Activation of MAP Kinase (ERK1/2) in human neonatal colonic enteric nervous system

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Abstract

The aim of this study was to examine MAP kinase (ERK1/2) activation in the human neonatal colonic enteric nervous system. For this, we investigated by immunocytochemistry the cellular localization of phosphorylated ERK1/2 (P-ERK) in a series of normal human colon samples removed from newborns and in patients with intestinal obstruction such as Hirschsprung's disease (HSCR), stenosis and atresia. We checked the presence of P-ERK in the 3 distinct histological layers of normal colon. P-ERK was detected in the colonic mucosa, in the enteric nervous system and in endothelial cells. In the mucosa from normal colon, P-ERK was detected at the upper part of the crypt, while P-ERK activation in epithelial cells is altered in HSCR, stenosis and atresia. In the normal colon, strong P-ERK staining was detected in myenteric and submucosal enteric plexuses. Using confocal microscopy analyses, we observed that P-ERK staining was localized in enteric glial cells and not in enteric neurons. Strong P-ERK staining was also observed in plexuses from stenosis and atresia whereas in HSCR, hypertrophic nerve fibres were not stained.

The motility of the gastrointestinal tract is ensured by the correct coordination of the visceral smooth muscle cells and the autonomous enteric nervous system. The enteric nervous system (ENS) originates from neural crest cells that migrate from the dorsal region of the neural tube and colonize the whole gut to establish its innervation.[1] At week 4, the neural crest-derived cells migrate into the gut mesoderm layer and colonize the gut along a cranio-caudal wave. These cells reach the hindgut by week 7, form two cluster rings and finally will differentiate into enteric neurons and glial cells.[2]

Intestinal obstructions in infants comprise a wide group of heterogeneous diseases with clinical symptoms ranging from simple constipation to intestinal occlusion. Intestinal obstruction may be due to mechanical diseases such as stenosis, stricture, atresia or to non-mechanical diseases of which Hirschsprung disease (HSCR) is a particular case. HSCR is due to an absence of the enteric nervous system along certain lengths of the bowel. In addition to the absence of ganglion cells, which constitutes the diagnostic criteria, HSCR is characterized by the presence of numerous hypertrophied nerve fibres. Despite the low prevalence, 1/5,000 live births[3], HSCR is the best described of the congenital neural abnormalities of the intestinal tract and has been widely studied.[3] The discovery that HSCR was due to a failure of migration of cells derived from the neural crest cells, has lead to the identification of molecular and cellular events essential for the normal development of the enteric nervous system (ENS).[1]

MAPK extracellular signal-regulated-kinases, ERK1 (p44) and ERK2 (p42) are pivotal compounds of intracellular phosphorylation cascades from the cytoplasm to the nucleus recruited for growth factor signal transduction.[4] Several signalling pathway ligands such as Fibroblast growth factor (FGF), Epidermal growth factor (EGF), Platelet-derived growth factor (PDGF) or Transforming growth factor β (TGFβ) are know to initiate receptor tyrosine kinase (RTK)[5] and mitogen-activated protein kinase (MAPK) activation.[4] After extracellular stimulation, ERK1/2 are both activated by dual phosphorylation (termed P-ERK)

by the mitogen-activated kinase MEK1 and 2.[5] Such phosphorylation strongly increases ERK1/2 activity, induces its nuclear translocation and subsequently triggers specific gene expression programs. In addition, activated ERK1/2 in neurons can stay in the cytoplasm to act locally.[6]

Since published studies concerning MAPK activation report the detection of ERK1/2 in cultured intestinal epithelial cell lines[7], central neurons[8] and prospective and nascent hepatic endoderm of mouse embryo[9], we investigated the cellular localization of P-ERK by immunocytochemistry and immunofluorescence in the 3 distinct histological layers of normal human colon samples, paying particular attention to enteric plexuses. Also, we checked the presence of P-ERK in the enteric nervous system in cases of intestinal obstruction such as HSCR, stenosis and atresia.

