Sodium/hydrogen-exchanger-2 modulates colonocyte lineage differentiation

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Abstract
Aim: The sodium/hydrogen exchanger 2 (NHE2) is an intestinal acid extruder with crypt-predominant localization and unresolved physiological significance. Our aim was to decipher its role in colonic epithelial cell proliferation, differentiation and electrolyte transport.

Methods: Alterations induced by NHE2-deficiency were addressed in murine nhe2−/− and nhe2+/+ colonic crypts and colonoids, and NHE2-knockdown and control Caco2Bbe cells using pH-fluorometry, gene expression analysis and immunofluorescence.

Results: pHi-measurements along the colonic cryptal axis revealed significantly decreased intracellular pH (pHi) in the middle segment of nhe2−/− compared to nhe2+/+ crypts. Increased Nhe2 mRNA expression was detected in murine colonoids in the transiently amplifying/progenitor cell stage (TA/PE). Lack of Nhe2 altered the differentiation programme of colonic epithelial cells with reduced expression of absorptive lineage markers alkaline phosphatase (iAlp), Slc26a3 and transcription factor hairy and enhancer-of-spli1 (Hes1), but increased expression of secretory lineage markers Mucin 2, trefoil factor 3 (Tff3), enteroendocrine marker chromogranin A and murine atonal homolog 1 (Math1). Enterocyte differentiation was found to be pHi dependent with acidic pHi reducing, and alkaline pHi stimulating the expression of entocyte differentiation markers in Caco2Bbe cells. A thicker mucus layer, longer crypts and an expanded brush border membrane zone of sodium/hydrogen exchanger 3 (NHE3) abundance may explain the lack of inflammation and the normal fluid absorptive rate in nhe2−/− colon.

Conclusions: The results suggest that NHE2 expression is activated when colonocytes emerge from the stem cell niche. Its activity increases progenitor cell pHi and thereby supports absorptive enterocyte differentiation.
1 | INTRODUCTION

The processes of cell proliferation, differentiation, migration and apoptosis are coordinated in the mammalian intestinal epithelium to a cell turnover time of 4–6 days.\(^1,2\) Proliferation occurs at the base of the colonic crypt, where intestinal stem cells (ISC) produce 14–21 transient amplifying cells that give rise to absorptive enterocytes, secretory goblet and enteroendocrine cells, which fully differentiate while migrating towards the surface epithelium. The proliferation of the ISC is strongly influenced by the Wnt signalling pathway, while the interaction of the Wnt and the Notch signalling pathway is decisive for the lineage differentiation programme.\(^3,4\) Two basic helix-loop-helix transcription factors Hes1 (hairy and enhancer of split 1) and Math1 (or Atoh1 (atonal homolog 1)) that are controlled by the Wnt/Notch signalling pathways regulate the switch between the absorptive and secretory lineages of the intestinal epithelium.\(^5\)

Many other factors that regulate intestinal proliferation and differentiation processes have been recently discovered.\(^6\) The role of ion transporters that regulate pH\(_i\), ion and volume homeostasis has not been addressed in this context, with one recent exception that revealed highly interesting results. Strubberg et al\(^7\) demonstrated that the loss of the CFTR anion channel resulted in increased intestinal stem cell (ISC) proliferation and Wnt/β-catenin signalling via a pH-dependent stabilization of the plasma membrane association of the Wnt transducer Dishevelled. These data suggest that CFTR serves as an acid loader in ISCs, and that CFTR-induced acidification is important in physiological ISC turnover.

Several members of the NHE (SLC9A) family of Na\(^+\)/H\(^+\) exchangers, namely NHE1-4 and potentially NHE8, are expressed in the plasma membranes of epithelial cells in the gastrointestinal tract, with differential segment- and membrane-specific localization, and by nature serve as acid extruders (base loaders).\(^8,9\) In the human distal colon, NHE2 mRNA is expressed along the crypt axis.\(^10\) Immunohistochemical staining in murine colon shows that Nhe2 protein is maximally expressed in the luminal membrane near the crypt base and the middle of the crypt.\(^11\) Consistent with this expression pattern, the highest Na\(^+\)/H\(^+\) exchange activity with an NHE2-typical pharmacological inhibitor profile was found in the lower part of the crypts.\(^12\) This is the part of the colonic epithelium where proliferation and lineage differentiation take place.

The present study investigates the effects of NHE2 depletion on epithelial pH\(_i\) in the colon of nhe2\(^{-/-}\) mice, as well as on intestinal epithelial cell proliferation and lineage differentiation both in the colonic cell line Caco2Bbe and in the murine mid-distal colon. Undifferentiated, highly stem cell-enriched colonoids, transiently amplifying/progenitor-enriched colonoids and differentiated enterocytes/goblet cell-enriched colonoids from the mid-distal colon of nhe2\(^{-/-}\) and nhe2\(^{+/+}\) mice, were generated and used to answer the question about the expression pattern and function of NHE2 and other acid and base extruders during epithelial differentiation. In a search for compensatory mechanisms, in vivo colonic perfusion studies and in vivo mucus accumulation and surface pH studies were performed in nhe2\(^{-/-}\) and nhe2\(^{+/+}\) mice.

2 | RESULTS

2.1 | Steady-state pH\(_i\) is significantly altered along the colonic crypt axis in nhe2\(^{-/-}\) mice

Fluorometric analysis of the colonocyte steady-state pH\(_i\) along the crypt axis in isolated crypts from nhe2\(^{-/-}\) and nhe2\(^{+/+}\) mid-distal colon revealed a gradual increase of the steady-state pH\(_i\) from more acidic values in the base area to more alkaline values in the crypt mouth in both nhe2\(^{+/+}\) (Figure 1A) and nhe2\(^{-/-}\) (Figure 1B) colon. While no significant difference was found at the cryptal base and the crypt mouth, the steady-state pH\(_i\) was significantly more acidic in the lower to middle segments of nhe2\(^{-/-}\) compared to nhe2\(^{+/+}\) colonic crypts (Figure 1C).

2.2 | Reciprocal regulation of the acid extruders NBCn1, NHE1 vs NHE2 during murine colonoid differentiation

The results from Figure 1 suggest that acid extruders other than NHE2 may play a more prominent role in pH\(_i\) regulation in the crypt base and the crypt mouth. To investigate possible differences in the expression pattern of different acid extruders along the crypt axis, a protocol was established to generate murine colonoids with a high number of stem and proliferating cells, and then to subject these colonoids to a two-step differentiation
programme to obtain transiently amplifying/progenitor cells, and differentiated colonocytes. The degree of stemness, proliferation and differentiation was assessed by measuring the expression of a panel of marker genes (Figure 2A-F) and by immunohistochemistry of selected proteins (Figure 2G,H). The stem cell-enriched (SCE) colonoid culture was characterized by high expression of Lgr5 and Ki67 (Figure 2A,B). Withdrawal of the stem cell-stimulating factors (CHIR99021, valproic acid and Y27632) for period of 2–3 days allowed generation of colonoid culture with low Lgr5 expression, reduced but still substantial Ki67 expression and high Wdr43 expression (Figure 2C) that was defined as TA/PE (transit-amplifying/progenitor cell enriched). By eliminating the presence of Wnt3a and R-spondin and reducing the amount of Noggin in the culturing medium further differentiation of the colonoids was induced, leading to increased expression of Dra, iAlp and Muc2 as marker genes for differentiated enterocytes and goblet cells (Figure 2D-F). The established model was then used to study the mRNA expression of the currently known acid extruders/base loaders in the intestinal epithelium. Cfr, NBCn1 and NHE1 were highly expressed in the stem cell-enriched colonoids (Figure 2I,J,L). Initiation of differentiation in the colonoids resulted in a significant decrease in the mRNA expression of Cfr, NBCn1 and NHE1, whereas NHE2 mRNA expression significantly increased in the transiently amplifying/progenitor cells (Figure 2M), and NHE3 mRNA expression significantly increased in the differentiated enterocytes (Figure 2N). NBCe1 was expressed at relatively low levels and did not change during differentiation (Figure 2K).

