KIT receptor-positive cells in the bovine corpus luteum are primarily theca-derived small luteal cells

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Abstract

The tyrosine kinase KIT receptor, the proto-oncogene CD117, plays a key role in growth and maturation of oocytes and follicles. Relevant data are sparse for the corpus luteum (CL). We first confirmed the presence of KIT mRNA and KIT protein in bovine CL homogenates. We then localized KIT-positive (KIT\(^+\)) cells in CL sections by immunohistochemistry. At the CL stage of early development, the former theca transforming into capsule/septa showed a strong band-like KIT\(^+\) immunoresponse. In addition, CD45\(^+\) leukocytes in septa included subpopulations of CD45\(^+\)/KIT\(^+\) and CD14\(^+\)/KIT\(^+\) leukocytes as validated by double immunofluorescence localization. At the early secretory stage, KIT\(^+\) cells appeared within the septa/capsule region and in the periphery of the CL parenchyma, there forming a complex network. This was separate from the capillary bed as determined by double staining for CD117 and FVIII-related endothelial cell antigen (FVIIIr). The KIT\(^+\) network coincided with cells positive for cytochrome P450 17\(\alpha\)-hydroxylase, a thecal cell-specific enzyme. The late secretory stage was defined by an advanced manifestation of the KIT\(^+\) network in the CL periphery. At the stage of regression, the KIT\(^+\) network was absent. The CL of pregnancy expressed high levels of KIT mRNA and KIT protein uniformly throughout pregnancy. The KIT\(^+\) immunolocalization revealed small fibroblast-like cells, luteal cells with granules, and clusters of large luteal cells with staining of the cell membrane. We conclude that a majority of KIT\(^+\) cells in the bovine CL are primarily theca-derived small luteal cells, and that a minority represent KIT\(^+\) leukocytes, in some cases KIT\(^+\) monocytes.

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Introduction

The KIT receptor, i.e., the proto-oncogene CD117, belongs to the family of tyrosine kinase growth receptors (Taylor & Metcalfe 2000, Rönnstrand 2004). The extracellular region of the transmembrane KIT receptor contains the binding site of the KIT ligand (stem cell factor, mast cell growth factor) and the domain for KIT dimerization after ligand binding. Various signaling pathways are triggered, e.g., phosphatidylinositol 3\(^\prime\)-kinases, Janus family kinases, Ras/Map kinases, giving rise to multiple cross connections between the pathways downstream of the KIT receptor. Because a coherent response at the gene level results from differently converging signals, the KIT ligand complex controls diverse processes. These mainly comprise cell migration, proliferation, and differentiation as well as suppression of apoptosis during hematopoiesis, melanogenesis, and gametogenesis. These processes are fundamentally disturbed in knockout mice deficient of the KIT gene and mildly affected in mutations of the intracellular receptor kinase domain, or of the Mfg gene, which encodes the KIT ligand (Besmer et al. 1993, Driancourt et al. 2000).

The fetal ovary is extremely sensitive to the KIT ligand complex. In gene-deficient mice, migration of germ cells into the genital ridge, oogenesis, and initiation of folliculogenesis fail or are impeded (Driancourt et al. 2000, Reynaud et al. 2001). The postnatal ovary appears to require the KIT ligand complex at particular steps of follicle growth and maturation. Blocking experiments in premature and mature mice with the anti-KIT antibody ACK2 indicate that the development of primordial follicles into preantral follicles is dependent on the KIT ligand complex (Yoshida et al. 1997). Preantral follicles continue to grow under KIT blockade, whereas antral follicles do not develop by lack of follicular fluid formation. Because superovulated mice develop CL despite the presence of blocking KIT antibody, it is said that the CL might be KIT independent. However, the KIT mRNA has been found in the ovine and porcine CL (Gentry et al. 1998, Brankin et al. 2004), which points to a possible KIT-dependent function. Hence, studies of the localization of the KIT protein in the CL are necessary.
Because the CL encompasses diverse cell types, i.e., steroidogenic and vascular cells, fibroblasts, and leukocytes, it is of special interest to identify KIT expression to particular cell types. Small luteal cells are likely candidates, because they are known to be derived from the theca where KIT transcription takes place (Driancourt et al. 2000). In addition, isolated thecal cells secrete an increased amount of progesterone under KIT stimulation when compared with unstimulated controls (Parrott & Skinner 2000).