Materials and Methods

Human tissues

Tissue samples were obtained from 19 newborns, aged 12 days to 2-months old and are summarised in Table 1. Normal samples were right colon specimens from 5 neonates undergoing ileocolic resection for congenital cystic duplication of the terminal ileum. In eight patients, after HSCR was diagnosed, rectosigmoïdectomy was performed and full-thickness large bowel specimens were collected in the aganglionic as well as in ganglionated zones. Full-thickness large bowel samples were also isolated from six patients undergoing segmental left colectomy for mechanical stenosis, in 4 cases of post-necrotizing enterocolitis (NEC) stenosis and 2 cases of atresia.

Immunohistochemistry

Paraffin embedded sections (3 μm thick) were immunostained by standardized automated procedures using a Dako autostainer (Universal staining system, Dakocytomation, Trappes, France), as previously described.[10] Antigen retrieval was achieved by heating at 98°C for 1h in 1mM EDTA (pH 9.0). P-ERK, (#4376, Clone 20G11, Cell Signaling, USA), a rabbit polyclonal antibody which detects phosphorylated forms of ERK1 and ERK2 MAP Kinases was used diluted 1:100 as primary antibody. Immunohistochemistry control experiments were performed by excluding the primary antibody (data not shown). In the competition experiments, phospho-p44/42 MAPK blocking peptide (Cell Signalling, Boston, MA, USA) was added to the primary antibody incubation, diluted 1:50.

For immunofluorescence, de-paraffined slides after antigen retrieval (1h at 98°C in 1mM EDTA, pH 9,0) were incubated 30 min at room temperature with P-ERK (1:100 dilution) and one of the primary antibodies: HuC/D (mouse, clone 16A11, Abcam, UK) diluted 1:20, synaptophysin (mouse, clone SY38, DAKO, Denmark) diluted 1:10, c-Kit (mouse, clone CD117, Zymed, USA) diluted 1:100, glial marker S100 (mouse, clone 4C4.9, Clinisciences, France) diluted 1:100 and α-Smooth Muscle Actin (mouse, clone 1A4, Sigma, USA) diluted 1:400. After washing, slides were incubated 30 min with Alexa 488 anti-mouse (Invitrogen, France, 1:2000 dilution) and Alexa 555 anti-rabbit (Invitrogen, France, 1:2000 dilution). Cells were rinsed again, mounted in FluorSave reagent (Calbiochem, Germany).

Tissues were visualized under a laser confocal microsope (Leica SP2). Fluorescence from each channel was captured sequentially to avoid cross-talk between channels. Excitation of Alexa 488 was from an argon laser (488 nm) and emitted light was collected through a band pass filter (512 nm). Alexa 555 was from a helium-neon laser (excitation 555 nm) and light collected via a band pass filter (beyond 560 nm). No cross-talk between channels was detected with these settings.

Results

Immunodetection of P-ERK in normal colon

Full-thickness large bowel specimens revealed, from the inside to the outside, (Fig. 1A): the mucosa, the submucosa and the muscularis externa, composed of inner circular and outer longitudinal smooth muscle layers. The enteric nervous system consisted of 2 types of plexuses: submucosal (Meissner's) and myenteric (Auerbach's) plexuses, characterized by the presence of ganglion cells, nerve fibres and glial cells.[11] P-ERK was detected in all layers of the normal colon (Fig. 1C) and the specificity of the staining is indicated in the absence of detectable positivity after pre-incubation with the blocking peptide (Fig. 1B). In the mucosa, P-ERK staining was observed only in the luminal surface of the epithelium (arrowhead in Fig. 1D), whereas the deep parts of the crypts were negative. In the submucosa and the muscularis plexuses, P-ERK was detected in both cytoplasmic and nuclear cellular compartments (arrows in Figs. 1E, F and 2J). P-ERK staining was also detected in capillary endothelial cells (arrowhead in Fig. 1E). Smooth muscle fibres of the intestinal wall and surrounding the vessels were not stained (Fig. 1F). The staining pattern of P-ERK in myenteric (Figure 2) and submucosal (Figure 3) plexuses was also investigated by immunofluorescence studies. Double labelling using P-ERK and HuC/D, a neuron specific RNA-binding protein, showed staining for HuC/D in nerve cell bodies (Figs. 2B, C and 3B, C) but not for P-ERK (Figs. 2A, C and 3A, C). Double labelling using P-ERK and c-Kit, an interstitial cells of Cajal marker, indicated no P-ERK/c-Kit colocalization in myenteric plexuses as c-Kit positive signals were only observed outside the plexuses (Fig. 2D-F). Double labelling experiments using P-ERK and synaptophysin, a presynaptic vesicle marker, demonstrated that the nerve fibers were stained by synaptophysin but were negative for P-ERK (Fig. 2G-I). In addition, synaptophysin-positive stainings corresponding to intramuscular nerves fibres were also observed in the circular muscularis layer whereas this staining was not observed with P-ERK

