Expression of bone morphogenetic protein signaling pathway players in the jejunum and colon of adult rats

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ABSTRACT

The bone morphogenetic protein (BMP) pathway, which plays a crucial role in the control of intestinal epithelial cell homeostasis, has been studied in mice and humans, leading to an understanding of its involvement in several intestinal pathologies. However, the expression and localization of the various actors (ligands, antagonists, receptors) of this pathway remain unknown in the rat intestine, although this species is widely used in pathophysiology studies. Here, we aimed to determine the expression and localization of the various players in the BMP pathway in the jejunum and colon of the rat using RT-qPCR and immunohistochemistry. BMP2, mainly localized in epithelial cells, was the most expressed ligand in the jejunum and colon in comparison with BMP4, BMP6 and BMP7. We showed for the first time that BMP7 was highly expressed in epithelial cells in both tissues. BMP2, BMP6 and BMP7 ligands were also present in the enteric nervous plexuses, as the BMP receptors and antagonists Noggin and Chordin-like 1. The expression of BMP antagonists and ligands in enterocytes and mature colonocytes could suggest a paracrine or autocrine feedback modulation at the cellular level. Finally, all the studied BMP actors were present in colonic vessel walls including GREM1, a BMP antagonist described as pro-angiogenic and also being a ligand for VEGFR receptors. These data provided a good correlation between the observations in rats compared to those in humans and highlighted the importance of the BMP pathway not only in the intestinal epithelium, but also in both the enteric nervous system and vascular system. Our work lays the foundations for further studies on the involvement of the BMP pathway in rat models of intestinal pathophysiology.

Key words: BMP pathway; ligands; antagonists; receptors; adult rat; jejunum; colon.

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Introduction

Bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily, are multifunctional cytokines that play a crucial role in regulating developmental processes such as embryogenesis, neurogenesis and bone formation.[1] These growth factors are also involved in the homeostasis and function of numerous tissues and organs during adulthood, such as the brain and the intestinal tract.[2,3] Various isoforms of BMP ligands have been described and subdivided into distinct subgroups according to their receptor specificity: BMP2/4, BMP5/6/7/8a/8b, BMP9/10, BMP12/13/14 and BMP15.[4] In the canonical BMP pathway, the signal transduction mechanism begins with the binding of a BMP dimer (homodimer or heterodimer) to an heterotetrameric complex composed of two type I and two type II receptors.[4] After the binding, the type II receptor (BMPR2, ACVR2a, ACVR2b) phosphorylates and activates the type I receptor (ACVR1, BMPR1a, BMPR1), which determines the specificity of further signal transduction. Here, the SMAD1/5/8 transcription factors act as effectors between the cytoplasm and the nucleus. These proteins are phosphorylated by type I receptor and recruit the SMAD4 common effector to form a transcriptional complex able to migrate and translocate into the nucleus to regulate directly or indirectly, *via* interaction with transcription factors, the different pathway targets: *Runx2, Id1, Id2, etc.*[5,6] Within a tissue, the BMP signaling pathway is modulated by the expression of specific ligands and receptors but also by the expression of antagonists that bind to BMP ligands and prevent them from accessing their receptors.[7] These antagonists include molecules such as Noggin, Chordin-like 1 and Gremlin (GREM1 and GREM2). Secretion of BMP ligands and antagonists in close proximity often leads to the formation of gradients that modulate the amplitude of BMP signaling. In the intestinal tract, BMP2 and BMP4 are the most frequently detected ligands in humans and mice.[8-10] BMP6 and BMP7, less often cited, are also thought to be present.[11,12] In addition, several studies reported that the antagonists GREM1, GREM2 as well as the BMPR2 receptor, were highly expressed, at least at the base of human colon crypts.[13,14] BMP signaling is involved in the morphogenesis of crypts and villi during intestinal development, as well as in the formation of the enteric nervous system and the muscular layers of the intestinal wall.[2,15-18] In adults, the BMP pathway takes part in the control of intestinal epithelial cell proliferation, differentiation and apoptosis, as well as in the regulation of the intestinal immune system, the enteric nervous system and intestinal motility.[2,19,20] As a result, deregulation of this signaling pathway is associated with the pathogenesis of several intestinal diseases, including colorectal cancer, inflammatory bowel disease (IBD), constipation and Hirschsprung's disease.[2,20-23] In colorectal cancer, for example, mutations in SMAD4 and BMPR2 have been implicated in the development of juvenile polyposis syndrome and in sporadic colorectal carcinoma.[24-26] Increased GREM1 expression is also thought to be responsible for a form of hereditary colorectal cancer.[27-29] The BMP4 ligand, on the other hand, may play a fundamental protective role in regulating inflammation in IBD.[30] Therefore, this signaling pathway is increasingly being explored to investigate the development of various intestinal pathologies. The study of these diseases (irritable bowel syndrome, IBD, colorectal cancer, *etc.*) requires preclinical models developed in various species, and in particular in rats. However, little is known about the presence and localization of BMP pathway players in the intestinal tract of this species. The aim of the present study was therefore to determine the expression and localization of the main players in the BMP pathway (ligands, antagonists, receptors) in the adult rat intestine.

Materials and Methods

Animals

Rat tissues were obtained from six-week-old Fischer males (Charles River, Saint-Germain-Nuelles, France). Animal experiment was authorized by the French Ministry for Higher Education, Research and Innovation (MESRI) in accordance with the local Ethic Committee evaluation (APAFIS#2023112014134680). Animals were euthanized by cervical dislocation. For RT-qPCR analyses, samples of jejunal and colonic tissue were recovered and placed in FastPrep® 2 mL Lysing Matrix tubes (MP Biomedicals, Illkirch, France) then placed in liquid nitrogen before being stored at -80°C until extraction. For immunohistochemistry (IHC) studies, jejunal and transverse colon samples were harvested and fixed in 10% neutral buffered formalin solution (Sigma Aldrich, L'Isle-d'Abeau Chesnes, France) for 24 h at room temperature before being embedded in paraffin.

RT-qPCR

RNAs were extracted from tissues with Tri reagent (Molecular Research Center, Cincinnati, OH, United States). Total RNA samples (1 µg) were then converted to cDNA using the iScript Reverse Transcription Supermix (Biorad, Marnes-la-Coquette, France). Primers for Sybr Green assay (ONEGreen® FAST qPCR Supermix, Ozyme, Saint-Cyr-l'Ecole, France) were designed with PrimerQuest Tool (Integrated DNA technologies) and are described in Table 1. Amplification was performed using a Quant

Table 1. Primer sequence of the genes for RT- qPCR analysis.

Gene	Forward primer	Reverse primer
BMP2	ACGGACTGCGGTCTCCTAAA	GGGAAGCAGCAACACTAGAAGA
BMP3	CAGCCGCAGGAACTCTTCAAA	CTCAGACTTGGTTAGAGAAGTCAGATTA
BMP4	GAGCCAACACTGTGAGGAGTTT	CTGGGATGTTCTCCAGATGTTCTT
BMP6	GACAGCGCTTTCCTCAACGA	CGTACTCCACCAGGTTCACAAA
BMP7	CTTCCCTCTGAACTCCTACATGAAC	GTGAACCAGTGTCTGGACGATAG
ACVR1	GAAGATGAGAAGCCCAAGGTCAA	GCCCTCACACACACACATGTAA
ACVR2a	AAGCAAGGTTGTTGGCTGGA	ACAATCAGTCCTGTCATAGCAGTT
ACVR2b	CTATTGCCCACAGGGACTTCAA	GGTCGCTCTTCAGCAGTACATT
BMPR2	TCCCAATGGATCTCTGTGCAAAT	ACCCAATCACTTGTGTGGAGAC
GREM1	GATCCACTGAGGTGACAGAATGAA	AGCAAAGCTCCTACGGTGTATG
Chordin-like 1	CCACCATGAACCAGTGGAAGAT	GCTGAGCTGAGCTTCTCCTTC
POLR2a	CGGCGTCCTGAGTCCG	AACTTGGGGGACTAATGGATCC

Studio 5 Real-Time PCR System (Applied Biosystems). Final data were normalized to the level of the RNA Polymerase II Subunit A (POLR2A) and analyzed with LinRegPCR Software.