Together with the results from Figure 1, the data support the concept that other pH regulators than NHE2 are the major acid extruders/base loaders in the stem cell niche, and that NHE2 expression is upregulated early during initiation of colonocyte differentiation.

2.3 | Extended proliferative zone and increased length of nhe2−/− colonic crypts

Immunohistochemical analysis of the proliferative marker Ki67 showed tightly packed proliferative cells located at the base of the nhe2+/+ colonic crypts. In the nhe2−/− colon, the proliferative cells were diffusely distributed along the lower part of the crypt (Figure 3A, lower panels). The total crypt length was significantly longer in mid-distal colonic crypts of nhe2−/− mice compared to the nhe2+/+ crypts of the same colonic region (Figure 3B). Since nhe2−/− mid-distal colonic crypts were longer than nhe2+/+ crypts, the proportion of the proliferative zone in the crypts was calculated as the percentage of the length of the proliferative zone related to the total length of the crypt and it was found to be significantly increased in nhe2−/− compared to the nhe2+/+ mice (Figure 3C). However, the total number of proliferating
cells was similar between the two genotypes (Figure 3D), suggesting that the number of proliferating cells per µm of crypt length is reduced in the nhe2−/− crypts. The mRNA expression of Lgr5, as a marker of the adult stem cells and a modulator of their activity, was not different between nhe2−/− and nhe2+/+ colonic crypts (Figure 3E).
2.4 | Reduced proliferative rate of nhe2−/− colonoids and NHE2-knockdown Caco2Bbe cells

To further investigate the consequences of the loss of NHE2 on proliferation, EdU assay was employed using nhe2−/− and nhe2+/− colonoids at day 2 and 5 of culture. A significantly decreased percentage of EdU-positive cells was observed in nhe2−/− compared to nhe2+/− colonoids at day 5 of culture, while no differences were detected at day 2 (Figure 4A-D). Colony-forming capacity was not different between nhe2−/− and nhe2+/− colonoids (Figure S1).

The effect of NHE2 silencing on cell proliferation was analysed on a previously established cell model of Caco2Bbe cells, with downregulated NHE2 expression by shRNA and lower pH (Figure 4E,F).14 NHE2-knockdown Caco2Bbe cells (C2Bbe/shNHE2) proliferated at a significantly lower rate until day 12, when the cells became confluent, compared to control cells (C2Bbe/pLKO.1) (Figure 4G). mRNA analysis revealed significantly reduced cMYC mRNA expression in the C2Bbe/shNHE2 compared to the control cells (Figure 4H). Since cMYC is part of the Wnt signalling pathway, we wondered if additional stimulation of this pathway can recover the proliferation rate observed in the C2Bbe/shNHE2 cells. The addition of the GSK3 inhibitor CHIR99021 and the histone-deacetylase inhibitor VPA was used to stimulate proliferation under similar conditions as in the colonoid cultures. The treatment increased the proliferation of both cell lines, however, the proliferative rate of the C2Bbe/shNHE2 cells remained lower compared to C2Bbe/pLKO.1 cells, when normalized to the proliferative rates of the two cell lines under baseline conditions (Figure 4I). The accelerated proliferation was accompanied by an increase of cMYC mRNA expression (Figure 4J) in both cell lines, although less in the C2Bbe/shNHE2 cells.

2.5 | Delayed differentiation of absorptive colonocytes in nhe2−/− colon and in Caco2Bbe/shNHE2 cells

The results in Figure 1 suggest that the NHE2-mediated increase in steady-state pH occurs when the proliferating cells move out of the stem cell zone. In this zone of the crypt, the switch from colonocyte proliferation to differentiation takes place.

To address the impact of NHE2-deletion on colonocyte differentiation intestinal alkaline phosphatase (iALP), as a frequently studied enterocyte differentiation marker expressed in both differentiated Caco2Bbe cells and in differentiated surface colonocytes was used.15 Immunohistochemical
staining of iALP in the mid-distal colon showed significantly lower fluorescence intensity in the nhe2−/− than nhe2+/+ brush border membrane (Figure 5A). Enzymatic ALP activity was assayed in scraped mucosa from mid-distal colon and showed a significantly reduced activity in nhe2−/− compared to the nhe2+/+ colon (Figure 5B). Taking into consideration that iALP is only expressed in differentiated enterocytes (Figure 2), the results suggest an altered differentiation pattern of nhe2-deficient colonocytes.

To confirm this finding in another model, iALP mRNA expression was assessed in C2Bbe/pLKO.1 and C2Bbe/shNHE2 cells. An increase in iALP mRNA expression (Figure 5D) and enzyme activity (Figure 5E) was observed in differentiated (14 days) compared to undifferentiated (3 days) C2Bbe/pLKO.1 control cells. In contrast, in the NHE2-silenced cells both mRNA expression and enzymatic activity of ALP, remained significantly lower compared to the control cells (Figure 5D,E).

Absorptive enterocytes derive from enterocyte precursor cells, whose fate is determined by the transcription factor Hes1.15,16 HES1 mRNA expression was significantly reduced in nhe2−/− compared to nhe2+/+ mid-distal
colonic crypts (Figure 5C) as well as in C2Bbe/shNHE2 cells compared to C2Bbe/pLKO.1 cells (Figure 5F). This suggests that the delay of absorptive enterocyte differentiation is likely because of alterations in the transcriptional regulation of the differentiation pathway.

2.6 | Shift of epithelial differentiation to the secretory lineage in nhe2\(^{-/-}\) colon

To investigate whether NHE2 deletion also affects goblet cell differentiation, the goblet cell number and the thickness of the adherent mucus layer were assessed. The number of goblet cells, visualized by Alcian blue/PAS staining (Figure 6A) and Muc2 immunostaining (Figure 6B), was significantly increased in nhe2\(^{-/-}\) compared to nhe2\(^{+/+}\) mid-distal colon (Figure 6D). Furthermore, the thickness of the adherent mucus layer was higher in the nhe2\(^{-/-}\) than in the nhe2\(^{+/+}\) colon (Figure 6C). The rise in goblet cell number was accompanied by an increase of Tff3 (trefoil factor 3 (a peptide secreted by goblet cells)) mRNA expression in nhe2\(^{-/-}\) mid-distal colon (Figure 6E).

The differentiation of secretory precursors into goblet or endocrine cell fate is regulated by ATOH1 and its homolog Math1 in mice.\(^{17-19}\) Math1 expression was significantly higher in nhe2\(^{-/-}\) compared to nhe2\(^{+/+}\) mid-distal colonic crypts (Figure 6F). This suggests a colonicocyte lineage differentiation shift in the absence of NHE2, which occurs at the level of the relevant transcription factors for the absorptive and secretory cell differentiation. Previous studies have shown that Atoh1 (Math1) is required for the differentiation of both enteroendocrine and Tuft cells.\(^ {17,20}\) Therefore, we have analysed the expression of Chga and Dclk1, as marker genes for enteroendocrine and Tuft cells correspondingly.\(^ {20}\) The results showed significantly increased expression of Chga, suggesting increased enteroendocrine differentiation in nhe2\(^{-/-}\) compared to nhe2\(^{+/+}\) colonic crypts (Figure 6G), while the expression of Dclk1 as a marker for Tuft cells was not altered (Figure 6H).

