Anoctamin 1 Positive Esophageal Interstitial Cajal Cells in Late Stage Human Embryos

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ABSTRACT

Interstitial cells of Cajal (ICCs) are located in various smooth muscle organs and act as pacemaker cells, or ensure neuromodulation or mechanosensory roles. The study aims to investigate functional states of human ICCs in morphogenesis, focusing on the anoctamin 1 phenotype. The investigation was performed in five late stage human embryos with lengths varying between 23 and 29 mm. Immunohistochemistry on paraffin embedded specimens was performed for a series of antibodies: α-smooth muscle actin (α-SMA), desmin, CD31, CD34, CD117/c-kit, DOG1, and nestin. Longitudinal and circular muscle layers were α-SMA+/desmin+/nestin+. An immature microvascular layer located in the inner submucosa was CD34+/CD31+/α-SMA+/nestin+; endothelial tip cells were supporting active processes of sprouting angiogenesis. A CD34+/CD31- mesenchymal network was found in the circular muscle layer. CD117/c-kit+ multipolar ICCs with dichotomizing processes were found mostly in the myenteric plexus layer; processes were configuring a network within the circular muscle layer where intramuscular ICCs were scarcely found. A strong DOG1+ reaction was found for the ICCs of the myenteric plexus layer apposed on the outer surface of the circular muscle layer, and for the intramuscular ICCs. The evidence of a sublayer of DOG1+ myenteric

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Key words: DOG1; nestin; endothelial tip cells; CD34; CD31; smooth muscle actin; embryology

INTRODUCTION

Interstitial cells of Cajal (ICCs) are resident cells of smooth muscle organs, in which they perform various functions (Rumessen and Thuneberg, 1982; Rumessen et al., 1982; Faussone-Pellegrini and Cortesini, 1985; Thuneberg and Peters, 2001; Timmermans, 2001; Ward and Sanders, 2001; Sanders, 2006), such as pacemaker, mediator of neurotransmission and mechanosensory roles (Faussone-Pellegrini et al., 1990; Faussone-Pellegrini and Thuneberg, 1999; Timmermans, 2001; Huizinga and Faussone-Pellegrini, 2005a).

CD117/c-kit is a transmembrane receptor tyrosine protein kinase (Young, 1999). CD117/c-kit antibodies reliably label ICCs (Young, 1999; Wu et al., 2000; Poole et al., 2004; Ciontea et al., 2005; Huizinga and Faussone-Pellegrini, 2005b; Popescu et al., 2005; Huang et al., 2009; Suciu et al., 2010; Rusu et al., 2011a,b). Networks built-up by ICCs are widely distributed within the intermuscular (ICC-MY), intramuscular (ICC-IM, ICC-DMP), and submucosal (ICC-SM) layers from the esophagus to the internal anal sphincter (Takaki, 2003). Recently, a novel cell type was described: the telocyte (Popescu and Faussone-Pellegrini, 2010). It was discussed, on a rat experimental model, that in transmission electron microscopy the standards for differentiating ICCs from telocytes are quite similar, and the peculiar morphologies of telocytes, the telopodes (long, slender, and moniliform cell processes), should make in transmission electron microscopy the difference; moreover, the structural association with smooth muscle cells should direct the diagnosis toward ICCs (Rusu et al., 2012).

Anoctamin 1 has also been described as a specific marker of the ICCs (Hwang et al., 2009; Takaki et al., 2010; Chen et al., 2011; Sanders et al., 2012). In mice embryos, c-kit positive ICCs were also anoctamin 1 positive (He et al., 2012). The anoctamin-1 (*ANO1*, commonly known as *DOG1*) gene expression was found to be differentially expressed in gastrointestinal tumors, as compared with other mesenchymal tumors. It has also been demonstrated that *ANO1* may be expressed in esophageal and head and neck squamous cell carcinoma (West et al., 2004; Carles et al., 2006; Miettinen et al., 2009; Lee et al., 2010).

It was shown that neural crest cells (NCCs) colonize the embryonic gut from week 4 to week 7, and further coalesce to form the myenteric and submucosal plexuses. Smooth muscle differentiation follows the NCCs colonization. ICCs emerge from the gut mesenchyme (Wallace and Burns, 2005).