An important process of developing mammalian CL is the ingrowth of capillary sprouts from the vascular thecal layer into the avascular granulosa layer (Smith et al. 1994, Schams & Berisha 2004). Growing vessels are considered a vehicle for the dispersion of steroidogenic cells throughout the CL (Murphy et al. 2001). According to the classical concept, granulosa cells develop into large luteal cells, while thecal cells become small luteal cells (O’Shea 1987). During CL formation, the proliferation of both luteal cell types is low. Obviously, the amount of luteinizing granulosa and thecal cells is adequate to perform an endocrine function during the estrous cycle. In the case of pregnancy, the question of luteal cell renewal is unclear. Potentially, new luteal cells differentiate from CL precursor cells into luteal cells under the appropriate stimulus. Such a precursor concept was postulated years ago (Niswender et al. 1985, 2000), but the precursor cells have not yet been found. Because bone marrow precursor/progenitor cells are characterized by KIT expression (Li et al. 2003, Fazel et al. 2006), we undertook to determine the presence or absence of KIT+ cells in the bovine CL and whether changes in pattern are detectable during the estrous cycle and during pregnancy.

**Results**

**KIT mRNA and protein in homogenates of the cyclic bovine CL**

The presence of KIT mRNA in bovine CL was demonstrated by RT-PCR analysis. The amplification product was a single band of the expected 465 bp lengths, verified for all CL stages (Fig. 1A). Western blots of CL homogenates showed a band at 148 kDa, which was the characteristic size of the KIT protein. The KIT signal seemed to be high in the CL of late secretory stage and low in the developing and regressing CL (Fig. 1B).

**KIT+ cells in the cyclic CL by single and double immunolocalization**

At the early developmental stage, infoldings of the former follicular wall were still apparent giving rise to parenchymal ‘lobules’ of luteinizing granulosa and thecal cells. Parts of the infolded thecal cell layer shaped the capsule and the others were incorporated between the ‘lobules’ and turned into connective tissue septa. Immunoreactivity of KIT in the former thecal cell layer appeared as a dense KIT+ band at the very periphery of the CL (Fig. 2A). In addition, the outer thecal layer not included in the remodeling process displayed islands of KIT+ cells, most probably leukocytes (compare Figs 2B and 5B). The KIT+ islands were often found in the vicinity of venules. Both theca-derived venules and arterioles repeatedly showed a KIT+ endothelium (Fig. 2B and C). The specificity of immunostaining was demonstrated by a preabsorption control, which abolished the reaction (negative control), and by KIT+ oocytes in primary follicles as positive control (Fig. 2D, inset).

At the early secretory stage, the KIT+ cell band of thecal origin was not detectable. It had segregated into KIT+ cells of fibroblast-like shape to be seen in the septum and its boundary to the parenchyma, and additionally into a prominent KIT+ network at the periphery of the CL parenchyma (Fig. 3A–D). There the KIT+ cells looked like small luteal cells, which often completely surrounded the pale, large luteal cells. Occasionally, KIT+ leukocytes were observed amid the luteal cells. At the late secretory stage, this KIT+ network conspicuously expanded towards the interior, yet stayed delimited from the inner CL. KIT+ cells were completely absent from the central region of the CL. The KIT+ fibroblast-like cells abundant in the septa of early secretory stage were replaced by a few scattered KIT+
cells in the late luteal CL (Fig. 3E). These changes in distribution of KIT+ cells were a reliable parameter to differentiate between the early and late secretory stages. Again, the endothelium of septal vessels exhibited KIT+ staining. All the described staining patterns disappeared by the use of preabsorbed KIT antiserum (not shown).

At the early regression stage, marked by the appearance of arterioles, the KIT+ network in the periphery of

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**Figure 2** Immunolocalization of KIT+ cells in paraffin sections of the bovine corpus luteum at the developmental stage using the polyclonal anti-KIT antibody A-4502. (A) A distinct band-like staining is observed in the very periphery of the corpus luteum (arrow) coinciding with the previous theca. (B) The outer thecal cell layer not included into the reshaping process shows KIT+ cell islands, most likely leukocytes, found close to a venule with moderate KIT+ endothelial cells. (C) The medium-sized arteriole in a septum clearly shows KIT+ endothelial cells. (D) KIT-positive staining is abolished when the KIT antibody is pre-incubated with an excess of blocking peptide (negative control). (D inset) KIT+ oocytes are noted in primary follicles (positive control). Scale bars as indicated.

