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RET receptor in the gut of developing cat

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ABSTRACT

RET receptor is a transmembrane protein which, together with the glial-cell-line derived neurotrophic factor family receptors alpha, forms a receptor complex upon activation by the glial-cell-line-derived neurotrophic ligands (GFLs). RET signaling is crucial for: (a) development of the enteric nervous system and kidney; (b) development of sympathetic, parasympathetic, motor, and sensory neurons; (c) postnatal maintenance of dopaminergic neurons; (d) spermatogenesis. In humans, RET mutations cause the Hirschsprung's disease, characterized by megacolon aganglionosis, and different types of cancer, the multiple endocrine neoplasia type 2A and type 2B and familial medullary thyroid.

In the earliest aged cat embryos studied (stage 9 according to Knopse), RET immunoreactivity (IR) was observed in few cells detected in bilateral rows extending latero-ventrally to the neural tube and dorsolaterally to the foregut. In the successive aged group (stage 11), RET IR was observed in few single or grouped epithelial cells of the anterior gut and in small clustered cells scattered in the mesenchyme around the anterior gut. From stage 14–22 (the last stage 22 includes foetuses around the birth), RET IR was seen in neurons and fibers of the enteric nervous system. The appearance and intensification of RET-IR in the gut occurred with cranio/caudal and external/internal directions during the development.

These results, thus, suggest the involvement of GFLs in the neuroblast migration, proliferation and differentiation. For a short period of development, these molecules might also act on some cells of the epithelium.

The tyrosine kinases receptor (RET) is characterized by the presence of an extracellular ligand-binding domain, a transmembrane segment and an intracellular region containing the tyrosine kinase catalytic domain. RET is alternatively spliced, producing at least two isoform: a short isoform, or RET9 and a long isoform, or RET51. RET, together with the glial-cell-line derived neurotrophic factor family receptors alpha (GFR alpha- 1–4) form a receptor complex upon activation by the glial-cell-line-derived neurotrophic ligands, which encompass glial-cell-line derived neurotrophic factor, neurturin, artemin and persephin. RET signaling is crucial for the development of the enteric nervous system (ENS), as suggested by studies conducted in *Ret* gene deleted mice (Schuchardt et al., 1994), in which the ENS fails to form. In humans, inactivating mutations in RET cause Hirschsprung's disease characterized by megacolon aganglionosis. In contrast, activating mutations give rise to different types of cancer, multiple endocrine neoplasia type 2A and type 2B, familial medullary thyroid (for a review see Runeberg-Roos and Saarma, 2007).

The RET appearance and the description of the distribution during gut development have been performed in early stages of humans (Attiè-Bitach et al., 1998), mouse (Pachnis et al., 1993; Avantaggiato et al., 1994; Young et al., 1999), rat (Tszuki et al., 1995), avian (Schiltz et al., 1999; O'Donnel and Puri, 2008) and zebrafish (Marcos-Gutièrrez et al., 1997) embryos and also its function appears to be phylogenetically conserved (Heanue and Pachnis, 2008; Burzynski et al., 2009). In this study we aim to investigate the presence of RET in the gut of developing cat, by using extensively aged samples.

Cat embryos aged according Knospe (2002) were removed from ovariohysterectomized pregnant queens, as described in our previous study (Lucini et al., 2008). Fixation was made by immersion in Bouin's fluid for 12–24 h at room temperature (RT). Embryos of stage 9 (15–17 days), 11 (17–18 days), 14 (21–23 days) and 16 (25–28 days) were fixed *in toto* whereas the foetuses of stage 19 (25–28 days) were fixed after decapitation. The gastroenteric apparatus was removed from foetuses at stage 22 (55 days approximately) and fixed. The specimens were dehydrated in ethanol series and embedded in paraffin wax, and then cut in 7 μm-thick sections.

Peroxidase–antiperoxidase (PAP) method (Sternberger, 1986) was used for immunohistochemical staining, as previously described by Lucini et al. (2008). The primary antisera employed were: (a) rabbit polyclonal antibody against C-terminus of RET isoform C (RET 9) of human origin (1:200; Sc-167 Santa Cruz Biotechnology); (b) goat polyclonal antibody against C-terminus of RET

