Pediatric Surgery International (2019) 35:9–14
https://doi.org/10.1007/s00383-018-4370-x

ORIGINAL ARTICLE

Abnormal Scn1b and Fxyd1 gene expression in the pulled-through ganglionic colon may influence functional outcome in patients with Hirschsprung's disease

Anne Marie O'Donnell[1] · Hiroki Nakamura[1] · Christian Tomuschat[1] · Naoum Fares Marayati[2] · Prem Puri[1]

Accepted: 18 October 2018 / Published online: 1 November 2018

Abstract

Purpose Smooth muscle cells are electrically coupled to ICC and PDGFRα$^+$ cells, to regulate smooth muscle contraction. Recent studies have reported that the voltage-gated sodium channel type 1β (*Scn1b*), and the chloride channel subunit, *Fxyd1*, are highly expressed by both ICC and PDGFRα$^+$ cells in the mouse colon. We designed this study to investigate the expression of the *Scn1b* and *Fxyd1* genes in the normal human colon and in HSCR.

Methods HSCR tissue specimens ($n=6$) were collected at the time of pull-through surgery, while control samples were obtained at the time of colostomy closure in patients with imperforate anus ($n=6$). qRT-PCR analysis was undertaken to quantify *Scn1b* and *Fxyd1* gene expression, and immunolabelling of Scn1b and Fxyd1 proteins were visualized using confocal microscopy.

Results qRT-PCR analysis revealed significant downregulation of *Scn1b* and *Fxyd1* genes in both aganglionic and ganglionic HSCR specimens compared to controls ($p<0.05$). Confocal microscopy revealed a reduction in Scn1b and Fxyd1 protein expression in both aganglionic and ganglionic HSCR colon compared to controls.

Conclusion *Scn1b* and *Fxyd1* expression was significantly downregulated in HSCR colon. These results add to mounting evidence suggesting that the pulled-through ganglionic segment of bowel in these patients is abnormal, despite the presence of ganglion cells.

Keywords Scn1b · Fxyd1 · ICC · PDGFRα$^+$ cells · Hirschsprung's disease

Introduction

Co-ordinated gut motility and function is not solely dependent on the form of innervation it receives but also on the cells that regulate neurotransmission along with the enteric nervous system (ENS). Smooth muscle cells (SMC), interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor α-positive (PDGFRα$^+$) cells make up the 'SIP syncytium', communicating with each other and the ENS to regulate peristalsis in the gastrointestinal tract. Hirschsprung's disease (HSCR) is a congenital condition, affecting 1:5000 live births, which is characterised by the absence of ganglia in the distal colon. The extent of the aganglionic segment varies between patients from a short segment to total colonic aganglionosis.

Voltage-gated sodium channels (Na_Vs) are responsible for the upstroke and propagation of action potentials in excitable cells, including cardiomyocytes [1]. Na_Vs consist of a single, pore-forming α subunit and two different β subunits. The β subunits are multifunctional cell adhesion molecules and channel modulators that have cell type and subcellular domain specific functional effects. Variants in *SCN1B*, the gene encoding the Na_v-β1 and -β1B subunits, are linked to atrial and ventricular arrhythmias, e.g., Brugada syndrome, as well as to the early infantile epileptic encephalopathy Dravet syndrome, all of which put patients at risk for sudden death [1].

The FXYD family proteins are tissue-specific auxiliary subunits of the Na, K-ATPase, the principal enzyme responsible for maintaining the distribution of Na and K

✉ Prem Puri
prem.puri@ncrc.ie

[1] National Children's Research Centre, Our Lady's Children's Hospital Crumlin, Dublin 12, Ireland

[2] Department of Psychology, Princeton University, Princeton, NJ, USA

ion concentrations across animal cell membranes [2]. Seven FXYD proteins have been identified in mammals. FXYD1, more commonly known as phospholemman, is the principal substrate of hormone-stimulated phosphorylation by cAMP-dependent protein kinases A and C (PKA, PKC) in heart sarcolemma [2].

The results of surgical resection of the aganglionic bowel, although lifesaving, are often disappointing in that dysmotility, enterocolitis and soiling may persist in many patients despite a properly performed pull-through operation. Therefore, the presence of ganglia does not necessarily mean that the enteric nervous system will function normally. Several authors have reported abnormalities in the SIP syncytium in the ganglionated bowel in HSCR [3–5]. Recent studies have reported that both the *Scn1b* and *Fxyd1* genes are highly expressed by ICC and PDGFRα$^+$ cells in the mouse colon. We therefore designed this study to investigate the expression of the *Scn1b* and *Fxyd1* genes in the normal human colon and in HSCR.

