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Distribution of TMEM100 in the mouse and human gastrointestinal tract - a novel marker of enteric nerves

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Abstract

Identification of markers of enteric neurons has contributed substantially to our understanding of the development, normal physiology, and pathology of the gut. Previously identified markers of the enteric nervous system can be used to label all or most neuronal structures or for examining individual cells by labeling just the nucleus or cell body. Most of these markers are excellent but have some limitations. TMEM100 is a gene at locus 17q32 encoding a 134-amino acid protein with two hypothetical transmembrane domains. TMEM100 expression has not been reported in adult mammalian tissues but does appear in the ventral neural tube of embryonic mice and plays a role in signaling pathways associated with development of the enteric nervous system. We showed that TMEM100 messenger RNA is expressed in the gastrointestinal tract and demonstrated that TMEM100 is a membrane associated protein. Furthermore TMEM100 immunoreactivity was restricted to enteric neurons and vascular tissue in the muscularis propria of all regions of the mouse and human gastrointestinal tract. TMEM100 immunoreactivity co-localized with labeling for the pan-neuronal marker protein gene product 9.5 (PGP9.5) but not with the glial marker S100β or Kit, a marker of interstitial cells of Cajal. The signaling molecule, bone morphogenetic protein (BMP) 4, was also expressed in enteric neurons of the human colon and colocalized with TMEM100. TMEM100 is also expressed in neuronal cell bodies and fibers in the mouse brain and dorsal root ganglia. We conclude that TMEM100 is a novel, membrane-associated marker for enteric nerves and is as effective as PGP9.5 for identifying neuronal structures in the gastrointestinal tract. The expression of TMEM100 in the enteric nervous system may reflect a role in the development and differentiation of cells through a transforming growth factor β, BMP or related signaling pathway.

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Author Contributions

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Keywords

TMEM100; enteric nervous system; PGP9.5; TGFβ signaling; BMP4

Normal gastrointestinal function depends on many cell types including nerves (Furness, 2006). Enteric nerves are required for coordinated gastrointestinal motility and the identification of markers for neuronal structures has contributed substantially to our understanding of the development, normal physiology, and pathology of the gut. Markers reported to label all neuronal structures include neurofilaments (Bjorklund et al., 1984) neuron-specific enolase (NSE) (Bishop et al., 1985), protein gene product 9.5 (PGP9.5, also known as UchL1) (Krammer et al., 1993), Hu C/D (also known as ANNA-1) (Lin et al., 2002), cuprolinic blue (Heinicke et al., 1987) and FluoroGold (Powley and Berthoud, 1991). Each of these markers meet the criteria proposed by Karaosmanoglu et al (Karaosmanoglu et al., 1996) in that they are i) neuron-specific, ii) label all neurons, and iii) are readily available. All have proven useful in the quantification of neuronal cell bodies (Hu C/D, cuprolinic blue and FluoroGold) and/or neuronal fibers (PGP9.5, NSE, neurofilaments) (Bjorklund et al., 1984, Heinicke et al., 1987, Powley and Berthoud, 1991, Krammer et al., 1993, Karaosmanoglu et al., 1996, Lin et al., 2002, Phillips et al., 2004, Ganns et al., 2006, Bernard et al., 2009).

There are limitations to the existing markers, including failure to label all neuronal structures as reported for PGP9.5 immunoreactivity in rat myenteric neurons (Eaker and Sallustio, 1994) and for Hu C/D in aged rat myenteric neurons (Phillips et al., 2004). In addition, in some conditions labeling is observed in non-neuronal structures of the gastrointestinal tract including Hu C/D expression in glia (Phillips et al., 2004) and cuprolinic blue labeling in mast cells (Heinicke et al., 1987) and fibroblast-like cells in the sub-mucosa (Holst and Powley, 1995). For the most part these limitations are surmountable and in some tissues absence of expression or ectopic expression can provide important information. For example, studies of NSE expression have revealed important information about the processes of neuronal maturation, because a switch in expression from non-neuronal enolase (αα isoenzyme) to the neuron-specific isoenzyme (γγ, NSE) correlates with differentiation of precursor cells into mature neurons (Schmechel et al., 1980). The finding that HuC/D expression is high in small cell carcinomas and is immunogenic has led to the recognition of paraneoplastic enteropathies in which the enteric nervous system (ENS) is damaged by the anti-Hu or ANNA-1 antibodies (King et al., 1999).

Our work characterizes TMEM100 immunoreactivity as a novel marker for enteric nerves. TMEM100 expression has been reported outside of the nervous system. TMEM100 expression has been associated with clinical stage in lung adenocarcinomas (Frullanti et al., 2012) and may be part of the bone morphogenetic protein (BMP) signaling pathway (Somekawa et al., 2012), which is involved in development of the ENS (Chalazonitis et al., 2004). TMEM100 is a gene at locus 17q32 encoding a 134-amino acid protein with two hypothetical transmembrane domains (amino acids 53–75, 85–107) (Moon et al., 2010). TMEM100, first identified as a transcript from the mouse genome (FLJ10970, (Kawai et al., 2001), is well conserved in vertebrates and is not structurally related to any known family of proteins in any species. There appear to be several splice variants or alternative gene transcripts. Several expression profile studies have identified TMEM100 transcripts in the pre-tubular aggregate in the renal vesicle of the developing kidney (Georgas et al., 2009) and associated with transcripts for activin receptor-like kinase I (ALK1), the gene mutated in hereditary hemorrhagic telangiectasia (Moon et al., 2010). TMEM100 is down-regulated in mice with pulmonary arterial hypertension induced by over-expression of dominant-negative BMP receptor type II (GEO: GDS2147). In the lungs of ALK1-deficient mice,

TMEM100 expression is down-regulated, implying its involvement in the ALK1/transforming growth factor beta (TGF-β) signaling pathway (Moon et al., 2010). Greater levels of TMEM100 are detected in the transition zone of the prostate when compared to the peripheral zone where the majority of aggressive tumors are detected (van der Heul-Nieuwenhuijsen et al., 2006). TMEM100 was also identified as a marker of apoptotic cell death after thapsigargin treatment of SH-SY5Y neuroblastoma cells. Co-treatment of the cells with FK506 (tacrolimus) prevented the transcription of TMEM100 in response to thapsigargin and decreased apoptosis of the cells (Yamazaki et al., 2011).

A genetically modified mouse strain has been generated that contains the lacZ reporter gene driven by the TMEM100 promoter. In embryonic mice, expression of the reporter was detected in the vascular endothelium, heart, mammary glands, notochord and the ventral region of the neural tube (Moon et al., 2010). TMEM100 knockout animals did not survive to birth (Moon et al., 2010).

These published reports on TMEM100 indicate that, while the function of TMEM100 is not known, this protein seems to play a role in cellular differentiation, particularly in the pathways activated by the TGFβ/BMP/activin family of signaling molecules. TMEM100 may have some function in the fate of neoplastic cells, however it is not known if TMEM100 is a membrane protein, and there are no reports on its cellular and sub-cellular distribution in adult tissues. With respect to the gastrointestinal tract, TMEM100 transcripts were expressed in the muscularis propria of mouse jejunum (Chen et al., 2007). Our aim was to determine if TMEM100 is a membrane protein and to report its distribution in the adult mouse and human gastrointestinal tract.

1. Experimental Procedures

1.1 Immunohistochemistry

Mice were maintained and the experiments were performed with approval from the Institutional Animal Care and Use Committee of the Mayo Clinic. Six to eight week old female BALB/c mice (Harlan Sprague-Dawley, IN, USA) and C57BL/6J mice (Jackson Laboratory, ME, USA) were killed by CO_2 inhalation. The use of human tissue for research was approved by the Institutional Review Board of Mayo Clinic. Normal human stomach (n = 3), jejunum (n = 3) and colon (n = 3) tissues were obtained from patients undergoing surgery for gastrointestinal cancers or having bariatric surgery (Mayo Clinic, MN, USA).