2.7 | NHE2 deletion affects the budding capacity and the expression of differentiation markers in murine colonoids

A significant difference in the budding capacity between the nhe2\(^{-/-}\) and nhe2\(^{+/+}\) colonoids was observed during their differentiation (Figure 7A,B). In addition, the expression changes in a panel of genes during differentiation confirmed the delayed differentiation of nhe2\(^{-/-}\) colonoids towards absorptive enterocytes. Both iAlp as well as Slc26a3 (Dra) (another marker for differentiated absorptive colonocytes) mRNA expression was significantly decreased in nhe2\(^{-/-}\) compared to nhe2\(^{+/+}\) colonoids (Figure 7F,G). Muc2 mRNA expression was already increased in the transiently amplifying cells, and the difference in differentiated cells did not reach the significance level because of considerable variability (Figure 7H).

We also assessed the expression changes of the proven or potential acid loaders Cftr\(^ {21}\) and Tmem16a\(^ {22}\) in the nhe2\(^{-/-}\) and nhe2\(^{+/+}\) colonoids. In the SCE colonoid cultures, a significant difference was observed in Cftr expression, with lower Cftr mRNA expression in the nhe2\(^{-/-}\) organoids (Figure 7I), while Tmem16a expression was significantly higher (Figure 7J). However, no difference was seen in the TA/PE and the DG/DE organoids. Plating efficiency was not different between the nhe2\(^{-/-}\) and nhe2\(^{+/+}\) colonoids (Figure S1).
2.8 | Alkalinization favours absorptive cell differentiation, independently of NHE2 expression

To address the causal relationship between the reduced pH$_i$ in intestinal epithelial cells lacking NHE2 and the reduced absorptive cell differentiation, the intracellular pH$_i$ of Caco2Bbe cells was manipulated by culturing the cells for 14 days in a medium with different pH$_e$ (pH$_e$ 6.8, 7.3 or 7.8), achieved via changing the bicarbonate concentration of the medium (Figure 8A-D). Alterations in the pH$_e$ caused differences in the steady-state pH$_i$ (Figure 8D), the proliferation rate of the cells (Figure 8E) and the transepithelial resistance (Figure 8F). The rate of differentiation of the Caco2Bbe cells with different pH$_i$ was analysed by comparing the mRNA expression level of iALP, sucrose isomaltase (SI), Slc26a3 (DRA) and NHE3 (Figure 8H-K). The results showed significantly reduced expression of all absorptive enterocyte markers in Caco2Bbe cells with more acidic pH$_i$. In contrast, alkalinization of the pH$_i$ in Caco2Bbe cells resulted in significantly increased mRNA expression of the aforementioned genes compared to control cells with pH$_i$ ~7.3. The mRNA expression of NHE2 was not altered upon manipulation of the cell pH$_i$ values (Figure 8G). These results suggest that NHE2 is influencing enterocyte differentiation via pH$_i$ regulation.

2.9 | Functional adaptive changes in the nhe2$^{-/-}$ colon

We wondered what the functional consequences of the delay in the absorptive enteroocyte differentiation may be. In vivo mid-distal colonic perfusion measurements to study fluid absorptive rates showed no significant differences between anesthetized nhe2$^{-/-}$ and nhe2$^{+/+}$ mice (Figure 9A). The addition of 10 µM tenapanor, a selective
FIGURE 6  Higher goblet cell number and thicker stratified mucus layer in nhe2−/− colon. (A) Alcian blue/PAS staining in Carnoy-fixed mid-distal colon of nhe2+/+ (upper panel) and nhe2−/− (lower panel) mice (n = 5, Scale bar = 75 µm). (B) Immunofluorescent images of Muc2-positive cells (goblet cells) in nhe2+/+ (upper panel) and nhe2−/− (lower panel) mid-distal colon (n = 4, Scale bar = 75 µm, Muc2 = Alexa 568, Nuclei = DAPI). (C) The tissue adherent mucus was observed in Carnoy fixed mid-distal colon after Muc2 staining and found to be thicker in the nhe2−/− (lower panel) compared to the nhe2+/+ colon (upper panel) (n = 3, Mucin = Alexa 488, Nuclei = DAPI, Scale bar = 75 µm). (D) The number of goblet cells, as Muc2-positive cells, was calculated per crypt, and found to be significantly higher in nhe2−/− compared to nhe2+/+ colon (n = 4, mean ± SEM, Mann-Whitney nonparametric t-test, *P < .05). (E) Increased mRNA expression of Tff3 (peptide expressed by goblet cells) in the nhe2−/− compared to nhe2+/+ colon confirmed the increased number of goblet cells. (F) Math1 (mouse analog to human ATOH1), as a transcription factor involved in secretory cell differentiation in intestine, was significantly increased in the nhe2−/− compared to the nhe2+/+ mid-distal colon. The differentiation of other secretory intestinal epithelial cells was addressed based on the mRNA expression of the corresponding marker gene (G) ChgA for enteroendocrine cells and (H) Dclk1 for Tuft cells (n = 6, mean ± SEM, Mann-Whitney nonparametric t-test, *P < .05, †P < .01)
Figure 7  Comparison of nhe2+/+ and nhe2−/− colonoids at different differentiation state. Representative images of (A) nhe2+/+ and (B) nhe2−/− colonoids incubated in different media for defined time period to generate stem-enriched cells (SCE) (2 days), transiently-amplifying/progenitor-enriched (TA/PE) (4 days) and differentiated enterocytes/goblet-enriched (DE/GE) (8 days) (Scale bar = 250 µm). Comparison of mRNA expression of (C) Ki67, (D) Lgr5, (E) Wdr43, (F) iALP, (G) Dra, (H) Muc2, (I) Cftr, (J) Tmem16a, (K) Nbcn1, (L) Nbc1, (M) Nhe1 and (N) Nhe3 (n = 4, 2 repetitions per n number, mean ± SEM, Mann-Whitney nonparametric t-test, *P < .05)
NHE3-inhibitor, along with a switch to a HCO₃⁻-buffered, CO₂-gassed perfusate enhancing NHE-mediated fluid absorption, revealed a small but significant difference in residual fluid absorptive rates between nhe2⁻/⁻ and nhe2⁺/⁺ mice (Figure 9A). Immunohistochemical analysis showed an expanded zone of NHE3 immunoreactivity which was extended into the cryptal mouth region in nhe2⁻/⁻ colon in contrast to nhe2⁺/⁺ mid-distal colon where NHE3 was restricted to an apical localization in the surface colonocytes (Figure 9B). These results demonstrate a functional compensation in the nhe2⁻/⁻ colon by crypt elongation and an extension of the epithelial zone marked by NHE3 brush border membrane expression to include the crypt mouth area.
Despite reduced iALP activity, nhe2−/− distal colon did not develop signs of inflammation (for example no increase in proinflammatory cytokine expression, no infiltration with haematopoetic cells, data not shown). We therefore wondered about possible compensatory mechanisms related to the weakened barrier function in the presence of reduced iALP activity. The in vivo accumulation of a mucus layer over time was assessed by measuring the distance between the epithelial surface and the top of a mucus layer using 15 μm fluorescing microspheres. A higher mucus accumulation rate was observed over the mid-distal colonic epithelium of nhe2−/− compared to nhe2+/+ mice (Figure 9D). The juxtamucosal pH, that is the pH directly above the surface of the epithelial cells, was not significantly different between nhe2−/− and nhe2+/+ colon (Figure 9C). These results may point to a compensatory protection by a thicker alkaline mucus layer in NHE2-deficient colon.