With regards to the esophageal ICCs morphogenesis in human, to our knowledge, only one study specifically deals with this topic (Radenkovic et al., 2010). In that study, antibodies against c-kit, neuron specific enolase, smooth muscle actin, and desmin were used. The results revealed a continuous layer of esophageal c-kit positive cells surrounding by week 7 the elements of the myenteric plexus (Radenkovic et al., 2010).

It was thus hypothesized that in late stage human embryos anoctamin 1 may also be a key marker of the esophageal ICCs, thus contributing to the pacemaker machinery. Therefore, an immunohistochemical study was performed to test that hypothesis. Additional markers were designed to evaluate the esophageal structure in late stage embryos.

MATERIALS AND METHOD

Five human embryos resulted from legal abortions were collected immediately postabortion. The lengths of these embryos varied between 23 and 29 mm, thus corresponding to late embryo stages (54–56 days) (Sadler and Langman, 2009). Approval for the present study was granted by the Bioethics Committee of the "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania.

Samples were fixed for 24 h in buffered formalin (8%) and were processed with an automatic histoprocessor (Diapath, Martinengo, BG, Italy) with paraffin embedding. Sections were cut manually at 3 μm, and were mounted on SuperFrost® electrostatic slides for immunohistochemistry (Thermo Scientific, Menzel-Gläser, Braunschweig, Germany).

Histological evaluations used 3-μm thick sections stained with hematoxylin and eosin.

Primary antibodies for CD117/c-kit (clone T595, Novocastra-Leica, Leica Biosystems Newcastle, Newcastle Upon Tyne, UK, 1:20), DOG1 (clone K9, Novocastra-Leica, Leica Biosystems Newcastle, Newcastle Upon Tyne, UK, 1:100), CD34 (clone QBEnd 10, Dako, Glostrup Denmark, 1:50), nestin (clone 10c2, Santa Cruz Biotechnology, Santa Cruz, CA, 1:500), α-smooth muscle actin (α-SMA) (clone 1A4, Dako, Glostrup, Denmark, 1:50), desmin (clone D33, Biocare Medical PM 036 AA, Biocare Medical, Concord, CA, 1:100) and CD31 (clone JC70A, Dako, Glostrup Denmark, 1:50) were used. Sections were deparaffinized, rehydrated, and rinsed in PBS buffer solution at pH 7.4 (for the nestin, CD34, CD31, α-SMA and desmin antibodies) and in TBS buffer solution at pH 7.6 (for the CD117/c-kit and *DOG1* antibodies). Retrieval by incubation in specific buffer was completed as follows: (a) for CD34: EDTA, pH 9; (b) for the other antibodies: 0.01 M citrate retrieval solution, pH 6. The standard ABC technique used a DAB protocol. Appropriate blocking of endogenous peroxidase was

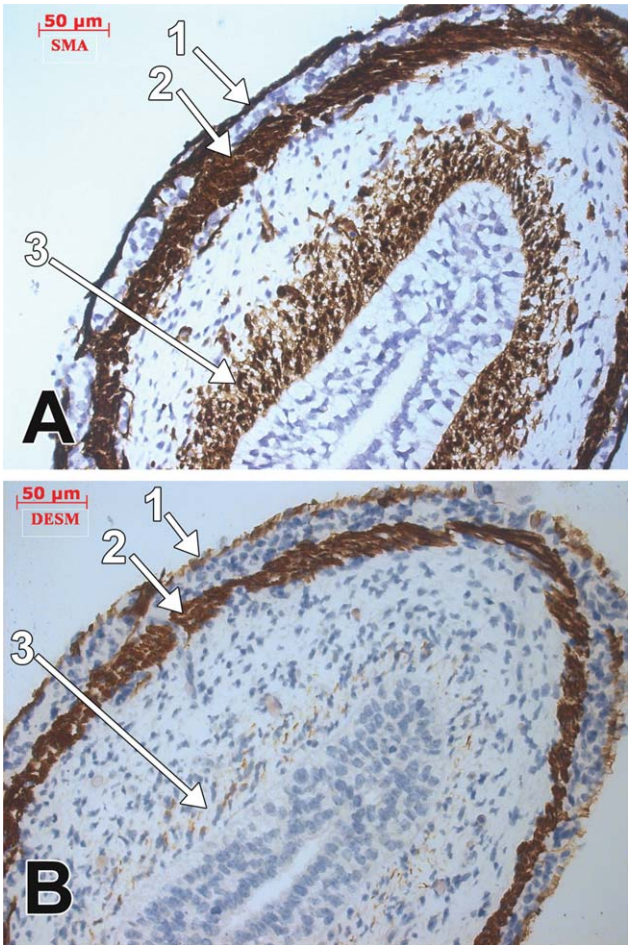

Fig. 1. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with α-SMA (A) and desmin (B) antibodies. 1. longitudinal muscle layer; 2. circular muscle layer; 3. lamina propria.

