Connexin 43 is Localized in Gizzard Smooth Muscle Cells during Chicken Development

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Smooth muscle cells are widely distributed in the digestive organs of chickens. They exist as single cells, but adhere to each other to function synchronously. In this study, the expression of the gap junction protein connexin 43 (Cx43) in chicken gizzards was investigated at embryonic days (E) 10, E15, and E18. Gizzards have an abundance of smooth muscle cells because of their thick muscle layers, which enable easy analysis of the cells. Morphological observations and expression patterns of smooth muscle markers were confirmed. Next, we observed where the markers were localized in the gizzard tissue at E10, E15, and E18. Finally, the expression pattern of Cx43 in primary cultured smooth muscle cells from E15 gizzards was investigated. The analysis revealed the expression and localization of Cx43 and calponin 1 in the smooth muscle layers, and 3D analysis revealed dynamic changes in the localization pattern of Cx43 from E10 to E15. Cultured smooth muscle cells confirmed that Cx43 was expressed in the cell membrane and cytosol. In conclusion, Cx43 expression was identified in chicken gizzards at E10, E15, and E18, which was localized differently during development. The expression was broad at E10, and became restricted at E15 and E18. Primary culture of smooth muscle cells showed that Cx43 was present in the cell membrane and cytosol. This suggests that Cx43 is actively translated into the cytosol at E10, forming a hexamer, and shuttling the cell membrane to function as a gap junction.

Key words: chicken embryo, differentiation, gizzard, smooth muscle cells

Introduction

Cells are the smallest functional units in living organisms. They migrate and proliferate during embryonic development and adhere to each other to form thick tissues.

Smooth muscle cells (SMCs) are one of the three major muscle tissues in vertebrate bodies. Unlike skeletal and cardiac muscles, smooth muscle is not categorized as striated muscle; rather, it functions as a population of single cells.

Interestingly, although they do not appear striated, they can contract synchronously when stimulated.

When cells adhere to each other, they form junctions to communicate, anchor, and occlude themselves. Gap junctions are communication junctions in which small molecules, such as ions, are sent and received between anchored cells (Nielsen et al., 2012). In addition, gap junctions play a role in the contraction of SMCs (Nielsen et al., 2012). In non-pregnant and prepartum rats, the junctions are small and few, occupying <0.001% of the cell membrane surface. However, this increases to 0.2–0.4% during labor and disappears immediately after delivery (Sakai and Garfield, 2004). The increase in gap junctions suggests that smooth muscle tissue acquires a contractile capacity.

Connexin 43 (Cx43) is the major protein that constitutes the gap junction of vascular SMCs (Blackburn et al., 1997). Cx43 expression and cell migration are closely associated. Increased Cx43 expression in late-stage tumors promotes metastasis by allowing migration, infiltration, intravasation, and extravasation (Aasen et al., 2016). This characteristic is
expected to be applicable to the tracking of cancer cells via their junctions (Bonacquisti and Nguyen, 2019).

Cx43 expression during embryogenesis has also been investigated. These investigations have shown expression patterns in the mouse ciliary body, rabbit stomach, and human kidney at various embryonic stages (Iwai-Takekoshi et al., 2018; Mokhtar and Hussein, 2019; Ráduly et al., 2019; Kosovic et al., 2020).

These approaches have revealed Cx43 expression in various species and tissues, however, it remains unknown whether Cx43 is expressed during gizzard embryogenesis in chickens. There are SMC markers, such as α-smooth muscle actin (ACTA2) or Calponin1 (CNN1), which are used during differentiation and development (Gomez et al., 2015; Liu and Jin, 2016); however, there are no gap junction proteins showing the status of SMCs. Therefore, this study was performed to investigate whether Cx43 expression is distinct in SMCs. To verify this, protein expression levels and their localization were examined.

