age were purchased from a poultry farm in Fuyang, China. Muscovy ducks were raised in the Animal Feeding Center, and all procedures on these animals were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals. Samples of tissue were taken from the glandular stomach, duodenum, jejunum, caecum, rectum, cloacae, and liver. Frozen sections of 5–8μm thickness of the above tissues were prepared on a Leica CM1900 Cryostat (Leica Biosystems GmbH, Nussloch, Germany) and mounted on poly-L-lysine-coated glass slides (Boster Biomart Inc., Wuhan, China).

Immunofluorescence: Immunofluorescence was performed according to the following routine method. Briefly, fresh tissue sections were fixed in pre-cooled 4°C acetone for 10 min then washed three times with pre-cooled 4°C phosphate-buffered saline (PBS). Tissue sections were blocked with 5% bovine serum albumin in PBS for 30 min at room temperature then incubated with a 1:1600 dilution of the mouse anti-MDIi antibody (Liu *et al*., 2014), at 4°C overnight. After incubation with the primary antibody, sections were washed three times for 5 min in PBS with 0.05% Tween-20. Sections were then incubated with a 1:400 dilution of Dylight-488-conjugated goat anti-mouse IgG antibody (EarthOx Inc., Millbrae, CA, USA) at room temperature for an hour. Subsequently, sections were washed three times with PBST for 5 min then coverslipped with a drop of anti-fade solution, Glycerin Jelly (Biohao Technology Co., Ltd., Beijing, China). Negative controls included sections where the primary antibody was replaced with normal mouse serum or diluent and where the secondary antibody was replaced with diluent. Fluorescence was visualized with the Leica micromanipulation fluorescence imaging system (Leica Inc., Nussloch, Germany).

MDIi expression was scored by the intensity of fluorescence: −, no fluorescence, no expression; ±, very weak fluorescence, no expression; +, weak fluorescence but clearly visible, weak expression; ++, bright yellow-green fluorescence, medium positive expression; +++, obvious very bright green fluorescence, strong positive expression.

RESULTS

High specificity and sensitivity of the immune-fluorescence method: High specificity and sensitivity of the MDIi antibody in various tissues were shown by immunofluorescent staining (Fig. 2-4). As MDIi was expressed at different levels in various cells, the amount of yellow-green fluorescence also varied. However, a lack of specific antibody binding was consistently evident in the normal mouse serum (Fig. 1a), secondary antibody-only (Fig. 1b), and primary antibody-only (Fig. 1c) controls. These controls were also negative in the other types of tissue tested (data not shown).

Localization of MDIi in glandular stomach tissue: MDIi expression in glandular stomach tissue is shown in Fig. 2(a-c). MDIi was abundantly and strongly expressed by what were suspected to be DCs, macrophages, and B lymphocytes of connective tissues in the simple tubular gland area of the lamina propria of the glandular stomach mucosa. In contrast, the mucosal epithelial cells of the glandular stomach had weak expression of MDIi. MDIi was also weakly expressed in epithelial cells of simple tubular glands, where it was located near the lumen surface. MDIi was not expressed in epithelial cells of compound tubular glands in the lamina propria, yet a small number of cells strongly expressed MDIi in the thin connective tissues around compound tubular glands. A few cells in the connective tissue of the thin submucosa expressed MDIi, while it was hardly expressed in the muscle layer. Thus, the lamina propria is the area of greatest MDIi expression.

Localization of MDIi in duodenal tissue: MDIi expression in duodenal tissue is shown in Fig. 2(d–f). MDIi was also abundantly expressed in cells of the lamina propria connective tissue in the mucosal layer of the duodenum, especially in cells of the enteraden-rich areas of lamina propria. MDIi showed weak expression in epithelial cells of the intestinal villus; however, it was located near the luminal surface of these cells. Interestingly, the MDIi expression in epithelial cells gradually increased from the intestinal villus to the enteraden. Few cells expressed MDIi in the lamina propria of the intestinal villus. However, many cells strongly expressing MDIi were evident in enteraden areas of the lamina propria. As these cells had different sizes and luminescence, it suggests different types of cells are present and expressing MDIi. Large numbers of small cells that highly expressed MDIi and fewer big cells that strongly expressed MDIi were seen. The expression of MDIi in the muscle layer and thin submucosa of the duodenum were similar to that of the glandular stomach. There were few cells strongly expressing MDIi in the thin serosal layer.