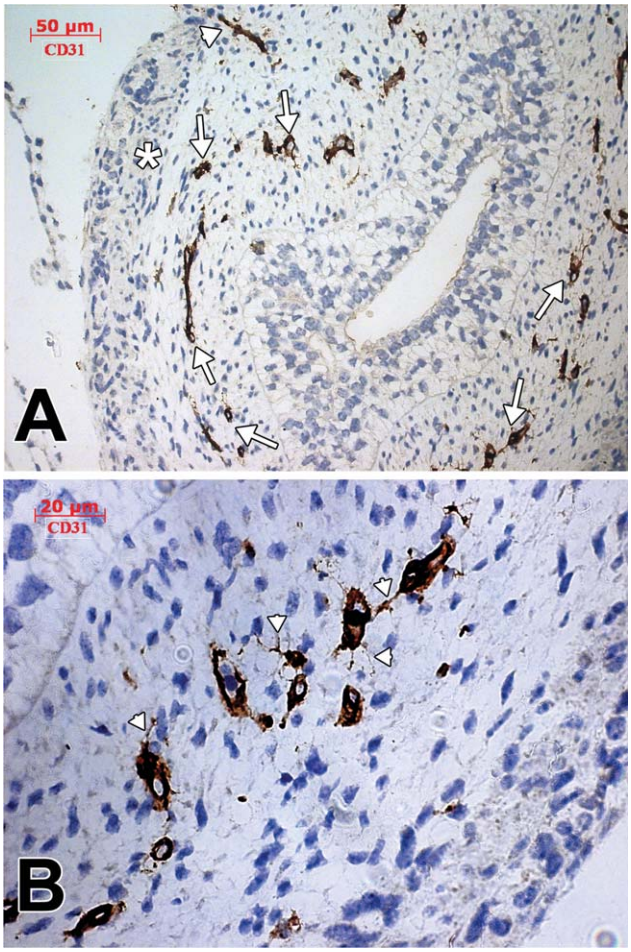

Fig. 2. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with CD31 antibodies. A. The microvascular layer identified with CD34 antibodies between the lamina propria and the submucosa is positively labeled with CD31 antibodies (arrows). A penetrating microvessel is indicated (arrowhead). The circular muscle layer (*) lacks immune positive elements. B. ETCs label with CD31 antibodies and project into stroma moniliform dichotomizing processes (arrowheads).

completed before immunolabeling (Peroxidazed 1, Biocare Medical, Concord, CA). Sections incubated with nonimmune serum served as negative controls. Sections were counterstained with Hematoxylin.

The microscopic slides were analyzed and micrographs were taken and scaled using a Zeiss working station, as previously described (Rusu, 2013).

RESULTS

In the late embryo stage, the lower esophagus presented a mucosa comprising a pluristratified epithelium and lamina propria, submucosa, and muscularis consisting, in turn, of an inner circular layer and an outer longitudinal layer. The longitudinal and circular muscle layers were positively labeled by α-SMA and desmin antibodies (Fig. 1) and were separated by a distinctive layer corresponding to the myenteric plexus. Moreover, α-SMA consistent positive labeling was associated with the lamina propria and the adjacent inner part of the submucosa; a discrete desmin-positive phenotype was corresponding to the strong α-SMA phenotype in this location (Fig. 1). In submucosa, α-SMA seemingly labeled also microvessels; however doubts were kept on this diagnosis.