(data not shown). In double labelling experiments using P-ERK and S100, a glial marker, nerve cells bodies were not stained. Stainings for both P-ERK and S100 colocalized in glial cells in myenteric (Fig. 2J-L) and submucosal plexuses (Fig. 3D-F). In addition, P-ERK was observed in some glial cell nuclei which were also positive for S100 (arrowhead in Fig. 2L). Taken together, these results indicate (i) specific P-ERK staining in enteric plexuses in normal colon, (ii) colocalization with S100 in enteric glial cells and (iii) not in nerve cell bodies or nerve fibres.

Immunodetection of P-ERK in intestinal obstruction

HSCR

The histological appearance of the colon in HRSC patients and the expression of P-ERK in ganglionated and aganglionated segments are shown in Figure 4. In aganglionated segments, submucosal and muscular hypertrophic nerve bundles typical of HSCR were not labelled with P-ERK (arrows in Fig. 4F, H), whereas P-ERK staining was observed in plexuses in the ganglionated segment (Fig. 4E, G). P-ERK was detected in capillary endothelial cells in both regions (arrowheads in Fig. 4E, F and H). It is interesting to note that, in the mucosa of both segments, the luminal surface of epithelium and also many cells deeper within the crypts were stained for P-ERK (arrowheads in Fig. 4C, D). This staining pattern was observed in 6 out of the 8 cases studied. Smooth muscle fibres from the longitudinal and circular muscularis were not stained in ganglionated segments, whereas in aganglionated regions, heterogeneous staining of the longitudinal layer was observed (data not shown).

Colonic stenosis and atresia

In cases of post-NEC stenosis, the calibre of the intestinal lumina was reduced and a fibrosis associated with mild inflammation in the submucosa was observed (Fig. 5A). In

atresia, these fibro-inflammatory changes extended in the remaining mucosa, with complete occlusion of the lumen (Fig. 5B). In both situations, plexuses were easily identified especially in the muscularis where they were much more numerous, like a string of beads (arrowheads in Fig. 5A, B). Strong P-ERK staining was detected in the submucosal and myenteric neuronal plexuses of both stenosis (Fig. 5E, G) and atresia (Fig. 5F, H). Endothelial cells were positive (arrowheads in Fig. 5E, F). In the mucosa of stenosis, P-ERK staining was detected throughout the glandular epithelium (arrowheads in Fig. 5C) whereas in that of atresia, no staining was observed as no epithelium was present (asterik in Fig. 5D). This staining pattern was observed in all cases studied. Circular and longitudinal smooth muscle layers remained unstained (Fig. 5D, G, H). Double labelling using P-ERK and α-SMA, a marker for smooth muscle cells, indicated no co-localization. However, both markers were detected in capillary vessels with α-SMA in the muscular wall (Fig. 5I, K) and P-ERK in endothelial cells (Fig. 5J, K).

Discussion

The aim of this study was to examine the MAP kinase (ERK1/2) activation in human normal colon, paying particular attention to enteric plexuses. In addition, MAP kinase activation was assessed in the enteric nervous system from patients with intestinal obstruction due to loss of enteric neurons (HSCR) and mechanical diseases (post-NEC stenosis and atresia). Colon samples were chosen from a homogeneous series of newborn patients so as to minimize variations due to age, which is critical for the maturation of the enteric nervous system.