Materials and methods

Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HSCR specimens from 6 patients who underwent pull-through surgery were studied. These specimens were divided into aganglionic and ganglionic specimens. We compared the most distal aganglionic segments with the most proximal ganglionic segments. HSCR patients were aged 6 ± 3 months old. No additional health issues existed in these patients. Colonic control samples included 6 specimens from patients who underwent colostomy closure following surgical correction of imperforate anus. Control samples were taken from patients who were 11 ± 4 months old. None of the imperforate anus patients had HSCR. Tissue specimens were either snap-frozen in liquid nitrogen and stored at − 80 °C for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at − 80 °C until use.

Immunofluorescence staining and confocal microscopy

Frozen blocks of HSCR colon and normal control samples were sectioned transversely at a thickness of 10 µm, mounted on SuperFrost® Plus slides (VWR International, Leuven, Belgium) and fixed with 10% buffered formalin for 5 min. Sections underwent cell membrane permeabilization with 1% Triton X-100 for 20 min at room temperature. After blocking with 10% normal goat serum (Sigma-Aldrich Ltd, Arklow, Ireland) for 30 min to avoid non-specific absorption, sections were incubated with primary antibodies; rabbit anti-Scn1b (Abcam, UK), mouse anti-fxyd1 (Abcam, UK), rabbit anti-PGP9.5 (Sigma-Aldrich, Ireland), rabbit anti-PDGFRα (Abcam, UK), mouse anti-PDGFRα (Abcam, UK), mouse anti-smooth muscle actin (Sigma-Aldrich, Ireland), mouse anti-huC/D (Santa Cruz, USA), rabbit anti-smooth muscle actin (Abcam, UK), rabbit anti-c-kit (Abcam, UK) and mouse anti-c-kit (Abcam, UK), all used at a dilution of 1:100 in PBS + 0.05% Triton X-100, overnight at 4 °C. Sections were then washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies; goat anti-mouse Alexa Fluor® 488, dilution 1:200 and goat anti-rabbit Alexa Fluor® 647, dilution 1:200, Abcam, UK, for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed, mounted, and coverslipped with Fluorescent Mounting Medium (DAKO Ltd, Cambridgeshire, UK). All sections were independently evaluated by two investigators with a LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

qRT-PCR

TRIzol reagent (Invitrogen) was used for the acid guanidinium-thiocyanate–phenol–chloroform extraction method to isolate total RNA from HSCR and control tissues ($n = 6$ for each group) according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at − 20 °C until further use. cDNA synthesis and quantitative polymerase chain reaction reverse transcription of total RNA was carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instructions. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20 µl per well. The following gene-specific primer pairs were used: Human Scn1b (Eurofins) sense primer 5′ ACA CCA GCG TCG TCA AGA AG and Human Scn1b (Eurofins) antisense primer 5′ GGC AGC GAT CTT CTT GTA GC, as well as Human Fxyd1 (Eurofins) sense primer 5′ AGA AAC ACC TGG AGC GAT GG and Human Fxyd1 (Eurofins) antisense primer 5′ ATT GGA AGT CTT GGC GGC AG. For normalization purposes, real-time RT-PCR was performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

GAPDH sense primer 5′ACA TCG CTG AGA CAC CAT GG and GAPDH antisense primer 5′ GAC GGT GCC ATG GAA TTT GC were used. After 5 min of initial denaturation at 95 °C, 55 cycles of amplification for each primer were carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression were normalized against the level of GAPDH gene expression in each sample. Experiments were carried out in duplicate for each sample and primer.

Statistical analysis

A one-way ANOVA was conducted to determine a statistically significant difference between aganglionic, ganglionic and healthy controls ($p < 0.05$). Data are presented as mean ± standard error. Specimens were classified into three groups: aganglionic ($n = 6$), ganglionic ($n = 6$) and normal controls ($n = 6$).

