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EXPRESSION OF ANOCTAMIN-1 IN HUMAN GASTROINTESTINAL TRACT DURING EMBRYONIC AND FETAL DEVELOPMENT

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Anoctamin-1 (ANO1, TMEM16A) is a transmembrane protein belonging to the ANO family that plays a role in the formation of calcium-activated chloride channels (CaCCs). It is involved in the regulation of physiological processes, including muscle contraction, gastrointestinal motility, secretion, and electrical excitability. Recent data also suggest that ANO1 is a specific marker for interstitial cells of Cajal (ICC). The aim of the paper was to examine the spatial and temporal distribution of ANO1 in the human stomach, small intestine, and large intestine during embryo-fetal development as a potential marker for the differentiation of ICC and smooth muscle cells (SMCs). As study material, we used samples from 2 embryos and 21 fetuses. The tissue samples were routinely processed into paraffin blocks, and 5 µm-thick sections were immunostained for ANO1. Our results showed that ANO1 expression appeared during the 8th week of embryonic development and persisted through the fetal stages. Epithelial, endothelial, and ICC cells consistently expressed ANO1 in all examined samples. SMCs showed strong ANO1 expression in the muscularis propria; however, by the 25th week, this immunopositivity was absent from the outer muscle layers in the stomach and large intestine. In conclusion, ANO1 can be considered a reliable marker for tracking the differentiation of SMCs and ICC during embryonic and fetal development.

Keywords: ANO1, TMEM16A, gastrointestinal system, development, interstitial cells of Cajal

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INTRODUCTION

Anoctamin 1 (ANO1, TMEM16A) is a plasmalemmal protein with eight transmembrane domains that belongs to the ANO family (ANO1-10). ANO1 is involved in the formation of calcium-activated chloride channels (CaCCs), which facilitate passive transport of chloride ions into the cytoplasm. These channels regulate physiological processes, such as muscle contraction, gastrointestinal motility, exocrine and endocrine secretion, and electrical excitability (1,2). ANO1 is expressed in epithelial cells in many organs, including the salivary glands, lacrimal glands, exocrine pancreas, bronchial tree, intestines, choroid plexus, and retina. It is also present in some types of smooth muscle cells (SMCs), interstitial cells of Cajal (ICC), vascular endothelial cells, and myocardium (3,4). It has been reported that abnormal ANO1 expression may be linked to the pathogenesis of diseases such as cystic fibrosis, hypertension, and gastrointestinal motility disorders (3,5). Additionally, ANO1 overexpression has been observed in many cancers, promoting tumorigenesis by influencing cancer cell proliferation, survival, and migration (6-9).

ANO1 has recently gained attention as a marker of ICC (10,11). These cells, originating from c-kit-positive mesenchymal precursors in the primitive gut, are observed at the end of embryonic development (12,13). They first appear in the esophagus and stomach, and then, following the rostrocaudal pattern of development, in the small and large intestines. Their development is closely associated with the colonization of the digestive tube by neural crest cells, which will eventually give rise to the neurons and glial cells of the myenteric and submucosal plexuses (14). ICC can first be identified by c-kit immunopositivity at the end of embryonic development, and by the 11th week, they surround the ganglia of the myenteric plexus (14-16).

The ICC are regarded as a crucial component of the enteric nervous system, providing the physiological basis for peristaltic movements (17,18). In response to cholinergic stimulation, ICC generate slow, non-oscillatory intestinal contractions that depolarize the ICC-smooth muscle cell network and convert the excitatory message from motoneurons into muscle contractions (1, 19). Spontaneous pacemaker activity generated by ICC and conducted to SMCs enables slow electrical waves and phasic contraction. In addition, ICC serve as stretch receptors and participate in the reflex peristalsis pathway due to the stretching of the digestive tube by food content (20). Available data suggest that ANO1 is expressed in all ICC classes, even those that do not contribute to slow-wave generation, implying that ANO1 may have an alternate function in these cells (21). The lack of differentiation and absence of ICC lie in the pathogenesis of many motility disorders in the gastrointestinal tract. Furthermore, mice lacking ANO1 were reported to have fewer proliferating ICC in culture, suggesting that ANO1 may also be involved in ICC proliferation (11,22). Lower or absent ANO1 expression has been reported in patients with gastrointestinal disorders, including diabetic gastroparesis, suggesting that this protein may play an important role in the pathogenesis of these conditions (23,24).

