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Presence of c-kit positive cells in fetal and adult bovine forestomachs

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Abstract

The interstitial cells of Cajal (ICC) have been reported to regulate gastrointestinal motility. We investigated the distribution and the morphological and morphometric characteristics of the immunohistochemical reaction against c-kit in the forestomachs of fetal, newborn and adult cows. The anti-c-kit reaction revealed different populations of ICC among age groups and organs. ICC were more numerous and smaller in fetuses. Larger ICC were identified in newborns, except for those in the rumen. During the earliest stages of development, ICC were abundant in the inner layer of the muscularis and were consistently associated with this layer. In all samples, ICC were found in the outer layer of the tunica muscularis. ICC were found between the two muscle layers in the omasum at all ages; however, they were identified only in the rumen of the adult. Our study demonstrated that ICC are present in the forestomach of bovines.

Key words: c-kit, Cajal cells, cattle, forestomachs, immunohistochemistry, interstitial cells, morphometry, rumen

Cattle and other ruminants possess four-chambered stomachs. The first three chambers (rumen, reticulum and omasum) collectively are known as the forestomachs. They contain a complex population of symbiotic bacteria, protozoa and fungi that use the host for nutrition and a suitable environment. The fourth chamber, the abomasum, has a mucosa with glands that secrete gastric juice. The forestomachs are lined by stratified aglandular epithelium that is adapted for absorption of microbial fermentation products. The biochemical activity of the stomach requires, among other factors, orderly sequential contraction of a thick muscular layer.

Prenatal development of the bovine stomach begins from a single spindle-shaped primordium, similar to non-ruminant mammals. The rumen and reticulum are formed in the proximal part of the primordium; in non-ruminants, this region gives rise to the fundus and corpus of the stomach. In some non-ruminants the distal portion of the primordium forms the pyloric portion of stomach, whereas in bovine species, this region is the abomasum. The omasum originates from the lesser curvature of the primordium and has no counterpart in non-ruminant species with a one-chambered stomach (Warner 1958, McGeady and Sack 1967). Therefore, the bovine stomach is considered a variation of the overall scheme of specialized mammalian stomachs that otherwise consist of homologous structures.

The proximal portion of the non-ruminant stomach responds with adaptive relaxation and minimal contractile activity during gastric filling. The contractile activity differs from the complex patterns of contraction of the rumen-reticulum for rumination, eructation, mixing and propulsion of contents (Sellers and Stevens 1966, Titchen 1976).

As part of the enteric nervous system, the wall of the forestomachs possess a rich intramural

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innervation that integrates local reflexes as in non-ruminants (Pfannkuche et al. 2002). Teixeira et al. (1998, 2000) described a complex architecture for the myenteric, submucosal and mucosal plexuses with regional differences in forestomachs of cattle fetuses. Motor innervation of the gastro-intestinal tract includes small cells of mesenchymal origin, the interstitial cells of Cajal (ICC) (Lecoin et al. 1996). ICC are located close to the nerve varicosities of the enteric nervous system and form a link between the nervous system and smooth muscle cells (Mazzone and Farrugia 2007) (Fig. 1).

According to Hanani and Freund (2000), ICC are located in association with the myenteric plexus (ICC-MP) at the border between the circular muscle and the submucosa (ICC-SMP) between the internal thin layer and the thick outer layer of the circular muscle (ICC-DMP); in the longitudinal muscle (ICC-LM) and in the circular muscle (ICC-CM). The latter localizations are referred to as intramuscular ICC (ICC-IM).

ICC express different molecules during their differentiation that can be demonstrated using immunohistochemistry (IHC). We used the anti-desmin antibody for staining the muscle cell and anti-vimentin antibody for characterization the ICC. Nevertheless, this method is not specific for ICC.

We used the anti-c-kit antibody because of its specificity and its adaptability for both frozen and paraffin sections (Vanderwinden et al. 1996). This technique has been used to detect ICC from the abomasum to the colon in the gastrointestinal tract of cattle (Stoffel et al. 2006) and the jejunum (Márquez et al. 2006). To our knowledge, however, there are no reports concerning ICC in the rumen, reticulum or omasum. Therefore, we investigated the distribution pattern and the morphological characteristics of c-kit positive cells in bovine forestomachs at different stages of development.