With regard to mucosa in normal colon, P-ERK staining was found to be restricted to the luminal part of the epithelium. In stenosis and, to a lesser extent in HSCR, P-ERK was in contrast detected throughout the epithelium. Despite the absence of data concerning the

expression of ERK in human colon, Boucher and colleagues demonstrated that constitutive activation of MEK/ERK signalling in a human intestinal cell line can produce either senescence or forced mitogenesis.[7] Our results are in agreement with those findings. In normal colon, P-ERK was detected in epithelial cells which are known to be much more differentiated and/or committed to undergo apoptosis[12], whereas in basal regions, proliferating cells were negative.

With regard to P-ERK detection in capillary vessels, our results are in agreement with a previous study performed on human brain blood vessels.[13] P-ERK staining was only detected in endothelial cells whereas smooth muscle cells from the media were unstained.

In spite of the fact that ERK1/2 activation in the central nervous system has been shown to be involved in many neuronal events including cell survival, synaptic remodelling and various specialized functions[14], to our knowledge, no data exist on the activation of ERK1/2 in enteric plexuses from normal human colon. With regard to enteric plexuses, our results are in agreement with previous findings in mouse embryos, where P-ERK was demonstrated in neural crest cells migrating away from the neural tube.[15] In plexuses from normal colon like in those from post-NEC stenosis and atresia specimens, our results indicate that ERK activation occurs in glial cells but not in neurons (Figure 2). Also, it is interesting to note that the remaining nerve fibres in HSCR aganglionated segments were not stained, suggesting the absence of ERK activation in these glial cells[16]. In addition, ERK activation was observed in some glial cell nuclei, similar to that of S100[17]. This indicates that, its function in these cells may be to modulate signal propagation to the nucleus and/or to stimulate gene expression in glial cells.