Immunohistochemistry

Immunostaining was carried out with ImmPRESS® HRP Horse Anti-Rabbit or Anti-Mouse IgG Polymer Detection Kit (MP 7801 or MP7802; Vector Laboratories, Les Ulis, France). Briefly, after a phase of tissue rehydratation, endogenous peroxidase was inhibited using BLOXALL® Blocking Solution (Vector Laboratories). After incubation in 2.5% normal horse serum for 20 min, primary antibodies properly diluted in Animal-Free Blocker solution (Vector Laboratories) were applied at 4°C overnight (Table 2). The next day, after washing slides, secondary antibody ImmPRESS Polymer Reagent (either anti-rabbit or anti-mouse) was added for 30 min. After several PBS baths, revelation was carried by Vector DAB (3,3'-Diaminobenzidine) substrate. The sections were then counterstained with hematoxylin (Biognost, Zagreb, Croatia) and mounted. Negative control tissue sections were prepared by omitting the primary antibody. Sections were examined by light microscopy (Leica DM 2000) using 10x and 20x objectives. Images were captured using a Leica DFC7000 T Digital Microscope Camera and Leica LAS X software. The analysis was carried out on the jejunum and colon of 6 animals. Each section of jejunum and colon was observed in its entirety, and 5 fields per section were meticulously analyzed.

The expression of each marker was assessed by a semi-quantitative method according to five levels: -, negative; +/-, very weak or patchy positivity; +, weak positivity; ++, moderate positivity; +++, strong positivity. Tables 3 and 4 report the average observations obtained for 6 rats.

Statistical analysis

Statistics analyses were finally performed using GraphPad Prism 10.2.3 (403). For RT-qPCR results, a one-way ANOVA (Tuckey or Dunnett's post-tests) was carried out to evaluate the difference in expression between the different actors of a same class (ligands / antagonists / receptors / effectors) in the jejunum and the colon.

Results

BMP ligands in rat intestine

We investigated the localization of BMP ligands in the jejunum and colon of 6 rats using IHC staining. IHC analyses are summarized in Tables 3 and 4. The presence of BMP2, 4, 6 and 7 was observed in the intestine of all rats.

Table 2. Primary antibodies used for immunostaining.

Antibody	Dilutions	Cat No.
BMP2	1:200	Abcam - ab14933
BMP3	1:200	GeneTex - GTX65951
BMP4	1:600	Genetex - GTX100875
BMP6	1:300	Clinisciences - CPA4353
BMP7	1:300	Clinisciences - CPA1101
ACVR1	1:1000	ProteinTech - 67417 - 1 - Ig
BMPR2	1:100	ProteinTech - 19087 - 1 - AP
GREM1	1:400	Bioss - bs-1475R
Chordin-like 1	1:50	Affinity - DF14928
Noggin	1:300	FineTest - FNab05782

Table 3. Immunohistochemical localization of BMP ligands, BMP receptors (ACVR1 and BMPR2) and antagonists in rat jejunum.

Rat jejunum		BMP2	BMP4	BMP6	BMP7	ACVR1	BMPR2	BMP3	Noggin	Chordin-like1	GREM1
Mucosa	Enterocytes	+++	+/-	+	++	+++	+++	+	+++	+	+/-
	Lamina propria	++	+/-	++	+	++	++	+	++	+	+/-
	Muscularis mucosae	0	0	0	0	0		0	0	0	0
Submucosa	ECM, mesenchymal and immune cells	0	++	0	0	0 (only few cells)	+/-	0	0	0	+/-
	Vessel walls					+	+	0	0	0	+/-
	Submucosa plexus	0	0	++	+	+	+	0	+	+	+
Muscular layers	Circular muscular layer	+/-	0	0	0	Few cells	0	0	0	0	0
	Myenteric plexus	+ (around)	0	+++	+/-	+	+/-	0	+/-	+/-	0
	Longitudinal muscular layer	+	0	0 (+)	+	++	++	Some cells	+	++	0

Jejunum segments were extracted from 6 adult rats, fixed and analyzed by immunohistochemistry. BMP3 acting as an antagonist has been classified with the latter. ECM, extracellular matrix; -, negative; +/- , very weak or patchy positivity; +, weak positivity; ++, moderate positivity; +++, strong positivity.

Table 4. Immunohistochemical localization of BMP ligands, BMP receptors (ACVR1 and BMPR2) and antagonists in rat colon.

Rat colon		BMP2	BMP4	BMP6	BMP7	ACVR1	BMPR2	BMP3	Noggin	Chordin-like1	GREM1
Mucosa	Colonocytes	+++	+/- surface colonocytes	+ surface colonocytes	+++	++	++	+	++	+	+/-
	Lamina propria	++	+	Few cells	0	+	+	+/-	++	+	+/-
	Muscularis mucosae	Diffuse	0	+	+/-	+	+	+	+	++	+
Submucosa	ECM, mesenchymal and immune cells	0	+	0	0	0	Some cells	0	0	0	0
	Vessel walls	+	0	+	+	+	+	+/-	+	+/-	+/-
	Submucosa plexus	0	0	++	+/-	+	+	0	+	+ (+/-)	0
Muscular layers	Circular muscular layer	+/-	A few cells	0 (+)	+/-	+/-	+/-	Some areas	+/-	+/-	+/-
	Myenteric plexus	++ (around)	0	++	++	(+/-)	(+/-)	0	+/-	+/-	0
	Longitudinal muscular layer	+	A few cells	0	A few cells	(+/-)	+	Some cells	+/-	+/-	0 Only a few cells

Colon segments were extracted from 6 adult rats, fixed and analyzed by immunohistochemistry. BMP3 acting as an antagonist has been classified with the latter. ECM, extracellular matrix; -, negative; +/- :, very weak or patchy positivity; +, weak positivity; ++, moderate positivity; +++, strong positivity.

BMP2

Representative images of the immunolabeling obtained using an anti-BMP2 antibody are shown in Figures 1 and 2. In rat jejunum, BMP2 ligands were highly expressed in villous enterocytes (Figure 1 a-c) and were also observed in some crypt enterocytes (Figure 1 a,b). An IHC labeling was detected in the lamina propria of all the sections observed. This BMP2 labeling was particularly intense under the villous enterocytes (Figure 1c). The anti-BMP2 antibody also revealed immunostaining around myenteric nervous plexuses and in the longitudinal muscle layer. Our data indicated a gradient of BMP2 immunostaining in colonocytes along the crypt, where the highest levels of BMP2 protein were observed in the upper third of the crypt, and were more diffuse at the base of the crypt (Figure 2 a,b). The lamina propria exhibited labelling, particularly in the lower third of the crypts. BMP2 immunostaining was also observed in muscularis mucosae and in vessel walls of the submucosa, but with lower intensity than in colonocytes (Figure 1c). As seen in the jejunum, immunostaining with BMP2 antibody was noted around the myenteric plexuses and in the longitudinal muscle layer (Figure 2 d,e).

