

Telocytes in the esophageal wall of chickens: a tale of subepithelial telocytes

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ABSTRACT The esophagus is a tubular organ which act as a passage for food from oral cavity to stomach. Telocytes (TCs) are a unique type of interstitial cell whose existence in many organs of various species still remains unknown. In the present study, we used transmission electron microscopy (TEM) and immunohistochemistry (CD34, Vimentin, PDGFR- α) to identify subepithelial TCs in the esophageal wall of chickens. TEM micrographs confirmed the presence of TCs in the lamina propria, tunica submucosa, and tunica muscularis muscular layer of the esophageal wall. A large population of TCs were observed just beneath the epithelial layer of the esophageal wall, and the TCs demonstrated structural heterogeneity, featuring various cell body shapes of cell bodies and

telopodes (Tps) with podoms, podomeres, and dichotomous branching. Furthermore, a large number of extracellular vesicles were found to be associated with TCs/Tps. Cellular extensions from TCs were observed in close proximity to blood vessels, immune cells, and mucosal glands. In the submucosa, Tps and immune cells were in very close contact. Immunohistochemical results showed that there were CD34+ cells, vimentin+ cells, and PDGFR- α + cells in the subepithelium, lamina propria, and mucosal glands of the chicken esophageal wall, which was consistent with the TEM results. Overall, our data confirmed the existence of TCs in the chicken esophagus and suggested that TCs might contribute to epithelial regeneration and tissue homeostasis.

Key words: telocyte, esophageal wall, chicken, TEM, immunohistochemical

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INTRODUCTION

Almost a decade ago, a novel type of interstitial cell known as telocytes (TCs) was serendipitously discovered (Popescu and Faussone-Pellegrini, 2010). Morphologically, TCs are relatively small cells with long, thin extracellular processes known as telopodes (Tps). Their cell bodies contain a nucleus that is large in size and surrounded by a small amount of cytoplasm that contains the mitochondria, endoplasmic reticulum, and Golgi apparatus. The Tps (long cellular prolongations), are made by comprise repeating thin (podomeres) and thick, dilated thick (podoms) segments. The podoms accommodate cell organelles like the endoplasmic reticulum, mitochondria, and caveolae, that drive protein synthesis and intercellular signaling. The number of Tps determines

the shape of the TCs, which range from piriform (single prolongations) to spindle (2 prolongations) and triangular (3 prolongations) (Popescu and Faussone-Pellegrini, 2010; Pellegrini and Popescu, 2011; Cretoiu and Popescu, 2014). Until now, TCs have only been reported to exist in the interstitial space (connective tissue) of various organs in many species. Furthermore, they are responsible for lots of various physiological functions depending upon the organs (Aleksandrovykh et al., 2017). To date, transmission electron microscopy (TEM) has been the “gold standard” for identifying TCs. However, although no single biomarker specific for TCs has been identified, proteins such as CD34, vimentin, and PDGFR- α , either individually or as combinations of CD34 and PDGFR- α or combinations of CD34 and PDGFR- α , have demonstrated to be reliable immune markers to target for the identification of TCs.

The esophagus is tubular organ that allows the passage of food from the oral cavity to the stomach. Histologically, most tubular organs in the body, especially those in the digestive system, comprise 4 basic tissue layers: the tunica mucosa, tunica submucosa, tunica muscularis mucosa, and tunica adventitia/serosa (also known as the tunica externa). The tunica mucosa is

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further divided into the epithelium, lamina propria, and muscularis mucosae (Young et al., 2013). TCs were first identified in the pancreas, so early thought was that they were mostly localized in the organs of the digestive system (Popescu and Fausone-Pellegrini, 2010). However, to date, TCs have been observed in almost every organ, regardless of the species. Our group was the first to report on the presence of TCs in the testes of turtles (Yang, et al., 2015a), naked mole rats (Haseeb et al., 2019), and rats (Liu et al., 2019), and we have reported on the presence of TCs in many different organs of reptilian and avian species (Ullah et al., 2014; Yang et al., 2015b; Yang et al., 2016; Gandahi et al., 2020). However, TCs have only been reported in the esophagus of mammalian species (i.e., wistar rat and human) (Rusu et al., 2012; Chen et al., 2013). Because of the clear differences in the digestive system between mammals and avian species, this served as an impetus for designing the present study, which identifies and reports TCs in the esophagus of chickens.

MATERIALS AND METHODS

Animals

Ten healthy adult chickens (3.5 months of age) were purchased from a poultry farm located in the Nanjing Jiangsu Province of China. The chickens were maintained in the lab and were fed ad libitum. The chickens were euthanized by cervical dislocation after intravenous administration of 3% (w/v) sodium pentobarbital. The esophagi were dissected from the chickens (4 months of age), and the tissue was immediately fixed by various procedures depending on the microscopic technique (details below). The animal protocols used in this study were in accordance with the guidelines of the Animal Research Institute Committee of Nanjing Agriculture University and were approved by the Science and Technology Agency of Jiangsu Province (Approval ID: SYXK (SU) 2020–0005). For animal welfare, all measures were made to minimize the suffering of the chickens.

Light Microscopy

For histological examination of the esophagus, tissue samples of the organ were fixed in 10% formalin in neutral buffer overnight. After fixation, the samples were embedded in paraffin wax and were cut into slices with thicknesses of 5 to 7 μm . The tissue sections were stained with hematoxylin and eosin (H&E) stain (Harry's hematoxylin for 2 min and 1% eosin for 30 s) and Masson separately. Finally, the tissues were analyzed under an Olympus BX53 microscope (Tokyo, Japan).