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Figure Legends

Fig. 1: Immunodetection of P-ERK in whole normal colon specimens. (A) H&E stained overall view of the intestinal wall: mucosa (asterix), submucosa (arrowhead), muscularis (arrow). (B) Negative immunostaining control using P-ERK and phospho-p42/44 MAPK blocking peptide. P-ERK immunostaining (C, D, E, F): (C) overall view of the intestinal wall, (D) mucosa: P-ERK staining in the luminal surface of the epithelium (arrowhead) and absence of staining in the deep parts of the crypts (arrow); (E) submucosa and (F) muscularis: staining in capillary endothelial cells (arrowhead), unstained ganglion cells nuclei (arrows). Scale bars: A, B, C, D: 300 µm; E, F: 50 µm.
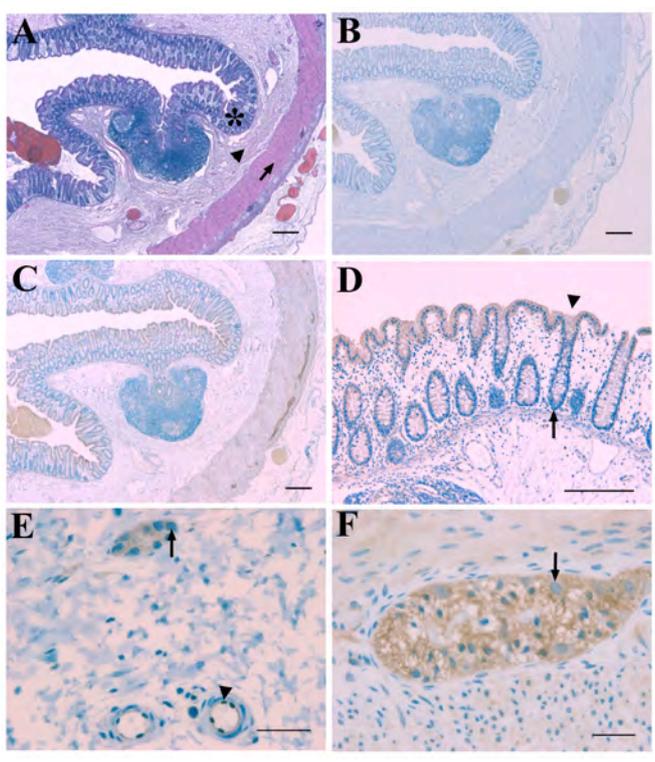
Fig. 2: Immunodetection of P-ERK, HuC/D, synaptophysin and S100 in normal colon myenteric plexuses. (A-C) Double labelling with P-ERK and HuC/D. (A) P-ERK: absence of staining in ganglion cell bodies (arrow), (B) HuC/D: staining of ganglion cell bodies, (C) P-ERK/HuC/D Merge. (D-F) Double labelling with P-ERK and c-Kit. (D) P-ERK, (E) c-Kit, (F) P-ERK/c-Kit Merge. (G-I) Double labelling with P-ERK and synaptophysin. (G) P-ERK: absence of staining in the nerve fibres (arrow), (H) synaptophysin: staining in the nerve fibres and absence of staining in ganglion cell bodies (arrow), (I) P-ERK/synaptophysin Merge. (J-L) Double labelling with P-ERK and S100. (J) P-ERK and (K) S100: staining in glial cell (glial nuclei, arrowheads) and absence of staining in ganglion cell bodies (arrows), (I) P-ERK/S100 Merge. Scale bar: 50 µm.
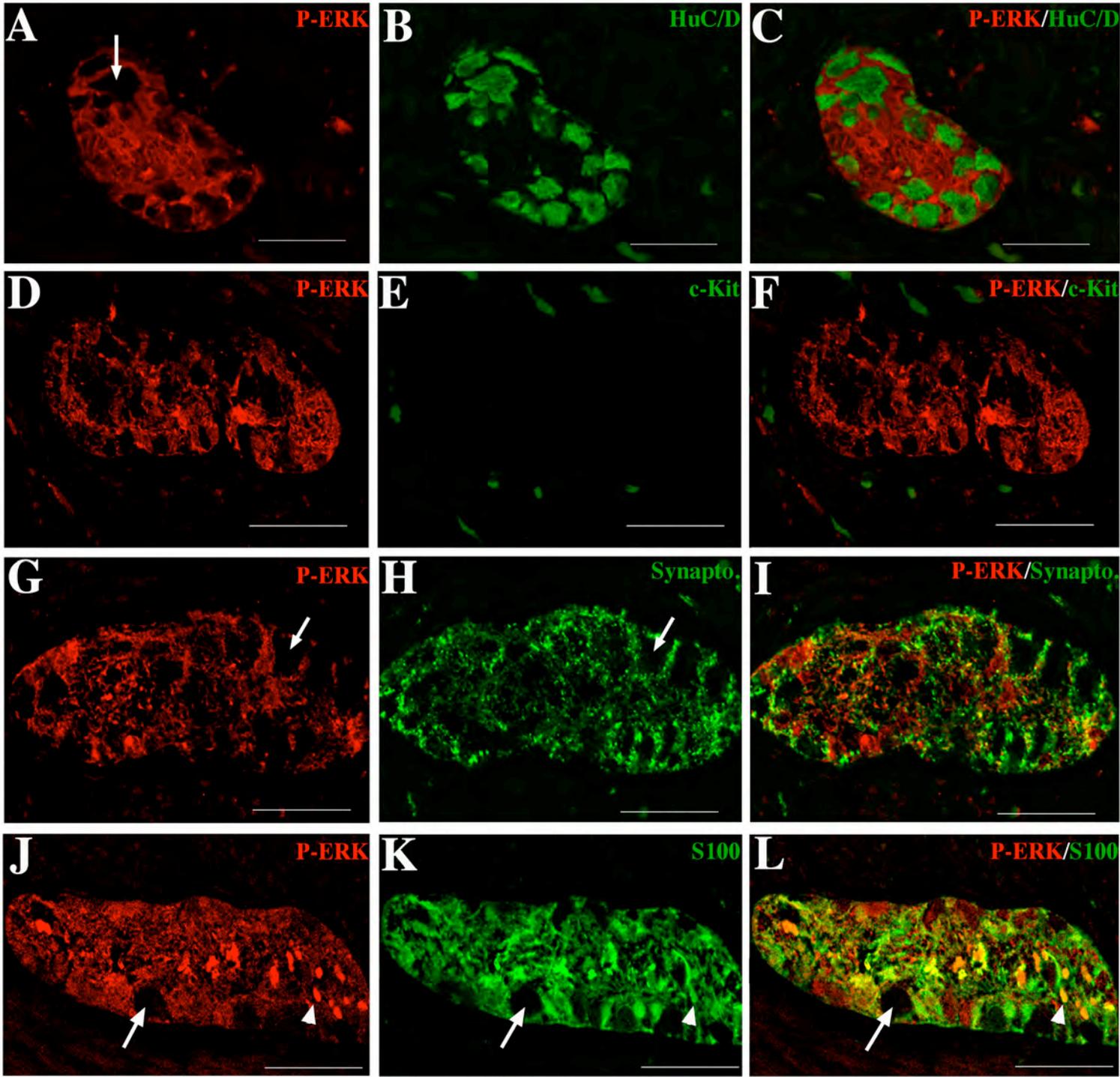
Fig. 3: Immunodetection of P-ERK, HuC/D and S100 in normal colon submucosal plexuses. (A-C) Double labelling with P-ERK and HuC/D. (A) P-ERK: absence of staining in ganglion cell bodies (arrow), (B) HuC/D, (C) P-ERK/HuC/D Merge. (D-F) Double labelling with P-ERK and S100. (D) P-ERK and (E) S100: staining in glial cell (arrowheads), (F) P-ERK/S100 Merge. Scale bar: 50 µm.
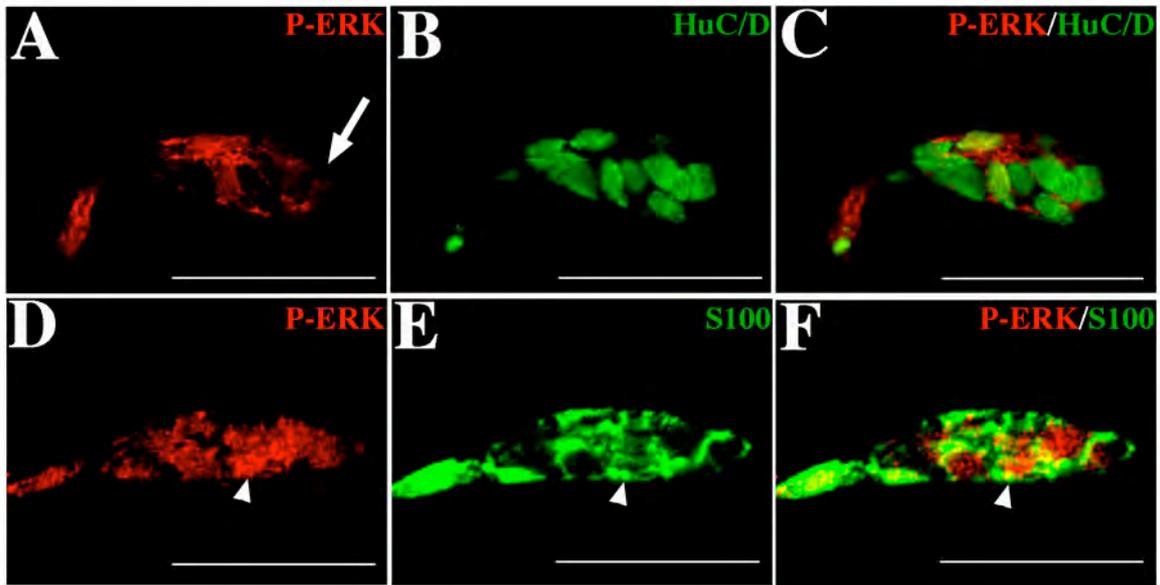
Fig. 4: Immunodetection of P-ERK in HSCR. Overall H&E view of the intestinal wall in (A) ganglionated and (B) aganglionic colon specimens. P-ERK mucosal staining in ganglionated colon (C) and aganglionic colon specimens (D): positive cells (arrowheads) at the luminal surface of epithelium and deeper within the crypts. P-ERK submucosal and muscularis staining in ganglionated colon (E, G) and aganglionic colon specimens (F, H): positive staining in capillary endothelial cells (arrowheads), absence of staining of hypertrophic nerve bundles (arrows). Scale bars: A, B, C, D: 300 µm; E, F, G, H: 50 µm.