Results

Immunofluorescence staining and confocal microscopy

Immunohistochemistry in conjunction with confocal microscopy revealed that the distribution of Scn1b and Fxyd1 was decreased in both the aganglionic and ganglionic HSCR colon compared to normal controls. Both Scn1b (Fig. 1) and Fxyd1 (Fig. 2) were found to be co-localised with ICC and PDGFRα$^+$ cells within the myenteric and submucosal plexuses, as well as within the smooth muscle layer.

qRT-PCR

The relative mRNA expression levels of *Scn1b* (Fig. 3) and *Fxyd1* (Fig. 4) were significantly decreased in both aganglionic and ganglionic HSCR specimens compared to normal controls ($p < 0.05$).

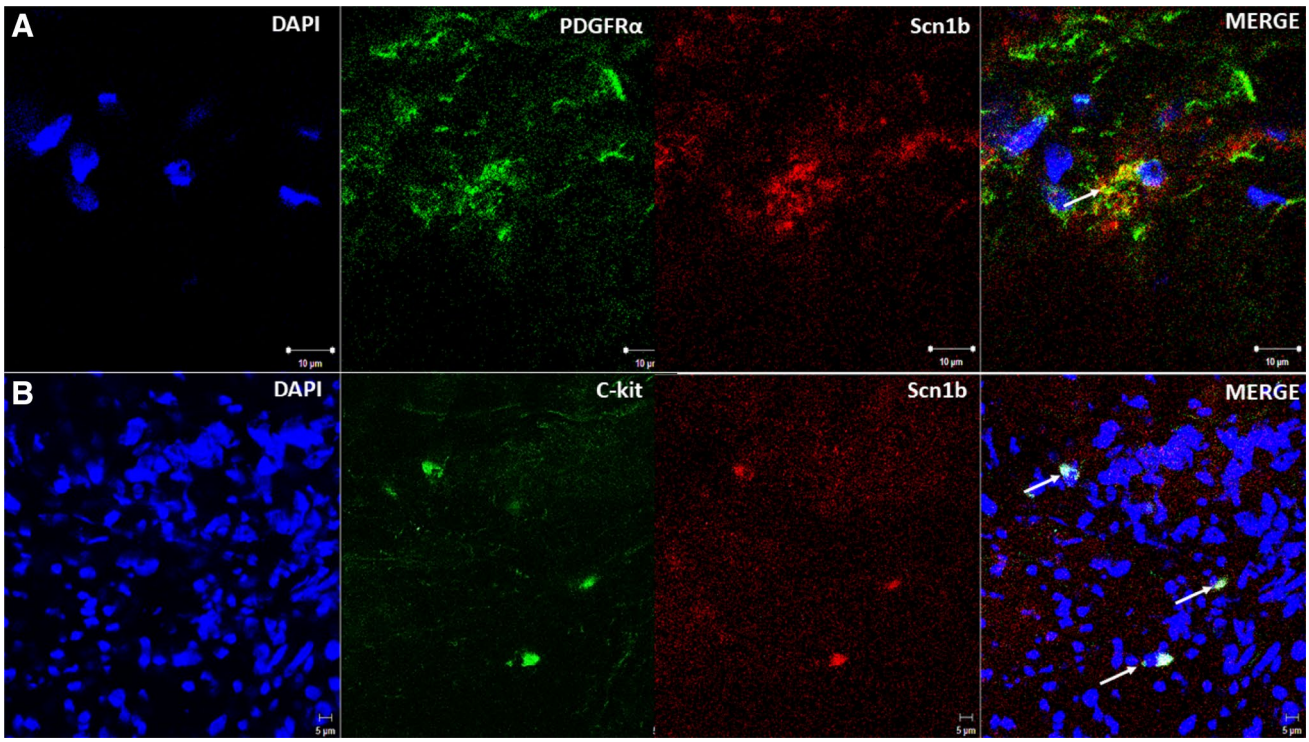

Fig. 1 Immunofluorescent staining of Scn1b found to be co-localised to ICC and PDGFRα$^+$ cells, within both the myenteric and submucosal plexuses, as well as within the smooth muscle layer of the ganglionic region. Nuclei were stained with DAPI (blue). Arrows show co-localisation