3 | DISCUSSION

The physiological function of NHE2 in the intestinal tract is frequently discussed as a putative apical salt and fluid absorptive mechanism.24,25 However, nhe2−/− mice show no diarrhea, no electrolyte imbalance and no decrease in jejunal fluid absorption in vivo26,27 and the diarrhoeal phenotype of nhe3−/− mice was not further aggravated in the nhe3−/−/nhe2−/− double knockout mice.27 With the recent development of a NHE3-specific inhibitor with selective intestinal action, we were able to unmask a very small contribution of NHE2 to colonic fluid absorption, only apparent after NHE3 inhibition and stimulation of fluid absorption by CO2-induced enterocyte acidification (Figure 9). The physiological significance of the high intestinal NHE2 expression levels in the basal part of the crypts therefore remained unexplained.

In a search for a suitable cell model to investigate the physiological functions of NHE2, we had previously studied a variety of intestinal cell lines and found that Caco2Bbe cells have high endogenous NHE2 mRNA expression levels.14 A Na+/H+ exchanger that is active in the plasma membrane at physiological pH is likely to have an impact on the steady-state pHi unless fully compensatory pHi regulatory mechanisms exist. Indeed, the steady-state pHi in Caco2Bbe cells after stable lentiviral shRNA-induced NHE2 silencing was found to be significantly decreased compared to empty-vector transfected Caco2Bbe/ pLKO.1 cells both during the proliferative state as well as after differentiation on filters (Figure 4F).14 Furthermore, overexpression of NHE2 in the RGM1 cell line, resulted in a strong increase in steady-state pHi.28

Proliferation is commonly thought to depend on or be associated with a high intracellular pH, particularly in tumour cells.29–32 A recent investigation demonstrated that an alkaline pH in ISC, caused by a lack of CFTR expression, was also associated with an increased proliferative rate of CFTR-deficient intestinal epithelium.7 After we had noticed a slower proliferative rate in Caco2Bbe/shNHE2 cells compared to mock-transfected cells,14 we had expected to also find a lower pH and a slower proliferative rate in the base of nhe2-deficient crypts. To our surprise, the cryptal base harbouring the stem cells was relatively acidic in both nhe2+/+ and nhe2−/− crypts, and not significantly different between the two genotypes (Figure 1). In contrast to the base of the crypt, the lower to middle cryptal region was significantly more alkaline in nhe2+/+ compared to nhe2−/− colon.

To investigate the expression pattern of NHE2, as well as other acid extruders/base loaders that counteract cellular acidification in the stem cell niche,33 along the cryptal axis and in differentiated epithelium, we established a technique of culturing murine colonoids with (a) high ISC content (SCE), (b) with a high content of transiently amplifying/progenitor (TA/PE) cells and (c) with a high expression of marker genes for differentiated enterocytes and goblet cells (DE/GE) (Figure 2). In the SC-enriched murine colonoids, high mRNA expression levels for the electroneutral NBCn1 and NHE1, but low NHE2 and NHE3 expression were observed. Induction of differentiation was accompanied by reduced expression of NBCn1 and NHE1, increased expression of NHE2 expression in the TA/PE and the differentiated colonoids and increased NHE3 expression only in the differentiated colonoids. This pattern of expression supports the concept that NHE2 is not an essential pHi regulator in the stem cell niche, but that NHE2 upregulation is an early event during progenitor cell proliferation. This finding explains the significantly lower steady-state pH in the lower to middle part of the nhe2−/− crypt, but not the base and the cryptal mouth (Figure 1).

Immunohistochemical staining with Ki67 showed that in nhe2+/+ colon, the proliferative zone was limited to the base of the crypts, whereas in nhe2−/− crypts, proliferating cells had spread from the base to the lower-middle part of the crypt (Figure 3). However, no differences in the number of proliferating cell nor the expression of Lgr5 were observed. The impact of NHE2 on proliferation was addressed in the nhe2−/− and nhe2+/+ colonoids at day 2 after seeding (when proliferative activity is very high), and showed similar percentage of EdU-positive cells in both genotypes. At day 5 of colonoid seeding, the percentage of EdU-positive cells had decreased by approximately half in the nhe2+/+, and by approximately two-thirds in the nhe2−/− organoids. This may point to a reduced
FIGURE 9  In vivo colonic fluid transport rates, NHE3 brush border abundance, mucus accumulation and surface pH in nhe2+/+ and nhe2−/− mice. (A) Measurement of net fluid transport in the nhe2+/+ and nhe2−/− mid-distal colon in the basal state, and after tenapanor (NHE3 inhibition) application. No difference was seen in the basal fluid absorption rate of nhe2+/+ and nhe2−/− distal colon, but a slight but significant difference was observed after NHE3 inhibition by tenapanor treatment (n = 3-4, mean ± SEM, Mann-Whitney nonparametric t-test †P < .01). (B) Expression of NHE3 detected by immunofluorescence in the colonic crypts of nhe2+/+ mice, revealed NHE3 localization at the surface area along the colonic crypts, while in the nhe2−/− mice NHE3 was extended downward to the deeper area of the crypts (n = 4, mean ± SEM, scale bar = 70 µm, NHE3 = Alexa568, Nucleus = DAPI). (C) Extracellular pH measured at the indicated time points near the epithelial surface after adding unbuffered saline with a pH of ~6 at time zero. No significant differences were observed between nhe2+/+ and nhe2−/− surface pH (n = 5, mean ± SEM, Mann-Whitney nonparametric t-test). (D) The thickness of the accumulated mucus was measured in vivo. Under basal and FSK-stimulating conditions, the thickness of the accumulated layer of mucus was significantly higher in case of nhe2−/− mice than in the nhe2+/+ counterparts (n = 5, mean ± EM, Mann-Whitney nonparametric t-test, ‡P < .001).
proliferation rate of the transiently amplifying cells and progenitor cells, which constitute the majority of cells in colonoids at day 5 of culture. Supporting evidence for this concept came from studies in the Caco2Bbe cell model. Caco2Bbe in their subconfluent state resemble absorptive enterocyte progenitor cells, and differentiate further in the weeks after confluence. NHE2-silenced Caco2Bbe cells displayed a lower steady-state pH than control cells, and proliferated significantly slower than the mock-transfected controls (Figure 4). They also displayed reduced cMYC expression. Culturing of the Caco2Bbe cells in a medium with low extracellular pH, resulted in more acidic pH, and caused a significant decrease in their proliferation rate as well, suggesting that one (if not the only) molecular mechanism how NHE2 silencing affects proliferation may be the effect of this manoeuvre on the steady-state pH.

The effect of NHE2-silencing or –deletion on the expression of the acid loaders CFTR and TMEM16a was also assessed both in C2Bbe/shNHE2 cells compared to C2Bbe/pLKO.1 cells, in freshly isolated nhe2−/+ and nhe2+/+ crypts (data not shown), and in nhe2−/− and nhe2+/+ colonoids (Figure 7). CFTR mRNA expression was downregulated during differentiation, while TMEM16a was upregulated, both in Caco2Bbe (data not shown) and in murine colonic organoids (Figure 7). No significant differences in CFTR- and TMEM16a mRNA expression was observed in NHE2-silenced Caco2Bbe cells and in isolated nhe2−/− and nhe2+/+ crypts. Slight alterations in the expression of Cfr and Tmem16a were detected in the stem cell-enriched state with lower Cfr, but increased Tmem16a expression in nhe2−/− compared to nhe2+/+ colonoids, while their expression levels were identical in the TA/PE and DG/DE colonoids of the two different genotypes. Therefore, changes in the expression levels of these bicarbonate exporters do not correlate with the observed differences in steady-state pH between nhe2−/− and nhe2+/+ mid-cryptal region, which is best explained by accepting a role of NHE2 itself on steady-state pH in this part of the colonic crypt.