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**Figure 3** Immunolocalization of KIT receptor-positive cells in paraffin sections of the bovine corpus luteum at the luteal and regressing stages using the polyclonal KIT antibody A-4502. (A) At the early secretory stage, the septum is rich in KIT+ cells and a network of KIT+ cells outlines the outer zone of the CL parenchyma; the inner zone lacks positive cells (star). (B) Similar to (A); KIT+ cells of various sizes are apparent. (C) At the late secretory stage, the network of KIT+ cells extends, yet stays delimited from the inner unstained zone. (D) Similar to (C); a few KIT+ cells have remained in the septum (arrows) when compared with (B). (E) The conspicuous network consists of KIT+ small luteal cells (arrows) surrounding large cells. (F) At the stage of regression, some KIT+ cells have the morphology of leukocytes (arrow). The large cells display a diffuse granular-like KIT+ immunoreponse, which here is barely detectable. Scale bars as indicated.
CL was missing (Fig. 3F). Instead, the now dominating cell type, the large luteal cells, acquired moderate KIT staining with pronounced cytoplasmic granules. The CL of regression also contained a few KIT+ leukocytes, which were scattered throughout the CL and often gathered in the vicinity of small blood vessels. The KIT+ staining including the 'granules' and the KIT+ leukocytes, disappeared by the use of preabsorption control (not shown).

At the secretory stage, large luteal cells were regularly surrounded by KIT+ small-sized cells (Fig. 3E). To identify their origin as steroidogenic thecal cells, double immunofluorescence localization was performed for KIT and CYP17. The results revealed a similar cell pattern at the early secretory stage (Fig. 4A and B). We observed some overlapping responses, which might be explained by KIT being primarily expressed at the cell membrane and CYP17 in the perinuclear zone (Fig. 4C). The specific staining for CYP17 was validated by CYP17-positive thecal cells in an antral follicle (Fig. 4D).

Double immunofluorescence localization was also performed to differentiate between KIT+ cells and FVIIIr expressing endothelial cells. These results convincingly showed two interwoven, but not overlapping networks in the periphery of CL parenchyma (Fig. 5A). As already shown by single immunostaining (Fig. 2B and C), KIT+ endothelial cells of arterioles, capillaries, and venules were restricted only to the capsule and the septa (not shown). We concluded that the KIT+ network represented a separate identity apart from the capillary bed of the CL and that KIT+ cells, being small luteal cells, were derived from steroidogenic thecal cells.

Single immunostaining with the KIT antibody marked beside luteal cells also leukocytes recruited into the CL, as we had identified by their morphology. Double immunofluorescence for the pan leukocyte marker CD45 and KIT antigen was performed to identify a subpopulation of KIT+ leukocytes in the CL. At the developmental stage, the CD45 staining detected all the leukocytes, which were preferentially localized at high density within the septal region (Fig. 5B). A subpopulation of CD45+ cells also displayed KIT+ staining as revealed by the orange–yellow color of the overlapping fluorochromes (Fig. 5B and C). The KIT+/CD45− luteal cells preferentially localized to the already described band-like pattern of the former theca. Some of the KIT+ leukocytes were identified as monocytes by positive staining for CD14 (Fig. 5D). At the CL stage of regression, the CD45+ leukocyte population contained only a few KIT+/CD45+ leukocytes (not shown).

**KIT message and protein in homogenates of the bovine CL of pregnancy, KIT immunolocalization**

Comparing early, middle, and late trimester of pregnancy, both KIT mRNA signal of 465 bp and the protein band of 148 kDa were present. The signals seemed to

![Image](https://example.com/image.png)