Materials and Methods

Chicken Embryos

Fertilized eggs from Boris Brown chickens (Itoshima Farmhouse UOVO, Itoshima, Japan) were incubated at 37.6°C in humidified incubators. The gizzards used for each experiment were removed from the embryos at embryonic days (E) 10, 15, and 18. The experiments were conducted in accordance with the “Guidelines for the Appropriate Conduct of Animal Experiments” of the Science Council of Japan, with ethical approval from the Institutional Review Board of Kyushu University (approval no. A20-168-1).

Cell Culture

The gizzards were removed from E15 chicken embryos and the smooth muscle layers were carefully removed. The smooth muscle parts were minced with scissors, 1 ng/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA) was added, and the mixture was shaken at 30°C for 30 min. Next, Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) containing 1X antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) and amphotericin B solution (HyClone Laboratories, Logan, UT, USA) was added and filtered through a cell strainer to remove myofiber fragments and tissue debris. After centrifugation at 800 rpm for 5 min, the supernatant was removed, and the precipitates were resuspended, washed, and centrifuged twice with media. The SMCs were seeded onto 35 mm cell culture dishes (Thermo Fisher Scientific) coated with laminin (Wako, Osaka, Japan), and incubated with media supplemented with 0.2% bovine serum albumin (BSA) and 5 ng/mL insulin-like growth factor 1 (Irvine Scientific, Osaka, Japan). For immunostaining, glass coverslips (Matsumami, Osaka, Japan) were placed in culture dishes and the cells were seeded onto them. All the cultures were grown at 37°C and 5% CO2.

Immunohistochemistry

At E10, E15, and E18, gizzard tissues were dissected and embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and liquid nitrogen was used to prepare frozen blocks for sectioning. A microtome was used to obtain 20 µm sections, which were fixed in 4% paraformaldehyde for 30 min and washed three times in phosphate-buffered saline (PBS). The sections were then treated with 0.2% (v/v) TritonX-100 (Sigma-Aldrich) for 20 min and washed three times with PBS. The sections were soaked in blocking solution (3% BSA in PBS) for 40 min. The primary antibodies were diluted in the blocking solution and incubated with the sections overnight at 4°C. After overnight incubation, the sections were washed three times with PBS before incubation with secondary antibodies diluted in blocking solution for 1 h. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1,000 dilution, Dojindo, Kumamoto, Japan) diluted in blocking solution for 15 min. The following primary antibodies were used: ACTA2 rabbit polyclonal antibody (Cat# ab5694, 1:500 dilution, Abcam, Cambridge, UK), CNN1 mouse monoclonal antibody (Cat# MAB1512, 1:200 dilution, Abnova, Taipei, Taiwan), and Cx43 rabbit polyclonal antibody (Cat# C6219, 1:2,000 dilution, Sigma-Aldrich). The following secondary antibodies were used: Alexa-Fluor-488-conjugated goat anti-rabbit IgG (#A-11008, 1:1,000 dilution, Invitrogen, Carlsbad, CA, USA) and Alexa-Fluor-647-conjugated goat anti-mouse IgG (#A-212235, 1:1,000 dilution, Invitrogen).

Cultured SMC Immunocytochemistry

Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. After fixation, the cells were washed three times with PBS and incubated with 0.2% (v/v) TritonX-100 for 20 min. Subsequently, a glass coverslip with cultured cells was placed on top of a 1.5 mm tube lid in a 6-well plate (Thermo Fisher Scientific), and the plate was blocked with 3% BSA in Tris-buffered organized saline (T-TBS; blocking solution) for 40 min. The primary antibodies were placed in a blocking solution and incubated overnight at 4°C. The cells were stained with DAPI diluted in blocking solution (1:1,000) for 5 min. The following primary antibodies were used: CNN1 monoclonal mouse antibody (Cat# MAB1512, 1:400 dilution, Abnova) and Cx43 rabbit polyclonal antibody (Cat# C6219, 1:400 dilution, Sigma-Aldrich). The following secondary antibodies were used: Alexa-Fluor-488-conjugated goat anti-rabbit IgG (#A-11008, 1:1,000 dilution, Invitrogen) and Alexa-Fluor-647-conjugated goat anti-mouse IgG (#A-212235, 1:1,000 dilution, Invitrogen).