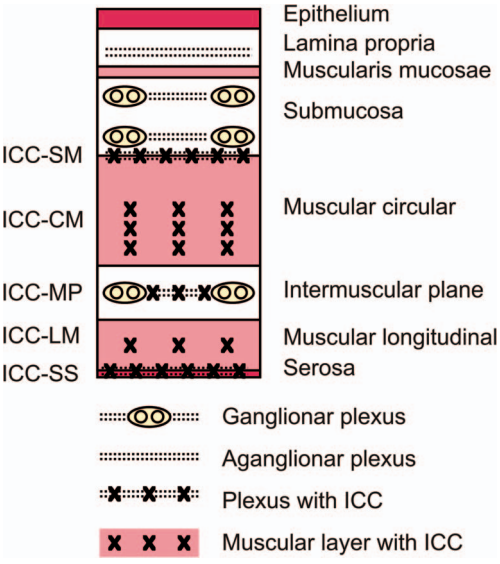

Fig. 1. C-kit cell identification by location in the stomach of mammals. ICC-SM, submuscular c-kit positive cells of the pyloric antrum; ICC-CM, ICC in the circular muscular layer; ICC-MP, ICC of the myenteric plexus; ICC-LM, ICC in the longitudinal muscular layer; ICC-SS, ICC of the subserous plexus.

Material and methods

Animals

Samples of stomach regions were obtained from healthy animals slaughtered (adults) or found (fetuses) in abattoirs and also from newborn calves that were sacrificed specifically for our study. We used ten Hereford steers. Animals were 2–3 years old, clinically healthy and pasture-fed, as verified by assessment of rumen contents. Ten 1 month newborn Holstein calves were included in our study; diets consisted of newborn ration mix and milk replacer. The ten fetuses were from dams of various breeds (Hereford, Aberdeen, Angus). Samples were taken from fetuses whose cephalo-caudal length was 60–85 cm from the front suture to the base of the tail, which corresponds to a gestational age of approximately 240 days (Youngquist and Threlfall 2006). All procedures were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, 1996, Washington, DC)

Samples

Samples of the rumen were taken from its pillars. Samples from reticulum and omasum were obtained from their parietal walls. All samples were fixed in 10% buffered formalin for 48–72 h, then processed according to standard histological techniques and embedded in paraffin. Sections 4 ± 1 µm thick were cut with a sliding microtome. All samples were stained with either hematoxylin and eosin or the Mallory trichrome technique.

IHC

Sections were mounted on positively charged slides (Starfrost, Knittel Glaser, Brunswick, Germany). Sections then were deparaffinized and hydrated. Sections were incubated with 0.03% H_2O_2 in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. Antigen retrieval was performed in citrate buffer solution (pH 6) at boiling