With CD31 antibodies (Fig. 2) endothelia were positively labeled: a distinctive microvascular layer was demonstrated in the inner part of the submucosa. Penetrating dichotomizing vessels were also identified. Endothelial cells projecting in the adjacent stroma thin moniliform processes were identified as endothelial tip cells (ETCs). The microvascular layer identified with CD31 antibodies was also CD34-positive; ETCs were also positively labeled by CD34 (Fig. 3). However, labeling with CD34 antibodies identified an outer positive network, mostly corresponding to the circular muscle layer (Fig. 3). Since in this location CD31 did not label any element, the network has been considered mesenchymal. Nestin-positive immune labeling was associated with the microvascular layer at the lamina propria – submucosa border (Fig. 4), and with the muscular coats identified with desmin and α-SMA antibodies, but

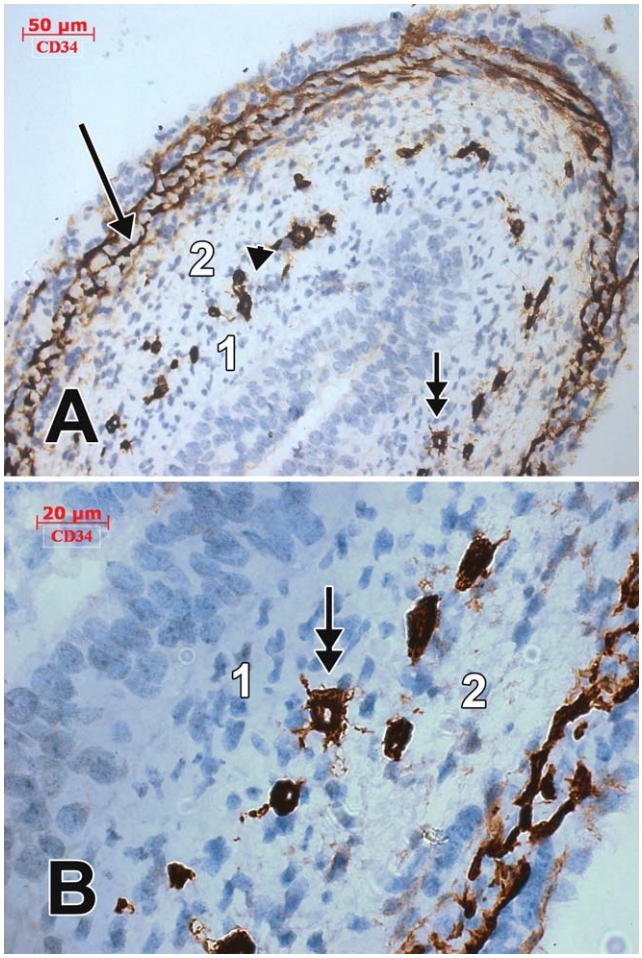

Fig. 3. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with CD34 antibodies. A. A mesenchymal network is identified within the circular muscle layer (arrow). A different microvascular layer (arrowhead) lies between the lamina propria (1) and the submucosa (2); ETCs are indicated (double-headed arrow in A and B).

with the myenteric plexus layer between the circular and longitudinal muscle layers.

CD117/c-kit positive labeling was associated mostly with the myenteric plexus layer, where ICCs with long dichotomizing processes were found (Fig. 5). These c-kit positive ICCs (ICC-MY) were configuring a complete circumferential network; they were sending long processes coursing circumferentially on the outer surface of the circular muscle layer, and processes which were penetrating in this muscle layer, with a radial or oblique course. Also c-kit positive cells and processes were found within the inner part of the circular muscle layer (ICC-IM). With *DOG1* antibodies, a weak positive reaction was found in the circular and longitudinal muscle layers (Fig. 6). A strong *DOG1* positive immune labeling was found for the ICC-MY (cell bodies and processes) applied on the outer surface of the circular muscle layer, and for ICC-IM scarcely distributed within the inner part of the circular muscle layer (Fig. 6).

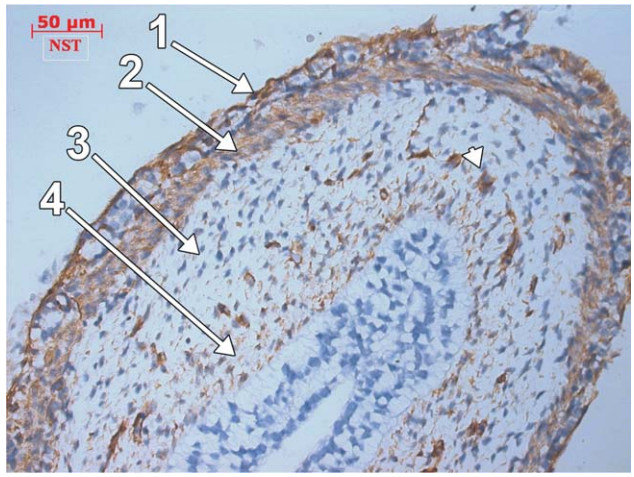

Fig. 4. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with nestin antibodies. 1. longitudinal muscle layer; 2. circular muscle layer; 3. submucosa; 4. lamina propria. The microvascular layer at the submucosa/lamina propria border is indicated (arrowhead).