Because NHE2 expression and NHE2-mediated increase in pH manifests itself as the cells move out of the stem cell region, we were interested if NHE2 may be required for epithelial cell differentiation. Using specific differentiation markers characterizing the two major cell lineages in the colon, we analysed the effect of NHE2 depletion on absorptive enterocyte and secretory goblet cell differentiation in mouse colon and in Caco2Bbe cells. ALP is an established differentiation marker both in murine colon and in Caco2Bbe cells. Its expression and activity was reduced in nhe2−/− mid-distal colon as well as in C2Bbe/shNHE2 cells compared to the respective controls (Figure 5). These results suggest a defective differentiation of NHE2-deficient progenitor cells to absorptive enterocytes. In contrast, an increased number of Muc2-positive goblet cells was observed in the nhe2−/− compared to nhe2+/+ mid-distal colon, which was accompanied by a thicker mucus layer (Figure 6) and a higher mucus accumulation rate in vivo (Figure 9). These results point to a shift in the differentiation programme in the nhe2−/− colon, from absorptive to secretory cell differentiation. When subjected to identical differentiation protocols, murine nhe2−/− colonoids expressed significantly less iAlp as well as Slc26a3 mRNA compared to nhe2+/+ colonoids (Figure 7). This demonstrates that the differentiation defect induced by NHE2 deletion is an intrinsic feature of the epithelium.

In the intestinal epithelium, lineage differentiation towards absorptive enterocytes is driven by the activation of Hes1, while secretory lineage differentiation towards goblet cells is driven by Math1 (murine Atoh1 homolog). Both are transcription factors that are activated as part of the Wnt and Notch signalling pathways. Our results demonstrate that Hes1 mRNA expression was decreased in nhe2−/− compared to the nhe2+/+ colonic crypts, while Math1 mRNA expression was increased (Figures 5 and 6). The same was seen in Caco2Bbe/shNHE2 cells compared to the respective controls, although these cells cannot (and did not) differentiate into goblet cells. These findings are consistent with the reported repressive action of Hes1 on Math1 (Atoh1) expression, and underline the importance of Hes1 for absorptive enterocyte differentiation. In an attempt to analyse the upstream participants of the signalling pathways which could regulate the expression of Hes1 and Math1 and colonocyte differentiation, a number of Wnt and Notch target genes were analysed (Figure S2). The results showed alterations in the expression of Notch1 and some of the Wnt ligands, receptors and downstream processors, which suggest that the Wnt and Notch signalling pathway may be differently regulated in absence of NHE2. However, taking into consideration the number of molecules participating in both Wnt and Notch signalling pathway and their interplay during regulation of the cell cycle, a simplified quantification of their mRNA expression cannot be used to judge the existence of potential dysregulations in either signalling pathway. A very elegant method was used by Strubberg et al., who crossed WT and CFTR-deficient mice with Dvl2-EGFP/ACTB-ttdTomato,-EGFP) and Luo/J(RosamT/mG) mice to demonstrate an increase in plasma membrane Dvl2-EGFP association, increased Wnt signalling and proliferation in CFTR-deficient organoids in relation to altered pH. Nevertheless, this model was not available to us.

It is not known whether NHE2 mediates its influence on intestinal epithelial differentiation via its influence on steady-state pH, or by other mechanisms. To
answer this question Caco2Bbe cells were cultured in media in which the pH₄ was adjusted to different values by altering the HCO₃⁻ concentration in order to change the pHᵢ. Although this manoeuvre is not able to fully mimic the selective change of the steady-state pHᵢ mediated by NHE2 silencing, culturing the Caco2Bbe cells in acidic pHᵢ resulted in decreased expression of marker genes for absorptive enterocyte differentiation, namely of NHE3, Slc26a3, iALP and SI, while incubation in alkaline pHᵢ medium resulted in increased expression of the same genes. NHE2 expression was not significantly affected by the alteration of the pHᵢ. This strongly favours the concept that the NHE2-mediated alkalization is the predominant mechanism that influences enterocyte differentiation. The importance of pHᵢ increase for the differentiation of different cell models has been shown before. Manipulation of the pHᵢ by adjusting the pHᵢ of the colonoid cultures was not feasible, because of the limitations of the culturing medium supplemented with number of growth factors that cannot be used with different HCO₃⁻ concentrations (since the medium is already buffered) and secondly the different pHᵢ would influence the activity of the growth factors and impact the growth/differentiation of the colonoids not associated to the pHᵢ.

We believe that NHE2 is particularly suited to mediate a pHᵢ increase into an alkaline range, because NHE2 has a very high proton affinity both of the intra- and extracellular proton binding site, which shifts its pHᵢ-dependence to the alkaline pH-range compared to the other NHEs. The reciprocal shift in expression levels for NBCn1 and NHE1 on the one hand, and the increase in NHE2 will therefore be sufficient to mediate the shift to more alkaline steady-state pHᵢ as the cells transit from stemm cell to transit amplying and later to differentiated stage.

A puzzling question is why the colonic epithelium employs an apically expressed NHE for the purpose of intracellular alkalization, a task that is assumed to be performed by the basolaterally expressed ‘housekeeping’ acid/base transporters. One reason may be that an increase of pHᵢ of the magnitude that we see as the cells move out of the base of the crypt will result in substantial proton export, which, were it to happen at the basolateral membrane, may result in a extracellular pHᵢ drop that could interfere with activity and binding of the many receptors expressed in the basolateral membrane and therefore alter number of cellular functions. Such candidates are the epidermal growth factor receptor (EGFR), pH-sensitive integrins or the calcium-sensing receptor (CaSR). The binding of the EGF ligand to the EGFR is strongly decreased with a drop of pHᵢ < 7, which will further impair the initiation of EGFR signalling cascade, that is involved in regulation of cell proliferation and differentiation. A number of integrins, which impact cellular behaviour via regulating adhesion signalling, are pH sensitive. The activity of CaSR could be largely influenced by a drop in the pHᵢ, since the receptor depends on an alkaline pH to be functionally activated and has been identified as an important driver for colonocyte differentiation by signalling both at the epithelial and subepithelial level. In addition, apical proton extrusion may help to recycle CO₂ by protonation of the HCO₃⁻ lost via CFTR, remove fluid from the crypt lumen, maintain cell volume and initiate sodium absorption, signals that may help in colonocyte differentiation.

In summary, our results suggest that the primary effect of NHE2 deletion or silencing on colonic epithelial function may be a compromised differentiation of colonocytes. The effect is subtle and the epithelium is able to compensate at least in respect to fluid absorption by an increased crypt length and an extended zone of NHE3 presence in the apical membrane. The consequences on other aspects of differentiation, such as barrier function and wound healing, need further study. The thicker mucus layer, probably a result of more goblet cells, but potentially an alternative mechanism to curb epithelial damage by pathogens, may explain why the strong reduction in iALP activity did not compromise epithelial integrity in this part of the gut. The changes may be more severe in other parts of the gastrointestinal tract like the gastric epithelium, or after injury, as has been described in the jejunum after ischaemia. Aihara et al recently studied gastric epithelial regeneration after acetic-acid induced ulceration and also concluded that NHE2 may be involved in cell differentiation in gastric epithelium, although the pathways were not clear. The reported importance of NHE2 for surface restitution in the jejunum and gastric epithelium would also be compatible with the concept that NHE2 modulates differentiation of the intestinal epithelium. Further studies into the regulation of NHE2 and its subsequent effect on downstream events in gastrointestinal epithelia are required to answer these remaining questions.