Current literature provides limited data regarding ANO1 expression patterns in the developing human gut tube. Therefore, this study aimed to examine the spatial and temporal distribution of ANO1 in the stomach and small and large intestines during embryo-fetal development. We evaluated its potential as a marker for distinguishing ICC from SMCs during their differentiation from common precursors.

METHODS

The study material comprised 2 human embryos and 21 human fetuses with gestational ages ranging from 8 to 25 weeks. Both embryos were at the 8th week of gestational age, while fetal samples included one each from the 10th and 14th gestational weeks; two from the 11th week; three each from the 15th, 17th, and 19th weeks; and four samples each from the 22nd and 25th gestational weeks. All specimens were obtained from the Center for Pathology and Pathological Anatomy, University Clinical Center Niš, Serbia, following legal abortions and premature births due to intrauterine fetal deaths. All procedures were conducted in accordance with ethical principles and were approved by the Ethics Committee of the University Clinical Center Niš (number 34794/3, date 1.10.2019).

Both sexes were represented in the sample, and no specimens had gastrointestinal disorders. Gestational ages were estimated by anatomical criteria according to the Carnegie Staging system, as well as by crown-rump length, head circumference, and foot length.

Each specimen was fixed in 10% neutral formalin for 24h and routinely processed into paraffin blocks. Tissue sections of 4 µm were cut using a microtome, mounted on slides, and subjected to both hematoxylin and eosin, as well as immunohistochemical staining. Hematoxylin and eosin staining was used to confirm the normal morphology

of all samples, consistent with their gestational age.

Immunohistochemistry

After deparaffinization in a thermostat and xylene, the tissue slides were rehydrated through decreasing concentrations of ethanol (100% and 96%) and distilled water. Following 30-minute heat-induced antigen retrieval, the tissue peroxidases were blocked with a 3% hydrogen peroxide solution for 10 minutes. The antibodies were applied overnight at 4°C. Staining continued the following day by using a secondary antibody conjugated with horseradish peroxidase for 30 minutes (Real EnVision System for visualization, Dako, catalogue number: K5007). Between the steps, the tissue slides were rinsed in EnVision FLEX wash buffer (pH = 7.4). Diaminobenzidine (DAB) served as the chromogen. Finally, the slides were dehydrated through a series of increasing ethanol concentrations (96%, 100%), cleared in xylene, and mounted with Canada balsam and coverslips.

The following antibodies were used for immunohistochemical staining: rabbit polyclonal antibody against ANO1 (Abcam, ab53212, 1:50), mouse monoclonal antibody against NSE (Dako, M0873, 1:100), and rabbit monoclonal antibody against desmin (Abcam, ab32362, 1:300).

Descriptive analysis

Three sections from each sample were analyzed using an Olympus BX50 light microscope (Olympus, Japan) equipped with a Leica DFC295 digital camera (Leica Microsystems, Germany) at the Department of Histology and Embryology, University of Niš Faculty of Medicine, Niš, Serbia.

RESULTS

Embryonic development

Our results showed that ANO1 was expressed in the stomach and small and large intestines at the 8th week of embryonic development (Figure 1A).

The muscularis propria of the stomach included both a broader inner circular layer and a thinner outer longitudinal muscle layer, which showed strong positivity for desmin. The outer longitudinal layer, consisting of 1–3 rows of cells, surrounded the inner layer along the entire circumference (Figure 2F). Developing myenteric plexuses, containing neuron-specific enolase (NSE)-positive cells, were identified between the two muscle layers (Figure 2D). ANO1-immunopositive cells were observed in both the inner and outer layers of the muscularis propria, while the large oval ganglionic cells in the myenteric plexuses were ANO1-immunonegative (Figure 1B). In the small and large intestines, only the inner muscle layer was present, containing ANO1-positive cells, while myenteric plexuses were absent (Figures 1C, 1D, 1F). ANO1-positive cells in the developing muscularis propria across all examined parts of the digestive tube formed a continuous layer and exhibited a pleomorphic appearance with euchromatic nuclei. Some cells were round and lacked cytoplasmic processes, while others adopted a spindle-shaped phenotype.