Whole Mounts—Whole mount preparations of the gastric body, jejunum, and proximal colon of BALB/c (n=3) and C57BL/6J (n=3) mice were immuno-labeled according to previously published techniques for fluorescence immunohistochemistry in paraformaldehyde-fixed tissue (Tharayil et al., 2010).

Cryosections—Human tissue fixed by immersion in 4% paraformaldehyde was immunolabeled as 15 μm cryosections using standard techniques including all necessary controls as described previously (Bernard et al., 2009). To confirm specificity of tissue labeling, each TMEM100 primary antibody was incubated with the respective immunogens for 24 hours prior to immunohistochemistry. In each experiment, samples were exposed to secondary antibody in the absence of primary antibody to test for non-specific staining. Secondary antibodies directed against IgG from a different species were used to control for the specificity of secondary antibodies in doubly labeled tissues to ensure that there was no cross reaction between secondaries targeting different species. (See Table 1)

Mouse brain and dorsal root ganglia (Balb/c N=2, C57BL/6J N=2) were removed and fixed by immersion in 4% paraformaldehyde in 0.1 mol L^{-1} phosphate buffer (pH 7.2). The next

day the tissue was washed 4 × 15 min with 1× 0.1 mol L^{-1} phosphate buffered saline (PBS, pH 7.2) then incubated in 30% sucrose in 1× PBS overnight before freezing and storage at −80 °C until needed. Fifteen micron tissue sections were cut and immunohistochemistry was performed with proper controls as described above. SlowFade® Gold Antifade Reagent with DAPI (Invitrogen, CA, USA) was used as a counterstain to detect nuclei.

1.2 Reverse Transcription PCR

The muscularis propria of the small intestine of adult BALB/c mice (n=3) was removed from the mucosa by dissection. RNA was extracted, transcribed and amplified from each tissue separately. Extraction of mRNA was done using RNA-Bee reagent (AMS Biotechnology, Abingdon, UK). Complementary DNA was synthesized using SuperScript® VILO™ cDNA Synthesis kit (Life Technologies, CA, USA). Taq polymerase-amplified PCR product was run on a 2% agarose gel. TMEM100 primers and cycles were as follows : forward, 5′- TGG ACT GCC TTT CTG TGA GCT TGC A-3′, reverse, 5′- GGT GAC CAC AAC TTC CCT CTT GGG G-3′; 94 °C for 3 min, 35 times at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, 72 °C for 2 min. The PCR product was cloned into the pCR™2.1-TOPO® vector (Life Technologies). Positive transformants were amplified, and plasmid DNA was extracted and purified using a MiniPrep kit (Qiagen, CA, USA). Plasmid DNA was sequenced by the Molecular Biology Core Facility at Mayo Clinic and confirmed identity to TMEM100.

1.3 Sub-cellular localization of TMEM100

Total internal reflection fluorescence (TIRF) microscopy of HEK293 cells transfected with TMEM100-GFP and fixed in 4% paraformaldehyde was used to determine the subcellular localization of TMEM100 protein. HEK293 cells were grown in T25 flasks in Minimum Essential Medium, 10% horse serum, non-essential amino acids, sodium pyruvate, and penicillin-streptomycin (Invitrogen). Cells were grown to about 75% confluency. C-terminal GFP-tagged ORF clone (Origene, MD, USA) of Homo sapiens TMEM100 transcript variant 2 was transfected in HEK293 cells with 1 μg of plasmid DNA and 3 μL of Lipofectamine 2000 reagent with OPTI-MEM I reduced serum medium (Invitrogen). After 24 h of exposure, the cells were dissociated using 0.05% trypsin-EDTA (Gibco, CA, USA), and re-plated on coverslips. Twenty-four hours after plating on coverslips, cells were fixed with 4% PFA at room temperature and quenched with ammonium chloride for 10 min. Transfected cells were then examined using TIRF microscopy. Cells that were mock-transfected without plasmid or not transfected were used as negative controls.

1.4 Image acquisition

Images of immunolabeling were collected using an Olympus FV1000 laser scanning confocal microscope. Confocal images were collected with ×60 1.2-NA or × 20 0.95-NA water objectives. TIRF images were collected on an Olympus attachment for the I×70 microscope fitted with a Quant EM: 512SC (Photometrics, AZ, USA) CCD camera EM system with ×100 1.35-NA oil immersion objective using the MetaMorph image processing program (version 7.3.2; Photometrics). All images were prepared for individual figures using Adobe Photoshop CS. No 3D reconstructions, deconvolution, surface or volume rendering, or gamma adjustments were performed.

2. Results

2.1 TMEM100 mRNA is expressed in the mouse gastrointestinal muscularis propria

Reverse transcription PCR of isolated mRNA from small intestinal muscularis propria of Balb/C mice using primers specific for TMEM100 amplified a product of the expected size

(N=3, Fig1). No product was amplified when reverse transcriptase was omitted during synthesis of complementary DNA (N=3, Fig1). The PCR product was cloned, and *E. coli* cells were transformed with the recombinant vector. Plasmid DNA from positive transformants was sequenced and the identity to TMEM100 was confirmed.

2.2 TMEM100 is restricted to the plasma membrane of transfected HEK293 cells

TIRF imaging of HEK293 cells transfected with the human transcript of TMEM100 demonstrated that much of the immunoreactivity is localized to within 100 nm of the imaging surface and therefore within the plasma membrane. Figure 2A shows the epifluorescence image of a transfected cell. This immunofluorescence is restricted to the region illuminated by the evanescent wave as visualized by TIRF (N=4, Fig2B) and therefore is restricted to within 100 nm of the imaging surface. Non-transfected and mock transfected cells had no fluorescence in the plasma membrane (data not shown).

2.3 Specificity of two different antisera against TMEM100

A number of controls were completed to ensure that the labeling observed in immunohistochemistry was detecting the target of interest, TMEM100. Double labeling with two different primary antibodies toward TMEM100 demonstrated the specificity of these antibodies toward their intended target. Mouse (Fig3A,D) and goat (Fig3B,E) anti-TMEM100 antibodies labeled the same structures in these preparations and clearly labeled neurons of the myenteric (N=3, Fig3A–C) and submucosal plexuses (N=3, Fig3D–F) in the human jejunum. Note that the goat anti-TMEM100 antibody labeled a subset of neurons and fibers more intensely (Fig3E, arrows), but this phenomenon was not seen with the mouse anti-TMEM100 antibody. It remains to be determined if this antibody may be detecting differences in levels of expression, or an isoform of TMEM100. Due to non-specific interactions with host IgG, the mouse anti-TMEM100 could not be used for investigation in mouse tissues, therefore uneven immunolabeling is apparent in the presented mouse data. Additional controls were performed to ensure specificity for TMEM100 with regard to each primary antibody. Pre-absorption of both primary antibodies with their respective immunogen decreased markedly the fluorescence signal (N=3, Fig3G–H, J–K). No signal was observed when the tissue preparations were incubated with only secondary antibody, demonstrating that there was no non-specific labeling by the secondary antibody (N=3, Fig3I,L). In addition, secondary antibodies directed against IgG from different species were used to control for the specificity of secondary antibodies in doubly labeled specimens (data not shown). HEK293 cells transfected with TMEM100-GFP were positively immunolabled with the mouse anti-TMEM100 antibody, while cells transfected with only GFP were not labeled (data not shown).

2.4 TMEM100 immunolabeling in the mouse and human gastrointestinal tract

Immunohistochemistry was done in mouse whole mounts and human cryosections to determine localization of TMEM100 immunoreactivity. Goat anti-TMEM100 antibody labeled the cell bodies of neurons, as well as the nerve fiber bundles connecting ganglia, and fibers innervating the longitudinal and circular muscle layer of the gastric body, jejunum, and proximal colon in two different strains of mice (N=3 for each strain, Fig4A–C). There was no difference in TMEM100 distribution between BALB/c and C57BL/6J mouse strains. TMEM100 labeling in full thickness cryosections of human stomach, jejunum, and colon was present in the myenteric plexus (N=3, Fig4D, arrows) and innervating nerve fibers of the muscle layers. In addition, TMEM100 immunoreactivity extended to the submucosal and mucosal areas of the ENS. The submucosal plexuses (Fig4D, arrowheads), along with fibers innervating the muscularis mucosa and mucosa were labeled clearly using the mouse anti-TMEM100 antibody (N=3, Fig4D).