BMP4

In the jejunum, BMP4 immunoreactivity (Figure 1 d-f) appeared mainly in the submucosa. A very weak immunostaining

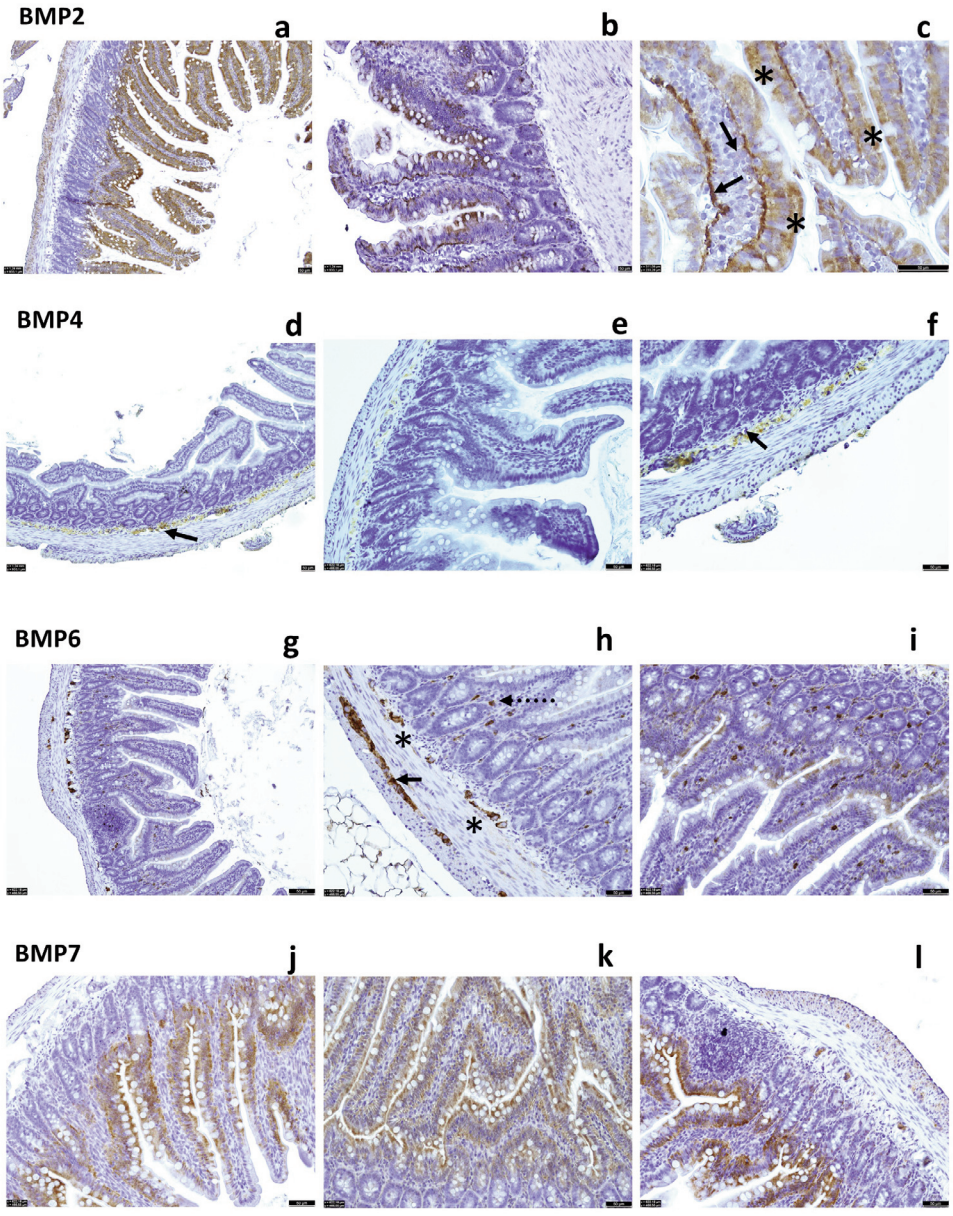

Figure 1. Immunohistochemical localization of BMP2 (**a,b,c**), BMP4 (**d,e,f**), BMP6 (**g,h,i**) and BMP7 (**j,k,l**) in rat jejunum. **c**) The arrows show intense BMP2 immunostaining beneath the enterocytes of the villi and the asterisks indicate villous enterocytes. **d,f**) The arrows show the submucosa. **h**) The arrow indicates a myenteric plexus, and the asterisks designate submucosal plexuses; the dotted arrow shows immunostaining of lamina propria cells. To determine the localization of BMP ligands, we performed immunohistochemical staining on jejunum sections from 6 different rats. Sections were examined by light microscopy (Leica DM 2000) using 20x and 40x objectives. Representative images are shown. Scale bars: 50 µm.

was also observed in the lamina propria. In the colon, BMP4 was expressed in the same locations but with more intense staining. In addition, immunolabeling for BMP4 was found in surface colonocytes (Figure 2 f-h).

BMP6

In the jejunum, BMP6 was moderately expressed in some villous enterocytes (Figure 1i). In contrast, a very strong immunoreaction for BMP6 was observed in cells of the lamina propria in both villi and crypts, as well as in myenteric and submucosal plexuses (Figure 1g-i). In the colon, the anti-BMP6 antibody immunolabelled mature surface colonocytes as well as submucosal and myenteric plexuses (Figure 2 i-k). In addition, we observed a weak and diffuse expression of BMP6 in muscularis mucosae and in submucosal vessel walls.

Jejunal expression of BMP7 was mainly localized in villous enterocytes, in some crypt enterocytes, as well as in submucosal plexuses and longitudinal muscular layer (Figure 1 j-l). Weak BMP7 immunostaining was also present in the lamina propria. In the colon, BMP7 was principally expressed in surface colonocytes, submucosal vessel walls and myenteric plexuses (Figure 2 l-n).