4 | MATERIAL AND METHODS

4.1 | Mice

The NHE2 (Slc9a2)-gene-deleted mouse strain was originally generated by the group of Gary Shull. All in vivo experiments were performed using P3-6-month-old nhe2⁻/⁻ and nhe2⁺/⁺ littermates, congenic on the FVB/N background. Genotyping was performed as described previously and verified by PCR (primers are listed in Table S1). Mice were killed humanely by cervical dislocation after light isoflurane anaesthesia; the colon was excised and washed in ice-cold PBS, pH 7.4 before further
processing. All experiments involving animals were approved by the Hannover Medical School Committee on investigations involving animals and an independent committee assembled by the local authorities.

4.2 | Cell culture

Caco2Bbe cells (CRL-2102™, ATCC) were cultured in Dulbecco’s Modified Eagle Medium DMEM (Gibco) containing 4.5 g/L D-glucose and Na-pyruvate supplemented with 10% FBS, 50 units penicillin/streptomycin, 1% non-essential amino acids. C2Bbe/shNHE2 cells with ~60% downregulated expression of NHE2 and the corresponding mock-transduced cells (C2Bbe/pLKO.1) were generated as described in Yu et al, 201914 and cultured in the same cell medium as wild type Caco2Bbe, but supplemented with 10 µg/mL puromycin.

In experiments where the intracellular pH was manipulated Caco2Bbe cells were cultured in DMEM medium containing 4.5 g/L D-glucose, but without sodium bicarbonate (DMEM, powder, high glucose, Gibco). The medium was supplemented with 10% FBS and 50 units penicillin/streptomycin. The pH of the medium was adjusted with different sodium bicarbonate concentration calculated based on the Henderson-Hasselbach equation as follows: (a) pH 6.8-6.1 mM NaHCO₃, (b) pH 7.4-19.2 mM NaHCO₃, (c) pH 7.8-60.6 mM NaHCO₃. Because of the severely reduced proliferation rate of cells cultured in pH 7.8, cells were incubated for 4 days in pH 7.3, then 3 days in pH 7.6 and 7 days in pH 7.8, to allow them to reach confluency and increase TEER.

4.3 | Murine colonoid culture

Mid colon (ca.3-6 cm from rectum) was removed from killed 12-week-old mice and ~1 cm of it was cut into small pieces, washed three times with DPBS and was then incubated in 10 mM EDTA/DPBS for 60 minutes at 4°C on a rotating shaker. Crypts were then released into 3 × 5 mL DPBS by pipetting, spun down at 150 g and seeded at a density of 30 crypts per 15 µL of 50% stem cell medium/ Matrigel (Corning). The medium composition was adapted from previously reported protocols with minor modifications.54,55 After 30 minutes incubation at 37°C 500 µL stem cell medium was added (Table 1). The medium was replaced after 48 hours with TA/PE cell medium and the colonoids were passaged every 5 days at a ratio of 1:3. Briefly, the colonoids were released from the Matrigel by pipetting in 1 mL medium, pooled in 5 mL and triturated with a 10 mL pipet equipped with a P200 tip. Colonoid fragments were spun down at 200 g and plated in Matrigel as described above. For the 2 step differentiation programme, colonoids were cultured for 2 days in stem cell medium, followed by 2 days of incubation in the TA/PE cell medium, before inducing differentiation on day 4 by changing to differentiation medium (Table 1). For the proliferation assay 10 µM 5-ethyl-2'-deoxyuridine (EdU) was added for the last 18 hours of culture. The colonoids were harvested on day 2, day 4 and day 7 for down-stream analyses.

L-WRN-conditioned medium was produced according to the manufacturer’s protocol. Noggin- and R-spondin 1-conditioned medium was produced in stably transfected HEK293T cells that were selected by the addition of 10 µg/mL Puromycin or 300 µg/mL Zeocin respectively. The conditioned medium was then produced by adding DMEM/F12 with 1x GlutaMAX and 10 mM HEPES..

4.4 | Isolation of colonic crypts for pHᵢ measurement and gene expression analysis

The mid-distal colon (beginning 2 cm distal from the caecocolonic junction and ending 1 cm before the anal verge),

| TABLE 1 composition of medium used for mouse organoids | Stem cell medium | TA/PE cell medium | Differentiation medium |
|--------------------------------------------------------|------------------|------------------|-----------------------|
| L-WRN-conditioned medium | 50 vol% | 50 vol% | — |
| Noggin-conditioned medium (0.1% 1M NaOH) | — | — | 15% |
| B-27 supplement | 1x | 1x | 1x |
| N-2 supplement | 1x | 1x | 1x |
| N-Acetyl-L-cysteine | 1 mM | 1 mM | 1 mM |
| Mouse EGF | 50 ng/mL | 50 ng/mL | 50 ng/mL |
| CHIR99021 | 3 µM | — | — |
| Valproic acid (VPA) | 1 mM | — | — |
| Y27632 | 10 µM | — | — |
| A83-01 | 0.5 µM | 0.5 µM | 0.5 µM |
where both NHE2 and NHE3 are highly expressed, was chosen for the experiments of this project. Colonic crypts were isolated from inverted mid-distal colonic segments using a Ca2+ chelation method described previously.56-58 Crypts were fixed on the coverslip with a polycarbonate membrane (pore size 3 μm, Osmonics) in a custom-built perfusion chamber.

4.5 | Steady-state pH<sub>i</sub> measurement

Steady-state pH<sub>i</sub> was measured using BCECF (5 μM) fluorescence at selected regions of interest along the colonic crypt, 14-day-old Caco2Bbe cells cultured on polyester transwell membranes (pore size 3 μm, area 0.33 cm<sup>2</sup>). The cells were pre-perfused with buffer A solution (121 mM NaCl, 19 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 0.5 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>x6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>x2H<sub>2</sub>O, 11 mM HEPES, pH7.4) for at least 20 minutes at 37°C gassed with O<sub>2</sub>/CO<sub>2</sub>. The emission signal was recorded for ca. 20 minutes, before the cells were perfused with two different pH calibration solution (20 mM NaCl, 100 mM K-gluconate, 21.5 mM KCl, 1.5 mM K<sub>2</sub>HPO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 10 mM HEPES, 10 mM D-glucose, 10 μM nigericin pH 7.0 or pH 7.4). The curve of the 495/440 ratio was reproduced from the stored images after background subtraction, followed by conversion of the 495/440 ratio values to pH<sub>i</sub> values.

In experiments where the Caco2Bbe cells were cultured in a medium with pH<sub>e</sub> 6.8 the cells were perfused in buffer A (135 mM NaCl, 5 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 0.5 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>x6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>x2H<sub>2</sub>O, 11 mM HEPES, pH6.7) and calibration buffers were kept the same, only the pH was varied to 6.8 and 7.2. Caco2Bbe cells cultured in pH<sub>e</sub> 7.8 were perfused in buffer A (80 mM NaCl, 60 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 0.5 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>x6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>x2H<sub>2</sub>O, 11 mM HEPES, pH6.7) and calibration was done with calibration buffer with pH7.4 and 8. Caco2Bbe cells cultured in pH<sub>e</sub> 7.3 were perfused in buffer A (80 mM NaCl, 19.1 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 0.5 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>x6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>x2H<sub>2</sub>O, 11 mM HEPES, pH6.7) and calibration was done with calibration buffer with pH7.0 and 7.4.