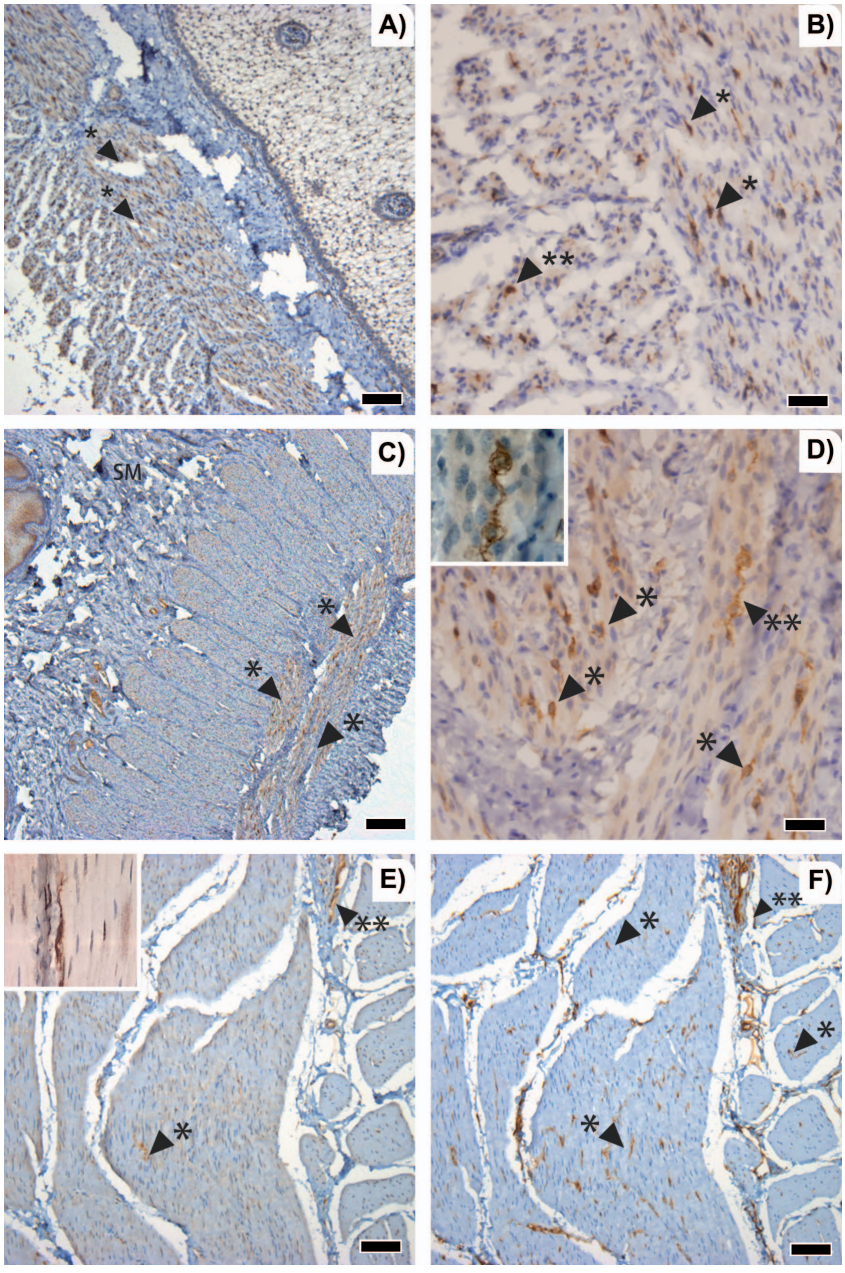

Fig. 2. IHC of the rumen. A) ◄★ Fetus c-kit positive cells. Bar = 100 μm. B) Higher magnification of (A); ◄★, c-kit positive cells in the internal oblique muscular layer; ◄★★, same cells in the longitudinal muscular layer. Bar = 25 μm. C) Newborn rumen. IHC c-kit positive cells. SM, extensive submucosal layer; ◄★, positive cells. Bar = 250 μm. D) Higher magnification of (C); ◄★, positive cells with fusiform shape; ◄★★, positive cells connected through their extensions. Bar = 25 μm. Inset: staining at the cell membrane. E) C-kit positive cells in the cow rumen; ◄★, positive cells in the external muscular layer; ◄★★, positive cells in the MP region. Bar = 100 μm. Inset: positive cells in the internal oblique muscular layer. F) IHC anti-vimentin in cow; ◄★, positive cells between muscular layers; ◄★★, positive cells between muscle bundles. Bar = 100 μm.

temperature in a microwave oven for 5 min. Slides were cooled at room temperature, washed with PBS, then incubated with 1% bovine serum albumin (BSA) to block nonspecific binding. Incubation with different primary antibodies was performed for 24 h at 4° C (Portiansky and Gimeno 1996, Portiansky et al. 1997, Zanuzzi et al. 2012). We used polyclonal c-kit (A4502; Dako, Glostrup, Denmark) monoclonal anti-swine vimentin, clone V9 (Dako) and monoclonal anti-swine desmin, clone DE-R-11 (Dako) antibodies. Labeled streptavidin-biotin (LSAB; Dakocytomation-LSAB2® System-HRP,

Table 1. Morphometric measurements of c-kit positive cells in the rumen of adult cows

Rumen	Area (μm^2)	Major axis (μm)	Minor axis (μm)	Perimeter (μm)
Fetus	36.27 ± 6.05[1]	13.42 ± 1.80[2]	3.58 ± 0.38	31.09 ± 4.78[3]
Newborn	64.69 ± 10.02[1]	12.83 ± 1.61[2]	6.58 ± 0.56	33.68 ± 3.80[3]
Adult	96.68 ± 16.02[1]	22.74 ± 2.99	5.56 ± 0.38	50.13 ± 5.64

[1]Area: significant differences among all groups.
[2]Major axis: significant differences between fetus and adults, and newborn calves and adults.
[3]Perimeter: significant differences between fetus and adults, and newborn calves and adults.