**Figure 4** Double immunofluorescence localization was conducted with cryosections of the bovine corpus luteum (early secretory stage) to co-localize KIT+ cells (Cy3 secondary, red) with cytochrome P450 17a-hydroxylase (CYP17, Cy2 secondary, green). (A and B) The KIT+ network (red) is comparable in arrangement with the CYP17-positive cells (green). (C) After merging (A) and (B), some overlapping zones are clearly depicted (arrow, yellow–orange). (D) The CYP17-positive thecal cells (arrows) of an antral follicle are considered as positive control. Nuclei are depicted in blue (DAPI stain). Scale bars as indicated.
At the morphological level, the CL of pregnancy displayed arterioles like the regressing CL. Large luteal cells with KIT+ granules dominated the parenchyma as we had already observed in the regressing CL. Low numbers of strongly KIT+, fibroblast-like cells were located between large, low-responsive luteal cells and loosely gathered in proximity to vessels of former septa. Unlike in CL of the secretory stage, these KIT+ cells did not form a network (Fig. 7A and B). We also found clusters of large cells depicted by a distinct KIT+ staining of the cell membrane. Neither small nor large KIT+ cells displayed the additional presence of CYP17 (results not shown). The pattern of immunostaining seemed comparable for early, middle, and late trimester. Preabsorption controls always displayed blank sections.

Finally, Table 1 summarizes location and identity of KIT+ cells in the bovine CL throughout the estrous cycle and pregnancy.

Discussion

The present study comprehensively describes the immunolocalization of KIT+ cells in the bovine CL and reports on changes in the distribution pattern and cell types during the estrous cycle and pregnancy. Findings are relevant to the physiological alterations of...
KIT+ endocrine cells in the bovine CL and to the recruitment of KIT+ leukocytes into the CL. Our results thus significantly add to the body of knowledge on KIT+ cells in the CL. In the sheep, KIT+ cells were detected in the CL by in situ hybridization without giving reference to the CL stage (Gentry et al. 1998). In the pig, different estrous cycle stages were examined, detecting low expression of KIT mRNA at the developmental stage and high expression at the secretory stage (Brankin et al. 2004). We are therefore careful about the current finding on a strong KIT mRNA signal in the CL of developmental stage as long as the quantitative assessment of KIT mRNA is missing. Our primers for RT-PCR analysis of KIT mRNA were based on GenBank accession no. D16680 as published by Kubota et al. (1994), thereafter confirmed by two other published bovine sequences (GenBank accession nos AF263827 and XM_612028). The amplified region of the bovine KIT gene is homologous to KIT of other species, but is different from related members of the platelet-derived growth factor receptor subfamily. Moreover, we also found the KIT protein in homogenates of the cyclic CL with likely elevated expression at the secretory stage and in the CL of pregnancy.

An immunohistochemical approach was used to identify the KIT-expressing cells and patterns in the bovine CL. The technique of Hoyer et al. (2005) was used, who carried out KIT antigen retrieval by microwave treatment at pH 9.0. It became evident that KIT+ cells exist in changing CL compartments. At the developmental stage, we found a strong KIT+ band-like pattern of expression in the former thecal layer. According to other groups, thecal cells of antral follicles express both KIT+ mRNA and KIT protein (Motro & Bernstein 1993, Parrott & Skinner 1997, Driancourt et al. 2000, Brankin et al. 2004). Obviously, the KIT expression persists after ovulation in the theca-derived capsule/septum in

**Figure 7** Immunolocalization of KIT+ cells in paraffin sections of the bovine corpus luteum of late trimester pregnancy using the polyclonal KIT antibody A-4502. (A) KIT+ cells (arrows) are scattered around the small septum (star). (B) Small KIT+ cells (arrows) are reminiscent of fibroblasts. (C) A cluster of large luteal cells shows KIT+ cell membranes (arrows). (D) A subgroup of large luteal cells display granule-like KIT+ structures (arrows). Scale bars as indicated.

**Table 1** Summary of location and identity for KIT+ cells in the bovine corpus luteum belonging to the four major cell types, i.e., luteal cells, vascular cells, fibroblasts.