4.6 | Cell proliferation of Caco2Bbe cells

C2Bbe/shNHE2 and C2Bbe/pLKO.1 cells were generated and maintained as described before.14 For proliferation experiments cells were seeded at a density of 1 × 10<sup>5</sup> on a 6-well plate, detached using Trypsin/EDTA and counted every 72 hours, for a period of 18 days. For each time point, two wells were counted. The experiment was repeated 3 times from three different cell passages. Cell proliferation after stimulation with CHIR99021 and VPA (Sigma-Aldrich) was analysed under the conditions described above. Namely, 1 × 10<sup>5</sup> cells were seeded on a 6-well plate and cultured for 48 hours. Before treatment, cells were starved overnight by decreasing the FBS concentration to 1% FBS to minimize the effect of other pro-proliferative factors. The culturing medium with 1% FBS, 3 μM CHIR99021 and 3 μM VPA was added to the cells for 24 hours before they were detached and counted. The experiment was repeated 5 times from three different cell passages.

The proliferation rate of the Caco2Bbe cells incubated in a medium with different pH<sub>i</sub> (6.8, 7.3 and 7.8) was assessed in a period of 14 days; using cells seeded in a density of 1 × 10<sup>4</sup> cells on a 24 well plate and counted every 48 hours.

4.7 | mRNA expression of differentiation markers in the mice colon and C2Bbe cells

The mRNA expression of different genes (primer sequences listed in Table S1) in nhe2<sup>−−</sup>/− and nhe2<sup>++</sup>/+ mice (colonic mucosa), murine colonoids and Caco2Bbe cells was analysed by qPCR using β-actin as a reference gene. The isolation of mRNA was performed using RNeasy mini kit, according to the manufacturer’s instructions (Qiagen). cDNA transcription and qPCR analysis were performed as described before.53

4.8 | Alkaline phosphatase (ALP) expression and enzyme activity in colonic mucosa and Caco2Bbe cells

Cells were seeded on the polyester transwell membranes (pore size 0.4 μm, area 0.33 cm<sup>2</sup>) at the density of 1.5 × 10<sup>3</sup> (C2Bbe/pLKO.1) and 3 × 10<sup>3</sup> (C2Bbe/shNHE2) per insert and cell lysates were collected at day 3, 8, 14 and 21 for mRNA and enzyme activity analysis. The isolation of mRNA, cDNA transcription and qPCR analysis were performed as described before.53 The activity of alkaline phosphatase (ALP) was analysed using the method described by Zucco et al.59,60 Briefly, cells were scrapped into 500 μL ALP collection buffer (10 mM Tris–HCl, 150 mM NaCl, pH 8.0) on ice, and 250 μL of the homogenate and 750 μL of pNPP solution (2.5 mg/mL pNPP dissolved in 100 mM diethanolamine, 150 mM NaCl and 2 mM MgCl<sub>2</sub>, pH 9.5) were transferred into reaction tube and incubated at 37°C. After 20 minutes, 200 μL was transferred to a 96-well plate containing 50 μL.
0.5 M NaOH to stop the enzymatic reaction. Enzyme activity was calculated with reference to a standard curve as described in Zucco et al. absorbance was read at OD405 in a microplate multifunctional reader BioTek® Eppendorf Epoch Reader, and calculated as mU/mg protein.

To measure the ALP activity in the colonic mucosa of nhe2+/+ and nhe2−/− mice, the proximal and distal segment were divided, opened longitudinally and washed thoroughly with PBS to remove the luminal contents. The mucus was then scraped using Whatman paper, stored in 500 μL ALP collection buffer (10 mM Tris–HCl, 150 mMNaCl pH 8.0), and then incubated for 30 minutes on ice. The homogenates were centrifuged at 15 000g for 5 minutes at 4°C, and the supernatants were collected. The endogenous activity of ALP was further measured as described for the Caco2Bbe cells. Protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific).

4.9 | Immunohistochemical analysis of nhe2−/− and nhe2+/+ colon and colonoids

The colon from nhe2−/− and nhe2+/+ mice was excised, opened along the mesenteric border, and ‘Swiss rolls’ were prepared by rolling up the colon from the anal verge, fixed with 4% paraformaldehyde and embedded in paraffin. Tissue sections (2 μm) were prepared, deparaffinized and rehydrated.

4.9.1 | Colonocyte proliferation

Proliferation of colonocytes was analysed using Ki67 immunostaining. Sections were prepared as described above, epitopes were retrieved by boiling in 10 mM Tris buffer (pH 9) with 0.5 mM EDTA, and then quenched with 50 mM NH₄Cl. Unspecific binding was blocked by 3% BSA. The sections were incubated overnight at 4°C with the Ki67 primary antibody (1:500, VP-K451, Vector Laboratories, United Kingdom), diluted in 1% BSA/ PBS with 0.1% Triton X-100, and detected using HRP-conjugated goat anti-rabbit secondary antibody (1:500, Dako). The sections were later dehydrated in graded alcohol and xylene and mounted in Eukitt mounting medium. Microscopy was performed on a Leica DMRE bright-field microscope equipped with a Leica DM300 digital camera.

4.9.2 | Enterocyte differentiation

ALP was used as a marker for enterocyte differentiation. Endogenous ALP in the colon of nhe2+/+ and nhe2−/− mice was analysed by immunofluorescence using VECTOR RED Alkaline phosphatase detection kit (SK-5100, Vector Laboratories) according to the manufacturer’s instructions. Sections were mounted in Fluoromount-G (SouthernBiotech) and images were acquired using Olympus FV1000.

4.9.3 | Goblet cell differentiation

Muc2 was used as a marker for goblet cell identification. Muc2-positive cells in the colon were stained as described above, using MUC2 (1:1000, sc-515032, Santa Cruz) primary antibody, further detected with Alexa Fluor 568 (1:500, A-11036, Invitrogen). Sections were mounted in Fluoromount-G (SouthernBiotech) and images were acquired using Olympus FV1000.

4.9.4 | Stratified mucus layer

In order to identify the mucus layer, distal colon was excised from nhe2−/− and nhe2+/+ mice immediately after killing and fixed in Carnoy fixative. Paraffin-embedded sections were then stained for mucin with MUC2 as described above using Alexa Fluor 488 as a secondary antibody (1:500, A-11034, Invitrogen) and DAPI for nuclei staining. Carnoy fixed sections were used to detect goblet cells with Alcian blue/PAS staining. Slides were deparaffinized as described above, and stained in alcin blue solution (pH 2.5) for 10 minutes, treated with periodic acid for 5 minutes and stained with Schiff’s reagent for 10 minutes. After rinsing in distilled water they were counterstained in nuclear fast red solution for 1 minute, followed by dehydration through a series of stepwise ascending alcohol and xylene and mounted in hydrophobic Eukitt mounting medium. Microscopy was performed on a Leica DMRE bright-field microscope.

4.9.5 | NHE3 membrane abundance

NHE3 distribution was detected immunofluorescently using anti-rat NHE3 antibody (1:500) (NHE31-A; Alpha Diagnostic International) and Alexa Fluor 568 as a secondary antibody and DAPI for nuclei staining. Sections were mounted in Fluoromont and images were acquired using Olympus FV1000.