ANO1 positivity was also observed in endothelial cells of developing blood vessels in the submucosa, as well as in epithelial cells of the pseudostratified epithelium in the stomach and small and large intestines.

The stomach during fetal development

In the 10th and 11th weeks of development, the wall of the stomach consisted of mucosa, submucosa, muscularis propria, and serosa. The epithelium was pseudostratified; however, it was lower than in the embryonic period and showed signs of differentiation. The epithelial cells displayed ANO1 positivity, and gastric glands were not yet formed (Figures 2A, 2B). Two muscle layers were discernible in the muscularis mucosa, both exhibiting strong desmin and ANO1 immunopositivity (Figure 2B). Elongated ANO1-positive cells surrounded myenteric plexuses, with cells showing strong NSE-positivity; however, they did not show ANO1 expression (Figures 2B, 2E). ANO1-immunopositive endothelial cells were also observed within the blood vessels.

Between the 14th and 20th weeks, all layers of the stomach were fully developed. The epithelium was simple columnar, and the gastric glands began to form. The epithelial ANO1 immunopositivity was evident, and the pattern of ANO1 expression resembled that seen at the start of the fetal developmental period.

In the 25th week of development, epithelial ANO1 immunopositivity was weak or absent from cells in the surface epithelia and gastric glands. The circular muscle layer exhibited strong ANO1 immunopositivity, whereas in the longitudinal layer, ANO1-immunopositive cells were rare. Highly flattened, elongated cells were observed in the longitudinal muscle layer and around the myenteric plexuses, consistently lacking ANO1 immunopositivity. The ANO1-positive cells formed a continuous single-cell layer around the margins of the myenteric plexuses. Endothelial positivity was evident in small blood vessels (Figure 2C).