Transmission Electron Microscopy

For TEM observation of the Esophageal, the esophagus samples were cut into 1 mm³ blocks, which were immersed in 2.5% glutaraldehyde in 0.01 M PBS (4°C,

pH 7.4) for 24 h. The samples were removed from the PBS and rinsed with PBS, after which they were fixed in a 1% osmium tetroxide solution for 1 h at 37°C. The postfixed samples were dehydrated with increasing concentrations of ethyl alcohol (75%, 85%, 95%, and 100%), infiltrated with a propylene oxide-Araldite mixture, and embedded in Araldite. The blocks were sectioned using an ultramicrotome (Reichert Jung, Vienna, Austria). The ultrathin sections of tissue (50 nm) were mounted onto copper grids and stained with uranyl acetate and lead citrate for 20 min each. Finally, ultrastructural examination of the esophageal tissue was conducted using a Hitachi H-7650 transmission electron microscope (Hitachi H-7650, Tokyo, Japan).

Immunohistochemistry (IHC)

The paraffined tissue sections were dewaxed by rinsing with distilled water, and the sections were incubated in 3% H₂O₂ at room temperature for 10 min. After wiping, the sections were rinsed with distilled water three times for 5 min each \times 3 times. The sections were subjected to thermal antigen repair and rinsed with PBS after cooling, after which the sections were dropped with 5% bovine serum albumin (BSA; Boster Bio Co., LTD, Pleasanton, CA) and blocking solution and incubated at 37°C for 30 min. The blocking solution was discarded, and appropriate amount of rabbit anti-CD34 (1:100 dilutions; catalog no. BS-0646R; Bioss Beijing, China), mouse anti-vimentin (1:100 dilutions; catalog no. MA5-11883; Thermo Fisher Scientific, Shanghai, China), rabbit anti-PDGFR- α (1:100 dilutions; catalog no. P16234; Abcam, Cambridge, UK) antibodies at 4°C overnight. After incubating with the primary antibodies, the sections were removed from the incubation solution and washed with PBS three times for 5 min each \times 3 times. After the sections were wiped, a biotin-labeled secondary antibody (KIT-5001, KIT-5004, MXB Biotechnology, China) was added to the x containing the tissue sections, which was incubated at 37°C for 1 h. The sections were washed with PBS solution three times for 5 min \times 3 times each. Following, 3,3'-diaminobenzene (DAB; Boster Bio Co., Ltd., Pleasanton, CA) was added to x to stain the tissue samples for periods of 5 to 30 min. The sections were then counterstained with hematoxylin. Tissue sections incubated in PBS served as a negative control. The stained sections were observed under a light microscope (BX53; Olympus, Tokyo, Japan) equipped with a camera (DP73; Olympus).

RESULTS

General Histology of the Chicken Esophagus

Histological analysis of the chicken esophagus tissue by H&E (Figure 1A, B) and Masson (Figure 1C, D) staining by light microscopy revealed that the esophagus contained a nonkeratinized stratified squamous epithelium superficial to the prominent lamina propria. The lamina propria is a thin layer of loose connective tissue

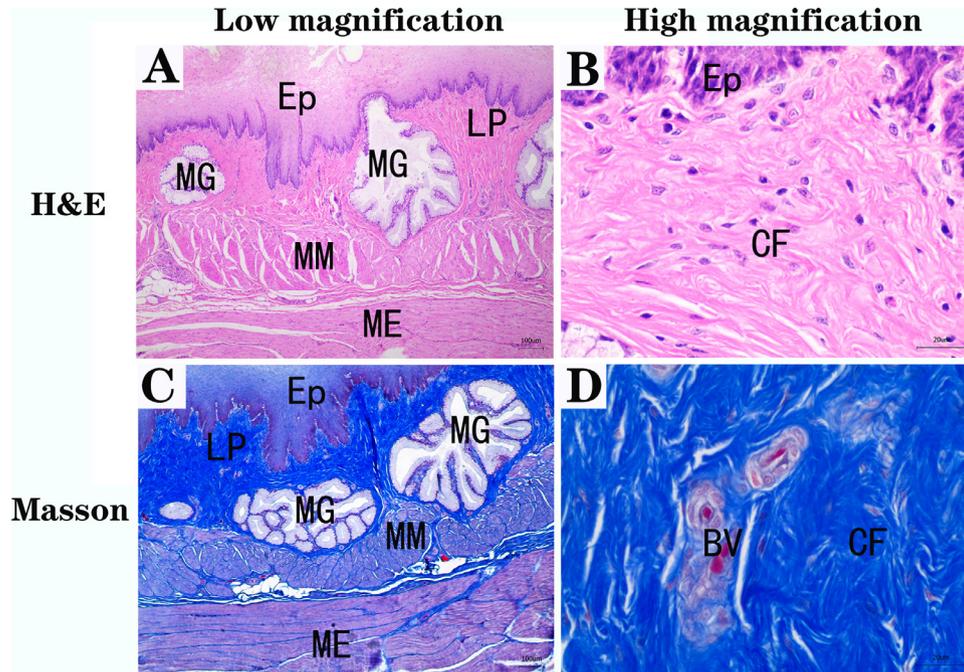


Figure 1. Normal histology of the chicken esophagus. Ep, epithelium; LP, lamina propria; MG, mucosal gland; MM, muscular mucosae; ME, muscular extrana; CF, collagen fiber; BV, blood vessel. Scale bar: A and C: 100 μm , B and D: 20 μm .

containing simple or branched tubular mucosal glands. Within the stratified squamous epithelium, the muscularis mucosa was composed of a single layer of longitudinal smooth muscle fibers, while the submucosa was composed of loose connective tissue. Within the tunica muscularis, inner circular and outer longitudinal layers covered by the adventitia, which is composed of loose connective tissue, were observed (Figure 1A–D).