Photograph and 3D Image Analysis

All immunofluorescence images were acquired using a Leica DMi6000 B fluorescence microscope and TCS SP8 confocal microscope. For the 3D images, 10 random points of the gizzard muscle layer were set up, and images were captured under the following imaging conditions: DAPI (PMT, laser 1%), ACTA2 (HyD, laser 25%), CNN1 (HyD, laser 4%), and Cx43 (HyD, laser 2%).

Protein expression levels in tissues were analyzed using Imaris ver9.6 (Oxford Instruments, Abingdon-on-Thames, UK). Data are presented as the volume of expressed Cx43 and CNN1 divided by the number of nuclei.

Western Blotting

Gizzard was subjected to protein extraction using TRizol
reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Whole cell extracts of cultured cells were collected in 1x sample buffer. Both the samples were incubated at 95°C for 5 min in a heating block. The samples were electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane using a TransBlot Turbo blotting system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% skim milk in T-TBS for 1 h. Primary antibodies were diluted with Can Get Signal Solution 1 (Toyobo, Osaka, Japan) and incubated with the membranes overnight at 4°C. The membranes were washed three times (10 min each) with T-TBS and further incubated with secondary antibodies diluted in Can Get Signal Solution 2 (Toyobo) at 25°C for 1 h. Next, the membranes were washed three times (10 min each) with T-TBS, and horseradish peroxidase (HRP) activity was measured using Amersham ECL Select (GE Healthcare, Chicago, IL, USA) and a Fusion chemiluminescence imaging system (Vilber-Lourmat, Lamirault, France). Bands were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The following primary antibodies were used: CNN1 monoclonal mouse (Cat# MAB1512, 1:100 dilution, Abnova), ACTA2 polyclonal rabbit (Cat#ab5694, 1:1,000 dilution, Abnova), desmin polyclonal rabbit (Cat#D8281, 1:10,000 dilution, Sigma-Aldrich), Cx43 rabbit polyclonal (Cat# C6219, 1:1,000 dilution, Sigma-Aldrich), and α-tubulin mouse monoclonal (Cat#T9026, 1:250 dilution, Sigma-Aldrich).

The following secondary antibodies were used: goat anti-mouse IgG/HRP (Cat# A4416, 1:5,000 dilution, Sigma-Aldrich) and swine anti-rabbit IgG/HRP (Cat# P0399, 1:5,000 dilution, Agilent, Santa Clara, CA, USA).

Statistical Analysis

A one-way analysis of variance test with post-hoc Tukey-Kramer test was performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). The data are presented as the mean±standard error of the mean of 10 random spots from the stained gizzard section at E10, 15 and 18. Two independent experiments were performed to confirm the data. Statistical significance was set at *P*<0.05 and **P**<0.01, indicated by * and **, respectively.

Results

Expression of the Smooth Muscle Markers in Gizzards is Upregulated from E10 to E18

To examine the morphological distribution of muscle layers during chicken development, gizzards at E10, E15, and E18 were stained with hematoxylin and eosin (H&E; Fig. 1A). The proventriculus (PV) is displayed on the left-hand side of the gizzard. Smooth muscle layers (sm) were detected on both sides of the inner epithelial layer (arrow). Smooth muscle marker expression was also examined. Three gizzard samples were collected at E10, E15, and E18, and western blotting was performed. Three markers, CNN1, ACTA2, and desmin, were examined (Fig. 1B). These genes were continuously expressed from E10 to E18. ACTA2 and desmin are early markers of SMCs, and they are also expressed in matured SMCs. CNN1 is a late marker. This expression suggests that there were already mature SMCs at E10, and the
expression was activated towards E15. The expression of these markers was sustained until E18.