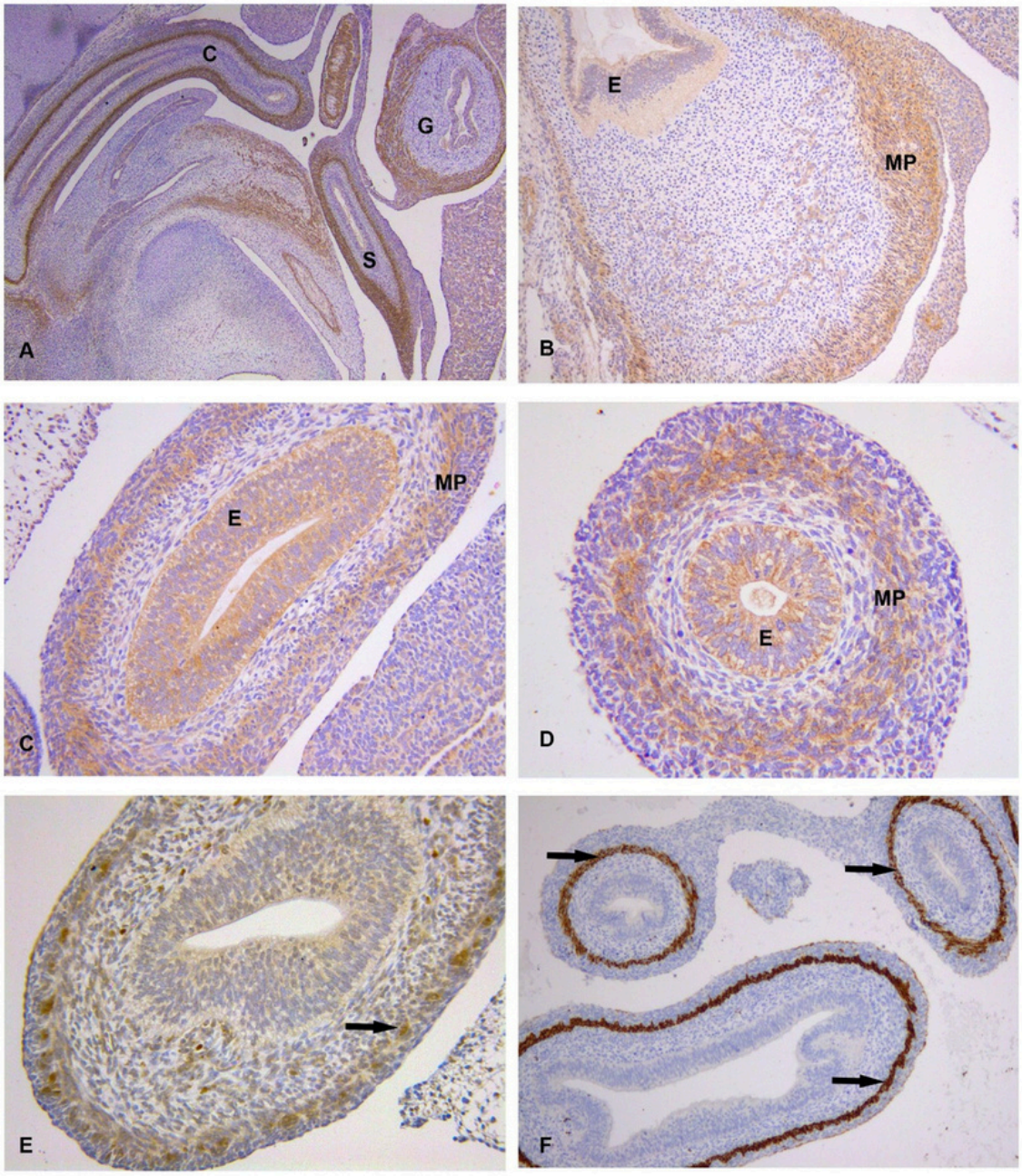

Figure 1. *A) Panoramic view of ANO1 immunopositivity in small and large intestines in late embryonic period at 8th week, x40, G – stomach, S – small intestine, C – large intestine; B) ANO1 immunopositivity in epithelial cells and muscularis propria in stomach at 8th week of development, E – epithelium, MP – muscularis propria, x100; C) ANO1 immunopositivity in epithelial cells and cells of inner layer of muscularis propria in small intestine at 8th week of development, E – epithelium, MP – muscularis propria, x200; D) ANO1 immunopositivity in epithelial cells and cells of the inner layer of muscularis propria in the large intestine at 8th week, E – epithelium, MP – muscularis propria, x200; E) NSE-immunopositivity in ganglionic cells of myenteric plexus in small intestine (black arrow) at 10th week, x200; F) Desmin immunopositivity in SMCs in small and large intestine (black arrows) at 8th week, x125.*