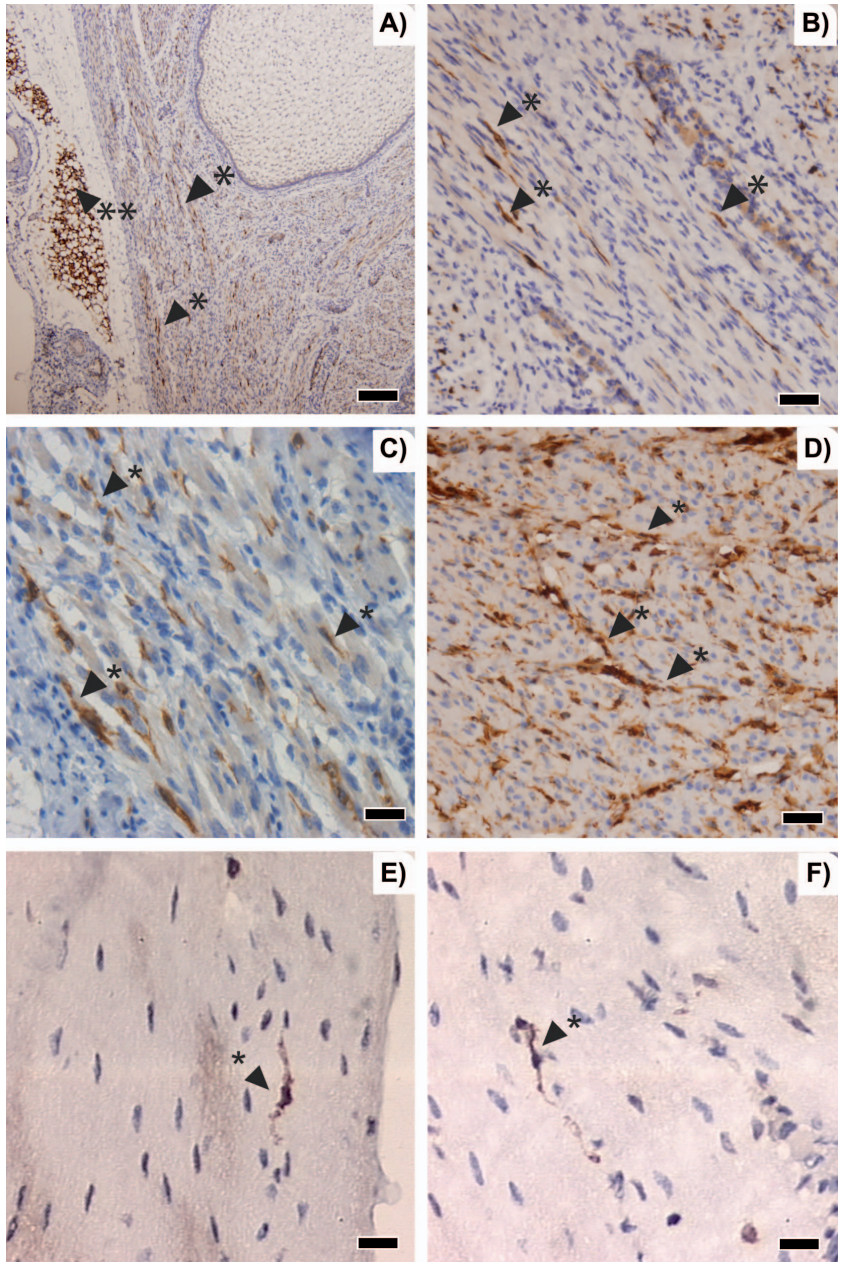

Fig. 3. C-kit IHC of the reticulum. A) ◄★, fetus c-kit positive cells in both muscle layers; ◄★★, positive cells in adipose tissue. Bar = 100 μm. B) Higher magnification of (A); ◄★, c-kit positive fusiform cells with long extensions between muscular layers. Bar = 25 μm. C) Newborn rumen. ◄★, C-kit positive cells. Bar = 25 μm. D) ◄★, anti-vimentin positive cells between smooth muscle bundles. Bar = 25 μm. E) C-kit positive cells in the cow reticulum; ◄★, isolated fusiform positive cells with extensions. Bar = 25 μm. F) ◄★, c-kit positive cell bodies in the reticulum of the cow. Bar = 25 μm.

Dako), goat anti-rabbit and goat anti-mouse immunoglobulin (Dako) were used as amplification and detection systems. The chromogen was 3, 3′ diaminobenzidine (DAB). Mayer's hematoxylin was used for counterstaining. Sections were observed by light microscopy. Cells stained dark brown from the oxidation of DAB were identified as positive. Nuclei were stained by the hematoxylin.

Considering the difficulty of counting highly branched individual ICC, the concentration of c-kit positive cells was estimated subjectively as follows: (−) none, (+) few, (+ +) many, (+ + +) abundant.

Image analysis

For morphometric studies, 10 images of each slide were captured using a microscope (Olympus BX50 system microscope, Tokyo, Japan) at various magnifications and a digital video camera (Olympus DP71, Japan) connected to a computer. Images were processed using the Image-Pro Plus v6.3 software (Media Cybernetics, Silver Spring, MD) with an RGB depth of 24 bits, and saved in TIFF format. The spatial calibration yield was either 0.13 (100 x objective) or 0.32 (40 x objective) μm/pixel. ICC were characterized quantitatively based on their area (μm^2), major and minor axes for the aspect ratio of the object, and perimeter (μm).

Statistics

Results were analyzed using analysis of variance (ANOVA) and Student's t-test. The Bonferroni correction was used to control family-wise error rate and to compare the results obtained in different sections of gastrointestinal tract, especially in the myenteric plexus region. Values for $p \leq 0.05$ were considered statistically significant.

Results

Samples stained with hematoxylin and eosin and Mallory trichrome showed structures typical of each organ at each developmental stage.

Rumen

C-kit labeled cells in fetuses were evenly distributed among muscle fibers of both the transversae and longitudinal layers (Fig. 2A). The stained cells were elongated, oriented in the direction of the muscle cells, had an oval or triangular shape and short extensions that rarely contacted other cells (Fig. 2B).