Localization of MDIi in jejunum tissue: MDIi expression in jejunum tissue is shown in Fig. 2(g–i). MDIi was abundantly expressed in the mucosal layer of the jejunum. Epithelial cells of the intestinal villus weakly expressed MDIi; however, the expression level was obviously higher than that of the duodenum. MDIi was also polarly expressed near the luminal surface in epithelial cells of the intestinal villus. Isolated cells among the epithelial cells of the intestinal villus were found to highly express MDIi, while a few cells in the lamina propria connective tissues of intestinal villus strongly expressed MDIi. The epithelial cells of enteradens strongly expressed MDIi; with polar localization of MDIi in these cells. The trend of increasing MDIi expression in epithelial cells from the intestinal villus to the enteraden in the jejunum was the same as that in the duodenum. More large cells strongly expressed MDIi in lamina

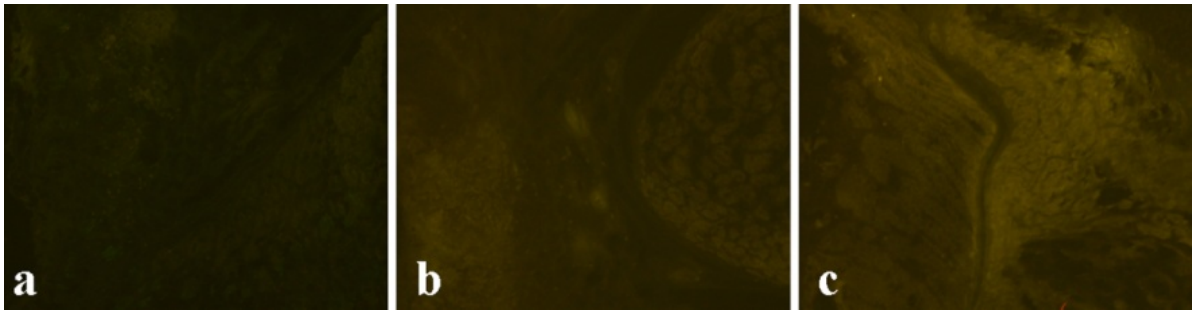

Fig. 1: Representative figures of the negative controls for MDIi expression in glandular stomach tissue. a) Normal mouse serum control, b) Secondary antibody-only control and c) Primary antibody-only control. 100×.