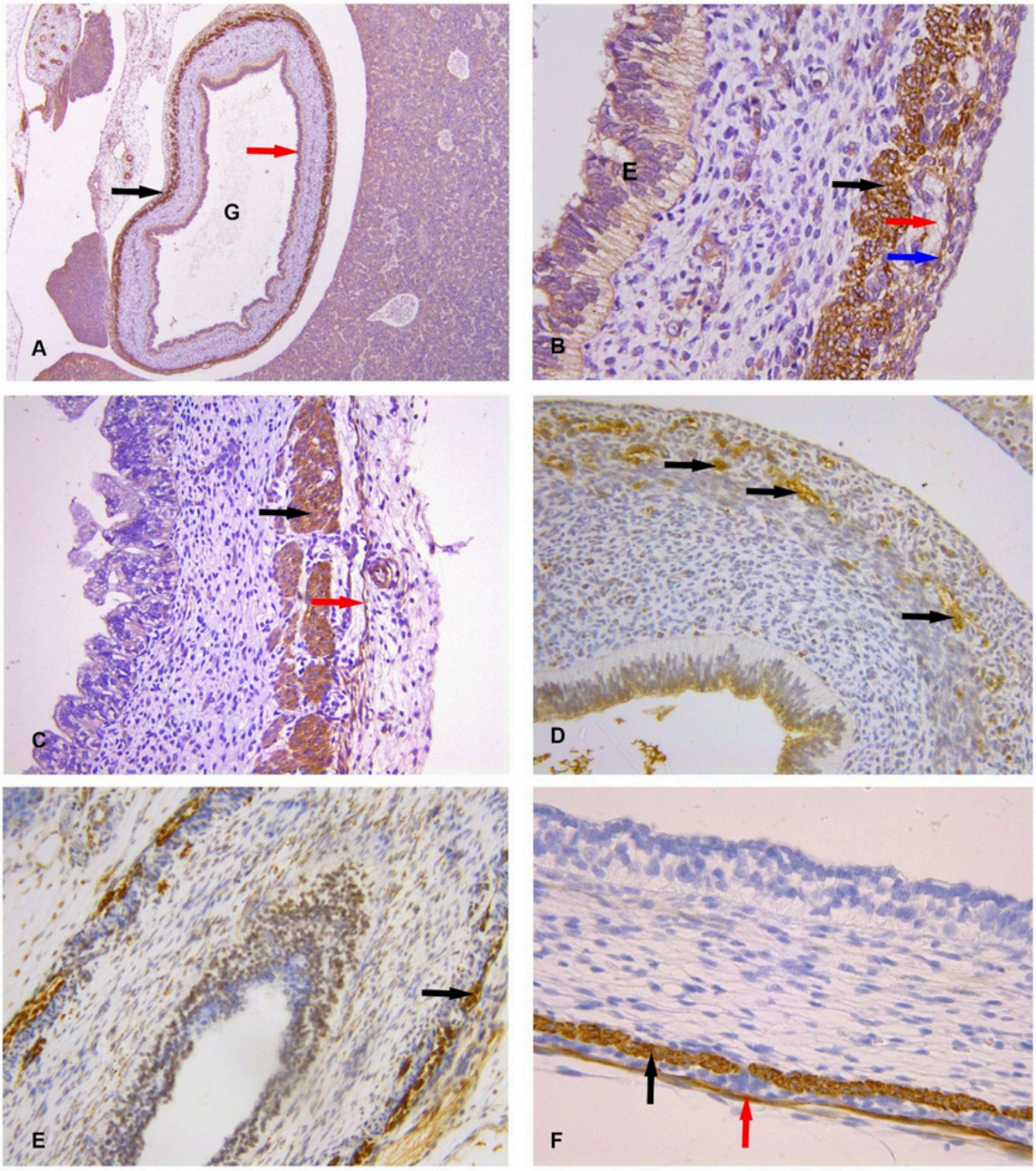

Figure 2. *A) ANO1 immunopositivity in cells of muscularis propria (black arrow) and epithelial cells (red arrow) in the stomach at 10th week of development, x40; B) ANO1 immunopositivity in the stomach in the 10th week of development. ANO1 immunopositivity is observed in epithelial cells (E), endothelial cells of submucosal blood vessels, smooth muscle cells in muscularis propria (black arrow), and elongated cells surrounding the myenteric plexus (blue arrow) (corresponding to ICC). The cells of the myenteric plexus (red arrow) do not show ANO1 immunopositivity, x400; C) ANO1 immunopositivity in the stomach at 22nd week of development. Epithelial cells show low or absent ANO1 expression. Smooth muscle cells of inner muscle layer (black arrow) and elongated cells around the myenteric plexus (corresponding to ICC) (red arrow) show strong ANO1 positivity, which is absent from smooth muscle cells in outer muscle layer, x250; D) NSE immunopositivity in ganglionic cells of myenteric plexus (black arrows) in stomach at 8th week of development, x300; E) NSE immunopositivity in ganglionic cells of myenteric plexus (black arrow) in stomach at 11th week of development, x200; F) Desmin immunopositivity in smooth muscle cells in inner (black arrow) and outer layers (red arrow) of muscularis propria in stomach at 8th week of development, x500.*

Small and large intestine

In the 10th and 11th weeks of development, the primordia of short intestinal villi began to appear in the small intestine, where intestinal glands were also visible. In contrast, they remained absent in the colon (Figure 3A). The thin outer longitudinal muscle layer showed strong desmin positivity observable in both the small intestine and the proximal colon (Figure 3B). Where both muscle layers were present, clearly distinct NSE-immunopositive myenteric plexuses were observed between them (Figure 1E). The epithelium in both the small and large intestines was pseudostratified, though its height was reduced compared to the 8th week, and showed signs of differentiation. Plasmalemmal ANO1 immunopositivity was present across all epithelial cells. ANO1-positive cells were visible within both the circular and longitudinal layers of the muscularis propria. These cells appeared as thin, flattened, elongated structures with bipolar morphology. Flattened, strongly ANO1-positive cells completely enclosed the ANO1-negative myenteric plexuses, forming a continuous belt.