2.5 TMEM100 immunoreactivity is colocalized with the pan-neuronal marker PGP9.5

Antibodies to the pan-neuronal marker PGP9.5 were used to label all neurons and nerve fibers. Results from tissues doubly labeled with antibodies to TMEM100 and PGP9.5 confirmed that TMEM100 immunoreactivity was localized in all areas of the human and mouse enteric nervous system. Double labeling in human tissue revealed colocalization of TMEM100 and PGP9.5 in the myenteric plexus (N=3, Fig5A–C), fibers innervating both longitudinal (N=3, Fig5A–C, arrows) and circular muscle (N=3, Fig5D–F), the submucosal plexus (N=3, Fig5G–I), and fibers innervating the muscularis mucosa and mucosa (N=3, Fig5J–L). TMEM100 also colocalized with PGP9.5 in mouse tissues (n=6, Fig5M–O). All PGP9.5 positive structures were positive for TMEM100.

2.6 TMEM100 is not expressed in glia or interstitial cells of Cajal

S100β, a glial-cell-specific marker, was used to determine if TMEM100 localization was isolated to nerves. The close relationship that exists between neurons and glial cells brings these two cell types into close proximity, but double-labeled tissue with primary antibodies to TMEM100 and S100β demonstrated that TMEM100 is isolated to neuronal tissue (N=3, Fig6A–C, asterisks). Interstitial cells of Cajal (ICC) were examined closely by doubly labeling tissue with TMEM100 and anti-Kit antibodies. Double-labeling showed that there was a very close relationship of TMEM100-positive structures and Kit-positive ICC in the intramuscular regions of gut muscle (Fig6G–I, arrows), while this close relationship did not exist with ICC in the plane of the myenteric plexus (N=3, Fig6D–F). There were no TMEM100 positive structures that were also positive for Kit and S100β.

2.7 TMEM100 and BMP4 are co-localized in enteric neuronal cell bodies of the human colon

BMP4 labeling was present in adult human enteric neurons. Co-labeling of TMEM100 and BMP4 was isolated to neuronal cell bodies of the ENS. In cryosections of human colon, confocal slices showed that neuronal cell bodies identified by large round euchromatic nuclei (Fig7, arrows) displayed immunoreactivity for TMEM100 and BMP4 in both the myenteric (N=3, Fig7A–C) and submucosal plexuses (N=3, Fig7D–F). Weak BMP4 immunoreactivity extended to the neuropil of the enteric nerves; however, BMP4 immunoreactivity was confined largely to the neuronal cell bodies.

2.8 TMEM100 labels arterial endothelial cells and neurons of the central and peripheral nervous system

TMEM100 immunolabeling was found outside of the ENS. Arterial endothelial cells within the human submucosal vasculature were positive for TMEM100 (N=3, Fig8A–D, arrows). TMEM100 labeling in endothelial cells was present, but weaker than labeling found in the PGP9.5-positive nerve fibers innervating the muscle layers of the same vessel (Fig8A–D, arrowheads). In the central nervous system, labeling with the mouse anti-TMEM100 antibody colocalized with PGP9.5 in neural cell bodies throughout the mouse brain, an example of which is shown for the mouse cerebral cortex (n=4, Fig8E–H). It was particularly noted that neurons of the locus coeruleus labeled very intensely for TMEM100 in comparison to cell bodies in other regions of the brain, consistent with differences in expression levels between neuronal subtypes. In double labeling experiments, TMEM100 immunoreactivity was excluded from GFAP-positive glial cells in the brain (data not shown). In the peripheral nervous system, TMEM 100 immunoreactivity was detected in all neuronal cell bodies of the dorsal root ganglia. Variation in brightness of the labeling was also observed in these neurons but the goat anti- TMEM100 antibody labeled all structures and cell bodies that were positive for PGP9.5 (n=4, Fig8I–L). Neural fibers were more intensely labeled with TMEM100 in comparison to PGP9.5 (n=4, Fig8I–L, arrowheads).

3.1 Discussion

In the present study, we demonstrated that TMEM100 is a pan-neuronal marker of mouse and human enteric nerves. The use of two different anti-sera and strict immunohistochemical controls confirmed the specificity of the data presented. The mRNA for TMEM100 was also amplified from the muscularis propria of Balb/C mice. The data presented here show TMEM100 as being expressed in all enteric nerves, as it colocalizes with the pan-neuronal marker PGP9.5 throughout the gastrointestinal tract. In addition, TMEM100 immunoreactivity did not co-localize with markers for glial cells (S100β) or interstitial cells of Cajal (Kit). TIRF imaging of HEK293 cells transfected with TMEM100 demonstrated that TMEM100 is a plasma membrane protein. We also demonstrated co-localization of TMEM100 with BMP-4 in the cell bodies of neurons within the sub-mucosal and myenteric plexuses of the human colon.

This work shows that TMEM100 meets the criteria first proposed by Karaosmanoglu et al (Karaosmanoglu et al., 1996) for a marker of enteric nerves. In the gastrointestinal tract, TMEM100 appears to be present in all neurons, and at least three reliable antisera are available currently (we tested a third antiserum raised in goat, which gave very similar results so do not show the data). Using the lots of antibodies available to us, TMEM100 immunoreactivity is at least as good as PGP9.5 in identifying enteric nerves. There was some variation in the intensity of the labeling, and it is not clear whether these variations reflect differences in the quantity of the protein in sub-types of neurons or in neurons under different types of regulation. This work was done exclusively on paraformaldehyde-fixed tissue that was not paraffin-embedded, we have not tested the antisera on paraffin-embedded, formalin-fixed specimens.

Few data exist on expression of TMEM100 in non-gastrointestinal tissues. We found expression of TMEM100 in both mouse brain and dorsal root ganglia. There are some detailed studies on the changes in gene expression of TMEM100 during renal development (Georgas et al., 2009) and on the contribution of TMEM100 to vascular morphogenesis (Somekawa et al., 2012). The apparent expression of TMEM100 in the ventral neural tube close to the neural crest of developing mice (Moon et al., 2010) suggests that this protein is present in some cells which differentiate into enteric neurons and, as shown by our data on the colocalization of TMEM100 and BMP4, is congruent with the overlap of TMEM100 expression and mediators of Notch/BMP/TGFβ/activin signaling in several tissues (Georgas et al., 2009, Somekawa et al., 2012). Thus TMEM100 may be an indicator of cells differentiated along specific pathways; a concept supported by the observation of reduced levels of TMEM100 transcripts in less differentiated cells such as more invasive lung adenocarcinomas (Frullanti et al., 2012) and in regions of the prostate where more aggressive forms of cancer originate (van der Heul-Nieuwenhuijsen et al., 2006).

The Notch, BMP, TGFβ, and activin pathways are of interest because of the involvement of TMEM100 in the TGFβ pathway of vasculogenesis and the implication of TMEM100 being involved downstream from the BMP 9/10-activin receptor-like kinase 1 ALK-1 pathway (Somekawa et al., 2012). Treatment of human umbilical artery endothelial cells with BMP 9/10 resulted in increased expression of TMEM100 by mRNA microarray analysis, and these effects were inhibited by knockdown of ALK1, BMP type II, and mothers against decapentaplegic homolog 4 (Somekawa et al., 2012) – all of which play roles in TGFβ signaling. These initial studies of the TMEM100 function imply a potentially important role of TMEM100 in the TGFβ signaling pathway required for proper embryogenesis of the vasculature.