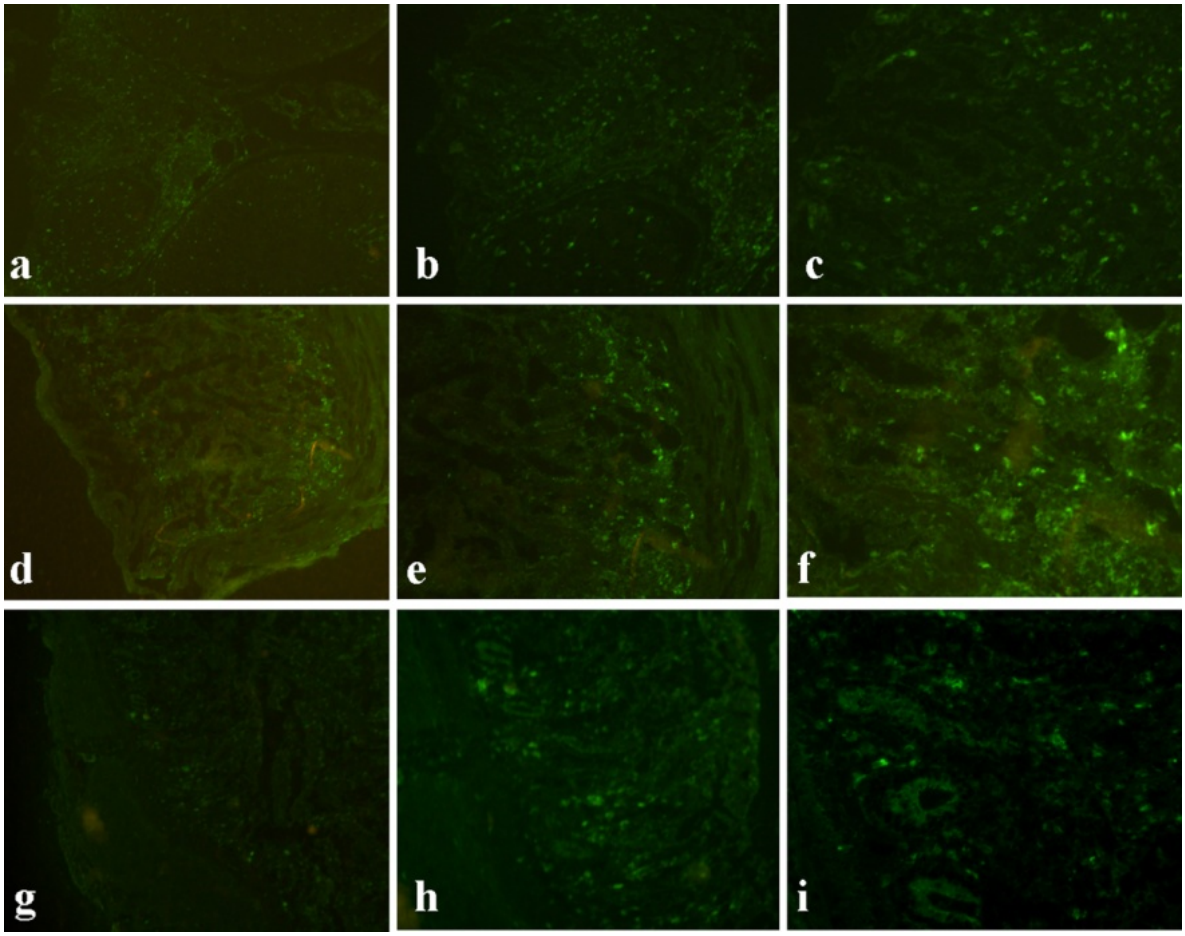

Fig. 2: The expression of MDIi in tissues of the glandular stomach (a–c), duodenum (d–f), and jejunum (g–i). Glandular stomach mucosa: a) The epithelial cell layer, the simple tubular gland area, and compound tubular gland area of the mucosal lamina propria, b) The simple tubular gland area, compound tubular gland area, and connective tissues of the mucosal lamina propria and c) The simple tubular gland area and connective tissues of mucosal lamina propria. Duodenum: d) Tissues of the walls of the duodenum and duodenum lumen, e) The mucosal layer, submucosa, and muscle layers of the duodenum and f) The intestinal villus and enteraden of mucosal layers of the duodenum. Jejunum: g) Tissues of the walls of the jejunum and jejunum lumen, h) The mucosal layer, submucosa, and muscle layer of the jejunum and i) The intestinal villus and enteraden of the mucosal layer of the jejunum. a, d and g=50×; b, e and h=100×; c, f and i=200×.

propria connective tissues among enteraden in the jejunum. The MDIi expression level in enteraden areas was also greater than that in the intestinal villus areas.

Localization of MDIi in cecum tissue: MDIi expression in cecum tissues, which were randomly selected from the cecum wall, is shown in Fig. 3(a-c). MDIi was mainly expressed in the mucosa layer. MDIi was weakly expressed and polarly distributed near the luminal surface in epithelial cells of the mucosa layer in the cecum. A few big cells strongly expressing MDIi were distributed in the lamina propria of the mucosa.

Localization of MDIi in rectal tissue: MDIi expression in rectum tissue is shown in Fig. 3(d, e). MDIi-expressing cells were mainly distributed throughout the mucosa and