<table>
<thead>
<tr>
<th>CL stage</th>
<th>Capsule/septum</th>
<th>Peripheral zone</th>
<th>Inner zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>Band-like cell pattern, groups of poly- and mononuclear leukocytes, endothelial cells</td>
<td>Scattered poly- and mononuclear leukocytes</td>
<td>None</td>
</tr>
<tr>
<td>Secretory stage</td>
<td>Fibroblast-like cells, endothelial cells</td>
<td>Developing network of small luteal cells, a few leukocytes</td>
<td>None</td>
</tr>
<tr>
<td>Early</td>
<td>A few fibroblast-like cells, endothelial cells</td>
<td>Stabilized and delimited network of small luteal cells, a few leukocytes</td>
<td>None</td>
</tr>
<tr>
<td>Late</td>
<td></td>
<td>Luteal cells with granules, leukocytes</td>
<td>As in the peripheral zone</td>
</tr>
<tr>
<td>Regression</td>
<td></td>
<td>Large luteal cells with granules, cell clusters with positive cell membranes, fibroblast-like cells</td>
<td>All over large luteal cells with granules</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
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</tbody>
</table>
fibroblast-like cells and in small luteal cells. In situ hybridization studies in the ovine CL also detected KIT mRNA in small luteal cells (Gentry et al. 1998). During the secretory stage of the bovine CL, KIT+ fibroblast-like cells reside in the septum close to blood vessels and might also be recruited into the parenchyma, probably under KIT ligand stimulation. Recruitment of KIT+ stromal cells to primary follicles after KIT ligand stimulation has been found in the cortex of the adult bovine ovary (Parrott & Skinner 2000).

Apart from vascular cells, the thecal layer consists of steroidogenic cells and fibroblasts, both of which are candidate cells for the small KIT+ cells in the bovine CL. We here clearly show by double-immunostaining of KIT and the thecal cell-specific enzyme CYP17 that the KIT+ network in the peripheral zone of the CL was derived from the steroidogenic thecal cells. The presence of CYP17-positive cells in the CL’s periphery seems to be a ruminant-specific feature (Conley et al. 1995).

In the bovine CL of secretory stage, the small-sized KIT+ cells form a peripheral network surrounding the large luteal cells. Using double-immunostaining of the CL, we verified that the FVIIIr-positive microvascular bed is separate from the KIT+ network. Since neovascularization of the CL has its origin in the invading theca, the intimate vicinity of both networks would allow for a molecular crosstalk between small KIT+ luteal cells and sprouting endothelial cells. It is conceivable that this cellular arrangement is engaged in the replenishment of capillaries in the CL. Recent studies of induced angiogenesis identified perivascular fibroblasts and smooth muscle cells with stromal-derived factor 1 expression being responsible for the retention of recruited proangiogenic cells in peripheral tissues (Ruiz de Almodovar et al. 2006). Among other retention candidates, KIT and KIT ligand are also discussed (Quesenberry et al. 2005, Grunewald et al. 2006). In the inner CL zone, which is free of KIT+ cells, different factors might be necessary to stabilize the microvascular bed. For example, angiopoietin-1 and its specific receptor Tie-2, which control blood vessel stabilization and are abundant in the mid-luteal phase of the human CL, might be involved (Sugino et al. 2005).

The complete disappearance of the KIT+ network at the stage of regression, characterized by degradation of the capillaries, also indicates a KIT function in neovascularization of CL. Examination of the localization of the KIT+ network suggests that early in luteolysis, the KIT+ cells undergo programmed cell death, or luteolytic factors, e.g., prostaglandin F2α, vasoactive peptides, etc. (Schams & Berisha 2004), influence KIT expression and processing.

In the CL of pregnancy, large luteal cells are dominant, which is indicative of a high steroidogenic output. Some of them display a KIT+ cell membrane, possibly indicating de novo induction of KIT. Alternatively, the KIT+ large cells have originated from small KIT+ cells, which reside close to the small septum. We could not confirm this transformation by the presence of CYP17 positivity in KIT+ cells (unpublished) and therefore deduce that the thecal cell-specific marker is missing in the CL of pregnancy. This is in line with the literature describing lack of CYP17 expression in the bovine CL (Rodgers et al. 1986, Conley et al. 1995). Our observation revisits the discussion of whether small thecal cells remain small cells or alternatively transform into large luteal cells (Niswender et al. 1985, 2000). The small KIT+ cells with a rather diffuse immunostaining might represent theca-derived cells and contribute to cell renewal in pregnancy.