The TGFβ signaling pathway has also been shown to be vital in the development of the ENS during neurogenesis. BMPs are involved in development of other organs and play critical roles in early induction, migration, and patterning of cells in the neural crest (LaBonne and Bronner-Fraser, 1999, Sela-Donenfeld and Kalcheim, 1999, Goldstein et al., 2005, Chalazonitis and Kessler, 2012). In vitro concentration-dependent activity of BMP-2 and BMP-4 increases neuronal differentiation and affects neuronal survival of purified enteric progenitors (Chalazonitis et al., 2004). The BMP/TGFβ pathway is remarkably complex but has been shown to be vital in generation and maintenance of the ENS; moreover the BMP/TGFβ pathway has now been implicated in the maintenance and cell death of enteric neurons (Sela-Donenfeld and Kalcheim, 1999, Chalazonitis et al., 2004, Goldstein et al., 2005). The importance of BMP/ TGFβ signaling has been well documented in the developing embryo; however, its role in the adult organ and the signaling molecules involved downstream has yet to be closely analyzed.

As the importance of this pathway begins to become clearer, identifying additional signaling molecules may prove vital to fully understanding its role in adult tissue. Now that we have demonstrated that TMEM100 is localized to the ENS of the mouse and human gut, its functional role in these cells and potential link to the TGFβ signaling pathway can begin to be elucidated. In these regards it will be useful to know the sub-cellular distribution of TMEM100 and whether that distribution changes during cell activation. Our data locate the majority of the immunoreactivity to the plasma membrane, which is consistent with the prediction of transmembrane spanning domains in the structure of the protein (Moon et al., 2010). However, the labeling is not uniform, it seems to localize to tube-like structures projecting from transfected HEK293 cells, but the nature of these structures remains unclear.

3.2 Conclusion

TMEM100 is a novel, membrane-associated marker for enteric nerves that appears to be as useful as existing markers for enteric nerves. It also appears to be a pan-neuronal marker. Its expression in enteric nerves may reflect a role in the development and differentiation of the cells. Identifying the signaling pathways involving TMEM100 may yield important data concerning enteric neuropathies.

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Abbreviations

TMEM100	transmembrane protein 100
PGP9.5	protein gene product 9.5
TGFβ	transforming growth factor beta
ENS	enteric nervous system
BMP	bone morphogenetic protein
ALK1	activin receptor-like kinase I
NSE	neuron specific enolase
Kit	tyrosine protein kinase Kit (CD117)

TIRF total internal reflection fluorescence

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Highlights

- TMEM100 mRNA is present in the muscularis propria of Balb/c mice.
- TMEM100 is a membrane-associated protein in TMEM100 transfected HEK293 cells.
- Anti-TMEM100 antibodies labeled enteric neural cell bodies and fibers.
- TMEM100 colocalizes with bone morphogenetic protein-4 in the human proximal colon.
- TMEM100 expression is present in neurons of the central nervous system.

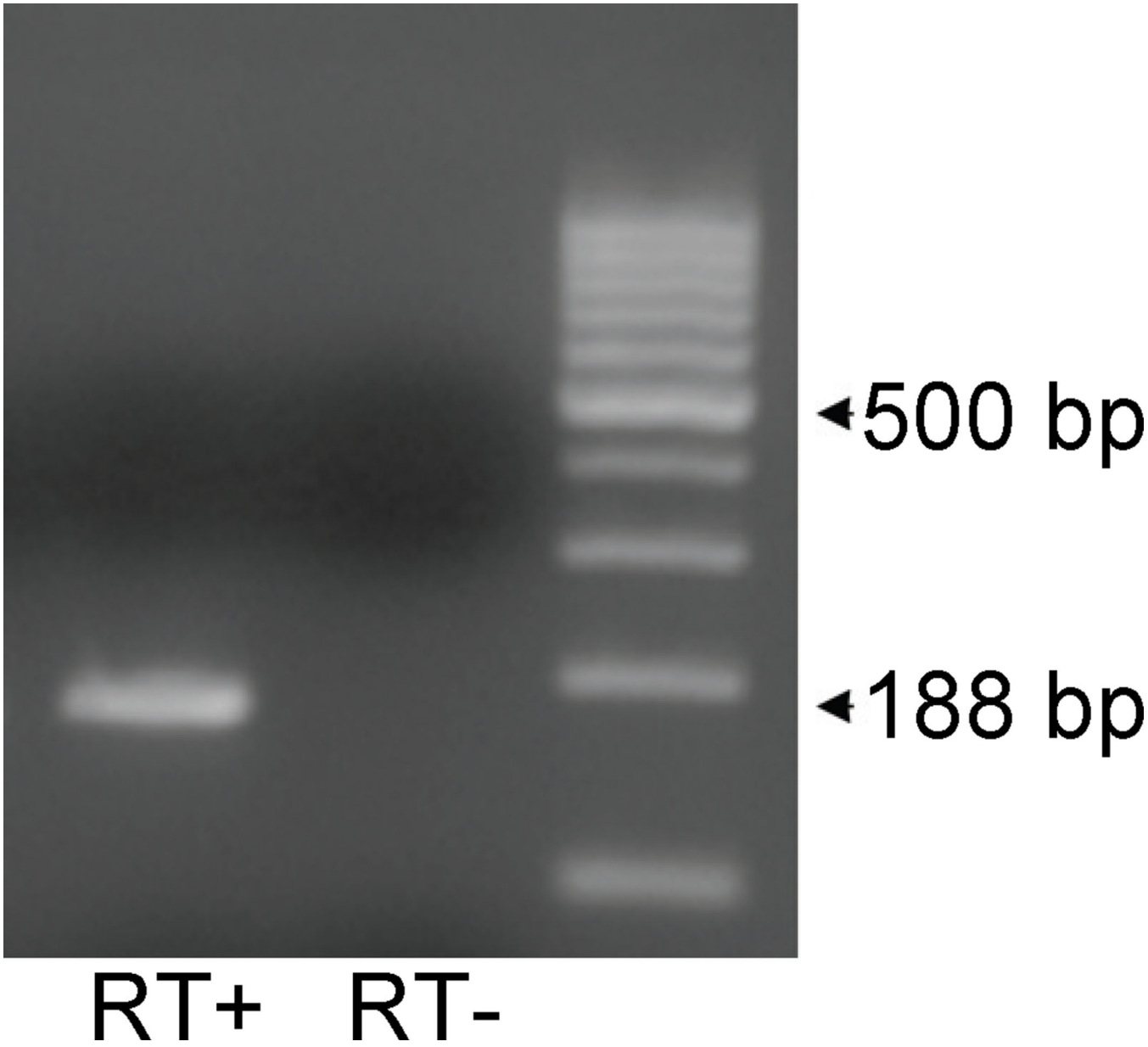

Fig. 1.
TMEM100 messenger RNA is present in the mouse muscularis propria of the small intestine. A product of the expected size was amplified using the reaction containing reverse transcriptase but no product was amplified when reverse transciptase was omitted. The expected band size was 188 base pairs and was confirmed by DNA sequencing (N=3). Image is representative of three independent experiments.