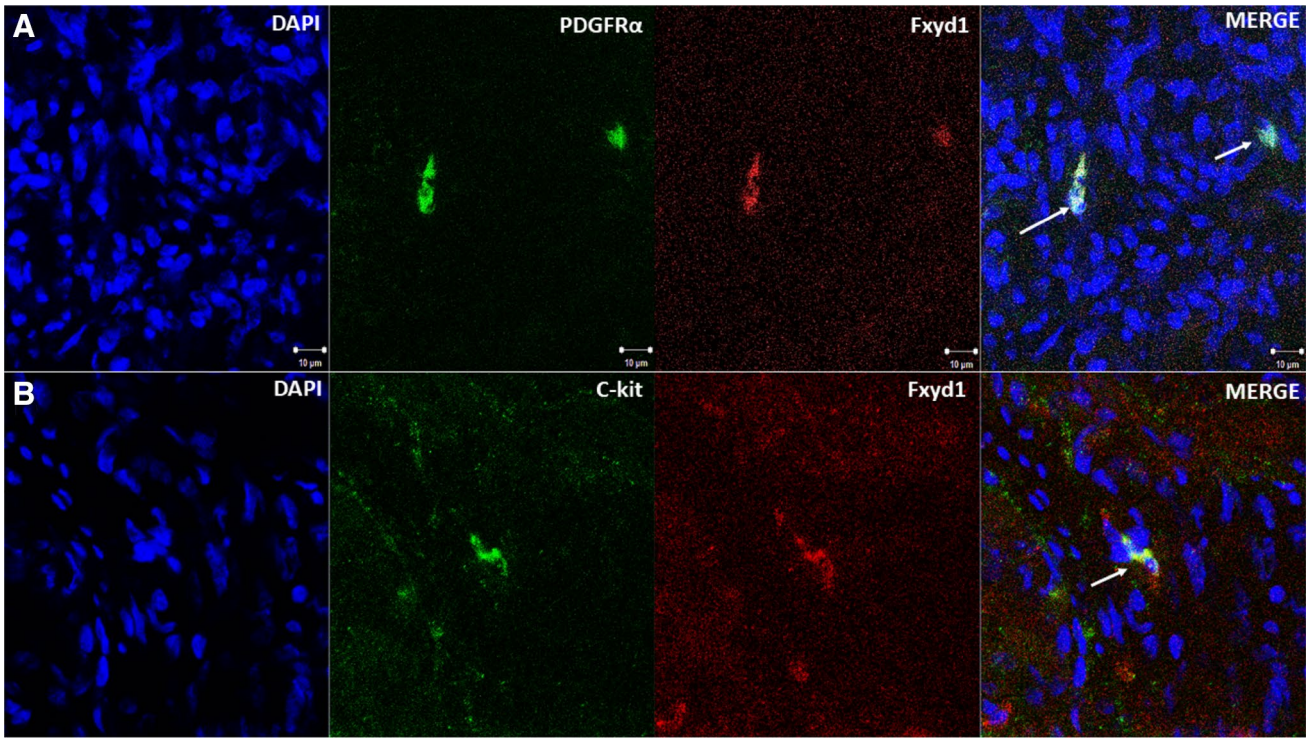

Fig. 2 Immunofluorescent staining of Fxyd1 found to be co-localised to ICC and PDGFRα^{+} cells, within both the myenteric and submucosal plexuses, as well as within the smooth muscle layer of the ganglionic region. Nuclei were stained with DAPI (blue). Arrows show co-localisation