C-kit positive cells were distributed uniformly within the muscular layer of the rumen of newborn calves (Fig. 2C). These cells were characterized by a prominent nucleus, oval body and two long extensions that connected through their ends with other cells of the same type (Fig. 2D). The plasma membrane was clearly delineated (Fig. 2D, inset).

Few c-kit positive cells were observed in adult cows. These cells were located between muscle fibers, and had a narrow and elongated body with terminal ends that were long and thin (Fig. 2E). Table 1 shows the morphometric measurements from those cells. Also, vimentin IHC showed few positive cells, most of which were observed between muscle fibers (Fig. 2F).

Reticulum

Many c-kit positive cells were observed in the myenteric plexus region and in the muscular tunic, mainly in the longitudinal layer (Fig. 3A) of the fetal reticulum. These cells were spindle shaped and usually bipolar (Fig. 3B).

In the newborn calf, anti-c-kit IHC showed many positive cells throughout the thickness of the muscular layer and in the myenteric plexus of the organ. These cells showed two or three extensions that were distributed throughout the layers to form chains or rows of positive cells between muscle fibers (Fig. 3C). Anti-vimentin IHC demonstrated a rich network of cells between muscle fibers (Fig. 3D).

Few c-kit positive cells were observed in the reticulum. These cells had a fusiform body and long and tortuous extensions (Fig. 3E). Table 2 shows the morphometric measurements of these cells. Vimentin IHC showed a pattern similar to that observed with c-kit IHC (Fig. 3F).

Table 2. Morphometric measurements of c-kit positive cells in the reticulum of adult cows

Reticulum	Area (μm^2)	Major axis (μm)	Minor axis (μm)	Perimeter (μm)
Fetus	39.54 ± 3.82^1	12.76 ± 0.97	4.08 ± 0.25	29.82 ± 1.88
Newborn	51.50 ± 4.96^2	13.02 ± 1.27	5.17 ± 0.28	31.28 ± 2.60
Adult	44.27 ± 6.05	12.61 ± 0.89	4.58 ± 0.41	29.32 ± 2.16

Significant differences in area: [1]fetus vs. newborn and [2]newborn vs. adult.