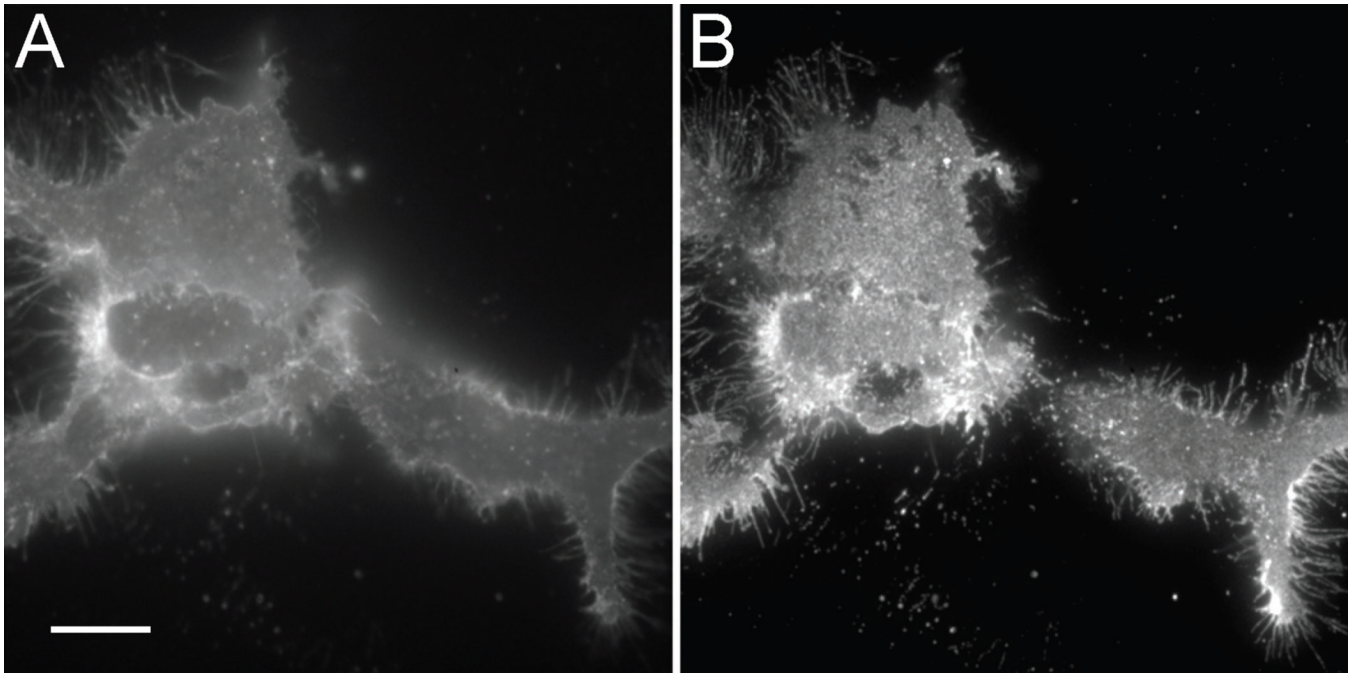

Fig. 2.
Transfection of TMEM100 human transcript in HEK 293 cells; fluorescence (A) and TIRF (B) imaging showing that much of the immunoreactivity is localized to within 100 nm of the imaging surface and therefore, within the plasma membrane (N=4). Scale bar A–B: 10 μm

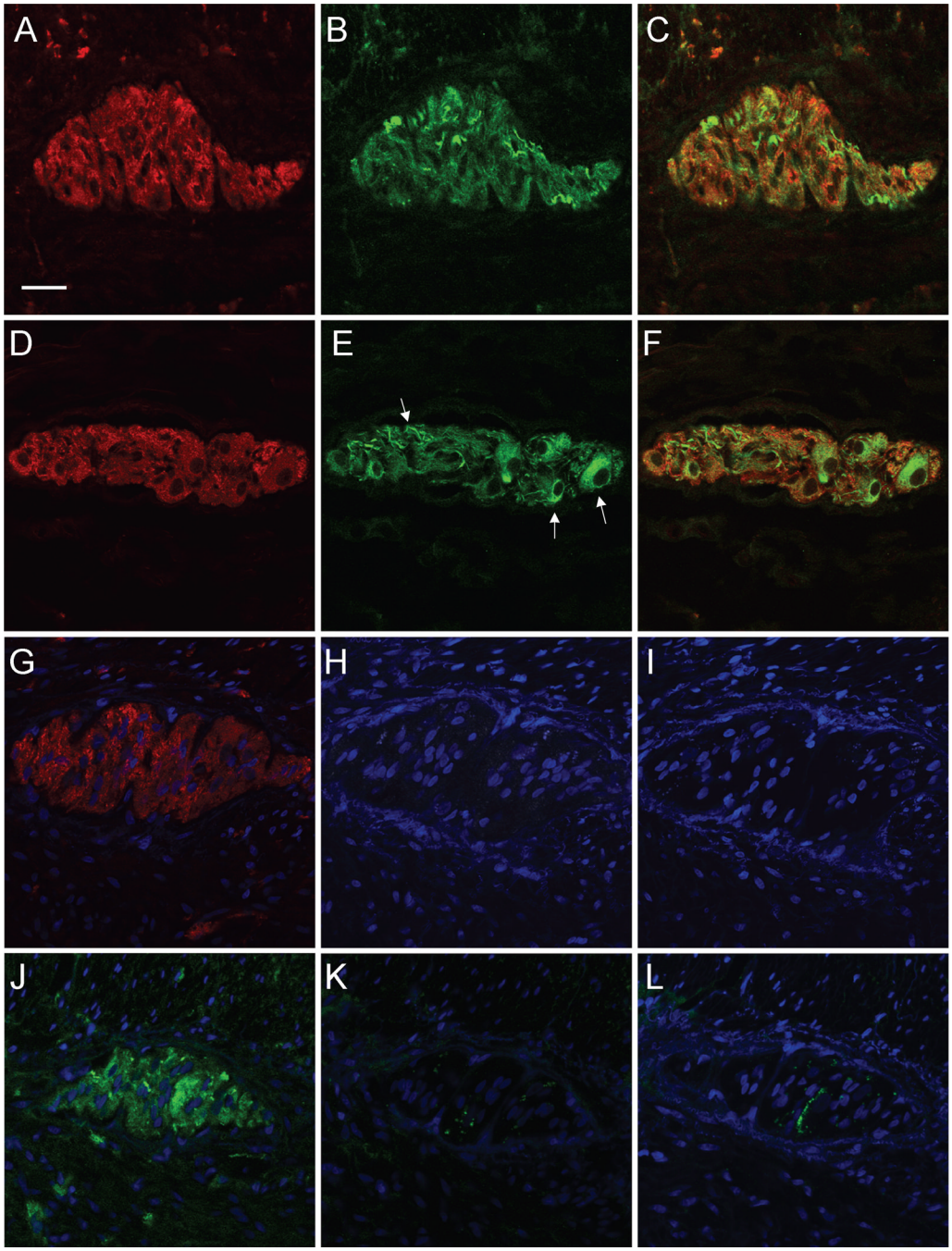

Fig. 3.

Positive and negative controls demonstrate specificity of two different antisera against TMEM100 in human jejunum cryosections. Co-localization of TMEM100 immunoreactivity in myenteric and submucosal ganglia using two antibodies raised in different species. Labeling using mouse (red, panels A,D) and goat (Alexa 647 - pseudo colored green, panels B,E) anti-TMEM100 is shown, colocalization is shown in yellow (C,F) (myenteric A–C, submucosal D–F). The goat anti-TMEM100 antibody labeled a subgroup of neurons and fibers more intensely (E, arrows). Pre-absorption using the immunogen demonstrated that the labeling was not falsely positive. Adjacent 15 μm cryosections are shown from the myenteric plexus region. Immunolabeling was only detected with mouse anti-TMEM100

(G) and goat anti-TMEM100 (J), while samples in which the primary antibodies were preabsorbed (H,K) and samples in which only secondary antibody was added (I,L) were negative. A–L are representative images of TMEM100 distribution as seen in gastric body, jejunum, and colon (N=3). Scale bar: 20 μm.