**ACTA2 and CNN1 are Expressed in Smooth Muscle Layers of the Developing Gizzard and PV**

To confirm the localization of smooth muscle markers in the developing gizzard, immunostaining with ACTA2 and CNN1 antibodies was performed. DAPI was used to trace the location of the nuclei (Fig. 2C, G, and K). ACTA2 and CNN1 generated signals at E10, E15, and E18. The ACTA2 signal was broadly detected in the gizzard muscle layers and PV throughout development from E10 to E18 (Fig. 2A, E, and I). CNN1 expression was also detected at all three stages (Fig. 2B, F, and J). ACTA2 and CNN1 expression overlapped with that of smooth muscle layers in the gizzard (Fig. 2D, H, and L). The signal was stronger at the edge of the staining toward the glandular epithelium, and there was a thin layer, which was both ACTA2 and CNN1 negative at E10 (Fig. 2D, inside the dashed line).

Furthermore, CNN1 expression was relatively weak at the boundary between the smooth muscle layer and the glandular epithelium. However, both ACTA2 and CNN1 positive cells expanded and attached to the inner glandular epithelium at E15 and E18 (Fig. 2H and L, arrow). The area positive for DAPI but negative for ACTA2 and CNN1 was no longer observed in the gizzards at E15 and E18 (Fig. 2H and L).

**Cx43 is Expressed in the Smooth Muscle Layers of the Developing Gizzard**

SMCs were not yet fully distributed in the gizzard at E10, however, they became ACTA2 and CNN1 positive at E15 and E18. There was a gap between the glandular epithelium and boundary of the ACTA2 positive area (Fig. 2A and D). Next, it was investigated whether cell-to-cell contact was important for the maturation of SMCs, and Cx43 expression and localization was examined (Fig. 3). Western blot analysis revealed that Cx43 was expressed in developing gizzards, and its expression increased with embryonic development (Fig. 3A). To examine its distribution in the gizzard, immunohistochemistry was performed at E10, E15, and E18 (Fig. 3B–M). Cx43 expression was detected in the SMC layers (Fig. 3B and E). The signal was undetected in the gap between the edge of the SMC and glandular epithelial regions at E10 (Fig. 3E, dashed line). However, Cx43 positive cells were expanded and attached to the inner glandular epithelium at E15 and E18 (Fig. 3I and M). The glandular epithelium is shown in Fig. 3I and M (arrow).

**3D Analysis Captured Cx43 Localization in the SMC Layers**

Cx43 is a gap junction protein and should be located in the cell membrane, therefore, a 3D analysis of the muscle layer was performed. Ten points on the stained SMC layers at E10, E15, and E18 (Fig. 3E, I, and M) were selected, and 3D analysis was performed using a higher magnification. Scattered Cx43 signals were broadly detected at E10 (Fig. 4A and G). These areas of expression overlapped with those of CNN1 (Fig. 4B and H). Surprisingly, Cx43 expression was restricted to E15 and E18 (Fig. 4C, E, I, and K). In contrast, the areas of expression remained broad for CNN1 (Fig. 4D, F, J, and L). Graphs quantifying the relative expression showing the volumes of expressed Cx43 (Fig. 4M) and CNN1 (Fig. 4N) were produced. The data suggest that Cx43 has the highest expression at E10, and its expression is lowered at E15 and E18. It was well distributed over the SMC layers at E10, and the proteins were shuttled to the restricted area to function at E15 and E18.

**Cultured SMCs Express Cx43 in the Cell Membrane**

Cx43 was localized in the chicken gizzard in the 3D analysis (Fig. 3M). However, Cx43 was not located in the cell. Therefore, the localization of Cx43 in culture plates was examined. The cells were cultured for 3 days (d3) and analyzed by immunostaining and western blotting. Western blotting confirmed the expression of Cx43, CNN1, and the other SMC markers, ACTA2 and desmin (Fig. 5). The expression of these markers was upregulated upon culturing. The fluorescence signals of Cx43 and CNN1 at d3 were first determined by immunostaining using antibodies against Cx43 and CNN1 (Fig. 6A–D). Cx43 signals were detected as dots and CNN1 signals were broadly expressed in the cytosol (Fig. 6A–D). These signals were observed along the cell surface and in addition to the nucleus, and serial dot staining was observed between the two cell types (Fig. 6A, arrows). Next, 3D analysis was performed using the same samples (Fig. 6E–L). The 3D images allowed for observation of the cells from different viewpoints. This confirmed that the dots were aligned at the boundary between cells (Fig. 6E and I, arrows).