The results of RT-qPCR confirmed the expression of BMP2,

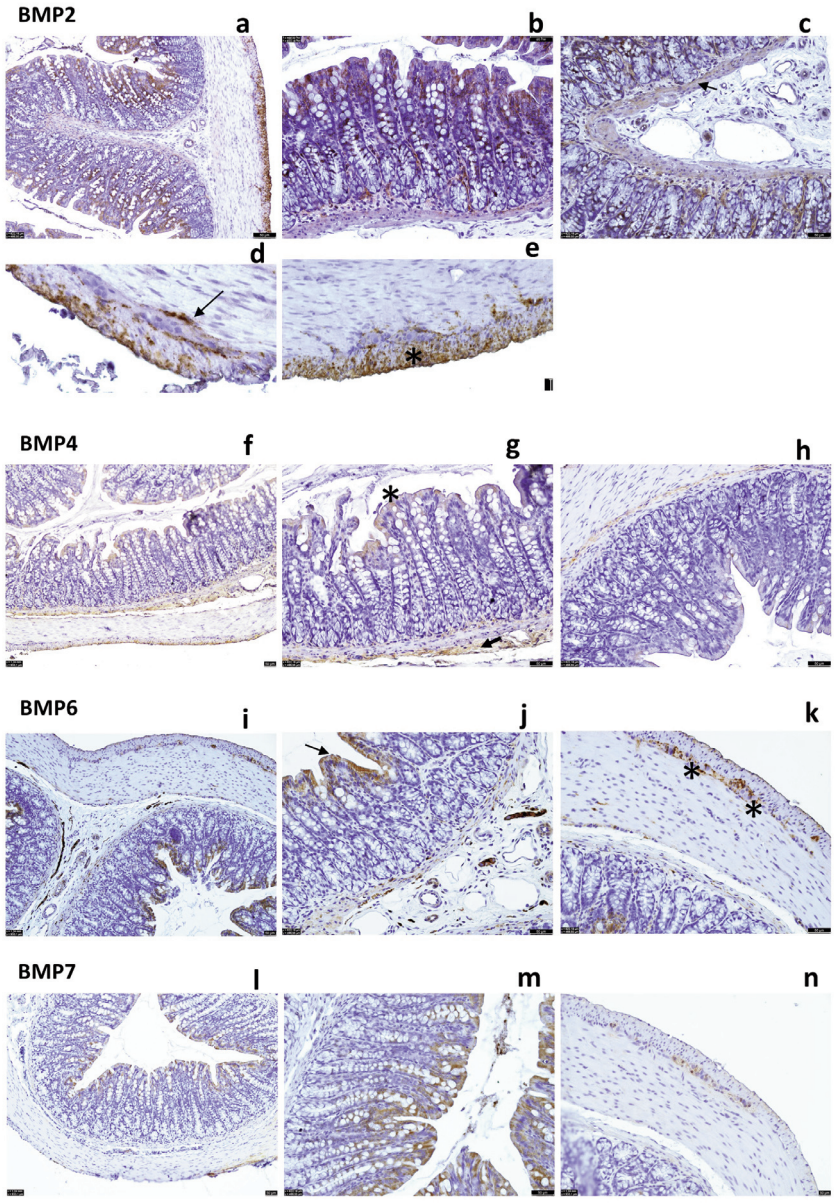

Figure 2. Immunohistochemical localization of BMP2 (**a,b,c,d,e**), BMP4 (**f,g,h**), BMP6 (**i,j,k**) and BMP7 (**l,m,n**) in rat colon. To determine the localization of BMP ligands, we performed immunohistochemical staining on colon sections from 6 different rats. **c**) The arrow shows the muscularis mucosae. **d**) The arrow indicates immunostaining of BMP2 localized around a myenteric plexus. **e**) The asterisk shows the longitudinal muscularis layer of the colon. **g**) The asterisk indicates surface colonocytes and the arrow shows the submucosa. **j**) The arrow indicates mature surface colonocytes. **k**) the asterisks show myenteric plexuses. Sections were examined by light microscopy (Leica DM 2000) using 20x and 40x objectives. Representative images are shown. Scale bars: 50 µm.

Bmp4, Bmp6 and Bmp7 mRNA in both the jejunum and colon tissues of rats. Overall, we observed a similar expression profile for each ligand between the two localizations (Figure 3). In the jejunum and in the colon, *Bmp2* ligand was the most highly expressed ligand compared with *Bmp6* (approximately 5-fold higher in the jejunum and 8-fold higher in the colon, $p<0.0001$), *Bmp7* (approximately 20-fold higher in the jejunum and 10- fold higher in the colon, $p<0.0001$) and *Bmp4* (approximately 20-fold higher in the jejunum and 28-fold higher in the colon, $p<0.0001$).

BMP receptors in rat intestine

We observed a very strong immunoreaction for ACVR1 and BMPR2, with identical localization for both receptors (Table 2 and Figures 4 and 5). In rat jejunum, the enterocytes appeared strongly labeled throughout the villi (Figure 4 a,d); some enterocytes in the crypts also expressed the receptors (Figure 4b). Strong immunostaining was present in the lamina propria, submucosal vessel walls and some submucosal cells (Figure 4e). Finally, immunoreaction for ACVR1 and BMPR2 was localized in submucosal plexuses and longitudinal muscle layer. In the rat colon, we noted lower expression of both receptors. BMPR2 and ACVR1 immunostaining was observed in colonocytes of the upper third of the crypt and in surface colonocytes (Figure 5 a,e). Both receptors were also detected in lamina propria, muscularis mucosae and submucosal nerve plexuses (Figure 5). Data from RT-qPCR confirmed the expression of type I and type II BMP receptors in the jejunum and the colon (Figure 3). The highest and lowest expressions were respectively attributed to *Acvr2b* and *Acvr2a* in the two localizations. Otherwise, the relative mRNA expression for each receptor was globally the same whether located in the colon or in the jejunum.

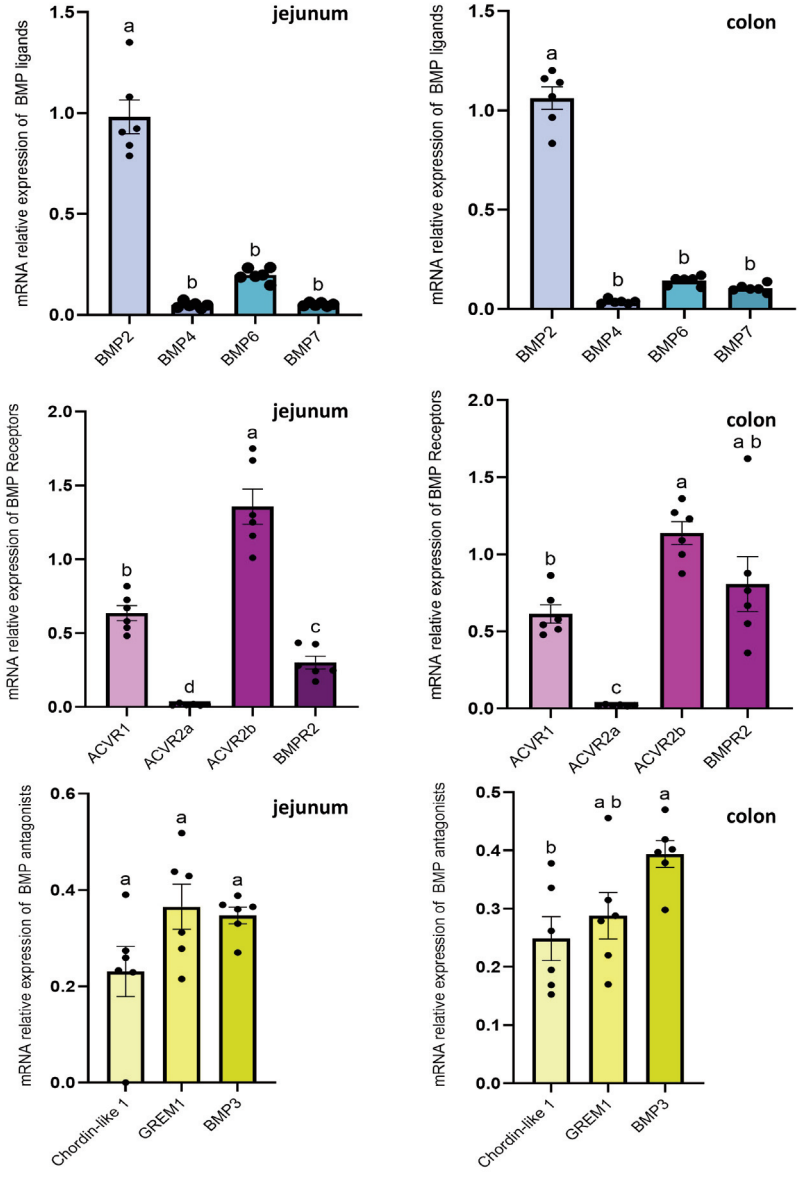

Figure 3. mRNA relative expression of BMP ligands, BMP receptors and BMP antagonists in rat jejunum and colon, normalized with *Polr2a gene*. Tissues were extracted from 6 adult rats. Statistical analysis: one-way ANOVA with Tuckey's post-test, $p<0.05$.