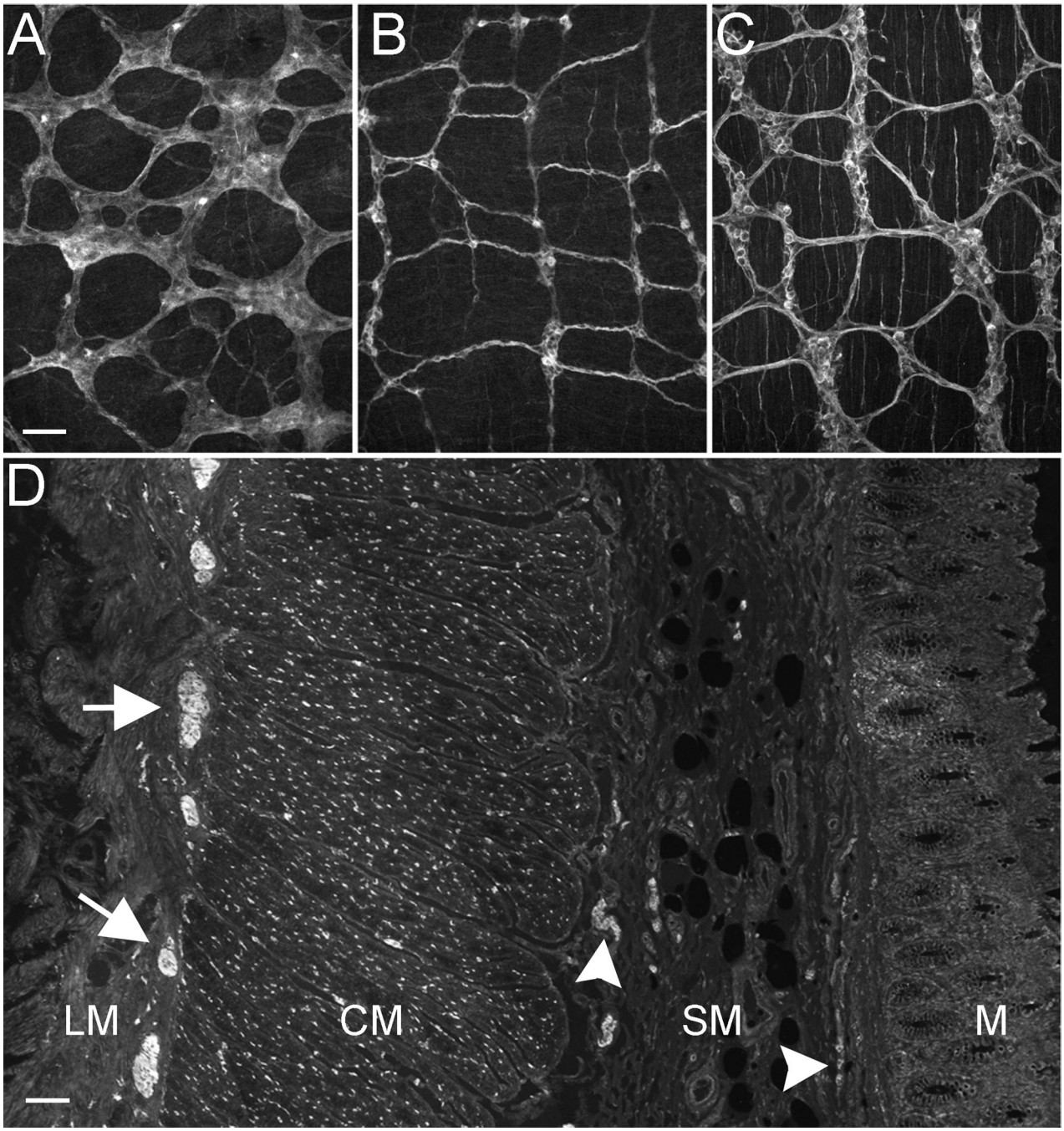

Fig. 4.

Micrographs showing immunolabeling of TMEM100 in mouse whole mounts and human cryosections. Confocal image stacks of TMEM100 immunoreactivity in the myenteric plexus region of mouse gastric body (A), jejunum (B), and proximal colon (C). TMEM100 immunoreactivity shown as a low power tiled image across the thickness (D) of the human proximal colon. Note labeling of what appear to be nerve fibers in all layers of the tissue as well as myenteric (arrows) and submucosal ganglia (arrowheads) - (longitudinal muscle - LM, circular muscle -CM, submucossa -SM, mucosa –M). A–D are representative images of TMEM100 distribution as seen in gastric body, jejunum, and colon (Mouse N=6, Human N=3). Scale bars A–C: 25 μm, D: 50 μm.

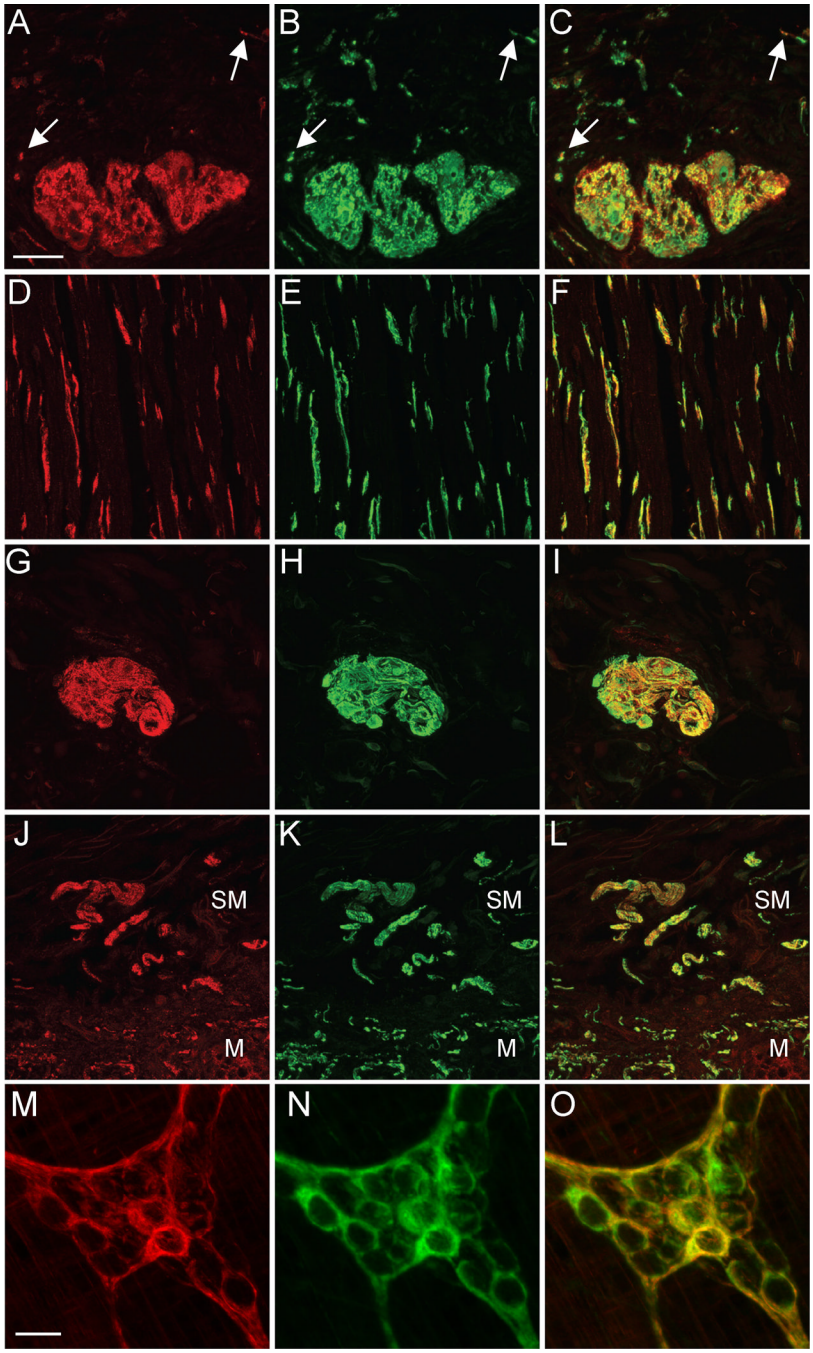

Fig. 5.

TMEM100 (A,D,G,J,M) co-localized with the pan-neuronal marker PGP9.5 (B,E,H,K,N) in all areas of the ENS. Shown in yellow (C,F,I,L,O), the co-localization of TMEM100 and PGP9.5 was found in the neural cell bodies and neural fibers in the longitudinal muscle (A–C, arrows), myenteric plexus of the human (A–C) and mouse (M–O), circular muscle (D–F), submucosal plexus (G–I), and along the mucosal border (J–L, submucossa – SM, mucosa - M) in the gastric body, jejunum, and proximal colon of human (N=3) and mouse (N=6). Stacks of 8–10 confocal images collected using a 60× objective. These data are representative images of TMEM100 distribution as seen in human and mouse gastric body, jejunum, and colon. Scale bars A–L: 20 μm, M–O 10 μm.