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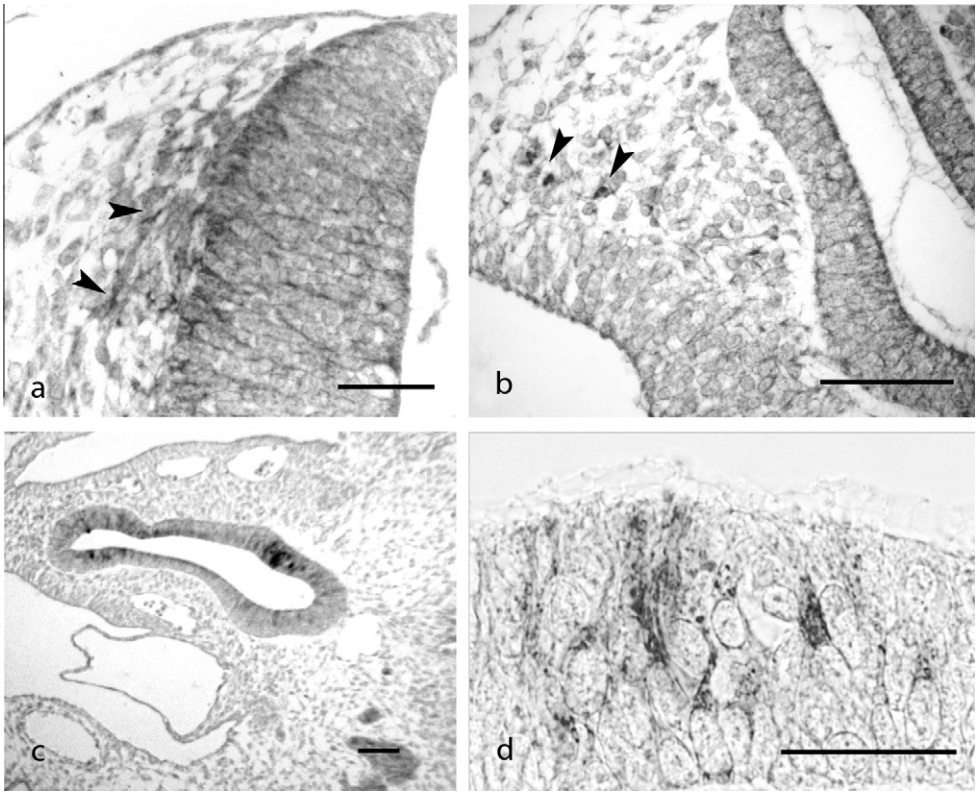

Fig. 1. RET IR in the gut of early cat embryos. Stage 9: positive cells (arrowheads) aligned in a row placed laterally to neural tube (a) and to the foregut (b). Stage 11: single or grouped epithelial cells of the foregut at low (c) and high (d) magnification. Scale bar = 50 μ a–c, Scale bar = 25 μ d.

isoform A (RET 51), a of human origin (1:200; Sc-1291 Santa Cruz Biotechnology).

Immunocytochemical stainings were photographed using a Leica microscope DM RA2 connected to a Leica DC 300F camera for light microscopy and stored in a Leica IM 1000 archive.

Immunocytochemical staining specificity was tested by successively substitution of the primary or secondary antiserum or the PAP complex with normal serum or PBS in repeated trials. Adsorption controls were also made by using homologous (RET, added to antiserum up to 25 μg, sc167 P Santa Cruz Biotechnology) and heterologous (Fibroblast growth factor receptor 1, added to antiserum up to 50 μg ml, sc-121 P Santa Cruz Biotechnology) antigen.

The antiserum to the short isoform RET 9 (RET C19) worked well on sections of all stages embryos, whereas the antiserum to the long isoform of RET 51 (RET T20) did not stain any embryos section. So, for simplicity's sake, we report in the test RET IR as immunoreactivity to the short isoform RET 9.

In the embryos at stage 9 RET IR was observed in few cells detected in bilateral rows extending in the mesenchyme latero-ventrally to the neural tube (Fig. 1a) and dorso-laterally to the foregut (Fig. 1b). The cells were elongated in shape and showed weak RET IR in the perinuclear cytoplasm or in the cytoplasmic processes.

In the embryos at stage 11 RET IR was observed in few single or grouped epithelial cells (Fig. 1c and d). They were mainly elongated in shape and showed positivity in the perinuclear cytoplasm or in the basal and apical regions (Fig. 1d). Moreover, RET IR was also detected in grouped small, round cells scattered in the mesenchyme of the anterior gut (data not shown).

In the embryos at stage 14, RET IR was detected in nervous cells of the gastro-intestinal wall (Fig. 2a–c). Specifically, in the gastric region (Fig. 2a) RET IR was observed in neurons localized along the two main plexuses, the myenteric and the submucous plexus. In the intestinal wall (Fig. 2b and c) RET immunopositive cells were prevalently detected in the myenteric plexus and rarely also in the submucous plexus.

In the embryos at stage 16, RET IR was observed in the two main plexuses of both gastric and intestinal walls (Fig. 2d and e). The myenteric and submucous plexuses appeared formed by ganglia with numerous intensely RET positive neurons joined by positive fibers.

In the foetuses at stage 19 and 22, RET IR appeared in intensely stained neurons and fibers of the myenteric (Fig. 2f and g) and submucous ganglia, and in fibers decurring along the myocells of the circular muscle layer or between the myenteric and submucous plexuses by crossing the circular muscle layer.

No reaction was observed in controls performed by substituting the primary antibodies with PBS, normal serum or antibody adsorbed by the homologous antigen. On the contrary, the reaction was not modified by the substitution of primary antibodies with antibodies adsorbed by heterologous antigen.

This study reports the presence of RET IR in the cat gastrointestinal apparatus during development, from 15–17 days of gestation to approximately 55 days of gestation. RET receptor was present: (a) in cells scattered in the mesenchyme around neural tube and gut primordium at very early stage; (b) transiently in cells of anterior intestinal epithelium of earlier embryos and (c) steadily in developing ENS.

In all studied embryos, only the short isoform of RET was detected. These findings are consistent with the results previously reported in the cat pancreas (Lucini et al., 2008) and in accordance with previous data reporting that RET9 and RET51 do not associate with each other (Attiè-Bitach et al., 1998; Tsui-Pierchala et al., 2002; Scott et al., 2005). Moreover mice lacking RET51 seem to be normal, whereas mice lacking RET9 have kidney abnormalities and enteric aganglionosis (for a review see Sariola and Saarma (2003), Heanue and Pachnis (2008)).