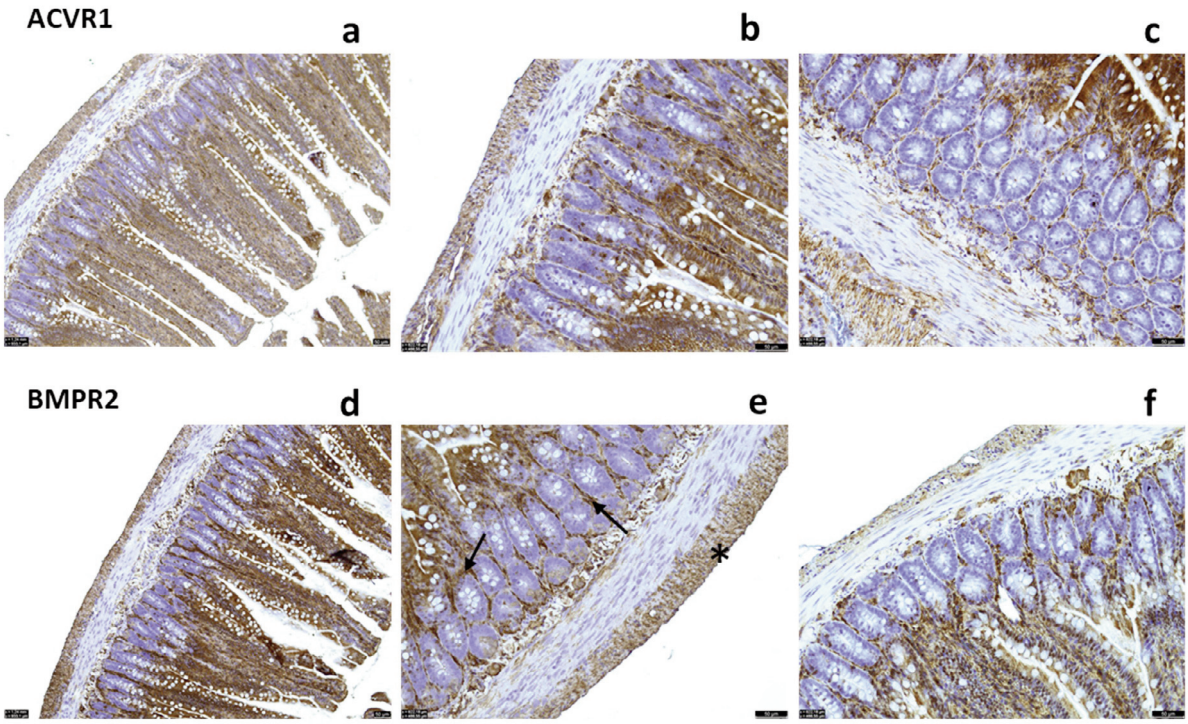

Figure 4. Immunohistochemical localization of ACVR1 (**a,b,c**) and BMPR2 (**d,e,f**) receptors in rat jejunum. **e**) The arrows show strong immunostaining in the lamina propria; the asterisk designates the longitudinal muscularis layer. To determine the localization of BMP receptors, we performed immunohistochemical staining on jejunum sections from 6 different rats. Sections were examined by light microscopy (Leica DM 2000) using 20x and 40x objectives. Representative images are shown. Scale bars: 50 μm.

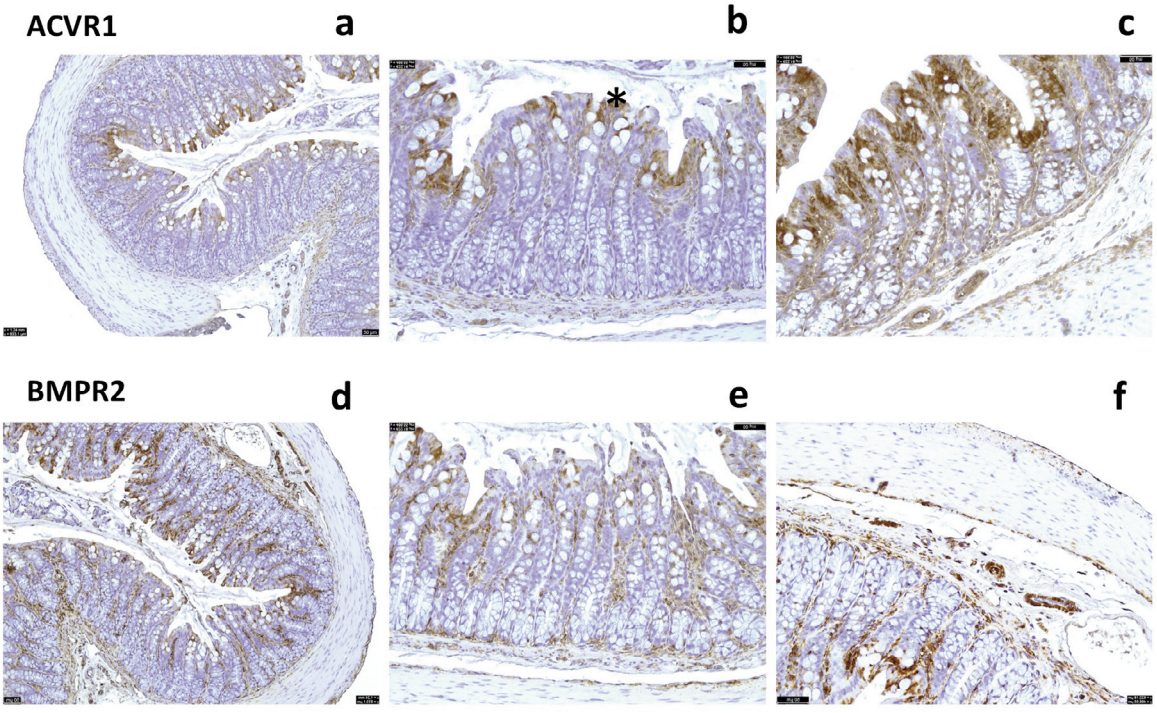

Figure 5. Immunohistochemical localization of ACVR1 (**a,b,c**) and BMPR2 (**d,e,f**) receptors in rat colon. **b**) The asterisk shows surface colonocytes. To determine the localization of BMP receptors, we performed immunohistochemical staining on colon sections from 6 different rats. Sections were examined by light microscopy (Leica DM 2000) using 20x and 40x objectives. Representative images are shown. Scale bars: 50 μm.

BMP antagonists

BMP3

The ligand BMP3, which has been classified as a BMP antagonist, was described here.[31] A moderate immunoreaction for BMP3 was observed in villous enterocytes (Figure 6 a-c). However, rare villus and crypt epithelial cells expressed this ligand very strongly. Their triangular or pyramidal shape, with the frequent presence of apical or basal cytoplasmic processes, suggests that they may be enteroendocrine cells (Figure 6b). Immunostaining was also present in some lamina propria cells. In the colon, BMP3 expression was present in colonocytes of the upper third of the crypt and in surface colonocytes (Figure 7 a-c). Epithelial cells expressing BMP3 very intensively were also observed at different crypt levels (Figure 7d). Finally, weak immunolabeling of BMP3 was observed in the lamina propria and in the muscularis mucosae.