DISCUSSION

In this study, evidence was brought on the esophageal microvascular developmental morphology in late stage embryos. Taking into account the CD31 and CD34 labeling, it appears that a microvascular layer, supplied by penetrating vessels, lies at the borderline between lamina propria and submucosa, and it is immature, as proven by the ongoing processes of sprouting angiogenesis supported by ETCs. The layer can be important when investigations are conducted in angiogenesis dependent diseases, such as Barrett's esophagus (a premalignant condition of the lower esophagus), as it may provide further proliferating endothelial cells of new blood vessels. These cells may serve as an important source for diagnostic markers specific for the evolution of Barrett's epithelium (Auvinen et al., 2002). It is known that ETCs conduct sprouting angiogenesis (Stanescu et al., 2012; Phng et al., 2013; Rusu et al., 2013c); the ETCs processes invade the stromal compartment by a different stage migration, project filopodial, and moniliform processes, and are under the influence of the stromal cell compartment (Rusu et al., 2013a). It was demonstrated that ETCs label with CD34 antibodies (Siemerink et al., 2012; Stanescu et al., 2012; Rusu et al., 2013a). This study shows that ETCs also label with CD31 antibodies. It is known that nestin, which is a type VI intermediate filament protein, labels various tissues during embryogenesis, such as muscle fibers, hepatocytes, and renal progenitors (Krupkova et al., 2010). Nestin also labels neural crest stem cells and endothelial cells in newly formed blood vessels (Rusu et al., 2013b). This is consistent with our findings of nestin-positive labeling of the microvessels at the submucosa-lamina propria border. Conversely, immunoreactivity for α-SMA and nestin, found at the border of lamina propria and submucosa, may suggest the existence of an immature lamina muscularis mucosae. Moreover, the external muscle coats nestin-positive labeling seemed not unusual, as long as transient nestin expression was found in immature

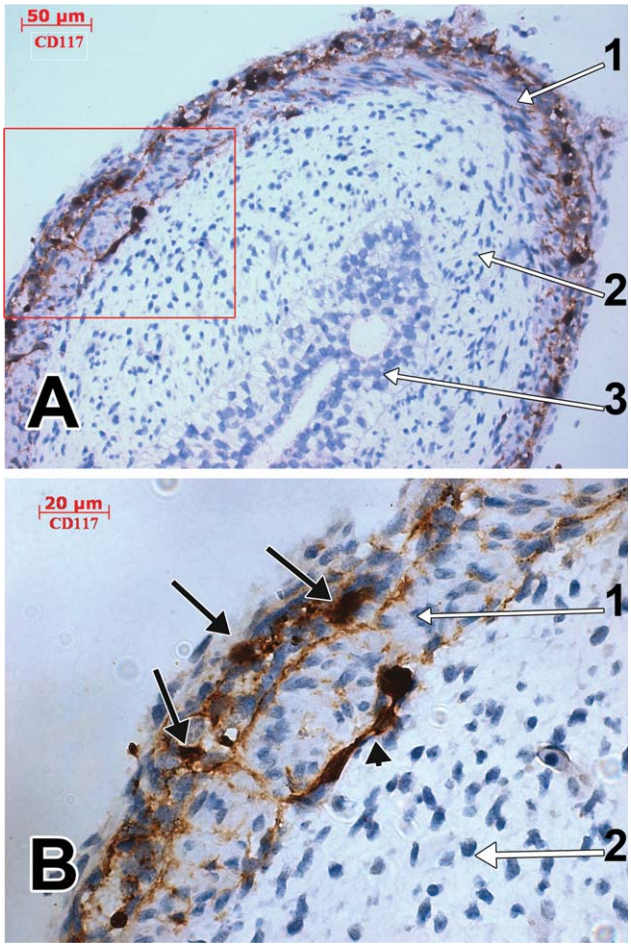

Fig. 5. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with CD117/c-kit antibodies. Inset in A is evaluated at a higher magnification (B). CD117/c-kit positive interstitial Cajal cells correspond topographically to the myenteric plexus (B, black arrows) but also lay on the submucosal boundary of the circular layer (B, black arrowhead). 1. circular layer of tunica muscularis; 2. lamina propria; 3. epithelium.