**Discussion**

**Gizzard SMCs are Proliferated and Differentiated from E10 to E18**

Our data confirmed that differentiated SMCs proliferated and formed smooth muscle layers in the gizzard between E10 and E18. The smooth muscle layers were thickened (Fig. 1A) and ACTA2 and CNN1 were expressed (Fig. 2). This expression has previously been confirmed at the earlier developmental stages, from E4.5 to E7; (McKey et al., 2016) however, our results showed expression and cell proliferation at later stages. The expression of these markers occurs during development. It is reasonable to assume that thickening of the muscle layers requires expression of these markers. ACTA2 and desmin are expressed during the early stages of SMC differentiation (Gomez et al., 2015). CNN1 is a late-phase marker (Liu and Jin, 2016).

Interestingly, SMCs did not reach the glandular epithelium at E10, as no SMCs were observed at the boundary (Fig. 2D). However, the area was DAPI-positive. This means that there are cells, but not SMCs. This may be because immature SMCs are present, and they mature by E15. A previous report examined its morphology of the gizzards (Kofuji and Inoue, 2002); however, no report has identified the characteristics of these cells, and further analysis is required.

**Cx43 Expression is Detected in Developing Gizzard SMCs**

Cx43 is widely expressed in vertebrates (White and Paul, 1999). However, its expression in the digestive organs of developing chicken has not yet been reported. Our examination confirmed that Cx43 colocalized with CNN1 in the gizzard. 3D analysis revealed that it was exclusively ex-
Fig. 2. Fluorescent immunostained images of serial sections from gizzards at E10 (A–D), E15 (E–H), and E18 (I–L). Gizzards were co-stained with anti-ACTA2 and anti-CNN1 antibodies. Nuclei were visualized by DAPI. Green: ACTA2; red: CNN1; blue: DAPI. Both sides of the inner epithelial layers are shown (arrow).
Fig. 3. (A) Proteins were extracted from gizzards at E10, E15, and E18, and Cx43 was analyzed by western blotting. The total protein content was confirmed by \( \alpha \)-tubulin. (B–M) Fluorescent immunostained images of serial sections from gizzards at E10 (B–E), E15 (F–I), and E18 (J). Gizzards were co-stained with anti-Cx43 and anti-CNN1 antibodies. Nuclei were visualized by DAPI. Green: Cx43; red: CNN1; blue: DAPI. White arrow: glandular epithelial boundaries; white arrowhead: glandular epithelial region. Both sides of the inner epithelial layers are shown (arrow).
Fig. 4. Fluorescent immunostained images of serial sections from gizzards at E10 (A, B, G and H), E15 (C, D, I and J), and E18 (E, F, K and L). Gizzards were co-stained with anti-Cx43 and anti-CNN1 antibodies, and nuclei were visualized by DAPI. The images were taken stereoscopically with a confocal microscope. Graphs quantifying the relative expression levels of the number of expressed Cx43 (M) or the volume of expressed CNN1 (N), standardized by the number of nuclei (DAPI).

Scale bars: (A–F): 10 µm; (G–L): 20 µm.
Fig. 5. The proteins were then extracted and analyzed by western blotting for the SMC differentiation markers CNN1 and ACTA2, the muscle differentiation marker desmin, and the cell adhesion factor Cx43. The total protein content was confirmed by α-tubulin.