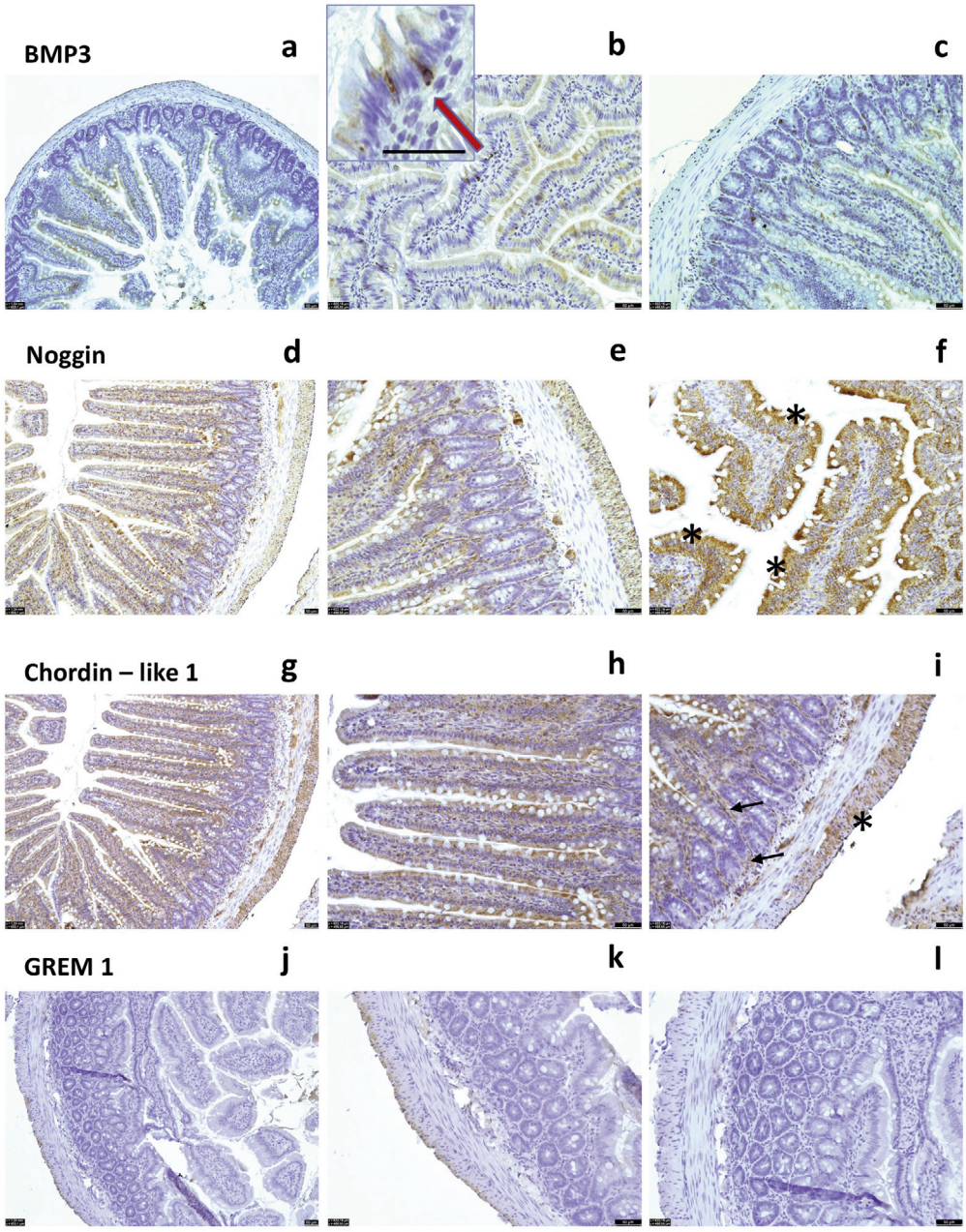

Figure 6. Immunohistochemical localization of BMP3 (**a,b,c**), Noggin (**d,e,f**), Chordin-like 1 (**g,h,i**) and Grem1 (**j,k l**) in rat jejunum. **f**) The asterisks indicate villous enterocytes. i) The arrows show the lamina propria and the asterisk indicate the longitudinal muscularis layer. To determine the localization of BMP antagonists, we performed immunohistochemical staining on jejunum sections from 6 different rats. Sections were examined by light microscopy (Leica DM 2000) using 20x and 40x objectives. Representative images are shown. Scale bars: 50 μm.

Noggin

In the jejunum, the anti-Noggin antibody immunolabelled villous enterocytes and some crypt enterocytes (Figure 6 d-f). In the colon, Noggin was detected with high intensity in colonocytes of the upper third of the crypt and in surface colonocytes, as well as in some colonocytes in the rest of the crypt (Figure 7 e-g). Noggin was also localized in the lamina propria, submucosal vessel walls, submucosa and myenteric plexuses and in the longitudinal muscle layer of the jejunum and colon.

Chordin-like 1

Chordin-like 1 was detected in the majority of villous enterocytes, but not uniformly (Figure 6 g-i). Some crypt enterocytes also expressed this antagonist. In the colon, Chordin-like 1 was detected in colonocytes of the upper third of the crypts and in surface colonocytes (Figures 7 h,i). As observed with Noggin, Chordin-like 1 was localized in the lamina propria, in submucosa and myenteric plexuses and in the longitudinal muscle layer of the jejunum (Figure 6i) and colon.