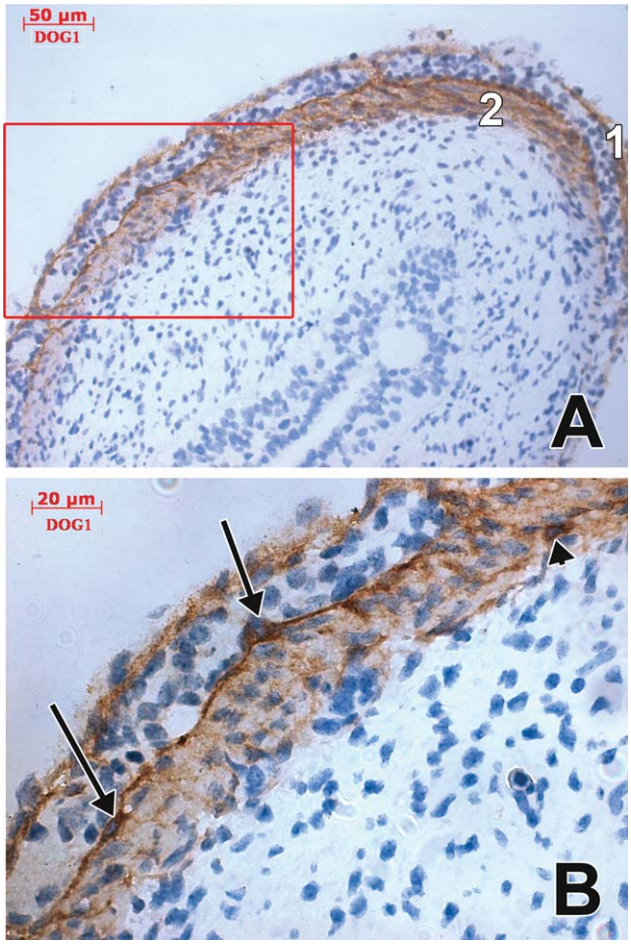

Fig. 6. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with DOG1 antibodies. Inset in A is evaluated at a higher magnification (B). 1. longitudinal layer of tunica muscularis; 2. circular layer of tunica muscularis. Immune positive cells are identified on the outer side of the circular muscle layer (arrows) and in the deep part of the circular muscle layer (arrowhead).

esophageal smooth muscle cells in early stages of development (Su et al., 2011).

It is actually established that ICCs originate from mesenchyme and not from the neural crest (Lecoin et al., 1996; Young, 1999; Timmermans, 2001; Wallace and Burns, 2005). ICCs and smooth muscle cells may have common precursors (Young, 1999; Timmermans, 2001). Our results support, as second time evidence, the findings of Radenkovic et.al. (2010) who demonstrated that in late stage embryos the layer of ICC-MY is morphologically defined in esophagus. Moreover, c-kit and *DOG1* labeling demonstrate that scarce intramuscular ICCs and networks are defined in this stage. This is comparable with the ICCs topography in adult esophagus, where these can be found within the muscle bundles, a few in their proximity, but the majority are found at the periphery of the bundles (Faussone-Pellegrini and Cortesini, 1985).

It was established that ICC-MY act as primary pacemaker cells (Takaki et al., 2010; Rusu et al., 2011a), and intracellular calcium dynamics play an important role in the pacemaking (Takaki et al., 2010). Intracellular calcium oscillations periodically activate plasmalemmal channels, such as Ca^{2+}-activated Cl^{-} channels (Takaki et al., 2010). Anoctamin 1 (*Ano1*, *TMEM16A, DOG1*) is a major component of the plasmalemmal Ca^{2+}-activated Cl^{-} channels (CaCCs) (Ferrera et al., 2010; Cipriani et al., 2011; Kunzelmann et al., 2011; Shimizu et al., 2013; Simon et al., 2013). In this regard, the evidence of a sublayer of *DOG1* positive ICC-MY is suggestive for a subpopulation of ICC-MY being qualified for pacemaking at this early developmental stage.

Further studies are however needed to explore the c-kit and *DOG1* phenotypes dynamics during the fetal and perinatal periods, and use of double label immunohistochemistry could add useful information.

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