ORIGINAL ARTICLE

microRNA overexpression in slow transit constipation leads to reduced Na\text{v}1.5 current and altered smooth muscle contractility

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ABSTRACT:

Objective This study was designed to evaluate the roles of microRNAs (miRNAs) in slow transit constipation (STC).

Design All human tissue samples were from the muscularis externa of the colon. Expression of 372 miRNAs was examined in a discovery cohort of four patients with STC versus three age/sex-matched controls by a quantitative PCR array. Upregulated miRNAs were examined by quantitative reverse transcription qPCR (RT-qPCR) in a validation cohort of seven patients with STC and age/sex-matched controls. The effect of a highly differentially expressed miRNA on a custom human smooth muscle cell line was examined in vitro by RT-qPCR, electrophysiology, traction force microscopy, and ex vivo by lentiviral transduction in rat muscularis externa organotypic cultures.

Results The expression of 13 miRNAs was increased in STC samples. Of those miRNAs, four were predicted to target SCN5A, the gene that encodes the Na\text{+} channel Na1.5. The expression of SCN5A mRNA was decreased in STC samples. Let-7f significantly decreased Na\text{+} current density in vitro in human smooth muscle cells. In rat muscularis externa organotypic cultures, overexpression of let-7f resulted in reduced frequency and amplitude of contraction.

Conclusions A small group of miRNAs is upregulated in STC, and many of these miRNAs target the SCN5A-encoded Na\text{+} channel Na1.5. Within this set, a novel Na1.5 regulator, let-7f, resulted in decreased Na1.5 expression, current density and reduced motility of GI smooth muscle. These results suggest Na1.5 and miRNAs as novel diagnostic and potential therapeutic targets in STC.

INTRODUCTION

GI motility is critical for the normal function of the human GI tract. Disruptions in GI motility are associated with several diseases or disorders with onsets that vary from acute, for example, postoperative ileus, to insidious, such as pseudo-obstruction. Slow transit constipation (STC), a chronic disabling disease hallmarkd by delays in colonic transit in the absence of outlet obstruction, may be refractory to
medication, resulting in a colectomy.1 2 The pathophysiology of STC is not well defined. Previous studies show a loss of enteric neurons3 and interstitial cells of Cajal (ICC)—the pacemakers of the gut.4,5 However, in the majority of cases, a histological analysis is not diagnostic,6 which may suggest functional pathology at the molecular level.

Ion channels are indispensable for electrical excitability and electromechanical coupling in GI smooth muscle. Therefore, tight regulation of ion channel expression and function are paramount for normal GI motility. Voltage-gated Na⁺ channels, in particular, are important for the generation and propagation of signals in electrically excitable cells, such as myocytes, cardiomyocytes and neurons.7 Even in GI smooth muscle, where Ca²⁺ signaling is fundamental for excitability and motility, Na⁺ channels contribute in a species-dependent fashion to the regulation of electrical and contractile function.8–13 A voltage-gated Na⁺ channel Na₉,1.5, encoded by the SCN5A gene, is traditionally considered to be a ‘cardiac’ Na⁺ channel,14 but Na₉,1.5 is expressed in human GI smooth muscle and ICC in jejunum and colon,10,15 where it is important for electrophysiological function11,16 and contractility.13 It is also present and functionally relevant in the intestinal smooth muscle of rat11 and myenteric neurons but not smooth muscle of mice.12,17

SCN5A mutations cause cardiac conduction disorders, ‘channelopathies’, such as long QT syndrome and Brugada syndrome,18 and SCN5A is the only non-structural gene implicated in dilated cardiomyopathy, a cardiac contractility disorder.19–21 Patients with SCN5A channelopathies and known cardiac arrhythmias have an increased prevalence of functional GI diseases,22 and conversely patients with IBS are more likely to have SCN5A channelopathies, with a greater percentage of patients with SCN5A channelopathies having constipation-predominant IBS.23 Furthermore, Na₉,1.5 block by ranolazine, a Food and Drug Administration-approved medication, is strongly associated with constipation24 due to the inhibition of Na₉,1.5 mechanosensitivity and reduced contractility of human colon smooth muscle.13