Identification and Localization of Telocytes in Chicken Esophagus

IHC analysis of the esophageal tissue corresponded with the results of transmission electron microscopy (Figures 2–4). The cell membrane was positive for CD34. There was a large number of CD34⁺ cells in the subepithelium of the chicken esophageal wall

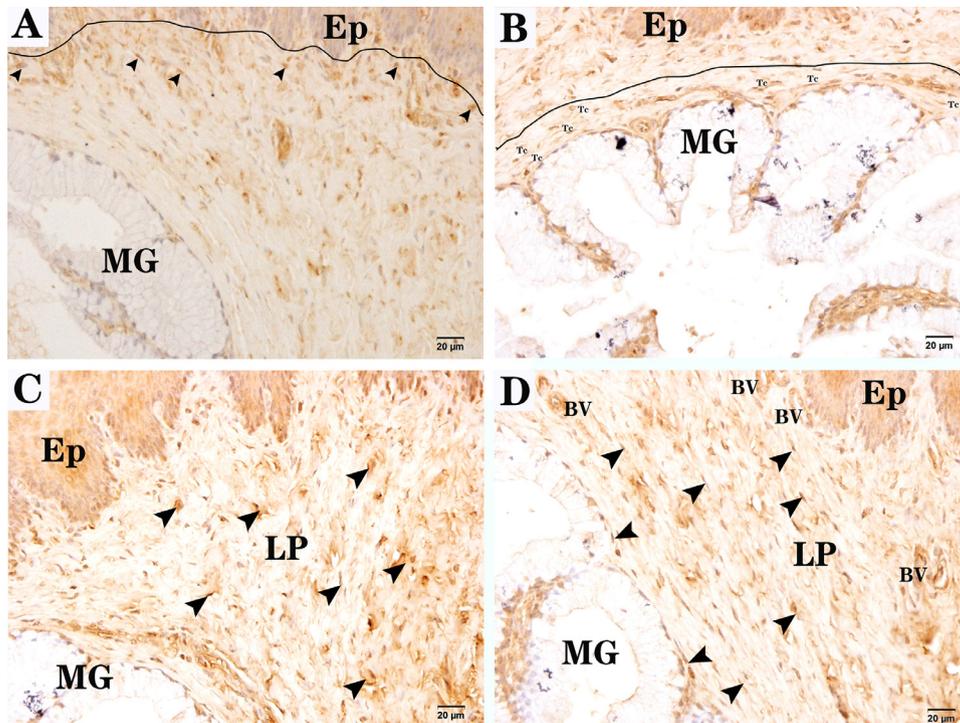


Figure 2. Immunohistochemical staining of CD34 in the chicken esophageal wall. (A) CD34⁺ TCs in the subepithelium of the chicken esophageal wall. The cell bodies were irregular and punctate; (B) elongated CD34⁺ TCs were present in large numbers around the mucosal glands of the chicken esophageal wall; (C) CD34⁺ TCs were scattered throughout the lamina propria of the chicken esophageal wall; (D) CD34⁺ cells were present in endothelial cells and blood vessels. Tc, telocyte; Ep, epithelium; LP, lamina propria; MG, mucosal gland; BV, blood vessel. Black arrow = CD34⁺ TCs. Scale bar: 20 μm .