4.9.6 | Colonoid staining

Colonoids were released from the Matrigel, centrifuged in 5 mL medium at 150g for 5 minutes at 4°C and fixed in
were performed by single-pass perfusion as previously indicated below. The abdomen was closed after the surgery, and then the mice were left to stabilize for 30 minutes for fluid equilibration after the surgical trauma. Haematocrit was measured using a haemocytometer (Hematokrit 210, Hettich) to ensure adequate fluid replacement.

Animals were maintained at 37°C using a heating pad and red lamp controlled by a thermometer in the abdominal cavity. Measurements of the colonic fluid absorptive rates were performed by single-pass perfusion as previously described for the jejunum. The amount of fluid absorption was assessed gravimetrically as described previously. The fluid absorptive rates were measured for a total period of 90 minutes with samples taken at an interval of 15 minutes. During the ‘basal absorption phase’ measurements, a solution containing 130 mM NaCl, 20 mM Hepes and 2 mM CaCl2, pH 7.4 was perfused. After 30 minutes, 10 µM tenapanor was added to the perfusate. This concentration of tenapanor was tested in concentration-response experiments previously and had proved to maximally inhibit intestinal fluid absorption rates. It is of note that tenapanor: (1) needs to penetrate the mucus layer and (2) that it slowly precipitates from neutral-alkaline solutions. The 10 µM concentration was chosen to ensure full NHE3 inhibition during the 30 minutes perfusion phase. After 60 minutes, the solution was switched to CO2/HCO3− pH 7.4 containing 24 mM, 126 mM NaCl and 2 mM CaCl2 gassed with 5% CO2, and perfusion was continued for a period of 30 minutes. This manoeuvre causes a strong stimulation of NHE3-mediated fluid absorption. All values were expressed as millilitres of fluid absorbed per cm colonic length per hour (mL/cm/hr).

4.10 In vivo fluid absorptive measurements

The mice were anaesthetized by a spontaneous inhalation of isoflurane (Forene, Abbott Germany). The inhalation gas contained a mixture of ~10%-15% oxygen, ~85%-90% air and 2.0 ± 0.2% isoflurane with the use of an isoflurane pump (Univentor 1250 Anaesthesia Unit; AgnTho, Lidingö, Sweden). Once the mice slept, they received tracheal intubation and mechanical ventilation (MiniVent Type 845; Hugo Sachs Electronik, March-Hugstetten, Germany). A catheter was placed in the left carotid artery for continuous infusion of a solution of the following composition (mmol) to correct the systemic acid–base balance: 200 Na+, 100, 0.005 K+ and 0.005 Cl−, at a rate of 0.3-0.35 mL/hr. After making a mid-line incision in the abdomen, the middle (starting ~2.5 cm away from caecocolonic junction) and distal colon (last 1.5 cm) with intact blood supply were selected for the experiments. The middle colon was opened about 2 cm distal to the caecocolonic junction. A small polyethylene tube (PE-100; inner diameter 1 mm) with a distal flange was inserted and secured by a ligature, then the middle and distal colon was perfused gently to remove the contained faeces. The outlet tube (PE-200; inner diameter 2 mm) was put through the rectum and fixed 1 cm proximal to the anal canal. The mid-distal colon was perfused (Gilson minipuls evolution, Villiers, France) at a rate of 30 mL/hr with the solutions indicated below. The abdomen was closed after the surgery, and then the mice were left to stabilize for 30 minutes for fluid equilibration after the surgical trauma. Haematocrit was measured using a haemocytometer (Hematokrit 210, Hettich) to ensure adequate fluid replacement.

Animals were maintained at 37°C using a heating pad and red lamp controlled by a thermometer in the abdominal cavity. Measurements of the colonic fluid absorptive rates were performed by single-pass perfusion as previously described for the jejunum. The amount of fluid absorption was assessed gravimetrically as described previously. The fluid absorptive rates were measured for a total period of 90 minutes with samples taken at an interval of 15 minutes. During the ‘basal absorption phase’ measurements, a solution containing 130 mM NaCl, 20 mM Hepes and 2 mM CaCl2, pH 7.4 was perfused. After 30 minutes, 10 µM tenapanor was added to the perfusate. This concentration of tenapanor was tested in concentration-response experiments previously and had proved to maximally inhibit intestinal fluid absorption rates. It is of note that tenapanor: (1) needs to penetrate the mucus layer and (2) that it slowly precipitates from neutral-alkaline solutions. The 10 µM concentration was chosen to ensure full NHE3 inhibition during the 30 minutes perfusion phase. After 60 minutes, the solution was switched to CO2/HCO3− pH 7.4 containing 24 mM, 126 mM NaCl and 2 mM CaCl2 gassed with 5% CO2, and perfusion was continued for a period of 30 minutes. This manoeuvre causes a strong stimulation of NHE3-mediated fluid absorption. All values were expressed as millilitres of fluid absorbed per cm colonic length per hour (mL/cm/hr).

4.11 Measurement of surface (juxtamucosal) pH above the colonic epithelial cells in vivo

Colonic epithelial surface pH measurements were performed following the same protocol as recently described. Briefly, the mid-distal colon of the anaesthetized, blood-gas and arterial blood pressure-controlled mouse was exteriorized with an intact blood supply and mounted on a custom-made perfusion. After stabilization, the chamber was perfused with unbuffered saline, pH−6, containing free 5 µM carboxylic acid SNARF-1 (Molecular Probes), and the perfusion was stopped before images were collected. SNARF-1F was excited at 780 nm, and the emission was collected at 580 nm (523-605 nm) and 640 nm (610-700 nm), using a 2-photon laser scanning microscope with an upright Leica TCS SP2 confocal microscope with a 20× water immersion objective and a MaiTai Ti:sapphire-pulsed laser (Spectra-Physics, Darmstadt). The ratio of 640/580 was converted into the pH using an in vitro calibration curve as described before.

4.11.1 Measurement of mucus accumulation in vivo

The same colonic segment as described above was chosen, exteriorized with intact blood supply, and the mucus exposed under the two photon microscope objective,
covered with 1 mL of unbuffered saline, and fluorescent polystyrene beads (1.0 × 10^6 beads mL^-1; FluoSpheres® Polystyrene Microspheres (Darmstadt), 15 μm, Crimson Fluorescent 625/645 Invitrogen, F-8839) were gently placed over the saline and allowed to settle by gravity for 5 minutes. These large beads were chosen so that they would stick to the surface of the mucus and would not sink in. The beads were placed at different time points to assess the build-up of the mucus over time (0, 30 and 60 minutes post-surgery) and the surface was measured by simultaneous scanning at 488 and 633 nm in x-y planes along the z-axis A total of 50 stacks were taken to cover the distance in the z-direction, starting from the visualization of beads at the top followed by the surface at the bottom. The 488 nm wavelength was used in reflection mode to visualize the tissue, while 633 nm was used to excite the Alexa 633-labelled fluorescent beads. Further details and illustrations of the technique have been previously described.53

4.11.2 Statistics

Data are presented as means ± SEM. n indicates the number of each nhe2−/− and nhe2+/+ mice, or C2Bbe/pLKO.1 and C2Bbe/shNHE2 cells, that is, n = 1 is one pair of each. The Mann-Whitney test (mice and colonoids, Caco2Bbe cells) and two-way ANOVA were used for statistics. Values of P < .05 were considered significant (*P < .05, †P < .01, ‡P < .001).

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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