However, channelopathies are relatively rare. More frequently, diseases are caused by altered ion channel expression,25 especially because changes in ionic current as little as 1% can lead to fatal diseases.26 Therefore, ion channel expression is tightly regulated by multiple mechanisms, including at the epigenetic, mRNA and protein levels. microRNAs (miRNAs) are a class of small non-coding RNAs that modulate gene expression at the post-transcriptional level and are involved in the pathogenesis of many diseases.27 Ion channel densities, including Na₉,1.5, are frequently regulated by miRNAs, and this mechanism is associated with cardiac conduction disorders.28 In the GI tract, miRNAs regulate ion channel densities and contribute to functional GI disorders.29,30 Since Na₉,1.5 current density is involved in GI smooth muscle function, where Na₉,1.5 inhibition leads to constipation and delayed transit, we hypothesised in this study that miRNAs might regulate Na₉,1.5 density in STC.

METHODS

Human colon samples and RNA/miRNA extraction

The Mayo Clinic Institutional Review Board approved the use of human colonic tissue obtained as surgical waste. Colon muscularis externa was dissected from human colon tissue from patients undergoing colon resections for colon cancer (table 1). Tissue was flash-frozen in liquid nitrogen and stored at −80°C until use. Total RNA, including the miRNA fraction, was extracted using miRNeasy mini kit (Qiagen) according to manufacturer instructions.

| Patient | Sex | Age (years) |
|---------|-----|-------------|
| CTRL 1  | Female | 58 |
| CTRL 2  | Female | 48 |
| CTRL 3  | Female | 64 |
| CTRL 4  | Female | 49 |
| CTRL 5  | Female | 69 |
| CTRL 6  | Female | 43 |
| CTRL 7  | Female | 52 |
| CTRL 8  | Female | 54 |
| CTRL 9  | Female | 58 |
| CTRL 10 | Female | 62 |
| STC 1   | Female | 54 |
| STC 2   | Female | 51 |
| STC 3   | Female | 54 |
| STC 4   | Female | 61 |
| STC 5   | Female | 54 |
| STC 6   | Female | 50 |
| STC 7   | Female | 55 |
| STC 8   | Female | 60 |
| STC 9   | Female | 61 |
| STC 10  | Female | 54 |
| STC 11  | Female | 60 |

CTRL, control; STC, slow transit constipation.

Quantitative PCR (qPCR) arrays

Reverse transcription to prepare cDNA for mature miRNA profiling was carried out using the miScript II RT Kit (Qiagen) and 400 ng of total RNA according to the manufacturer instructions. The qPCR arrays used were the miScript miRNA PCR Array Human miFinder 384HC (MIHS-3001Z, Qiagen). These continuous data were analysed using Qiagen software and significance was assigned if p<0.05.

qRT-PCR

Reverse transcription reactions for mRNA of interest were conducted using the SuperScript VIVO cDNA Synthesis Kit (Life Technologies). Reverse transcription reactions for mRNA of interest were done using the MicroRNA RT Kit and specific MicroRNA primers (TaqMan, ThermoFisher), according to specifications. qPCR was done on LightCycler 480 system with SYB RGreen1 Master Mix (Roche Applied Science). Results were calculated as expression relative to the housekeeper (hypoxanthine phosphoribosyltransferase 1 (HPRT1)) using 2^(-ΔCT), and statistics on these categorical data were done using Mann-Whitney with significance assigned if p<0.05.

Cell lines

Primary human smooth muscle cells (HuSMCs) were dissociated from the jejunum of a patient (female, 52) undergoing conversion to Roux-en-y gastric bypass surgery, and were immortalised by transformation with the SV40 large T antigen.31 HuSMCs were cultured in complete Clonetics SmGM−2 Smooth Muscle Growth Medium-2 (Lonza) containing 1% antimycotic-antibiotic (Gibco).

miRNA transfection

HuSMCs were transfected using LIPOFECTAMINE RNAiMax reagent (Invitrogen) and 50 nM of miRNA mimics (Thermo Fisher) in Opti-MEM according to the manufacturer instructions.
The cells were incubated for 48 hours before RNA/miRNA extraction and electrophoresis experiments.