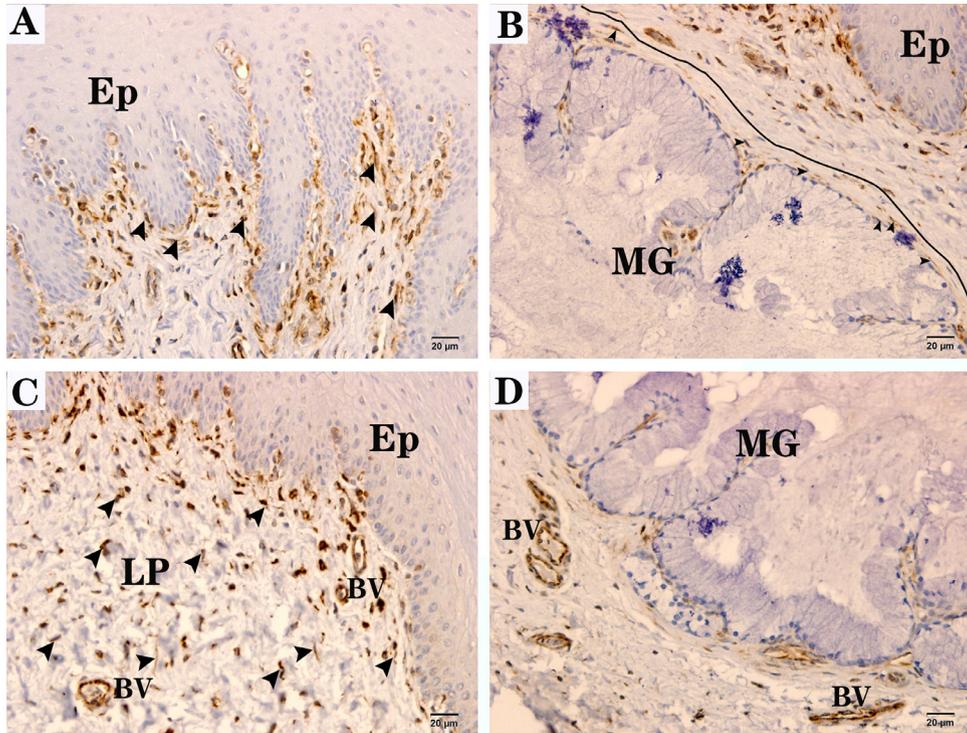


Figure 3. Immunohistochemical staining of vimentin in the chicken esophageal wall. (A) Vimentin⁺ TCs in the subepithelium of the chicken esophageal wall; (B) vimentin⁺ TCs around the mucosal glands of the chicken esophageal wall; (C) vimentin⁺ TCs scattered throughout the lamina propria of the chicken esophageal wall; (D) vimentin was present in the blood vessels in the esophageal wall. Ep, epithelium; LP, lamina propria; MG, mucosal gland; BV, blood vessel. Black arrow = vimentin⁺ TCs. Scale bar: 20 μ m.

(Figure 2A). In addition, there was a large number of elongated CD34⁺ TCs around the mucosal glands of the chicken esophageal wall, and these cells formed long cellular processes (Figure 2B). A large number of

CD34⁺ TCs was also scattered throughout the lamina propria of the chicken esophageal wall (Figure 2C). The blood vessels and endothelial cells stained positive for CD34 (Figure 2D).

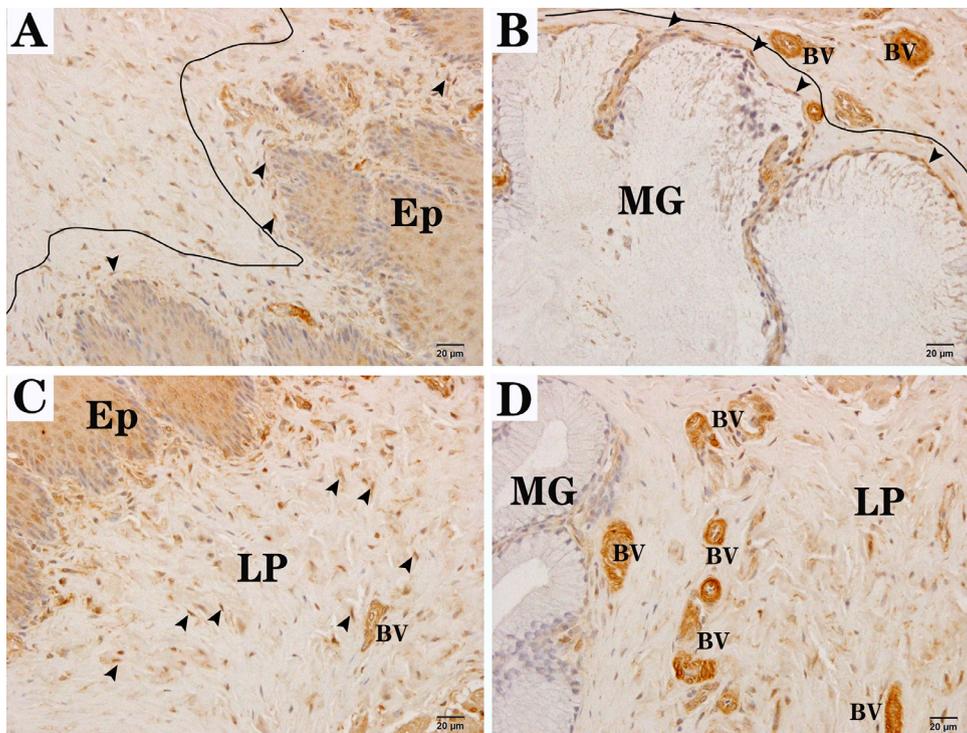


Figure 4. Immunohistochemical staining of PDGFR- α in the chicken esophageal wall. (A) PDGFR- α ⁺ TCs in the subepithelium of the chicken esophageal wall; (B) elongated PDGFR- α ⁺ TCs around the mucosal glands of the chicken esophageal wall; (C) PDGFR- α ⁺ TCs were scattered throughout the lamina propria of the chicken esophageal wall; (D) the blood vessels stained positive for PDGFR- α . Ep, epithelium; LP, lamina propria; MG, mucosal gland; BV, blood vessel. Black arrow = PDGFR- α ⁺ TCs. Scale bar: 20 μ m.