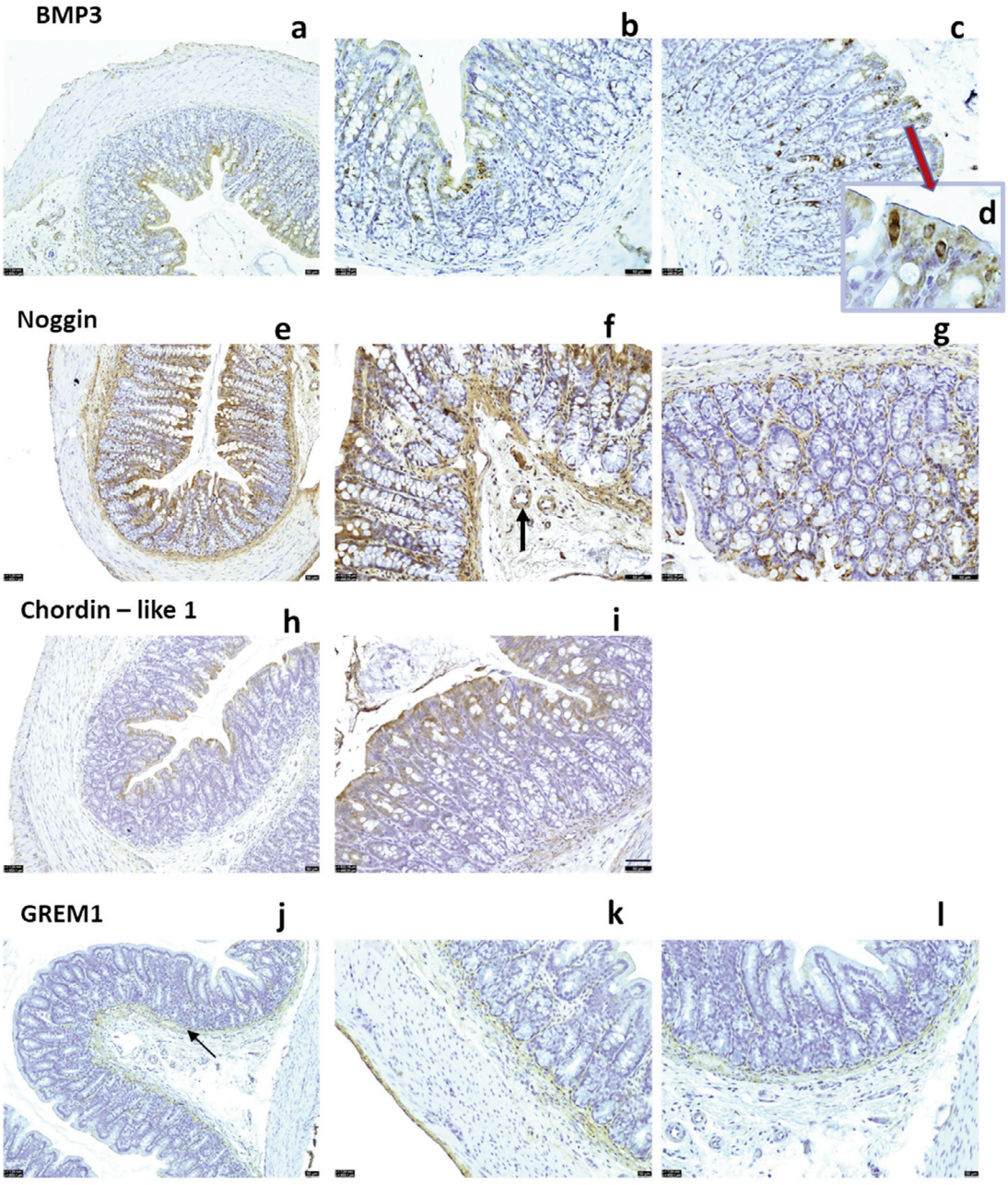

Figure 7. Immunohistochemical localization of BMP3 (**a,b,c,d**), Noggin (**e,f,g**), Chordin-like 1 (**h,i**) and Grem1 (**j,k,l**) in rat colon. **f**) The arrow shows the immunostaining of a vessel wall in the submucosa. **j**) The arrow shows muscularis mucosae. To determine the localization of BMP antagonists, we performed immunohistochemical staining on colon sections from 6 different rats. Sections were examined by light microscopy (Leica DM 2000) using 20x and 40x objectives. Representative images are shown. Scale bars: 50 µm.

GREM1

A very low expression of GREM1 was observed in the lamina propria and in submucosal plexuses of the jejunum (Figures 6 j-l). In the colon, a moderate immunoreaction for GREM1 was also localized in the lamina propria (Figures 7 j-l). In contrast, more intense immunostaining was observed in the muscularis mucosae. The antagonists analyzed in RT-qPCR (*Bmp3, Grem1 and Chordin-like 1*) were also expressed in the jejunum and the colon (Figure 3). The gene expression analysis of BMP antagonists did not reveal any difference in expression between the 2 localizations. In the colon, *Bmp3* expression was significantly higher than that of *Chordin-like 1* whereas in the jejunum there was no difference between the three antagonists.

Discussion

In this study, we were interested in the expression and localization of BMP pathway players in the intestinal and colonic walls of the rat, a species widely used in studies of intestinal pathology but whose BMP signaling pathway has been little explored to date.

BMP2 and BMP4 are the two most studied ligands of the BMP pathway in human and mouse intestines. The study of these two ligands in rats showed that the level of *Bmp2* mRNA in small intestine and colon tissues was much higher than that of *Bmp4* but also of *Bmp6* and *Bmp7*, and that this ligand was mainly localized in villous enterocytes and mature colonocytes. These data are consistent with the detection of BMP2 in human and mouse colonocytes[8,32] and associated to a primary role of BMP2 in terminal differentiation and apoptosis of epithelial cells.[8,33]. We also observed a strong expression of BMP2 in the lamina propria of rat intestine, as reported in mice in a study by Berkova *et al*.[33] Furthermore, we detected BMP2 around the myenteric nervous plexuses and in longitudinal muscle layer of the rat intestine. This localization was already observed in a study conducted by Honoré *et al.,*[34] where BMP2 was reported to be highly expressed in the cytoplasm of some myenteric plexus cells and also in the longitudinal muscular layer in the jejunum of control animals, while its expression was decreased in diabetic rats. The involvement of BMP2 in the Hirschsprung's disease, a congenital disease related to digestive obstruction and enteric nervous system dysregulation finally supports the importance of the BMP pathway in the development and function of enteric nerve cells.[21] All these data suggest that the role of BMP2 in the intestinal wall could be much broader than that usually attributed to it in the control of epithelial differentiation. On the contrary, although BMP4 also represents one of the most studied intestinal BMP ligands, our RT-qPCR and IHC data revealed that it was not the most abundant member of this family in the rat intestine. In human and mice intestines, BMP4 production is attributed to intravillus and intercrypt mesenchymal population, especially in subepithelial fibroblasts, which allow them to play a crucial role in epithelial stem cells proliferation and daughter cell differentiation.[35,36] Our immunostainings showed that BMP4 ligand was also expressed by mesenchymal cells of the submucosa of rat intestine and almost absent in jejunal cells or colonocytes (only weak expression in a few surface colonocytes). The difference in localization between BMP2 and BMP4 is consistent with the fact that these two ligands have distinct roles in the differentiation program of intestinal epithelial cells. Indeed, while BMP2 leads mature enterocytes at the top of the villi, BMP4 is in charge of central enterocytes. More specifically, it has been shown that BMP2 at the villus tip can influence adhesion features of epithelial cells and immunoregulation, while BMP4 has, has a role in the lipid absorption and metabolism.[33]

Regardless of the species, BMP6 and BMP7 are two little-studied ligands in the intestinal wall. Interestingly, we showed for the first time that BMP7 was highly expressed by rat villous enterocytes and mature colonocytes and, to a lesser extent, by small intestinal lamina propria cells. A parallel can be done with BMP2, which had a very similar epithelial localization. Moreover, in the study by Berkova *et al*.[33] performed on murine organoids, the colocalization of BMP2 and BMP7 was already identified and associated with similarity of function between the two ligands. Interestingly, many studies also reported that BMP7 can heterodimerize with BMP2 and that these heterodimers were more potent than BMP7 or BMP2 homodimers.[37,38] Noggin for which we have shown a strong epithelial expression, was also reported to have a less antagonistic activity on BMP2/BMP7 heterodimers than on homodimers.[39] The co-localization of these two ligands in the intestinal epithelium could thus reinforce the BMP pathway. The detection of BMP6 was rather moderate in rat enterocytes and colonocytes. BMP6 has already been studied and described as a regulator of hepcidin production related to iron metabolism. *In vivo* in mice and in an *ex vivo* model of mouse intestinal tissue, BMP6 ligand production was found to be increased in enterocytes at the villous tip in response to luminal iron sensing. BMP6 was then delivered into the portal circulation to regulate hepcidin in the liver.[11] However, what has never been shown is the strong presence of BMP6 in submucosal and myenteric plexuses suggesting a role of this ligand, as BMP2, in the regulation of the enteric nervous system. This hypothesis is consistent with the fact that BMP6 is known to be expressed by neurons, oligodendrocytes and astrocytes in the central nervous system, where it plays an important role in inhibiting neurogenesis and enhancing the protective effect of neurotrophins.[40-42]