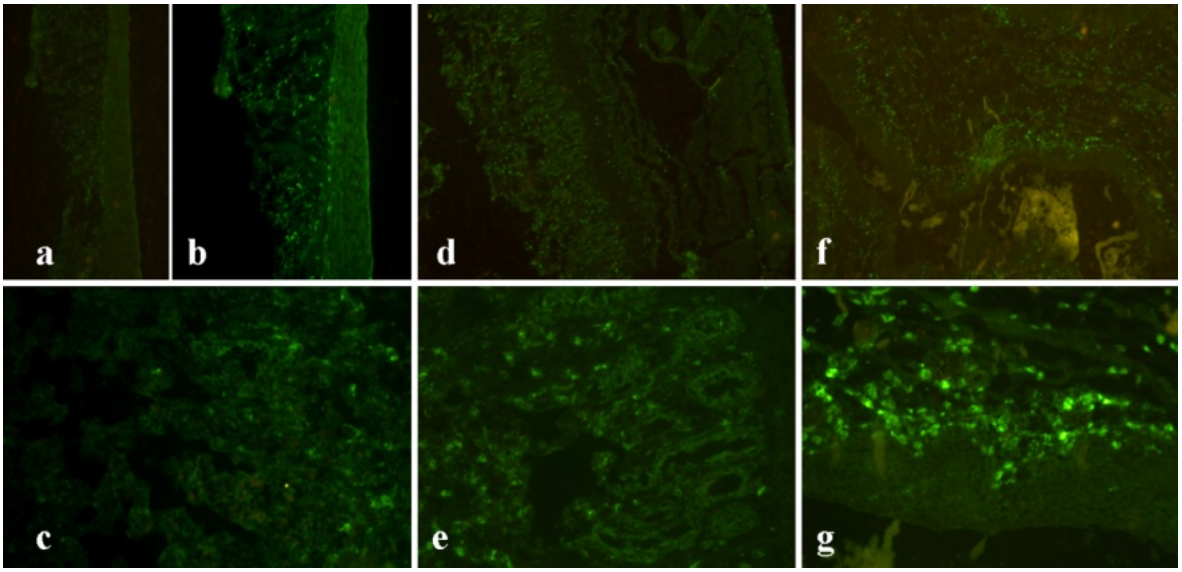

Fig. 3: The expression of MDIi in tissues of caecum (a–c), rectum (d, e), and cloaca (f, g). a-b) Tissues of the wall of the caecum, c) The mucosal layer of the caecum, d) Tissues of the wall of the rectum, e) The intestinal villus and enteraden of the mucosal layer of the rectum, f) Tissues of the mucosal layer of the cloaca and g) The epithelial cell layer and connective tissues of the lamina propria of the cloaca. a, d and f=50×; b=100×; c, e and g=200×.

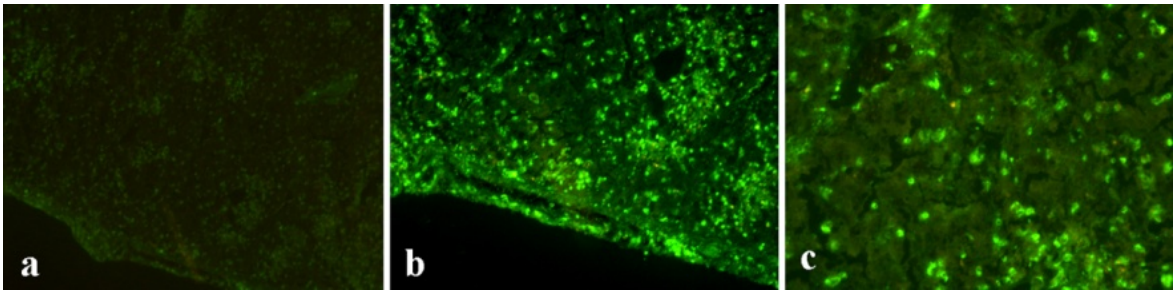

Fig. 4: Expression of MDIi in liver tissue. a) Low magnification view of liver tissue, 50×, b) Tissues of the liver including the portal area and liver capsule, 100× and c) Tissues of the portal area, hepatic cords, and blood sinus of the liver, 200×.

submucosa layer of the rectum. Epithelial cells weakly expressed MDIi on the surface of the rectum mucosa, but highly in enteraden areas. MDIi was polarly expressed near the luminal surface of epithelial cells. There were a substantial number of large cells strongly expressing MDIi scattered throughout the lamina propria connective tissues of all mucosa layers. The submucosa of the rectum was much thicker than that of the small intestine, and there were also large and scattered cells strongly expressing MDIi. Only a few cells strongly expressing MDIi were located in the thin connective tissues of the muscularis mucosae and rectum wall muscle layer. MDIi expression in enteraden areas was also greater than that in intestinal villus areas.

Localization of MDIi in cloaca tissue: MDIi expression in cloaca tissue is shown in Fig. 3(f, g). The cloaca was divided into the coprodeum, urodeum and proctodeum. The proctodeum was selected for further study in this experiment. Stratified epithelial cells of the proctodeum were all shown to weakly express MDIi. However, plenty of large cells strongly expressed MDIi in the lamina propria of the mucosa and these cells were more concentrated near the stratified epithelium in mucosal lymphoid tissue areas.

Localization of MDIi in liver tissue: Many large cells that strongly expressed MDIi were distributed throughout the liver; however, these cells were more concentrated around the portal area of the hepatic lobule (Fig. 4a-c). According to the shape or position of cells in tissues of the liver, these cells might be KCs, mononuclear cells, or DCs in hepatic sinusoid or perisinusoidal spaces rather than hepatocytes. However, all hepatocytes also expressed MDIi, albeit weakly. Hepatic sinusoidal endothelial cells also expressed MDIi at a low level. The liver capsule contained abundant cells strongly expressing MDIi.

DISCUSSION

The digestive system is the largest *in vivo* structure in contact with the environment outside of the body, and so may face extraneous pathogens at any time. Thus, it is necessary to have a strong defense system present in the digestive tract. Therefore, the mucosa, submucosa, muscularis and serosa of the wall of the digestive tract form a physical barrier to prevent pathogens and food antigens from intruding into deep tissues. Owing to long-term co-evolution, a variety of microorganisms in the digestive tract have formed a mutually beneficial microorganism ecosystem that directly kills invading

extraneous pathogens (Kamada *et al*., 2013; Power *et al*., 2014). However, these natural barriers may incompletely resist all threats in the digestive tract. The presence of Ii in the mucosa of the digestive tract indirectly reflects that mucosal immunity may be present as a back-up system as outlined below.