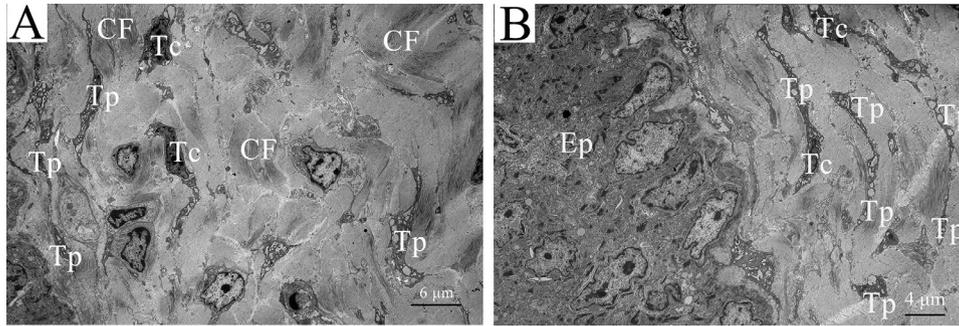


Figure 5. Transmission electron micrographs of the chicken esophagus. (A) TCs were scattered throughout the CFs; (B) TCs were distributed layer-by-layer beneath the epithelium. Tc, telocyte; Tp, telopode; Ep, epithelium; CF, collagen fiber. Scale bar: 4 μ m.

Vimentin was identified in the cytoplasm of the TCs. The vimentin staining results were consistent with the CD34 staining results (Figure 3). There was a large number of vimentin⁺ cells located beneath the epithelium and were punctate and irregularly distributed (Figure 3A). There was a large number of vimentin⁺ TCs with slender, elongated cell bodies around the mucosal glands of the chicken esophageal wall (Figure 3B). In addition, vimentin⁺ TCs were scattered throughout the lamina propria of the chicken esophageal wall (Figure 3C). The blood vessel walls of blood vessels within the esophagus also stained positive for vimentin (Figure 3D).

The PDGFR- α staining results were consistent with the CD34 and vimentin staining results (Figure 4). A large number of PDGFR- α ⁺ cells was located just beneath the epithelium, and the cells were punctate and irregularly distributed. The TCs under the epithelium were distributed in clusters and interwoven, forming a network with the neighboring TCs (Figure 4A). There was a large number of PDGFR- α ⁺ TCs with elongated, slender cell bodies around the mucosal glands of the esophageal wall (Figure 4B). Furthermore, a large number of PDGFR- α positive TCs was scattered throughout the lamina propria of the esophageal wall (Figure 4C), and the blood vessels stained positive for PDGFR- α (Figure 4D).

Due to the lack of commercially available poultry antibodies on the market, we further analyzed the tunicae/layers of the esophageal wall to identify TCs at the utero-vaginal junction using the “gold standard” (TEM) for identifying TCs. The TEM images indicated the presence of TCs within the lamina propria and submucosa and between the bundles of smooth muscle fibers in the muscularis mucosae and muscular layer (Figures 5–7). The TCs and their nuclei were clearly visible, and they featured long cellular projections in the vicinity of collagen fibers. TCs were widely distributed between the collagen fibers in throughout the lamina propria in a continuous or discontinuous manner between the collagen fibers, and their morphologies were irregular (Figure 5A). A large number of TCs was also distributed layer-by-layer beneath the epithelium (Figure 5B). The TCs in the lamina propria were located beneath the epithelial cells as subepithelial TCs (Figure 6A). Furthermore, they were distributed freely throughout the loose

connective tissue and were in close proximity to immune cells, blood vessels (Figure 6B, and D), and mucosal glands (Figure 7B). Moreover, TCs were observed in the muscularis mucosae and muscular layer between the smooth muscle’s cells groups (Figure 6C and 9A).

Morphologically, the cell bodies of the TCs varied in shape (e.g., pyriform-shaped, spindle-shaped, triangular, and quadrangular) and featured small amounts of cytoplasm surrounding the nucleus (Figures 6–8). The nuclei were moderately heterochromatic at the periphery (Figure 8A, and B). In addition, numerous cellular prolongations (telopodes) protruded from the cell body, showing over-lapping, convoluted, and tortuous processes as part of podomeres (thin segments) (Figure 8C, and D). Furthermore, well-differentiated podoms (bead-like regions) were observed. The podoms that were confined to the mitochondria, endoplasmic reticulum, and vesicles (Figure 8E and F).

The Tps, whose tortuous cross-sections are indicated by the white arrows in Figure 9A and D, were easily confused with extracellular vesicles. The white triangular arrows around TCs point to the tortuous cross section of Tp (Figure 9A and D). TCs and their cellular prolongations with various shapes and sizes were observed in many secretory granules and extracellular vesicles in the lamina propria having various shape and size (Figure 9B and C). The extracellular vesicles were observed in close proximity to the cell bodies (Figure 9B) and end points of the Tps (Figure 9C). The extracellular vesicles were clear and translucent. Moreover, the TCs showed both hetero and homo cellular contacts, were connected not only to other TCs via contact between the Tps to one another (Figure 9C) but also and Tps to immune cells (Figure 6D), blood vessels (Figure 6B), and plasma cells (Figure 7A).