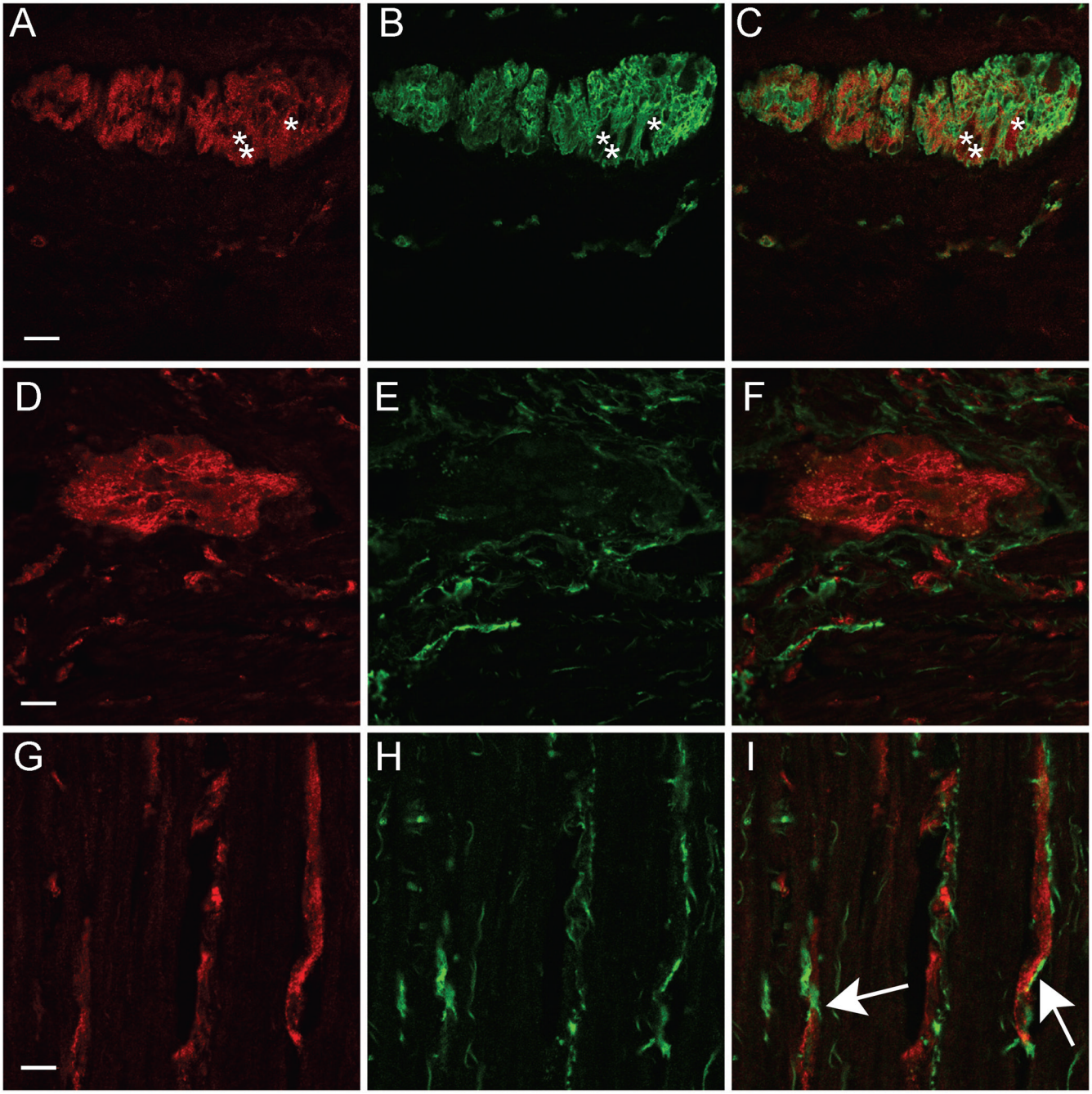

Fig. 6.

TMEM100 immunoreactivity did not co-localize with markers for glial cells (S100β) or interstitial cells of Cajal (ICC, Kit). Double-labeling of human jejunum with TMEM100 (A) and S100β (B) showed no co-localization. Asterisks mark neuronal cell bodies positive for TMEM100 immunoreactivity, but negative for S100β. Myenteric plexus labeled with TMEM100 (D) did not co-localize with Kit-positive ICC in the plane of the myenteric plexus. (E). Merged image of TMEM100/Kit (F). TMEM100 (G) labeled neural fibers come into close proximity with ICC-IM (H) in the intramuscular region of the human jejunum, but the immunoreactivity was in separate structures. Arrows indicate areas of close association

in the merged image (I). These data are representative images of TMEM100 distribution in human gastric body, jejunum, and colon (N=3). Scale bars A–F: 20 μm, G–I: 10 μm.

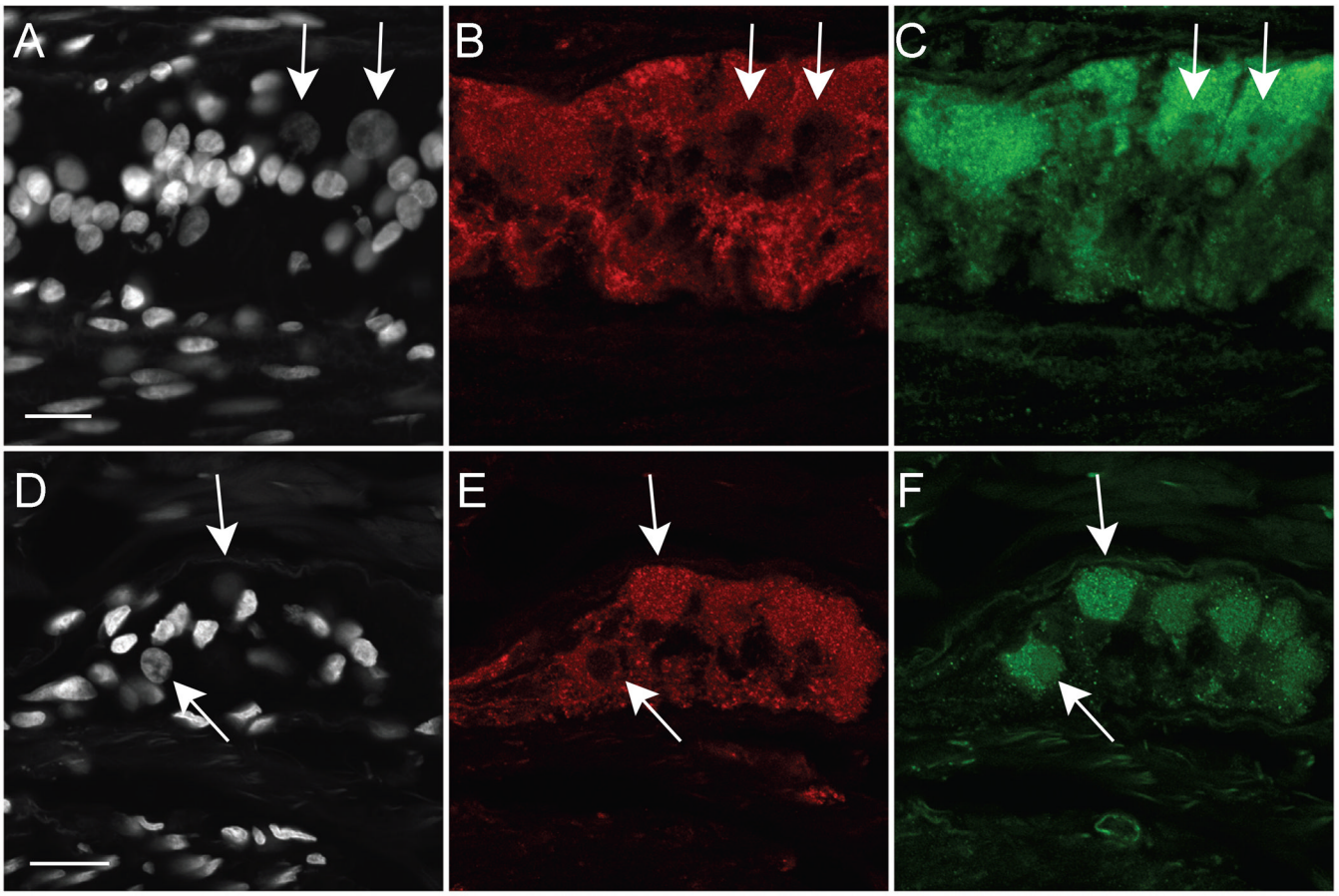

Fig. 7.