miRNA modulation of SCN5A expression
HEK293 cells were plated in six-well plates for 72 hours in minimum essential medium (MEM), supplemented with 10% fetal bovine serum and penicillin/streptomycin, then transfected by Lipofectamine 3000 (Invitrogen) with SCN5A full-length 3’ UTR (2259bp) reporter vector (HmiT016601-MT05, Genecopoeia) and/or 50nM of let-7f miRNA mimic (hsa-let-7f-5p, Life Technologies) in a total volume of 2.4 mL per well. After 24 hours, Opti-MEM was replaced with fresh MEM. At 48 hours, conditioned MEM media was collected into 1.5 mL tubes, and Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) activities were measured by Secretre-Pair Dual Luminescence Assay (Genecopoeia). Luminescence was read on a plate reader within 10 min at 1 s integration time. Normalised luminescence units (NLUs) were calculated by the equation: NLU = (GLucX – GLuc_X′)/ (SEAPX – SEAP_X′), in which GLuc or SEAP are activities (arbitrary units) of luciferase or SEAP in conditioned media, _UTR is conditioned media from cells transfected without the SCN5A 3’ UTR reporter vector and X is conditioned media from cells transfected without (control) or with miRNA mimic (let-7f).

Protein extraction and western blotting
HuSMC were gently washed twice with ice-cold phosphate-buffered saline and collected by scraping the plates. Cells were pelleted at 4°C for 5 min and used for protein extraction and immunoblotting as previously described (see online supplementary methods).

Electrophysiology
Data were recorded on an Axopatch 200B amplifier with pClampfit software. Whole-cell Na⁺ currents were elicited by stepping from the holding potential at −120 mV to 30 ms test pulses at −80 through +20 mV. Peak Na⁺ currents at the −20 mV step were normalised to whole cell capacitance. Solutions used are described in online supplementary methods.

Traction force microscopy
Traction analysis was conducted as previously described (see online supplementary methods).

Lentiviral transduction
Muscularis externa was dissected from the jejunum of 6–8 weeks old Sprague Dawley rat as previously described. Tissues were transduced with non-targeting or let-7f lentiviral particles (Dharmacon) with a multiplicity of infection of 5. At day 0 and day 5, about 2 min long movies were recorded at a rate of 14 frames/second using a DP22 camera (Olympus) connected to a SZ61 stereomicroscope (Olympus) and used for contractility measurement and spatial–temporal mapping.

Contractility measurements
ImageJ software was used to import and convert images for contractility measurements as described in online supplementary methods.

Spatial–temporal mapping
Individual frames over 90 s were extracted as grey-scale images from each video and enhanced in MATLAB (2015a, MathWorks).

RESULTS
Multiple miRNAs predicted to target SCN5A are overexpressed in STC
We examined 372 of the most common miRNAs in the miRBase database by a qPCR miRNA array to identify miRNAs for further investigation from four female patients and three age and sex-matched controls. We found that 13 of 372 miRNAs (3.5%) were threefold to eightfold upregulated in STC tissues (figure 1A; table 2). None of the examined miRNAs were significantly downregulated in STC. An unsupervised hierarchical cluster analysis based on the expression patterns of the 13 identified miRNAs using average linkage separated them into two major clusters (figure 1B). Interestingly, both clusters contained miRNAs that are known regulators of SCN5A, the gene encoding for the mechanosensitive ion channel NaV1.5, a well-known player in multiple GI motility disorders. The first cluster, with 10 of the 13 identified miRNAs, included two members of the miR-98 family (let-7e and let-7f), and the second included two members of the miR-200 family (miR-200b-3p and miR-429). Those miRNAs are predicted or previously shown to modulate NaV1.5 expression by binding to the 3’UTR of the SCN5A mRNA (figure 1C). These results suggest that a specific miRNA set is enriched in STC, and >30% of these may target SCN5A.