DISCUSSION

Telocytes in the Esophagus

After the discovery of TCs by electron microscopy, while many other methods have been used to identify TCs and elucidate their physiological roles, electron microscopy still serves as the best method for identifying TCs (Cantarero et al., 2016). Therefore, we used TEM to identify TCs in the esophagi of chickens, and these

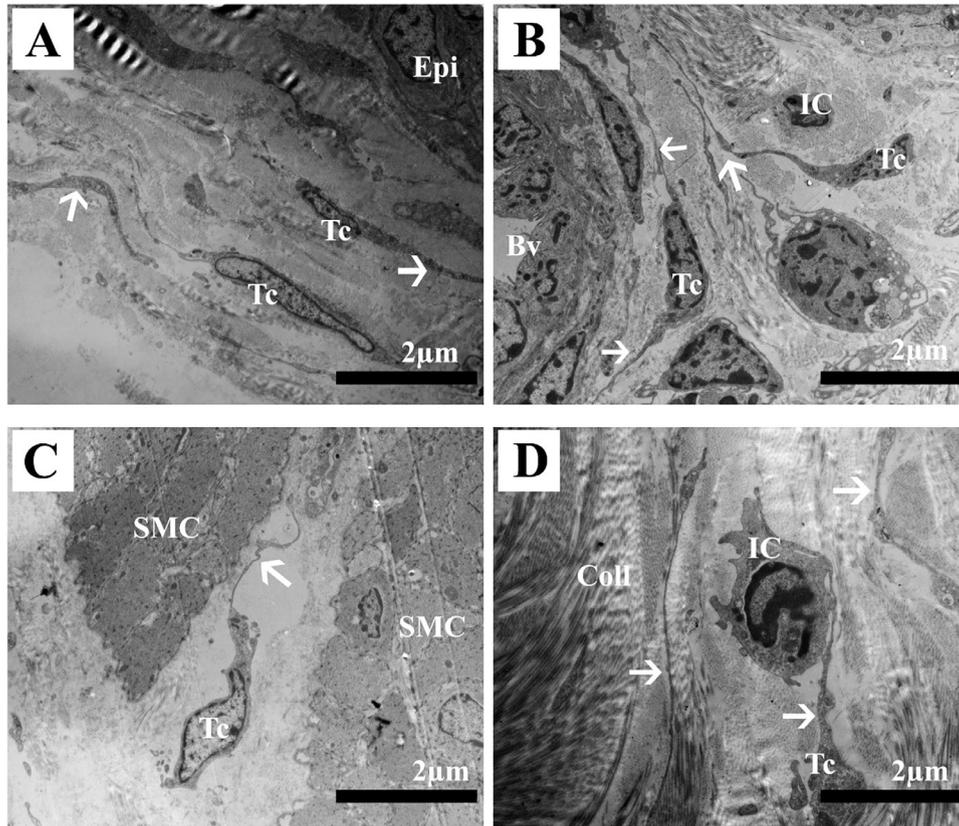


Figure 6. Transmission electron micrographs of the chicken esophagus. (A) The TC population just beneath the epithelial cells; (B) morphologically heterogeneous population of TCs around the blood vessels; (C) TCs in the muscular layer; (D) TCs in the tunica submucosa were in having cellular contact with immune cells. Epi, epithelium cell; Tc, telocyte; Bv, blood vessel; SMC, smooth muscles cell; IC, immune cell; CF, Collagen fiber. Scale bar: 2 μm .

results were validated by conducting IHC analysis by looking at CD34, Vimentin, and PDGFR- α . We found that the IHC results were consistent with the TEM results. TCs were identified and differentiated from adjacent cells according to previously set criteria (Popescu and Fausone-Pellegrini, 2010). Previously, TCs were only identified in the esophagi of Wistar rats and humans. However, our study is the first to identify TCs in chicken esophagi. The study performed in Wistar rats reported that TCs were present in all 4 layer/tunicae (tunica mucosa, tunica submucosa, tunica muscularis, and tunica adventitia) of the rat esophagus (Rusu et al.,

2012). In contrast, TCs were only discovered in 3 layers of the human esophageal wall, reported TCs in 3 layers namely the mucosa, submucosa, and muscular layer (Chen et al., 2013). Similarly, in present study, TCs were found predominately in the lamina propria, submucosa, and muscular layer of the chicken esophagus.

Previously, IHC analysis of developing embryos of white rabbits showed that TCs were present in all layers of the developing esophageal wall (Ibrahim et al., 2019). Furthermore, taking into consideration the other organs of the digestive system, TCs are commonly found in the lamina propria and muscular layer (Carmona et al.,

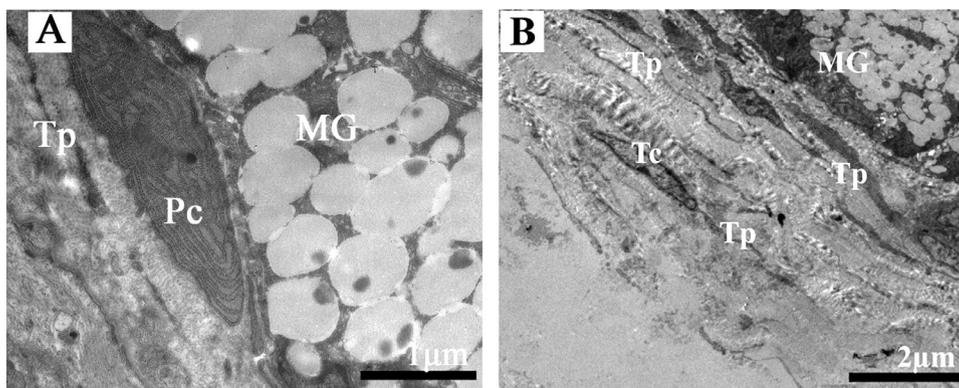


Figure 7. Transmission electron micrographs of the chicken esophagus. (A) Tps near to plasma cell. (B) Population of TCs near the mucosal glands. Tc, telocyte; Tp, telopode; MG, mucus gland; Pc, plasma cell. Scale bars: (A) 1 μm , (B) 2 μm .