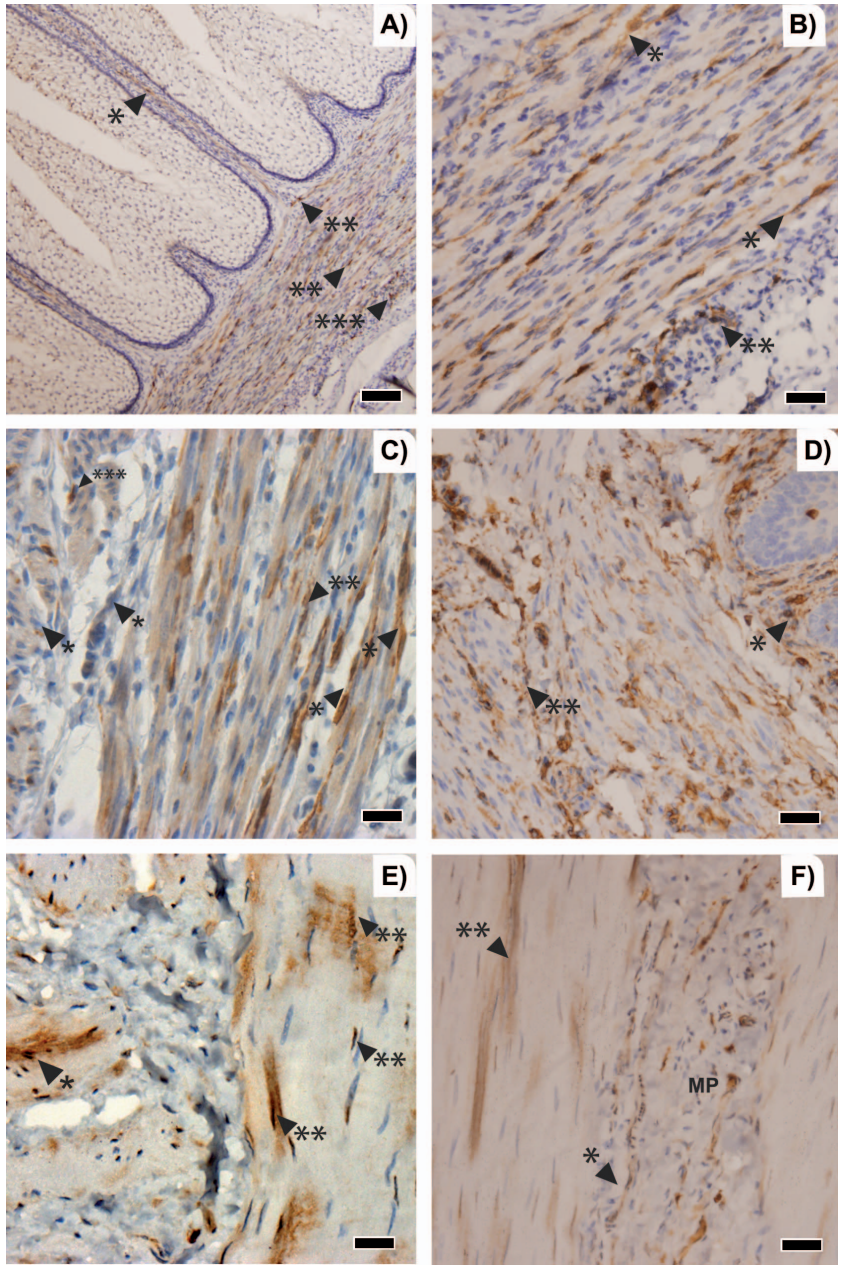

Fig. 4. C-kit IHC of the omasum. A) ◄★, fetal c-kit positive cells in the axis of the lamina; ◄★★, positive cells in the circular muscular layer; ◄★★★, positive cells in the myenteric plexus. Bar = 100 μm. B) Higher magnification of (A). ◄★, c-kit positive cell extension; ◄★★, c-kit positive cell close to the myenteric plexus. Bar = 25 μm. C) Newborn omasum. ◄★, c-kit positive cells showing extensions in contact with each other; ◄★★, c-kit positive cell body; ◄★★★, c-kit positive cells in the longitudinal muscular layer. Bar = 25 μm. D) ◄★, positive anti-vimentin cell in the axis of the lamina; ◄★★, extensions of a positive cell in the circular muscular layer. Bar = 50 μm. E) Positive c-Kit cells in the cow omasum. ◄, positive cells in the circular muscular layer; ◄★, positive cell in the interlaminar muscle layer. Bar = 25 μm. F) C-kit IHC in cow. ◄★, positive cells surrounding the myenteric plexus; ◄★★, positive staining in the muscle layer. Bar = 25 μm.

Omasum

C-kit IHC showed positive cells in all muscular layers in the omasum of fetuses. Positive cells were bipolar and relatively large compared to the adult. They were observed along the axis of the laminae of the omasum and many of them surrounded the nodes of the myenteric plexus (Fig. 4A). Extensions of the cells contacted each other to form a line (Fig. 4B).

In the newborn calf, c-kit positive cells were located in both muscular layers and around the myenteric plexus. Many of these cells formed

Table 3. Morphometric measurements of c-kit positive cells in the omasum of adult cows

Omasum	Area (μm²)	Major axis (μm)	Minor axis (μm)	Perimeter (μm)
Fetus	44.67 ± 4.08[1]	14.08 ± 1.13[2]	4.22 ± 0.36	32.65 ± 2.02
Newborn	69.42 ± 7.18	16.84 ± 1.03	5.41 ± 0.37	39.38 ± 1.89
Adult	49.38 ± 4.58[1]	16.25 ± 1.23	3.99 ± 0.30	35.70 ± 1.92

[1]Significant differences in area: newborn vs. fetus and adult.
[2]Significant differences in major axis: fetus vs. newborn and adult.

chains by way of their processes. These cells were interspersed between muscle fibers (Fig. 4C). Vimentin KHC showed positive cells in all muscle layers and in the axis of the laminae of the omasum. These cells were fusiform with thin extensions (Fig. 4D).

In the omasum of adult cows, c-kit positive cells were observed in the laminae of the organ,