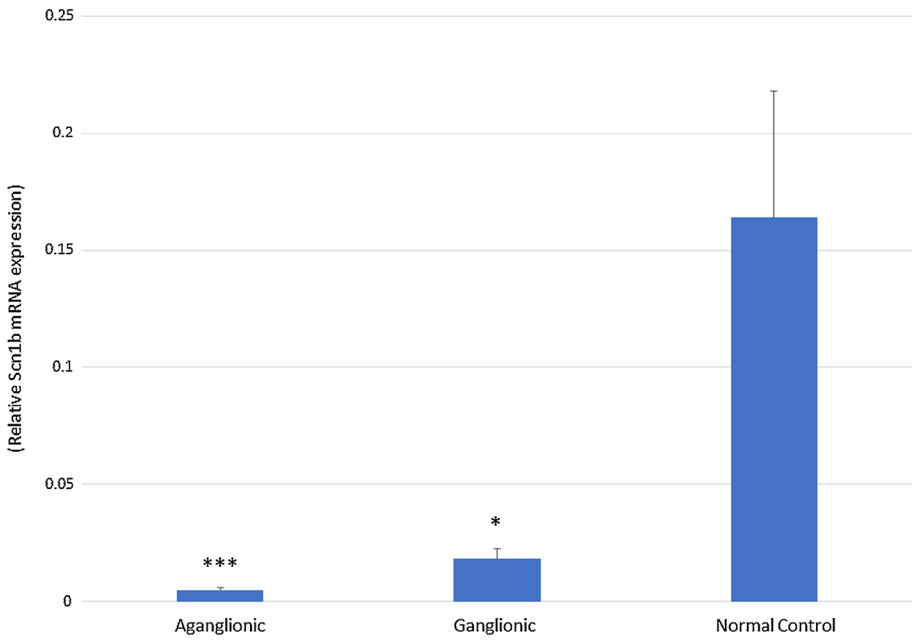

Fig. 3 qRT-PCR revealed significantly decreased relative mRNA expression levels of *Scn1b*, in the aganglionic and ganglionic HSCR specimens ($n=6$) compared to normal control tissue ($n=6$). Results are presented as mean ± SEM ($*p<0.05$, $***p<0.001$)

Discussion

A disruption of neural crest cell migration during the early stages of embryonic development is thought to be the main cause of HSCR, with neural crest cells failing to complete their cranio-caudal colonization of the gastrointestinal tract [6]. In addition to a lack of ganglia, many studies have documented deficiencies in smooth muscle proteins, extracellular matrix molecules, ion channels and various

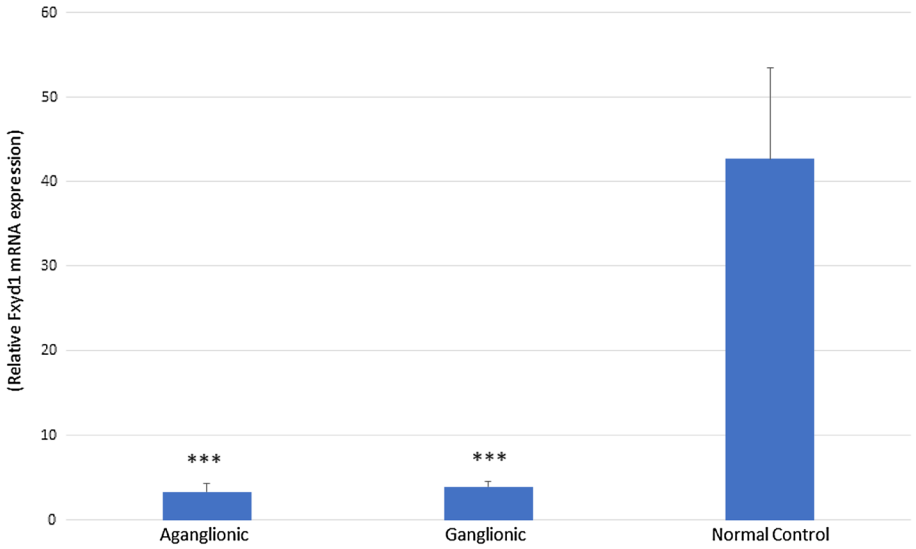

Fig. 4 qRT-PCR revealed significantly decreased relative mRNA expression levels of *Fxyd1*, in the aganglionic and ganglionic HSCR specimens ($n=6$) compared to normal control tissue ($n=6$). Results are presented as mean ± SEM (***$p<0.001$)

other important molecules in HSCR colon [3, 5, 7, 8]. The goal of surgical treatment for HSCR is to enable the affected child to have regular spontaneous bowel motions without soiling. Advances in the management of HSCR afford most patients a satisfactory outcome following a properly performed pull-through operation. However, a substantial cohort of patients continues to have persistent bowel dysfunction despite adequate resection of the aganglionic bowel segment. The postoperative bowel dysfunction includes enterocolitis, constipation, and incontinence [9–11]. Postoperative enterocolitis has been reported in 6–20% of patients and its incidence is unrelated to the timing of definitive surgery. Constipation and soiling have been reported to occur in 11–35% of patients after pull-through operations [9]. While a proportion of these patients are found to have a treatable pathology such as strictures, residual aganglionosis or transition zone, the majority have no identifiable cause for their ongoing bowel dysfunction.