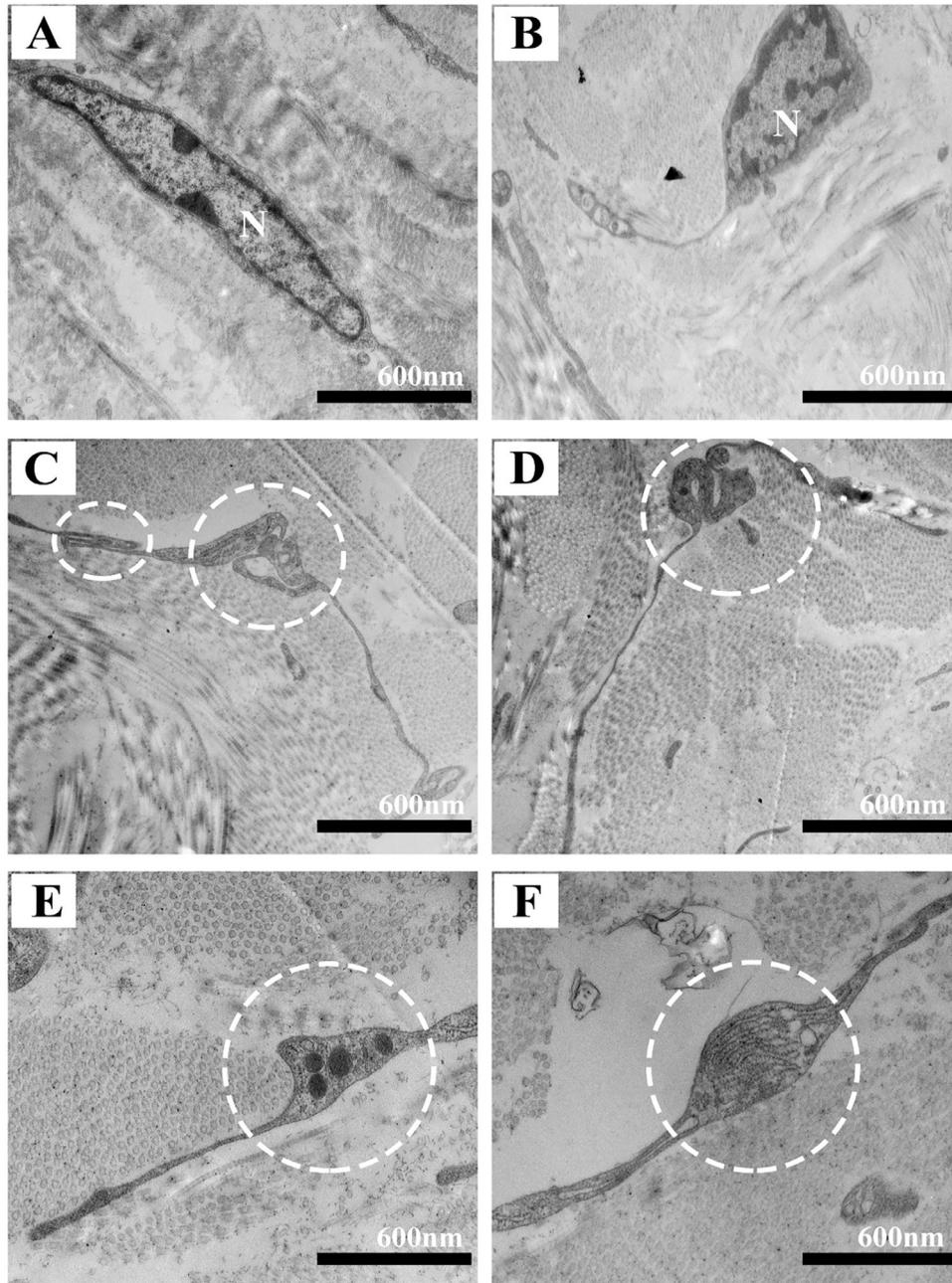


Figure 8. Ultrastructure of telocyte cell bodies and telopodes in the chicken esophagus. (A and B) Diverse morphologies of the TC cell bodies; (C and D) convoluted telopodes, marked by circles; (E and F) typical podoms featuring mitochondria and endoplasmic reticulum, marked by circles. N, nucleus. Scale bar: 600 nm.

2011; Cretoiu et al., 2012; Zhang et al., 2016; Liang et al., 2019; Vannucchi 2020). Therefore, based on these analyses, TCs are a type of interstitial cell that is retained in the esophageal wall of many animals and present study confirm their presence in esophagus of chicken. Morphologically, TCs vary in shape and size (Cretoiu et al., 2017). We also observed in this study that the cell bodies of the TCs had different shapes, and their Tps were long, convoluted, and moniliform. TCs cells have mesodermal origin. To date, no specific biomarker of TCs has been identified, but multiple antibodies, including anti-CD34, anti-CD117/c-kit, anti-PDGFR- α/β , and anti-VEGF. Both iNOS and vimentin can mark TCs in different organs of different species. Our experimental results showed that there was a large

number of CD34⁺, vimentin⁺, and PDGFR- α ⁺ cells in the esophageal wall of chickens. PDGFR- α was also co-expressed with CD34 in esophageal, stomach, and small/large intestine cells, suggesting that these cells constituted a mesenchymal cell population.