Figure 1 Thirteen of 372 miRNA examined are differentially expressed in slow transit constipation. (A) Heatmap plotting negative Ct values from the miRNA qPCR array organised by increasing p values. The box indicates the ones with p<0.05. The expression level of miRNAs is colour-coded as indicated in the legend. (B) Blowup of highlighted group of miRNAs, with hierarchical clustering analysis on the left. (C) Map of SCN5A mRNA 3’-UTR with binding sites for miRNAs obtained from multiple prediction algorithms (online supplementary table 1) and literature. In the boxes are position and sequence of target region complementary to each miRNA seed. In red are miRNAs identified by qPCR array screening. (D) SCN5A mRNA expression is reduced in STC smooth muscle. Data are median±IQR. n=10–11. *p<0.05, Mann-Whitney test. CTRL, control; miRNA, microRNA; qPCR, quantitative PCR; STC, slow transit constipation.

Displacements, frequency and coordination were measured as detailed in online supplementary methods.
We found no difference in expression between the STC (figure 2B,C). We also tested the expression of two negative controls. The first is miR-155, a miRNA that was not predicted differentially expressed in the array screening. Neither miRNA was significantly altered in STC. We expanded our cohort and collected tissue from seven additional patients with STC and seven age-matched controls. We found a robust decrease of \( \text{SCN5A} \) from seven additional patients with STC and seven age-matched controls. Expression of miR-429 (B) and Let-7f (C) is increased in STC. Data are median±IQR, n=10–12, \( *p<0.05 \), Mann-Whitney test. CTRL, control; miRNA, microRNA; qPCR, quantitative PCR.

Table 2 List of miRNA differentially expressed in slow transit constipation compared with controls

| miRNA       | Fold change | P value (t-test) |
|-------------|-------------|-----------------|
| hsa-miR-429 | 8.28        | 0.005983        |
| hsa-miR-32-5p | 6.79     | 0.004771        |
| hsa-miR-10b-5p | 6.71     | 0.004041        |
| hsa-let-7e-5p | 6.56     | 0.0042515       |
| hsa-let-7f-5p | 6.46     | 0.0046753       |
| hsa-miR-150-5p | 6.34     | 0.0046574       |
| hsa-miR-15b-3p | 6.26     | 0.0048789       |
| hsa-miR-25-3p | 6.23     | 0.0042496       |
| hsa-miR-454-3p | 4.97     | 0.0047989       |
| hsa-miR-7-5p | 4.76     | 0.03512         |
| hsa-miR-152-3p | 4.62     | 0.004643        |
| hsa-miR-146a-5p | 4.57    | 0.026612         |
| hsa-miR-200a-3p | 3.25    | 0.020789         |

Results of the statistical analysis carried out on the data obtained by the miRNA qPCR array screening using the SABioscience analysis software (http://pcrdataanalysis.sabiosciences.com/mirna). miRNA predicted to bind to the 3′-UTR of SCN5A is given in bold. miRNA, microRNA; qPCR, quantitative PCR.

These results prompted us to examine if SCN5A expression is altered in STC. We expanded our cohort and collected tissue from seven additional patients with STC and seven age-matched controls. We found a robust decrease of SCN5A mRNA expression by about 35% in STC (figure 1D). We used this expanded cohort to validate the array results by quantitative reverse transcription PCR of individual miRNAs. We selected differentially expressed miRNAs that were predicted to target SCN5A: miR-429 and miR-200a-3p from one cluster and let-7f from the other. We found no difference in expression between the STC and control groups for miR-200a-3p, likely due to low expression of this miRNA in our samples (figure 2A), but we confirmed significant differential expression for let-7f and miR-429 (figure 2B,C). We also tested the expression of two negative controls. The first is miR-155, a miRNA that was not predicted to target the 3′-UTR of SCN5A and was not differentially expressed in our array screening but reported to be affected in some pathologies of mechanosensitivity. The second control was miR-219, a miRNA that targets SCN5A, but was not differentially expressed in the array screening. Neither miRNA was differentially expressed between the control and STC samples (figure 2D,E).

Figure 2 The expression of let-7f miRNA is significantly increased in STC. (A) miR-200a is not differentially expressed between CTRL and STC. Expression of miR-429 (B) and Let-7f (C) is increased in STC. Expression of miR-155 (D) and miR-219 (E) is not altered in STC. Data are median±IQR. n=10–12, \( *p<0.05 \), Mann-Whitney test. CTRL, control; miRNA, microRNA; STC, slow transit constipation.