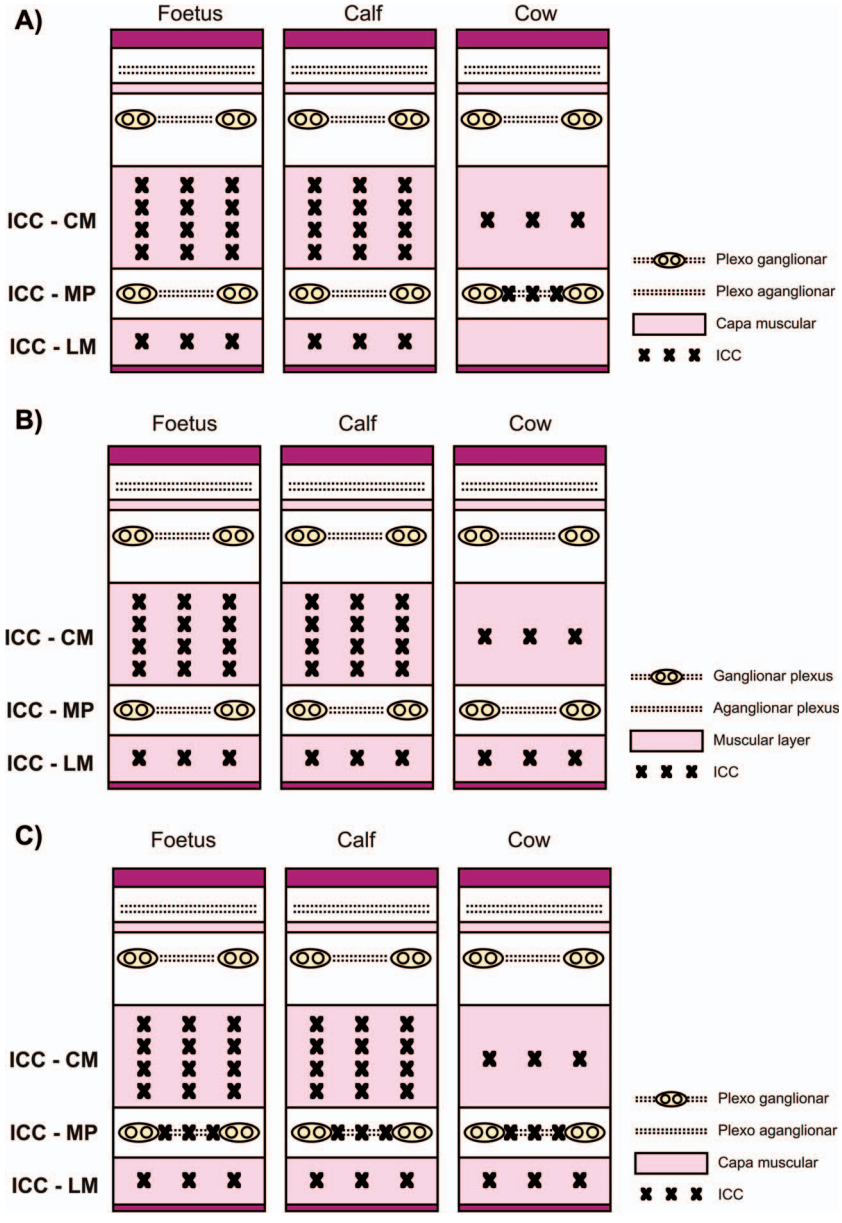

Fig. 5. Distribution of ICC in the forestomachs of fetus, newborn and cow. A) Rumen. B) Reticulum. C) Omasum.

in the muscular layer and close to the myenteric plexus. Immunoreactivity was diffuse; therefore, morphological patterns were difficult to distinguish (Fig. 4E, F). Table 3 shows the morphometric measurements of the c-kit positive cells.

C-kit IHC showed different populations of ICC among all ages and organs studied; these populations are depicted graphically in Fig. 5. In all organs of all three ages examined, c-kit positive cells were positive also with vimentin and negative with desmin; however, vimentin also stained other structures, while desmin identified muscle cells.

Discussion

We have demonstrated the presence of c-kit positive cells in bovine forestomachs at all developmental stages studied. Earlier studies of mice (Maeda et al. 1992), guinea pigs (Komuro et al. 1996), rats (Ishikawa et al. 1997), humans (Rømert and Mikkelsen 1998), dogs (Horiguchi et al. 2001), cats (Morini et al. 2004) and horses (Hudson et al. 1999) among other species have shown that c-kit immunostaining, together with cellular morphology and localization, enabled identification of these cells as ICC (Faussone-Pellegrini and Thuneberg 1999). Vimentin KHC was positive in the same areas as c-kit positive cells, although the vimentin staining was observed over a larger area, because fibroblasts contain vimentin intermediate filaments (Torihashi et al. 1993). Consequently, vimentin antibody is not suitable for identifying ICC cells specifically.

The presence of ICC in the forestomachs of fetus and newborn calves is consistent with the prenatal detection of ICC in the mouse (Faussone-Pellegrini1984, Burns et al. 1996), rat (Faussone-Pellegrini et al. 1996), guinea pig (Komuro 1999, Beckett et al. 2005) and human (Kenny et al. 1999). In the stomachs of these species, the ICC were restricted to the periphery of the myenteric plexus. We found positive cells scattered throughout the muscular layers of the rumen and reticulum. In cattle, ICC were fusiform with extensions at each end, similar to ICC-IM of adult human stomach, but different from the human fetus, which are oval (Torihashi et al. 1999). This observation suggests that ICC-IM differentiate early in cattle.

Two sub-populations of ICC were detected in the rumen of the adult cow, which according to their location might be considered ICC-IM and ICC-MP. ICC-IM were more dispersed in adults animals than in juvenile stages. The shape and location of ICC-MP were comparable to ICC-MP in the distal stomach of guinea pigs (Burns et al. 1997) and humans (Radenkovic et al. 2009). The proximal stomach of non-ruminant mammals is a storage structure, which has only tonic activity; however, ruminants exhibit propulsive activity in all gastric compartments (Titchen 1976, Sellers and Stevens 1966). Therefore, rumen ICC-MP may have a pacemaker function for the distal stomach of mammals (Hagger et al. 1997). Torihashi et al. (1999a) reported that ICC-MP do not appear in the proximal stomach of non-ruminant mammals. If the rumen-reticulum is considered the counterpart of the fundus and body of the single stomach as proposed by Warner (1958), however, ICC-MP could correspond to the population described by Christensen et al. (1992) in the body of the stomach of cats, dogs, opossum, rats, guinea pigs and rabbits. The reticulum showed c-kit positive cells that could be considered ICC-IM similar to those in the aglandular portion of the mouse stomach (Ward et al. 2004). The density of this subpopulation in the newborn calf was much greater than in adult animals.