Possible Role of Telocytes in the Esophagus

The actual function of TCs is still largely unknown. While many different physiological or cellular activities that are influenced by TCs have been proposed, direct evidence of these is still lacking in many cases. At present, the known functions of TCs entail the maintenance of homeostasis in certain tissues, cellular communication,

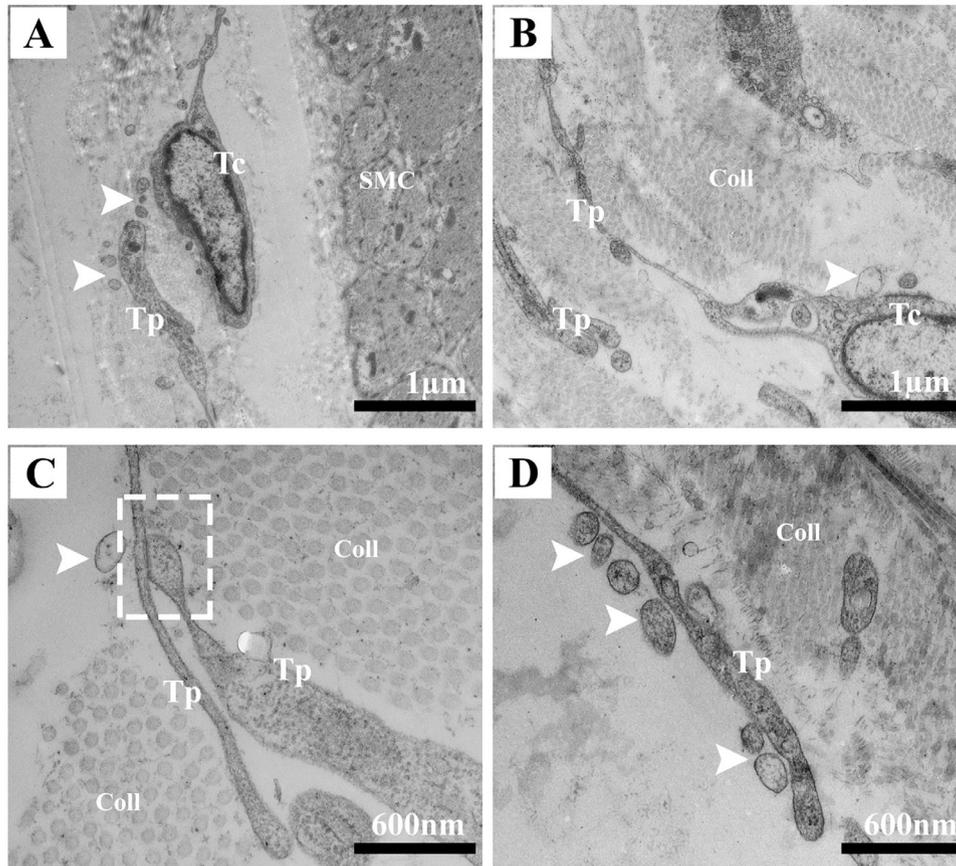


Figure 9. Extracellular vesicles associated with the telocytes. Tc, telocyte; Tp, telopode; SMC, smooth muscles cell; Coll, collagen fiber. The arrow is pointing to the extracellular vesicles in B and C and to the tortuous cross-section of Tp in A and D. Scale bars: A and B: 1 μm , C and D: 600 nm.

and the release of extracellular vesicles and tissue regeneration are the most known functions of TCs (Cretoiu et al., 2016; Kondo and Kaestner, 2019). In our study, we observed that there was a direct connection between the Tps of TCs and the release of extracellular vesicles and connection between Tps were observed clearly (Figure 5). Many other studies revealed that TCs enabled cellular commination via both direct and indirect contact (Faussonne-Pellegrini and Gherghiceanu, 2016), which was supported by our data. These studies also showed that TCs were in close contact with other resident cell types in the interstitium, such as immunocytes and myocytes, as well as structures, such as blood vessels, nerve vessels, and mucosal glands (Faussonne-Pellegrini and Gherghiceanu, 2016). The imaging data in our study also revealed that TCs and their Tps were in close contact with various different cells and glands in the chicken esophageal wall, which could enable the regulation of homeostasis in the tissue.

Epithelial tissue has ability to quickly and continuously renew itself. Due to frequent wear and shedding, they are replaced by poorly differentiated cells in the epithelium through mitosis to keep the number of epithelial cells constant and intact. One recent study explored the potential roles that TCs play in intestinal epithelial renewal (Shoshkes-Carmel et al., 2018), while other studies revealed that TCs were associated with tissue regeneration (Bei et al., 2015). Our data revealed a

population of TCs just beneath the epithelial lining (Figures 2–5), which indicated that it was possible that TCs played a role in esophageal epithelial renewal, but further investigation is warranted.

This study was the first to confirm the presence of TCs in an avian esophagus, which were identified by TEM and immunohistochemical methods in different regions of the chicken esophageal wall. We also revealed the ultrastructure of TCs and their close contacts with other cell types, such as immune cells. Our results suggested that TCs might play a role in epithelial regeneration and tissues homeostasis. This study will aid in elucidating the physiological roles of TCs in not only avian esophagi but also other tissues of various animals.

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The handling of chickens and study procedures were in accordance with guidelines of the Animal Research Institute Committee of Nanjing Agriculture University. All the protocols were approved by the Science and

Technology Agency of Jiangsu Province (Approval ID: SYXK (SU) 2020–0005).

DISCLOSURES

The authors declare no conflict of interest.

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