Figure 3 HuSMCs have characteristics similar to GI smooth muscle cells. (A) Representative images of cell tractions’ colour maps generated by the same HuSMC in basal conditions (left) and response to carbachol (right). The colours correspond to magnitudes of traction forces generated as indicated in the colour bar. The RMS traction forces generated are quantified in the graph. Data are median±IQR, n=7, \( *p<0.05 \), Mann-Whitney test. (B) HuSMCs (passage 6, P6) are highly enriched for mRNA for smooth muscle cell marker genes, including SCN5A and CACNA1C, compared with later passages (>10, P10), which lose smooth muscle phenotype (n=4). (C) HuSMCs express Na\(_{\text{v}1.5}\) protein by western blot. (D) Representative traces of Na\(_{\text{v}1.5}\) function directly and found robust Na\(_{\text{v}1.5}\) currents in HuSMCs (figure 3C). We used whole-cell voltage clamp to test HuSMC electrical excitability as indicated in the graph. Data are median±IQR, n=7, \( *p<0.05 \), Mann-Whitney test. (B) HuSMCs (passage 6, P6) are highly enriched for mRNA for smooth muscle cell marker genes, including SCN5A and CACNA1C, compared with later passages (>10, P10), which lose smooth muscle phenotype (n=4). (C) HuSMCs express Na\(_{\text{v}1.5}\) protein by western blot. (D) Representative traces of Na\(_{\text{v}1.5}\) function directly and found robust Na\(_{\text{v}1.5}\) currents in HuSMCs (figure 3C). Western blot experiments showed that HuSMCs also express Na\(_{\text{v}1.5}\) protein (figure 3C). We used whole-cell voltage clamp to test HuSMC Na\(_{\text{v}1.5}\) function directly and found robust Na\(_{\text{v}1.5}\) currents with density and kinetic properties comparable to freshly dissociated HuSMCs (figure 3D–G). These data suggested that HuSMC is a suitable SMC model to examine the effects of miRNAs’ regulation on Na\(_{\text{v}1.5}\) and SMC function.

Let-7f overexpression in HuSMCs significantly reduced SCN5A expression, Na\(_{\text{v}1.5}\) current density and cellular contractility

We first used a luciferase reporter driven by SCN5A 3′UTR to determine if let-7f suppresses SCN5A expression. We found that 48 hours after transfection, let-7f miRNA mimics decreased...
Overexpression of let-7f significantly reduced Na⁺ current density in HuSMCs and resulted in changes in the cells’ properties. (A) RT-qPCR show efficient delivery of miRNA after transfection of mimics in HuSMCs (means±SDs, n=6). (B) Representative patch-clamp traces for HuSMC±miRNA mimics. (C) Peak Na⁺ current densities were reduced after transfection with let-7f (means±SEMs, n=5–17 cells, *p<0.05 by a one-way analysis of variance with Dunnett’s post-test). (D) NLUs of HEK-293 cells transfected with SCN5A 3’ UTR vector alone (control) or cotransfected with the 3’ UTR vector and miRNA mimic (let-7f) (means±SEMs, n=6 transfections, *p<0.05 to same-plate controls by a two-tailed paired t-test) from cells transfected with the 3’ UTR vector alone (control). GLuc, Gaussia luciferase; HuSMC, human smooth muscle cells; miRNA, microRNA; NLU, normalised luciferase unit; UTR, compared with cells transfected with the 3’ UTR vector alone (control) or cotransfected with the 3’ UTR vector and miRNA mimic (let-7f) (means±SEMs, n=6 transfections, *p<0.05 to same-plate controls by a two-tailed paired t-test). We pursued the mechanism of miRNA regulation of smooth muscle contractility.

**DISCUSSION**

The goal of this study was to improve our understanding of STC. We focused on miRNAs because they control GI and vascular smooth muscle phenotype and function. 32–34 Previous studies in mice that entirely removed the ability of SMCs to generate miRNAs showed a dramatic smooth muscle dysfunction, hallmarking by decreases in vascular smooth muscle contractility 36 and bowel hypomotility and dilatation. 37 However, complete miRNA knockout made the clinical relevance of these studies unclear. In this study, we found in human STC colonic smooth muscle a surprisingly small subset of dysregulated miRNAs, suggesting functional roles of miRNAs in human GI smooth muscle contractility and dysmotility.