Between the 12th and 20th weeks of development, the intestinal villi were fully formed in the small intestine, and intestinal glands were present in both the small and large intestines. The epithelium consisted of simple columnar cells, which exhibited ANO1 positivity in the apical region and, to a lesser extent, in the lateral areas. In the small intestine, ANO1-immunopositive cells were found within both muscle layers, while in the large intestine, ANO1 immunopositivity was low or absent in the outer muscle layer. Elongated ANO1-positive cells were seen around the myenteric plexuses, whose ganglionic cells lacked ANO1 expression. ANO1-positive cells surrounding the myenteric plexuses were flat and elongated, and formed a complete belt encircling them. Endothelial cells also showed ANO1 immunopositivity in blood vessels (Fig 3C, 3D).

By the 25th week of development, the small and large intestines were fully developed. The muscularis propria comprised a broader circular layer and a distinct, separate outer muscle layer, both of which stained strongly for desmin. The pattern of ANO1 immunopositivity in the muscularis propria resembled that seen at the 20th week of development. In both the small and large intestines, the lamina muscularis mucosae was clearly visible and contained small, stellate, interconnected ANO1-immunopositive cells. These cells were also observed in the intestinal villi, in continuity with the lamina muscularis mucosae, corresponding to the cells of the Brücke muscle (Figure 3E).

DISCUSSION

Our results showed that the stomach and small and large intestines were discernible in histological slides at the end of embryonic development. Their mucosa was lined with pseudostratified epithelium, and the inner circular layer was present in the small and large intestines. In contrast, the thin outer longitudinal muscle layer was observed only in the stomach, where the myenteric plexuses were seen between the two muscle layers. By the 14th week, all histological features of the gastrointestinal tract were developed, including simple columnar epithelium, gastric and intestinal glands, and a well-defined two-layered muscularis propria (15,16,25,26).

ANO1 expression in the stomach and small and large intestines was evident at the end of the embryonic period in epithelial, endothelial, and smooth muscle cells in all examined parts of the gastrointestinal tract. In addition, elongated ANO1-positive cells, corresponding to ICC, were observed surrounding the myenteric plexuses in the stomach. With maturation and development of the outer muscle layer and myenteric plexus in the small and large intestines, ANO1 showed the same pattern of expression in its ICC. Ganglionic cells of the myenteric plexus consistently showed a lack of ANO1 expression in both embryonic and later fetal development. Although there are limited data related to ANO1 expression during the development of the gastrointestinal system, the studies report that c-kit, as a widely accepted marker of ICC, is expressed in the muscle precursors and ICC in the stomach and the proximal part of the duodenum at the end of embryonic development, suggesting that the differentiation of ICC and SMCc begins early during development (26-28). This shared positivity likely results from their common mesenchymal origin. Studies have shown that these two cell types arise from a common c-kit-positive mesenchymal precursor, with c-kit playing a crucial role in directing their differentiation toward SMCs or ICC phenotypes (12,14,29-31). Unlike c-kit-immunopositive cells, which are initially present only in the oesophagus, stomach, and proximal part of the duodenum and appear in other regions only during the 9th and 10th weeks of development, ANO1 positivity was present throughout the gastrointestinal system by the end of embryonic development. (16,27,28,32,33). We therefore suggest that ANO1 may be a potentially earlier or more specific marker for labeling common mesenchymal progenitors compared to c-kit. We observed ANO1 positivity in SMCs and ICC in tissue samples from the 25th week of development,