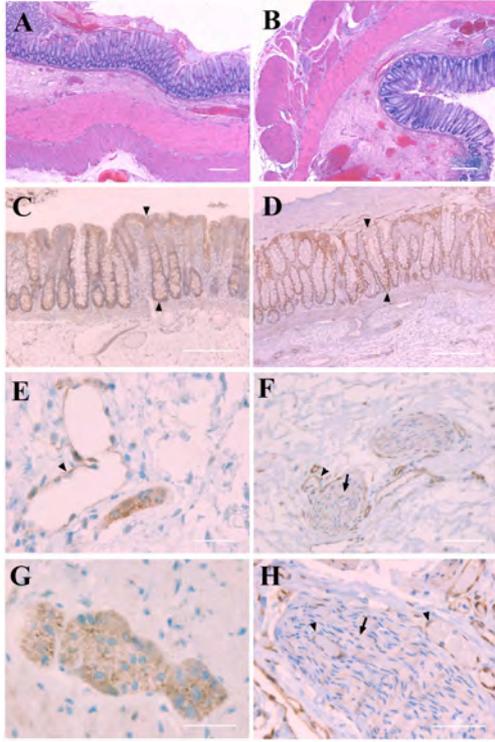
Fig. 5: Immunodetection of P-ERK in stenosis and atresia. Overall H&E view of the intestinal wall in stenosis (A) and atresia (B). (C) P-ERK mucosal staining in stenosis: positive cells (arrowheads) at the luminal surface of epithelium and deeper within the crypts. (D) P-ERK submucosal staining in atresia: lumen (asterix). P-ERK submucosal and muscular staining in stenosis (E, G) and atresia (F, H): positive staining in capillary endothelial cells (arrowheads), absence of staining in muscle fibres (arrows). (I-K) Double labelling with α-SMA and P-ERK. (I) α-SMA: staining of the visceral (arrow) and vascular (arrowhead) smooth muscle cells. (J) P-ERK: staining of enteric plexus (arrow) and endothelial cells (arrowhead). (K) α-SMA/P-ERK Merge. Scale bars: A, B: 150 μm, C-K: 50 μm.
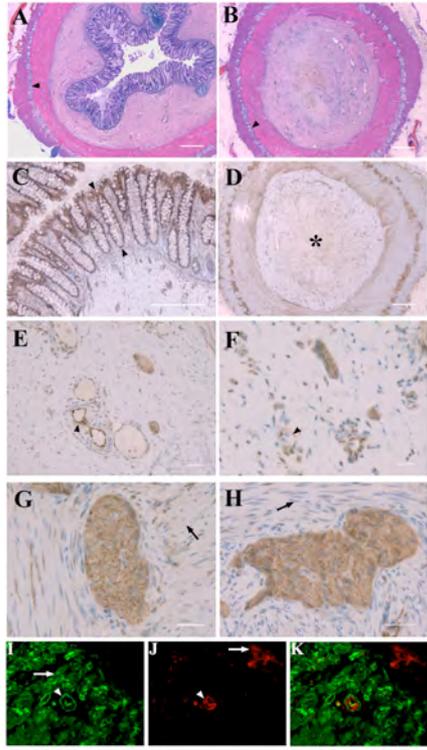
Table 1. Human neonate colon samples.

Normal colon	**5**
HSCR	**8[a]**
Stenosis	**4**
Atresia	**2**

[a] The eight samples from patients with HSCR were collected both in aganglionic and in proximal ganglionated colon.