The *SCN1B* gene encodes the sodium channel beta1 subunit (Navβ1) and sodium channel beta1b subunit (Navβ1b). *SCN1B* is comprised of large extracellular immunoglobulin-like domains, a single transmembrane-spanning segment, and intracellular C-terminal domains [12]. Scn1b has two kinds of transcripts, *SCN1B* and *SCN1Bβ*, which encodes Navβ1 and Navβ1b, respectively. Functional analysis indicated that β-subunit encodes by *SCN1B* involved in modulation of sodium channel gating and voltage dependence expression of sodium channel at the cell surface and cell adhesion [12]. Evidence over the past two decades has demonstrated that *SCN1B* subunits play critical roles in cardiac myocyte physiology, in which they regulate tetrodotoxin-resistant and -sensitive sodium currents, potassium currents, and calcium handling, and that Scn1b subunit dysfunction generates substrates for arrhythmias [1]. Variants in the *SCN1B* gene, encoding the splice variants Na_V-β1 and Na_V-β1B, are implicated in a variety of inherited pathologies including epileptic encephalopathy, Brugada syndrome, long-QT syndrome, atrial arrhythmias, and sudden infant death syndrome and epilepsy [13]. Fxyd1 is the first sequenced member of the FXYD family of regulators of ion transport, which in mammals, contains seven members (Fxyd1–Fxyd7). It is widely distributed with highest expression in cardiac and skeletal muscle [14]. FXYD1 produces chloride-activated currents and has been shown to colocalize with Na/Ca exchanger; with co-immunopreciptation experiments suggesting a direct interaction between the two proteins [14]. A study by Floyd et al. revealed high expression of Fxyd1 in the human gastrointestinal system (antral and oxyntic gastric mucosa, small intestine, and colon) [15]. The authors speculated that Fxyd1 associated with Na, K-ATPase may play an important role in regulating nutrient uptake in the gastrointestinal system [15]. Lee et al. have recently shown that the most predominant sodium channel gene expressed by murine colonic ICC was *Scn1b*, and a high expression of *Fxyd1* was also observed [16]. An additional study by Ha et al. investigating the transcriptome of murine colonic PDGFRα$^+$ cells revealed high expression of both *Scn1b* and *Fxyd1* genes [17]. Taken together, these studies suggest an important role for the *Scn1b* and *Fxyd1* genes in the functioning of the SIP syncytium.

The results of our current study showed statistically significant downregulation of the *Scn1b* and *Fxyd1* genes in both aganglionic and ganglionic HSCR specimens compared to controls. Confocal microscopy supported these results showing a reduction in Scn1b and Fxyd1 protein expression in both aganglionic and ganglionic HSCR colon compared to controls. Scn1b and Fxyd1 were found to be co-localised with ICC and PDGFRα$^+$ cells. These results add to mounting evidence which suggests that the pulled-through

ganglionic segment of bowel in these patients is abnormal, despite the presence of ganglion cells. The presence of ganglion cells alone can no longer serve as a standard for HSCR patients who continue to have persistent bowel symptoms despite a properly performed pull-through operation. Further research is necessary to fully understand the etiology of the disease and better success in its treatment. Better therapy, such as stem cell transplantation awaits advances in knowledge of ENS development, which is well advanced considering the success of several pre-clinical trials in animal models [18–20].