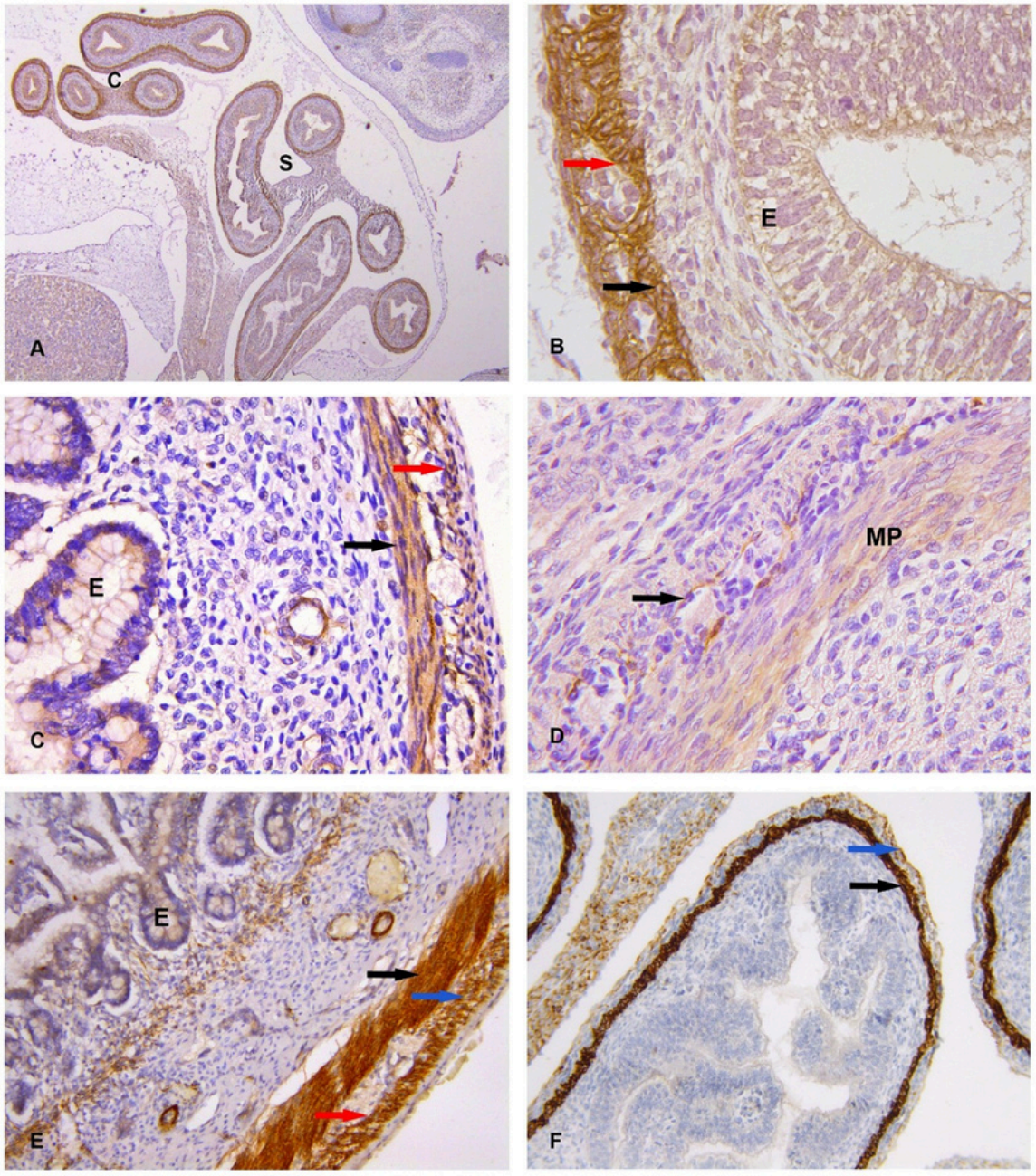

Figure 3. *A) Panoramic view of ANO1 immunopositivity in small and large intestine at 10th week of development, x40, S – small intestine, C – large intestine; B) ANO1 immunopositivity in epithelial cells (E), smooth muscle cells of muscularis propria (black arrow) and cells corresponding to ICC (red arrow) in large intestine at 14th week, x640; C) ANO1 immunopositivity in epithelial cells (E), smooth muscle cells of muscularis propria (black arrow) and cells corresponding to ICC (red arrow) in large intestine at 17th week, x400; D) ANO1 immunopositivity in smooth muscle cells of inner muscle layer (MP) and elongated cells around the myenteric plexus (corresponding to ICC) (black arrow) in large intestine at 19th week, x400; E) ANO1 immunopositivity in epithelial cells (E), smooth muscle cells of muscularis propria (inner layer – black arrow, outer layer – blue arrow) and lamina muscularis musosae, and in cells corresponding to ICC in small intestine (red arrow) in 25th week, x320; F) Desmin immunopositivity in smooth muscle cells of inner (black arrow) and thin, outer muscle layer (blue arrow) in small intestine at 10th week, x200.*