At present, a new concept is emerging that KIT+ cells from the bone marrow contribute to the self-healing potential of the body. These bone marrow-derived KIT+ cells migrate for angiogenic repair into a remote organ such as ischemic hindlimb, myocardium after infarction, and injured femoral artery, there secreting angiogenic cytokines (Li et al. 2003, Fazel et al. 2006, Wang et al. 2006). This regenerative mechanism may have a physiological counterpart in the vascularization of the CL. We have described that macrophage-like cells from bovine antral follicles proliferate in co-culture with granulosa cells (Spanel-Borowski & Ricken 1997). Others have isolated immature, hematopoietic cells from the ovarian murine stroma expressing high KIT levels. In culture, these cells retained long-term proliferative capacity and eventually differentiated into CD45+ monocytes/macrophages (Pascual et al. 2005). In the developing bovine CL, we have also reported on the correlation of microvessel sprouting and the recruitment of CD18-positive leukocytes into the capsule/septa area (Spanel-Borowski et al. 1997). Of note, 90% of all leukocytes have been defined as eosinophils, which do not colonize together with mast cells (Reibiger & Spanel-Borowski 2000). It can thus be deduced that <10% of the CL invading leukocytes are CD14+ monocytes containing a KIT+ subpopulation. It is assumed that these particular monocytes might be derived from the bone marrow, which contains KIT+ hematopoietic progenitor cells in the bovine system (Hikono et al. 2001). The KIT+ leukocytes might provide a local pool of progenitor cells, which could ensure recruitment of cells for angiogenesis, steroidogenesis, or production of CL-specific signals like growth factors, chemokines, etc.

Our findings convincingly demonstrate KIT+ small luteal cells, which have originated from the former thecal layer and which persist in the CL periphery. In addition, a subpopulation of KIT+ monocytes are detectable in the septum/capsule area of the bovine CL of early development. Further studies are needed to corroborate whether, similar to the promotion of follicular development by the KIT-KIT ligand complex (Carlsson et al. 2006, Hutt et al. 2006), the CL life cycle also depends on KIT and KIT ligand-expressing cells.
**Materials and Methods**

**Ovaries**

Ovaries with CL were removed from non-pregnant and pregnant cows immediately after death at the local abattoir. Cyclic CL stages were classified at the macroscopical level according to Ireland et al. (1980). Development, early and late secretory stage, and early regression were finally confirmed by immunostaining for factor VIII-related antigen (FVIIIir) to reveal differences in the microvascular bed (Ricken et al. 1995). For collection of CL during pregnancy, uterine horns were opened and the fetus removed for measurement of the crown–rump length (Sakumoto et al. 2000): trimester I, fetal crown–rump length 6–20 cm; trimester II, 25–45 cm; and trimester III, 50–80 cm. The samples were transported in PBS (pH 7.4) to the laboratory. Each CL was dissected free from cortical tissue; samples were either snap frozen in liquid nitrogen and stored at −80 °C or were pretreated with microwaves in 0.02 M Tris (pH 9.0) and then cooled to room temperature (RT). Endogenous non-specific binding sites were blocked by 0.05% SDS. After further homogenization by ultrasonification, samples were centrifuged twice at 16,000 × g (13,000 r.p.m.) at 4 °C for 15 min. Cell lysates were diluted 1:1 in sample buffer (250 mM Tris–HCl (pH 6.8) containing 4% SDS, 10% glycerol, and 2% β-mercaptoethanol) and denatured at 95 °C for 5 min. Protein content of cell lysates was determined by the bicinchoninic acid assay using bovine albumin as standard (Pierce, Rockford, IL, USA). Proteins (15 μg/lane) were separated by SDS–7.5% PAGE and transferred to a nitrocellulose membrane by electroblotting. Upon blocking non-specific binding sites with 5% non-fat milk for 30 min, blots were incubated with the 1:3000 diluted anti-KIT rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight. The final incubation was performed with a horseradish peroxidase-labeled donkey anti-rabbit secondary antibody (1:6000; Genovia, Hamburg, Germany) for 2 h. Immunoreactive proteins were visualized using a chemiluminescence kit and Hyperfilm (Amersham Pharmacia Biotech). Equal protein loading was confirmed by stripping the blots and reprobing with a monoclonal anti-GAPDH antibody (1:10,000; Research Diagnostics, Flanders, The Netherlands).