References

1. Edokobi N, Isom LL (2018) Voltage-gated sodium channel β1/β1B subunits regulate cardiac physiology and pathophysiology. Front Physiol 9:351. https://doi.org/10.3389/fphys.2018.00351
2. Franzin CM, Gong XM, Teriete P, Marassi FM (2007) Structures of the FXYD regulatory proteins in lipid micelles and membranes. J Bioenerg Biomembr 39(5–6):379–383. https://doi.org/10.1007/s10863-007-9105-y
3. Rolle U, Piotrowska AP, Nemeth L, Puri P (2002) Altered distribution of interstitial cells of Cajal in Hirschsprung disease. Arch Pathol Lab Med 126(8):928–933. https://doi.org/10.1043/0003-9985(2002)126%3C0928:ADOICO%3E2.0.CO;2
4. Coyle D, O'Donnell AM, Puri P (2015) Altered distribution of small-conductance calcium-activated potassium channel SK3 in Hirschsprung's disease. J Pediatr Surg. https://doi.org/10.1016/j.jpedsurg.2015.01.013
5. O'Donnell A, Coyle D, Puri P (2016) Deficiency of platelet-derived growth factor receptor-α-positive cells in Hirschsprung's disease colon. World J Gastroenterol 22(12):3335–3340
6. Young HM, Bergner AJ, Anderson CR, Enomoto H, Milbrandt J, Newgreen DF, Whitington PM (2004) Dynamics of neural crest-derived cell migration in the embryonic mouse gut. Dev Biol 270:455–473
7. O'Donnell AM, Coyle D, Puri P (2015) Decreased expression of hyperpolarisation-activated cyclic nucleotide-gated channel 3 in Hirschsprung's disease. World J Gastroenterol 21(18):5635–5640. https://doi.org/10.3748/wjg.v21.i18.5635
8. Rolle U, Nemeth L, Puri P (2002) Nitrergic innervation of the normal gut and in motility disorders of childhood. J Pediatr Surg 37(4):551–567
9. Menezes M, Corbally M, Puri P (2006) Long-term results of bowel function after treatment for Hirschsprung's disease: a 29-year review. Pediatr Surg Int 22(12):987–990. https://doi.org/10.1007/s00383-006-1783-8
10. Menezes M, Prato AP, Jasonni V, Puri P (2008) Long-term clinical outcome in patients with total colonic aganglionosis: a 31-year review. J Pediatr Surg 43(9):1696–1699. https://doi.org/10.1016/j.jpedsurg.2008.01.072
11. Mc Laughlin D, Friedmacher F, Puri P (2012) Total colonic aganglionosis: a systematic review and meta-analysis of long-term clinical outcome. Pediatr Surg Int 28(8):773–779
12. Yao H, Fan J, Cheng YJ, Chen XM, Ji CC, Liu LJ, Zheng ZH, Wu SH (2018) SCN 1Bβ mutations that affect their association with Kv4.3 underlie early repolarization syndrome. J Cell Mol Med. https://doi.org/10.1111/jcmm.13839
13. Watanabe H, Darbar D, Kaiser DW, Jiramongkolchai K, Chopra S, Donahue BS, Kannankeril PJ, Roden DM (2009) Mutations in sodium channel beta1- and beta2-subunits associated with atrial fibrillation. Circ Arrhythm Electrophysiol 2(3):268–275. https://doi.org/10.1161/CIRCEP.108.779181
14. Geering K (2005) Function of FXYD proteins, regulators of Na, K-ATPase. J Bioenerg Biomembr 37(6):387–392. https://doi.org/10.1007/s10863-005-9476-x
15. Floyd RV, Wray S, Martin-Vasallo P, Mobasheri A (2010) Differential cellular expression of FXYD1 (phospholemman) and FXYD2 (gamma subunit of Na, K-ATPase) in normal human tissues: a study using high density human tissue microarrays. Ann Anat 192(1):7–16. https://doi.org/10.1016/j.aanat.2009.09.003
16. Lee MY, Ha SE, Park C, Park PJ, Fuchs R, Wei L, Jorgensen BG, Redelman D, Ward SM, Sanders KM, Ro S (2017) Transcriptome of interstitial cells of Cajal reveals unique and selective gene signatures. PLoS One 12(4):e0176031. https://doi.org/10.1371/journal.pone.0176031
17. Ha SE, Lee MY, Kurahashi M, Wei L, Jorgensen BG, Park C, Park PJ, Redelman D, Sasse KC, Becker LS, Sanders KM, Ro S (2017) Transcriptome analysis of PDGFRα+ cells identifies T-type Ca^{2+} channel CACNA1G as a new pathological marker for PDGFRα+ cell hyperplasia. PLoS One 12(8):e0182265. https://doi.org/10.1371/journal.pone.0182265
18. Burns AJ, Goldstein AM, Newgreen DF, Stamp L, Schafer KH, Metzger M, Hotta R, Young HM, Andrews PW, Thapar N, Belkind-Gerson J, Bondurand N, Bornstein JC, Chan WY, Cheah K, Gershon MD, Heuckeroth RO, Hofstra RM, Just L, Kapur RP, King SK, McCann CJ, Nagy N, Ngan E, Obermayr F, Pachnis V, Pasricha PJ, Sham MH, Tam P, Vanden Berghe P (2016) White paper on guidelines concerning enteric nervous system stem cell therapy for enteric neuropathies. Dev Biol 417(2):229–251. https://doi.org/10.1016/j.ydbio.2016.04.001
19. Stamp LA, Young HM (2017) Recent advances in regenerative medicine to treat enteric neuropathies: use of human cells. Neurogastroenterol Motil. https://doi.org/10.1111/nmo.12993
20. McCann CJ, Thapar N (2018) Enteric neural stem cell therapies for enteric neuropathies. Neurogastroenterol Motil. https://doi.org/10.1111/nmo.13369