Because of their location and the close relation of their long extensions with smooth muscle cells, the remaining c-kit positive cells in the adult may have a mechanic-sensor function. There is evidence that ICC-IM regulate distension and maintain myogenic tone in the fundus of the stomach (Dixit et al. 2006). Mechanic sensors distributed in the esophagus, rumen, reticulum and reticular sulcus are described in cattle (Leek 1969, Falempin et al. 1978), but have not been linked to ICC. It has been suggested that ICC-IM are stretch receptors for vagal afferent nerves in the rat stomach (Powley et al. 2008). It has been shown that the myenteric neurons of the rumen and reticulum produce a specific neurochemical code (Pfannkuche et al. 2002). The gastric ICC-IM of other species, such as mouse (Chen et al. 2007a,b), possess receptors for these neurotransmitters. It is possible that ICC-IM of the reticulum may have such receptors as well.

According to their location and morphology, C-kit positive cells identified in the omasum of all age groups were ICC-MP and ICC-IM. Because the omasum is considered a derivative of the lesser curvature of the primitive stomach (Warner 1958, McGeady and Sack 1967), the area can be considered equivalent to that reported by Hirst et al. (2002), who described ICC-MP and ICC-IM in the lesser curvature of the mouse stomach. Although there is a decreasing population gradient of ICC toward the lesser curvature and fundus of the stomach (Christensen et al. 1992, Mazet and Raynier 2004, Song et al. 2005), we found that a greater proportion of immunostained cells appeared in the omasum compared to the rumen-reticulum. In mouse stomach, ICC-IM

of the lesser curvature regulates inhibitory neurotransmission (Burns et al. 1996). We speculate that the same action can be accomplished by the omasum, because its strong and prolonged contractions have a rate of motor activity independent of the reticulum-rumen and are inhibited by distension of the abomasum (Sellers and Stevens 1966).

We found a much larger population of ICC-IM in the forestomachs of fetuses and calves compared to adults. One possible explanation may be that these cells change phenotype. Torihashi et al. (1999b) showed that when blocking c-kit (CD117) receptor, the ICC do not disappear in areas where their distribution would be expected, but they transdifferentiate to acquire characteristics of smooth muscle cells. It is accepted that in the absence of stem cell factor or mutation of the c-kit receptor, ICC lose their phenotype, as shown in the mouse small intestine (Klüppel et al. 1998).

The development of the enteric nervous system is not uniform in the stomach of ruminants and it varies not only according to the compartment, but also within the same segment of a given region (Teixeira et al. 1998). In adults, the myenteric plexus is significantly less developed than in the suckling calf. This decline with age can be attributed to the additional development of forestomachs (Pfannkuche et al. 2003). Because ICC are located near nerve fibers, the decreased c-kit positive area of the adult is related to the decreased development of the nervous plexus as reported by Kitamura et al. (1986).

According to our morphometric analysis, the significant differences in the dimensions of immunolabeled cells in the forestomachs of fetuses and calves could be associated with continuing development of the muscular layer of the stomach of the latter. Significant differences also were observed between the rumen ICC of calves and cows; these appear to be related to the continuing development of the rumen in the adult. The relation between major and minor axes of ICC remained constant. At all ages studied, ICCs were elongated and had the fusiform phenotype of ICC-IM (Torihashi et al. 1995, Lin et al. 2007). Moreover, extensions of the cells located between muscle fibers increased in length with aging and, therefore, the length of the cell body decreased in proportion to the increased area occupied by the cellular projections. We found no morphometric data from other species to compare with our results, but the shape of the cells indicates that ICC correspond to the spindle phenotype described in humans and equines (Hudson et al. 1999, Torihashi et al. 1999a).

C-kit positive cells are present in the muscular layer of the forestomachs of cattle at all ages studied. The location, distribution and phenotype of these c-kit positive cells is similar to that described previously for ICC in the stomach of non-ruminants and therefore they are considered ICC.

Considering the physiological studies reported for other species, we speculate that ICC in cattle should also be involved in gastrointestinal motility as a link between nerve fibers and smooth muscle cells. The evaluation of the normal pattern of distribution of ICC in the muscular layer of the stomach of cattle could be an important element for studying motor digestive disorders of ruminants.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for its contents.

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