In the intestinal tract, the maintenance of epithelial stem cells in the crypt base is governed by both low activity of the BMP pathway and high activity of the Wnt pathway. Conversely, differentiation of epithelial cells along the crypt axis or crypt-villus axis is induced by an increase in the BMP signaling pathway in these cells, while the Wnt pathway decreases. BMP signaling gradient along the crypt-villus axis is ensured by the localization of ligands but also by the presence of BMP extracellular antagonists. Interestingly, these antagonists do not bind directly to BMP receptors but to ligands, and each antagonist binds specifically and preferentially to one or more BMP ligands.[43] GREM1 represents one of the most investigated antagonists of the BMP pathway in the intestinal tract, essentially because it plays a role in the development and progression of several pathologies including colorectal cancers[4.4,45] In rat intestinal tract, we found an expression of GREM1 in the lamina propria, the muscularis mucosae and the submucosa. These localizations are similar to those observed in human and mice colon tissues where GREM1 was found to be mainly produced by subepithelial fibroblasts and smooth muscle cells from the muscularis mucosae and the muscularis propria of the colon.[13,14,46] However, a study in mice by Dutton *et al*.[47] showed that, while fibroblasts and muscularis mucosae cells were the source of *Grem1* transcripts, GREM1 protein could also be detected in Paneth cells and transit amplifying cells at the base of the crypt, probably after uptake. In agreement with this result, we observed very low GREM1 immunoreactivity in jejunal epithelial cells and colonocytes, sometimes in surface colonocytes. Finally, we noticed a strong immunostaining of GREM1 in the wall of vessels. In the study of Liu *et al.,* [48] functional analyses of colorectal cancer transcriptomes showed that GREM1 silencing resulted in vascular endothelial growth factor (VEGF) inhibition, suggesting an important role of this antagonist in vascular homeostasis. GREM1 would be able to bind to vascular endothelial factor receptor 2 (VEGFR2) in endothelial cells *in vivo* and *in vitro* and to act

as a pro-angiogenic agonist.[49] In addition, GREM1 could also act by blocking the BMP pathway in the intestinal wall. Indeed, BMP ligands (excepted BMP4), BMP receptors and the other BMP antagonists evaluated in our work have been detected in colonic vessel walls. Only a previous study has evaluated the expression of BMP receptors in human colon tissues. ALK3 (*i.e.*, BMPR1a), ALK6 *(i.e.*, BMPR1b) and BMPR2 were found to be expressed in endothelial cells whereas ALK2 (*i.e.*, ACVR1) was not. By further *in vitro* investigations, the main hypothesis was to attribute to the BMPs a potential proangiogenic role related to the organization and differentiation of endothelial cells into a network.[50] It should be noted the importance to develop our knowledge concerning the role of the BMP pathway in the intestinal endothelium because a dysregulation could promote the progression of intestinal disease such as colorectal cancer or inflammatory diseases.

In our study, we focused on three other BMP antagonists: Chordin-like 1, Noggin and BMP3. Only the works of Kosinski *et al*.[13] and, more recently, of McCarthy *et al*.,[51] have reported the presence of Chordin-like 1, a BMP4 antagonist, in the gastrointestinal tract of humans and mice. Chordin-like 1 is described as being produced by i) myofibroblasts of the colonic crypt and muscularis mucosae in humans but also by ii) smooth muscle cells located near the base of intestinal crypts and called cells of the "superficial muscularis propria" in mice.[13,51] Our immunostainings highlighted the presence of the antagonist also in the colonic muscularis mucosae and in the lamina propria of both tissues. In addition, our study revealed a Chordin-like 1 expression in the myenteric nervous plexus, a localization never previously observed that suggests a potential role of this antagonist in the enteric nervous system. The expression of this antagonist in mature epithelial cells was more surprising and needs to be confirmed. For its part, the BMP antagonist Noggin had predominantly an epithelial localization both in the jejunum and the colon, consistent with what has been described in mature mouse colonocytes.[8] We also noted the presence of Noggin in the intestinal nervous plexus, consistent with a crucial role for this molecule in the development and control of the enteric neuron population.[18,5]. The specific role of BMP3 remains unclear, even if it is clearly described as a BMP antagonist.[23,31] Our immunostainings showed that BMP3 was expressed mainly by cells of the lamina propria in the jejunum and the colon. BMP3 was also detected in enterocytes and colonocytes which has already been shown in adult humans and mice. Indeed, in humans, BMP3 has been observed in the cytoplasm of colonocytes and in a specific type of epithelial cell called BEST4+ in the intestine and the colon.[53] Interestingly, we observed a very strong immunostaining of BMP3 also in specific epithelial cells. These cells that had an elongated pear-shape could be enteroendocrine cells, but this has not been verified. Even if a functional *Best4* (bestrophin 4) gene was identified in rats, BEST4+ cells were not found in the epithelium of the rat ileum.[54] In mouse colon, BMP3 expression has been attributed to crypt top fibroblasts when compared to crypt bottom fibroblasts related to a role in epithelial differentiation.[55] The same "crypt top" localization was given in intestinal tissue of transgenic mice in the study of McCarthy *et al*.[51] The role in epithelial differentiation could be indirect and explained by the co-expression with BMP2 and BMP3 ligands in mature epithelial cells since we know that BMP3 competes mainly with BMP2.[23,31]

We have demonstrated the expression of BMP antagonists (GREM1, BMP3, Chordin-like 1 and Noggin) and BMP ligands in the same cell types. Although this may seem surprising, it has already been observed in other tissues.[56] These results could indicate a paracrine or autocrine feedback mechanism for modulating BMP pathway activity at the cellular level. Regarding BMP receptors, we showed that, in addition to having an important epithelial localization, they were expressed in both submucosa and myenteric plexuses in the jejunum and the colon. The study by Brewer *et al*.[57] focused on the expression of BMPR1A in adult human colon tissues and concluded that this type I receptor was detected both in myenteric and submucosa ganglia and specifically in the cytoplasm of all neuronal cells. The same expression was found again in the study by Honoré *et al*.[34] conducted on diabetic rats. These data are consistent with both the binding of BMP ligands to type I and II epithelial receptor complex to promote colonocyte and enterocyte terminal differentiation and also with the presence of BMP ligands in enteric nervous cells.

In conclusion, the expression and localization of BMP pathway players found in the rat intestine show similarities with those of humans and mice, particularly for the ligands BMP2 and BMP4, the BMP receptors and the antagonists GREM1 and Noggin, suggesting that the BMP pathway could be explored in rat models of intestinal pathologies. The present work also provides important additional information to that available in the literature, notably concerning the presence of ligands and antagonists in intestinal layers other than the mucosa. In particular, we observed for the first time a strong expression of BMP6 in cells of the myenteric and submucosa plexuses and of BMP7 in epithelial cells. Our results further suggest and highlight the importance of the BMP pathway in the enteric nervous system of the rat because all the actors studied were found to be expressed sometimes highly in the different plexuses. These data will need to be deepened by studying the differences in expression of BMP pathway ligands and antagonists according to the expression of other receptors, of non-signaling membrane BMP pseudo-receptors such as BAMBI (membrane-bound inhibitor BMP and activin) and intracellular BMP antagonists (Smad 6/7). Finally, it could appear relevant to focus on the modulation of the BMP signaling pathway related to age, sex and nutritional status, which would represent a strategic way for innovative prevention and therapy for intestinal pathologies.

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