Fig. 6. Fluorescent immunostained images of cultured SMCs co-stained with anti-Cx43 and anti-CNN1 antibodies and nuclei were visualized by DAPI. (A–D). The 3D images of the cells were derived using a confocal microscope (E–L). The representative images are from two different viewpoints. Scale Bars: (A–D): 100 µm; (E–L): 15 µm. Cx43 expression of the two adhered SMCs. Th arrows show the dots aligned at the boundary between cells (A, E and I). (M) Proteins were isolated from SMCs contained in gizzards at E15 and cultured for 1 or 3 days (d1 and d3, respectively).
pressed in the smooth muscle layer at E10 (Fig. 4). Surprisingly, the expression pattern was altered from broad to highly restricted areas by E15 (Fig. 4). It can be considered that the expression decreased, however, western blot analysis showed that upregulated expression occurred as development progressed (Fig. 3A). Cx43 is expressed in skeletal muscle (Araya et al., 2005), and Cx43 localization in C2C12 myoblasts is altered during differentiation (Mcclure et al., 2019). The expression was broadly scattered when the cells were single cells, however, the localization was restricted after differentiation. This may happen to SMCs in the gizzard during development. The cultured SMCs confirmed that Cx43 was localized at the boundary between the two adhered SMCs (Fig. 6).

**The Role of Cx43 in SMCs**

Our study revealed that Cx43 is actively expressed during development, however, our analysis did not conclude that it changes significantly from E10 to E18. However, protein localization was restricted from E10 to E15 and E18. The reason for this phenomenon is yet to be determined. Previous reports have suggested that the phosphorylation of Cx43 can occur and alter its function (Yeganeh et al., 2012; Solan and Lampe, 2014, 2020; Leithe et al., 2018). Double bands were detected during western blotting, which may have led to localization changes and lowered the affinity of the Cx43 antibody in the embryonic gizzard. Although further analysis using the phosphorylated Cx43 antibody is essential, phosphorylation may affect the efficiency of staining because the unphosphorylated band is weaker at E10 and clear double bands are detected at E15 and E18. Cx43 within cell cultures produces a bright signal on cell membranes and in the cytosol. Therefore, isolating SMCs may have eased the reaction between cells and antibodies. Further analyses are required to investigate Cx43 function in gizzards. Cx43 forms a hexamer called connexon in the cytosol and is transported to the cell membrane. Concurrently, Cx43 in the cytosol is processed for lysosomal degradation (Berthoud et al., 2004). It has been revealed that the half-life of Cx43 is only one to five hours (Berthoud et al., 2004). These processes may be involved in the dynamic changes in localization during development. It is possible that Cx43 is actively translated at E10 and, simultaneously, may be actively sent for degradation. The antibody can detect Cx43 monomers and does not reflect the situation in the cytosol.

In conclusion, we found Cx43 expression in chicken gizzards at E10, E15, and E18, which is localized differently during development. The signals produced double bands in western blotting, suggesting that Cx43 can be phosphorylated in SMCs. This regulation may be involved in changing localization. ACTA2 and CNN1 are expressed in the early and late stages of SMC differentiation, respectively (McKey et al., 2016). Western blot analysis did not confirm the detailed localization of the SMC markers, therefore, immunohistochemistry was performed to determine where the markers were localized in the gizzard tissue. Based on the data presented, we confirmed the expression patterns and changes in the localization of Cx43 and CNN1 in detail using 3D analysis. Cultured SMCs predominantly expressed Cx43 on the cell membranes, however, it was also detected in the cytosol.

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**Author Contributions**

Kosuke Tokunaga designed the study, performed cell culture experiments, and drafted the manuscript. Shota Akimoto performed H&E staining, immunohistochemistry of the gizzard embryos, 3D analysis, and formatted the figures and legends. Machiko Aiba performed western blotting, smooth muscle cell culture experiments, and formatted the figures and legends. Mutsuki Nakagomi performed smooth muscle cell culture experiments. Takahiro Suzuki and Ryuichi Tatsumi designed the study. Mako Nakamura conceived and designed the study, and finalized the manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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