suggesting that these cells maintain ANO1 positivity even beyond the second trimester. In contrast, c-kit expression in SMCs is lost during the early stages of embryonic development. Interestingly, studies on adult human tissue show that ANO1 expression in the gastrointestinal tract is strictly limited to ICC and absent from SMCs (10,11,34). This shift in ANO1 positivity might result from the functional maturation of SMCs and the formation of other types of chloride channels in their plasma membrane (35). Available data also suggest that the deletion or pharmacological inhibition of ANO1 leads to disorders of gastrointestinal motility, resulting from disorganized and reduced contractility of smooth muscle cells (36-39). Currently, experimental efforts are underway to establish stem cell-based therapies for gastrointestinal disorders; however, most studies focus on differentiating ganglionic enteric cells from embryonic or induced pluripotent stem cells, while attempts to differentiate ICC are scarce (40-42). Dave et al. reported that the transplantation of murine ICC stem cells into mice with acute and chronic colitis reduced the severity of symptoms. These cells, whether homing in the colon or studied in vitro, showed the ability to suppress T-cell proliferation (43). Given that ANO1 is expressed in both ICC and their mesenchymal progenitors, it may be used in combination with other markers to identify specific time points during the differentiation of mesenchymal cells into ICC and SMCs, as well as their subsequent maturation.

Endothelial ANO1 positivity was a consistent finding in gastrointestinal tract blood vessels during embryonic and fetal development. Although ANO1 expression has been reported in endothelial cells in the brain, umbilical vein, and heart, we found no data on its expression in endothelial cells during development (44-47). The role of ANO1 in endothelial cells remains incompletely elucidated. Some data suggest that ANO1 promotes vasoconstriction and endothelial dysfunction by generating reactive oxygen species in endothelial cells (44). However, some authors report that activation of ANO1 channels induces vasodilatation and a consequent decrease in blood pressure (48,49). According to Garrud et al., CaCCs activation reduces cytoplasmic chloride ion concentration, thereby activating with-no-lysine kinase (WNK), which in turn stimulates transient receptor potential vanilloid 4 (TRPV4) channels and induces vasodilatation (48).

Our results show that ANO1 is expressed in the epithelial cells of the gastrointestinal tract. This immunopositivity was observed in the pseudostratified epithelium of the embryo and persisted, to a lesser extent, in the simple columnar epithelium of fetal samples. As biological membranes, epithelia play a crucial role in the secretion and absorption of fluids and electrolytes. The expression of ANO1 has been reported in intestinal epithelia, where it is assumed to regulate these processes, given the role of chloride ions in determining the direction of fluid or electrolyte secretion (34,39,50). The lower expression of ANO1 in intestinal epithelium may be explained by the fact that the primary anion channel responsible for chloride secretion in these cells is the cystic fibrosis transmembrane conductance regulator (CFTR) (51). Experimental data have shown an interaction between CFTR and CaCCs signalling pathways, further supported by reports from Benedetto et al. that ANO1 is crucial for the proper membrane function of CFTR (51,52). Furthermore, ANO1 depletion has been shown to reduce calcium-dependent chloride secretion in the small and large intestines of mice, resulting in mild mucosal oedema (50).

In conclusion, ANO1 is considered a reliable marker for monitoring the differentiation of SMCs and ICC during embryonic and fetal development. While ANO1 expression persists in ICC throughout the studied period, SMCs in the outer muscle layer of the stomach and large intestine lose ANO1 positivity by the 25th week of development. Given its early emergence during embryonic development, ANO1 may serve as a valuable biomarker for future studies investigating the differentiation of mesenchymal progenitors into SMCs and ICC lineages, as well as their subsequent maturation and in vitro cultivation.

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Authors' Contribution

Conceptualization, V.P. and G.R.; Formal Analysis, V.P.; Writing – original draft, V.P.; Writing – review & editing, A.V., M.J. and G.R.; Data curation, J.R. and G.R.; Methodology, B.K., D.M. and V.R.; Supervision, G.R. All authors have read and approved the published version of the manuscript.

Statement of Ethics

The study was reviewed and approved by the Ethics Committee of the University Clinical Center Niš, approval number 34794/3, issued on October 1, 2019.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed during this study are included within the published article.

Statement of Generative AI Technologies Use

No generative AI was used.

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