We pursued the mechanism of miRNA regulation of smooth muscle function. We focused on miRNA roles in regulating the availability of mRNA for ion channels, since ion channels...
Figure 5  Overexpression of Let-7f in rat small intestinal organotypic cultures results in altered smooth muscle contractility. (A) Representative images from transduced tissue showing expression of let-7f-GFP throughout its full depth. Scale bar sizes are indicated in the figure. (B) Representative contraction patterns using ImageJ. Horizontal scale bar=2.5 s, vertical scale bar=35 pixels. (C) Frequency of contraction from ImageJ analyses reported as cpm, *p<0.05, n=4, paired t-test. (D) Representative grid overlay with tracked ROIs and the centroid used for calculations. (E) Displacements in the horizontal (x, blue) and vertical (y, red) directions for each ROI in the representative tissue. (F) Frequency of contraction from spatial-temporal mapping reported as cpm. (G) Amplitude of contraction calculated as displacement of pixels. (H) Coordination metric measured as ratio of vectors pointing in the same direction. For F, G and H, data are median±IQR, *p<0.05, n=4, one-way analysis of variance with Dunn’s multiple comparison test. cpm, contraction per minute; CTRL, control; ROI, region of interest; NT, non-targeted.
are crucial for SMC function, and miRNAs are highly effective regulators of electical excitability and excitation–contraction coupling.45 46 For example, miRNAs regulated visceral sensation47 and cardiac motility48 by controlling ion channel mRNA density. In our miRNA set, we found that 30% of the miRNAs we identified were predicted to target SCN5A, which codes for a mechano-sensitive voltage-gated Na+ channel Na,1.5 that is associated with motility disorders characterised by compromised contractile properties of smooth muscle.10 11 Since miRNAs have hundreds of targets,49 genomic analyses are intrinsically limited,50 and functional assessment was required. We focused our investigation on let-7f for three reasons. First, it is a part of the mir-98 family, which was recently shown to be upregulated in STC in a separate study.51 Second, it was abundantly expressed in our samples. Third, it has been suggested that other members of the mir-98 family regulate SCN5A.52 We found that let-7f overexpression led to a decrease in SCN5A expression, Na,1.5 current density and electrical excitability in human SMC.

Given that decreased Na,1.5 is associated with a decrease in excitability and contractility in the human colon13 and rat model,14 we used rat organotypic cultures with lentiviral transduction of let-7f. We found that let-7f decreased smooth muscle contractility through a decrease in the amplitude and frequency of contractions, leaving the coordination index unchanged, similar to a myopathic process on manometry. While let-7f may have several cellular targets, our results suggest that let-7f may regulate smooth muscle function potentially via regulation of Na,1.5 in SMCs. In addition to let-7f, we found three miRNAs that target SCN5A mRNA by seed sequences that are spread through the 3′UTR, suggesting that their effects may be additive. In all, our novel findings have broad implications in other excitable systems where Na,1.5 plays vital roles, such as cardiac,17 53 vascular smooth muscle44 and sensory neurons,45 51 but also non-excitable cells54 and even cancer.55 56

There are intriguing mechanistic possibilities regarding how lower Na,1.5 density decreases GI smooth muscle excitability, including via Na,1.5 function as an ion channel or via Na,1.5 role as a central component of the macromolecular complex that participates in myocyte cellular structure.57 Interestingly, SCN5A mutations are not only linked to dilated cardiomyopathy, a disease characterised by impaired cardiac contractility, but also SCN5A is one of only few ion channel genes involved.19 58 SCN5A channelopathies may contribute to hypomotility in both smooth and cardiac muscles via decreased myocyte excitability.19 58 59 In GI smooth muscle, Na,1.5 contributes to the initial, fast-rising depolarisation of the slow wave in SMC, and it is important for the regulation of amplitude and duration of the slow wave.10 11 Key parameters for the activation of SMC L-type Ca2+ channels. It is possible that the let-7f-driven decrease in Na,1.5 current density may cause decreased SMC and cardiomyocyte contractility via an imbalance in the function of Na+/K+ pump or Na+/Ca2+ exchanger, like in vascular smooth muscle,61 ultimately leading to decreased [Ca2+]i. Another possibility is via Na,1.5 role as the central component of a macromolecular complex that contributes to myocyte structure by interaction with structural genes, like syntrophin and telethonin, both in the heart62 and GI smooth muscle.59 60 Consequently, reduced expression of Na,1.5 may remove a critical component that supports the cytoskeleton and intercalated discs in the cardiac cell63 or the cytoskeleton and the plasma membrane in SMC,16 leading to cell structure alterations and impaired contractility. In all, while Na,1.5 role in dysmotility is established, future studies need to determine its roles in disease.