TMEM100 and BMP-4 were colocalized in enteric neuronal cell bodies of the human colon. Confocal slices of human colon cryosections showing BMP4 positive neuronal cell bodies in the myenteric (A–C) and submucosal (D–F) plexuses (N=3). DAPI counterstain (A,D) displays nuclei and allowed for the identification of neuronal nuclei as large rounded and euchromatic. Neuronal nuclei are identified by arrows. All neuronal cell bodies were positive for TMEM100 (B,E) and BMP4 (C,F). Scale Bars A–C: 30 μm, D–F 40 μm.

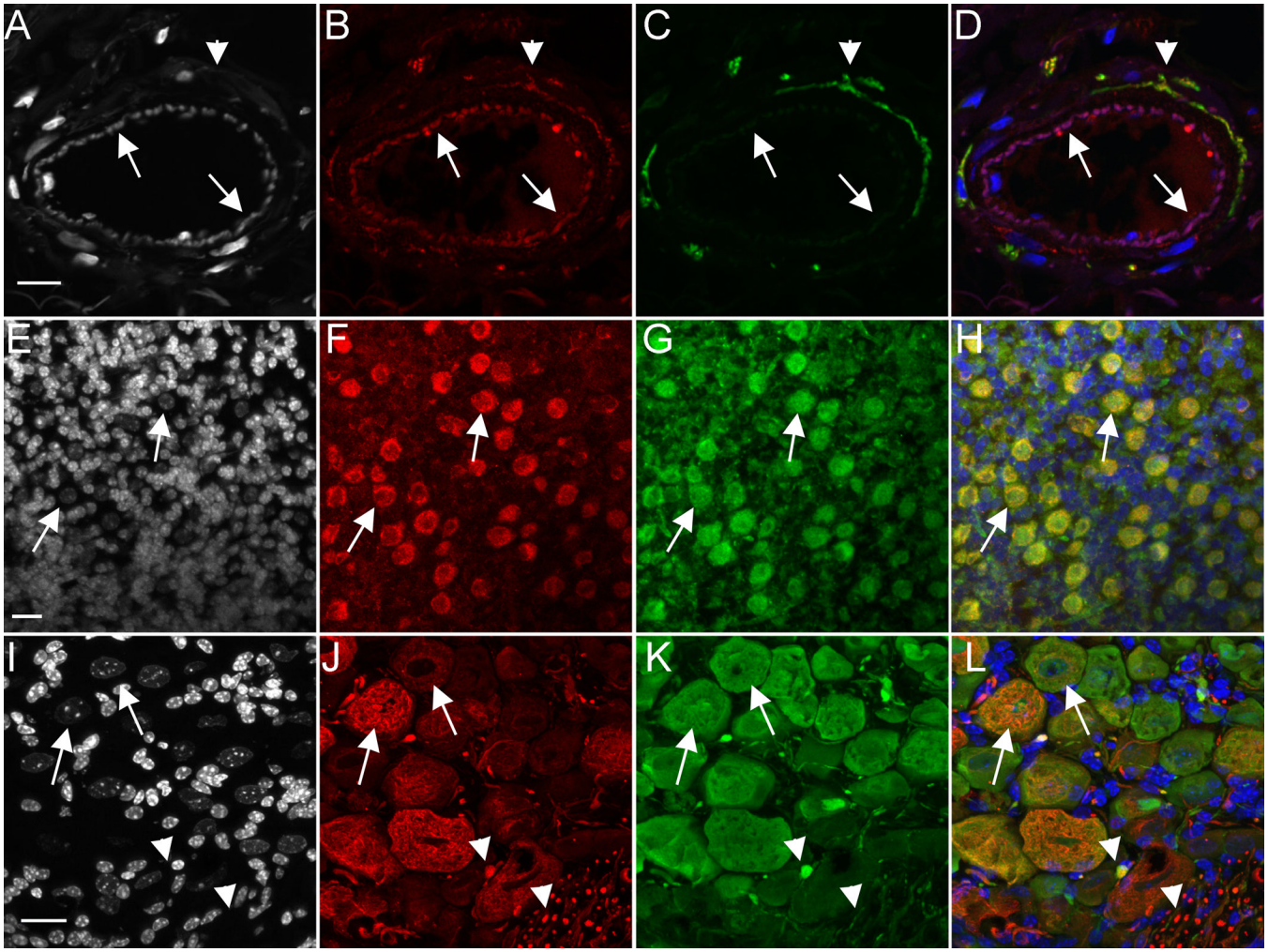

Fig. 8.

TMEM100 is located in tissues outside of the ENS. DAPI counterstain was used to show nuclei (A,E,I). TMEM100 was found in vascular endothelial cells (B, arrows) which were negative for PGP9.5 (C, arrows). Nerve fibers within the vasculature and submucossa were positive for both TMEM100 and PGP9.5 (B–C, arrowhead). Merged image (D, arrows) demonstrates TMEM100 distribution in vascular endothelial cells. Mouse anti-TMEM100 (F) and goat anti-TMEM100 (J) labeled neuronal cells bodies identified by large euchromatic nuclei (E,I, arrows) and colocalized with PGP9.5 (G,K) in both cerebral cortex (E–H) and dorsal root ganglia (I–L) of mouse cryosections (N=4). Merged images showing all neuronal cell bodies and fibers were positive for TMEM100 and PGP9.5 in the cerebral cortex (H) and dorsal root ganglia (L). Single confocal slice representative of TMEM100 labeling in vasculature of the human gastric body, jejunum, and proximal colon (A–D). Stacks of 8–10 confocal images representative of TMEM100 labeling in mouse brain and dorsal root ganglia. Scale bars A–D: 30 μm, E–H: 20 μm, I–L: 30 μm.

Table 1

Antibodies used for immunohistochemistry:

	Host	Concentration	Source	Catalog #
Primary Antibody				
TMEM100 polyclonal	Goat	1 μg mL^{-1}	Santa Cruz (CA, USA)	sc-165727
TMEM100 monoclonal	Mouse	5 μg mL^{-1}	OriGene (MD, USA)	TA500532
S100β monoclonal	Rabbit	1.0 μg mL^{-1}	Epitomics (CA, USA)	2017-1
PGP9.5 polyclonal	Rabbit	32 ng mL^{-1}	AbD Serotec (NC, USA)	7863-0504
Kit polyclonal	Rabbit	0.2 μg mL^{-1}	MBL (MA, USA)	566
BMP4 polyclonal	Rabbit	2.5 μg mL^{-1}	Abcam (MA, USA)	ab39973
Secondary Antibody				
AlexaFluor 647 anti-goat IgG	Donkey	2.5 μg mL^{-1}	Jackson (PA, USA)	705-605-147
Cyanin-3 anti-goat IgG	Donkey	2.5 μg mL^{-1}	Jackson	705-165-147
Cyanin-3 anti-mouse IgG	Donkey	2.5 μg mL^{-1}	Jackson	715-165-150
Fluorescein anti-rabbit IgG	Donkey	5.0 μg mL^{-1}	Jackson	711-095-152