**RNA isolation, cDNA synthesis, and RT-PCR analysis**

Frozen CL were crushed into powder with a pestle and mortar in liquid nitrogen, and total RNA was isolated using the peqGOLD RNAPURE isolation kit (PEQLAB GmbH, Erlangen, Germany). Genomic DNA was digested with 0.02 U DNase/μg RNA (Roche Molecular Biochemicals). RNA of 5 μg was taken to synthesize cDNA using a first-strand cDNA synthesis kit from Amersham Pharmacia Biotech by using of oligo-dT primers in a 15 μl reaction volume. RT-PCR technique was used to amplify a KIT mRNA fragment from bovine CL cDNA (Table 2). KIT primers were designed using the GenBank deposited Bos taurus sequences (no. D16680). Primers did not match to the sequences of the platelet-derived growth factor receptor subfamily. Each 25 μl amplification reaction contained 2.5 μl 10× concentrated PCR buffer (15 mM MgCl2), 0.3 U Taq DNA polymerase (Roche Molecular Biochemicals), 100 μM dNTPs (Perkin–Elmer, Weiterstadt, Germany), 0.1 μM of each primer, and 1 μl sample cDNA. According to our standard laboratory protocol, the product was amplified by 40 cycles as 46 bp amplicons. Amplified fragments were cloned into pGEM-T (Promega). Clones harboring cDNA inserts of expected sizes were isolated and sequenced. Subsequent identification of the cDNA inserts was obtained by interrogation of GenBank (BLAST search). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), amplified as 566 bp fragments, was used for equal loading. RT-PCR analysis was carried out without sample cDNA as negative control (not shown).

**Western blotting**

Crushed CL were homogenized in 1 ml ice-cold homogenization buffer (pH 7.4) composed of 20 mM HEPES, 1 mM EDTA, 0.2 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5% SDS. After further homogenization by ultrasonification, samples were centrifuged twice at 16,000 × g (13,000 r.p.m.) at 4 °C for 15 min. Cell lysates were diluted 1:1 in sample buffer (250 mM Tris–HCl (pH 6.8) containing 4% SDS, 10% glycerol, and 2% β-mercaptoethanol) and denatured at 95 °C for 5 min. Protein content of cell lysates was determined by the bicinchoninic acid assay using bovine albumin as standard (Biorad, Hercules, CA, USA). A monoclonal anti-GAPDH antibody (1:10,000; Research Diagnostics, Flanders, The Netherlands).

**Immunohistochemistry for KIT localization**

The technique of Hoyer et al. (2005) was performed with minor modifications using antigen retrieval and Envision method (DakoCytomation, Glostrup, Denmark). We selected the rabbit anti-human CD117 antiserum (A5402, DakoCytomation), because comparative evaluations attested this product’s high specificity and sensitivity (Lucas et al. 2003, Vakiani et al. 2005). Additionally, the antiserum was successfully applied to reveal KIT + Cajal cells in the bovine gut (Marquez et al. 2006).

Paraffin sections (7 μm) were mounted on poly-l-lysine coating glass slides, dewaxed, and washed 3 × with TBS buffer (0.05 M Tris (pH 7.6), 0.15 M NaCl) containing 0.01% Nonidet P-40 (TBS/ NP40). To unmask antigens, CL sections were pretreated with microwaves in 0.02 M NaCl containing 0.02 M Tris (pH 9.0) and 0.5 mM EDTA as follows: 800 W for 5 min, 100 W for 15 min, and then cooled to room temperature (RT). Endogenous

**Table 2** Bovine primer for mRNA detection of KIT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

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<td>TGCCAGCCCCAGCATCGAAG</td>
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*aAnnealing temperature used in reaction. bPCR product length.*

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peroxidase activity was quenched with 0.45% H$_2$O$_2$ in TBS/NP40 for 15 min at RT. Sections were washed 1× in TBS with 0.125% Tween (TBS/Tween), 2× in TBS, and incubated in 10% normal goat serum in TBS/NP40 at RT to block non-specific binding for 30 min. The primary antiserum was diluted (concentration 100 μg/ml) in PBS with 0.25% BSA (A-4378, Sigma–Aldrich) and applied at 4°C overnight. To control the specificity of KIT staining, the antiserum was incubated with 100× molar excess of the antigenic peptide (PP-1518, LAB VISION Corp., Fremont, CA, USA) at 4°C overnight, the immunoprecipitates were removed by repeated centrifugation, and the supernatants applied to sections. Next day, slides were successively washed 3× in TBS/Tween and TBS alone, and then incubated with DakoCytomation Envision System (Labelled Polymer-HRP Anti-rabbit, K4002; DakoCytomation) at RT for 30 min. Sections were washed and incubated with DakoCytomation Envision System successively washed 3 and the supernatants applied to sections. Next day, slides were...


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