This study also provides important clues regarding GI SMC biology. Let-7f and other STC-associated miRNAs target GI SMC genes in addition to SCN5A. Even when mature and fully differentiated, SMCs retain remarkable plasticity. SMCs can lose the contractile function in favour of proliferating capability in response to environmental signals.64 An imbalance between the two states can lead to disease.64 Indeed, altered expression of let-7g, another member of the mir-98 family, plays a key role in the switch from contractile to proliferative phenotypes in vascular SMC.65 In GI smooth muscle, miRNAs have been shown to work in conjunction with serum response factor, to drive a switch in smooth muscle phenotype in the opposite direction from proliferative to contractile.66 In a subset of myopathic chronic intestinal pseudo-obstruction, impaired GI motility may be a consequence of loss of contractile SMCs and reversion to a more immature and proliferative state.66 Our STC patient cohort was carefully selected, but we were not able to precisely control the location of the tissue we received for analysis. Therefore, our cohort is likely a subset of STC patients and tissues. Further investigation is required to generalise our findings and to establish the GI SMC relationships between proliferation and differentiation pathways in health and STC. These questions may be illuminated by further work in humans, especially as full-thickness biopsies for GI motility disorders come of age.68

The causes of miRNA dysregulation in STC are not known, and we can only speculate. An intriguing possibility is through diet, which plays a crucial role in altering the expression of miRNAs in the GI tract, with physiological consequences on intestinal transit.69 The mechanism may involve microbial-derived metabolites such as short-chain fatty acid and bile acids that modulate gut motility70 and indirectly alter miRNA expression. It is also possible that microbiome either dependently or independently of diet71 may directly alter miRNAs expression as recently proposed for some GI diseases.72

In summary, we found in the colonic smooth muscle of patients with STC an increased expression of a small subset of miRNAs. A subset of these target Na,1.5, leading to decreased HuSMC Na,1.5 current density, and GI smooth muscle contractility. Thus, our observations suggest a role for miRNAs in regulation of SMC function and the pathogenesis and pathophysiology of STC.

### Table 3 Summary of the spatial–temporal mapping analysis

|                          | Day 0 CTRL | Day 5 CTRL | Day 0 NT | Day 5 NT | Day 0 let-7f | Day 5 let-7f |
|--------------------------|------------|------------|----------|----------|-------------|-------------|
| Frequency (cpm)          | 32±25, 42  | 25±24, 31  | 26±22, 25| 24±24, 25| 32±26, 36   | 21±20, 23   |
| Amplitude (pixel)        | 1.3±1, 2.3 | 0.7±0.2, 2.9| 0.9±0.6, 1.5| 0.6±0.3, 0.7 | 2.2±1, 4.3 | 0.5±0.3, 2.1 |
| Coordination             | 0.6±0.5, 0.7| 0.7±0.5, 0.7| 0.5±0.4, 0.6 | 0.5±0.3, 0.7 | 0.6±0.5, 0.7 | 0.5±0.4, 0.6 |

*p<0.05, n=4, one-way analysis of variance with Dunn’s multiple comparisons test.

*cpm, contraction per minute; CTRL, control; NT, non-targeted.

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Contributors AM designed and performed experiments, analysed data and wrote the manuscript. PRS, VI, YH, MER, CA and AHJ performed experiments and analysed data; TO, FIM, SC, DT, RG, SIG and PD designed experiments and analysed data; CEB coordinated identification and retrieval of surgical tissue; RRC, DWL and HKC performed surgeries and provided tissues; AB and GF designed the research, analysed data and wrote the manuscript. All authors critically reviewed and approved the manuscript.

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