First, many cells with various sizes were found strongly expressing MDIi in mucosal lamina propria in connective tissues of the digestive tract. These cells were speculated to be macrophages, DCs and B cells according to their intrinsic characteristics in connective tissue. In humans, these cells act as professional APCs and frequently phagocytose and present antigens in the digestive tract (Smith *et al*., 2011; ten Broeke *et al*., 2013). Furthermore, macrophages and DCs assemble in mucosal lamina propria of the digestive tract to form "microbial-mucosa ecosystems" (Dupaul-Chicoine *et al*., 2013) for adjusting the immune response to symbiotic bacteria and protecting the host from pathogens (van Baarlen *et al*., 2013).

Second, epithelial cells of the gastrointestinal mucosa, the simple tubular gastric gland, and enteraden of the digestive tract of the Muscovy duck weakly expressed MDIi, which was polarly distributed near the luminal surface of these cells. The above characterization of MDIi expression and distribution in these epithelial cells agrees with that reported for the corresponding human cells (Barrera *et al*., 2005; Cuthbert *et al*., 2009). As Ii is associated with MHC II, the expression of MDIi by gastrointestinal epithelial cells suggest that these cells also have antigen-presenting functions in Muscovy ducks, in accord with the non-professional antigen-presenting functions of epithelial cells in other species (Buning *et al*., 2008; Metodieva *et al*., 2013). However, the polar distribution of MDIi in epithelial cells is obviously different from the uniform distribution in non-epithelial cells such as macrophages and DCs, which suggests that there is a difference in the transport mechanism of MDIi between these cell types. MDIi expression increases from mucosal epithelial cells to glandular epithelial cells in the intestinal wall. MDIi expression also gradually increased in mucosal epithelial cells from the beginning to the end of the digestive tract. These findings reveal that immune function is gradually strengthened, not only from the superficial to the deep, but also from the forepart to the posterior of the digestive tract. Epithelial cells weakly expressing Ii may closely reflect the physiological status of the body, because inflammation or cancer epithelial cells of the gastrointestinal mucosa express more CD74 than epithelial cells of corresponding normal tissue (Gold *et al*., 2010; Metodieva *et al*., 2013).

Similarly, the cells strongly expressing MDIi among hepatocytes were deduced to be KCs, DCs or lymphocytes. Therefore, the liver of the Muscovy duck has a very strong immune presence similar to the human liver (Crispe, 2009; Koch and Leffert, 2011; Assis *et al*., 2014). In the human liver, as the largest group of APCs, KCs are one of the most important cells regulating immunity by phagocytosing and presenting antigens (Liao, 2010). Thus, KCs have become a research hotspot in human hepatitis, liver cancer and liver transplantation in recent years (Chen *et al*., 2008). The hepatocytes and hepatic sinusoidal endothelial cells in Muscovy ducks also have antigen-presenting abilities because they weakly express MDIi, similar to Ii expression in the corresponding human cells (Koch and Leffert, 2011; Kim *et al*., 2012). Thus, although the liver is obviously an important organ for metabolism, it is also an immune organ with various immune mechanisms ready to deal with food and microbial antigens coming from peripheral and small intestine visceral blood.

Conclusion: Immunofluorescence was successfully established to detect the localization of Ii in tissues of the digestive system of the Muscovy duck. A large number of suspected macrophages and DCs strongly expressed MDIi in connective tissue of the mucosal lamina propria throughout the digestive tract. Furthermore, MDIi was weakly expressed and polarly distributed in epithelial cells of mucosa and glands in mucosal lamina propria. Hepatocytes and endothelial cells around the hepatic sinusoids also weakly expressed MDIi, while suspected KCs and DCs strongly expressed it. These discoveries may provide a base for exploring mucosal immunity of the digestive tract and the treatment of liver disease in poultry.

Acknowledgment: This study was supported by grants from the Natural Scientific Research Major Projects of Anhui Department of Education (#KJ2013A203), the major project of biology discipline construction in Anhui province (#[2014]28) and the characteristic specialty project of Food Science and Engineering in Anhui province (#2014tszy092). The Key Laboratory of Embryonic Development and Reproduction Regulation of Anhui Province are appreciated.

Author's contribution: SJL and WYY designed the research and wrote the paper. SJL and CW executed the experiment and analyzed the tissue samples. QSN contributed essential reagents. All authors approved final version of the manuscript.

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