

Research Article

Upregulated Na^+/H^+ -Exchange Protects Human Colon Cancer Tissue against Intracellular Acidification

Ninna C. S. Voss,^{1,2} Henrik Kold-Petersen,² Mikkel B. Henningsen,¹
Casper Homilius,¹ and Ebbe Boedtkjer ¹

¹Department of Biomedicine, Aarhus University, Ole Worms Allé 3, Building 1170, DK-8000 Aarhus C, Denmark

²Regional Hospital Randers, Skovlyvej 15, DK-8930 Randers, Denmark

Correspondence should be addressed to Ebbe Boedtkjer; eb@biomed.au.dk

Received 24 October 2018; Revised 3 January 2019; Accepted 17 January 2019; Published 29 January 2019

Guest Editor: Jinwei Zhang

Copyright © 2019 Ninna C. S. Voss et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Increased metabolism accelerates local acid production in cancer tissue. The mechanisms eliminating acidic waste products from human colon cancer tissue represent promising therapeutic targets for pharmacological manipulation in order to improve prognosis for the increasing number of patients with colon cancer. We sampled biopsies of human colonic adenocarcinomas and matched normal colon tissue from patients undergoing colon cancer surgery. We measured steady-state intracellular pH and rates of net acid extrusion in freshly isolated human colonic crypts based on fluorescence microscopy. Net acid extrusion was almost entirely (>95%) Na^+ -dependent. The capacity for net acid extrusion was increased and steady-state intracellular pH elevated around 0.5 in crypts from colon cancer tissue compared with normal colon tissue irrespective of whether they were investigated in the presence or absence of $\text{CO}_2/\text{HCO}_3^-$. The accelerated net acid extrusion from the human colon cancer tissue was sensitive to the Na^+/H^+ -exchange inhibitor cariporide. We conclude that enhanced net acid extrusion via Na^+/H^+ -exchange elevates intracellular pH in human colon cancer tissue.

1. Introduction

Current treatment options for colonic adenocarcinoma include surgery, radiation, and chemotherapy depending on the stage of disease but mortality rates remain considerable, particularly for patients with disseminated cancer.

Insufficient blood supply, elevated metabolism, and a shift from oxidative phosphorylation towards fermentative glycolysis acid-loads the intracellular compartment of cancer cells [1]. Despite the increased production of acidic waste in solid cancer tissue, intracellular pH (pH_i) of cancer cells is typically maintained equal to or above that of normal cells [2, 3]. In contrast, the extracellular compartment in cancer tissue is up to one pH-unit more acidic than in corresponding normal tissue [4]. Studies propose that compartmentalized regulation of pH in cancer tissue facilitates cancer cell invasion and metastasis [5, 6], which are the prime causes of cancer mortality.

Most existing data regarding acid-base regulation in cancer cells derive from cultured cell lines and only few

have looked at freshly isolated tissue. In order to exploit the therapeutic potential of acid-base transporters, it is essential to define the mechanisms of acid-base regulation in relevant human cancer tissue. In the current study, we evaluated pH_i regulation in freshly isolated crypts from human colon cancer and matched normal colon tissue in an attempt to reveal the mechanisms of acid-base transport in colonic adenocarcinomas and the adaptations taking place during colon carcinogenesis.

2. Materials and Methods

We sampled biopsies from human colon resections immediately after excision from patients undergoing colon cancer surgery at Regional Hospital Randers, Denmark [7]. Normal colon tissue was biopsied from the same surgical specimens at a minimal distance of 10 cm from the macroscopic tumor border. The sampling procedure was approved by the Mid-Jutland Regional Committee on Health Research Ethics (enquiry no. 157/2014). According to Danish legislation,

written informed consent was not required because the procedures involve excess resected tissue from a surgical procedure where all postsurgical tissue and data handling were anonymized. The biopsies were placed in ice-cold DMEM F12 (Gibco, Denmark) and kept on ice during transport (~30 minutes) to the Department of Biomedicine at Aarhus University. The study included biopsy-verified adenocarcinomas from patients, who had not received radiotherapy in the area or recent chemotherapy.

2.1. Preparation of Colonic Crypts. Human colon crypts were prepared as previously described [8] by placing small samples of biopsy material in Ca^{2+} -free Ringer solution (in mM: 130 Na^+ , 132 Cl^- , 5 K^+ , 1 Mg^{2+} , 5 pyruvate, 10 HEPES, 5 EDTA, and 5 glucose; adjusted to pH 7.4) in a 37°C water bath on a shaking table for 20 minutes. After vigorous shaking, the samples sedimented for 5 minutes before the supernatant was removed and the pellet was washed three times with DMEM.

2.2. Intracellular pH Measurements. Human colon crypts were loaded with 3 μM BCECF-AM (Invitrogen, Denmark) in 0.1% DMSO for 20 minutes. The crypts were placed in a custom-made chamber heated to 37°C. For experiments performed in presence of $\text{CO}_2/\text{HCO}_3^-$, bath solutions were bubbled continuously with 5% CO_2 /balance air. Loaded crypts were excited alternately at approximately 490 and 440 nm and emission light collected at 510 nm using a Nikon Diaphot 200 microscope (Nikon, Japan) equipped with an SRV CCD Retiga camera (QImaging, Canada) and VisiView software (Visitron systems, Germany) or using an Olympus IX70 wide-field microscope coupled to an EasyRatioPro fluorescence imaging system (Photon Technology International, USA). BCECF fluorescence ratios were calibrated to pH based on the high- $[\text{K}^+]$ nigericin method [9].

Intracellular acidification was achieved with NH_4^+ -prepulse technique [10]. Intrinsic intracellular buffering capacity was calculated from the pH_i change induced by washout of NH_4Cl in absence of $\text{CO}_2/\text{HCO}_3^-$ [11]. The contribution of $\text{CO}_2/\text{HCO}_3^-$ to intracellular buffering was calculated as 2.3 times the intracellular concentration of HCO_3^- [12]. Assuming that NH_3 and CO_2 are in equilibrium across the cell membrane, the acid load during NH_4^+ -prepulses and the intracellular concentration of HCO_3^- were calculated from the Henderson-Hasselbalch equation. We plotted the intrinsic buffering capacities derived from individual experiments as function of the corresponding pH_i values calculated as the mean of the pH_i before and after NH_4Cl washout. We investigated the recovery of pH_i from acidosis in the absence of bath Na^+ and then after Na^+ -containing buffer was returned to the experimental chamber. The pH_i recovery rate was quantified during the last 60 seconds before and for three consecutive 60-second periods after readdition of bath Na^+ . Net acid extrusion was calculated as the product of the pH_i recovery rate and the buffering capacity corresponding to the midpoint of the evaluated pH_i interval. Because pH_i recovery was very modest when crypts were exposed to 10 μM of the Na^+/H^+ -exchange inhibitor cariporide, net acid extrusion in these

experiments was quantified for a single 60-second period corresponding to the first pH_i recovery phase after addition of bath Na^+ in experiments without cariporide present.

The $\text{CO}_2/\text{HCO}_3^-$ -containing buffer consisted of (in mM): 127 Na^+ , 4 K^+ , 1.6 Ca^{2+} , 1.2 Mg^{2+} , 111.02 Cl^- , 22 HCO_3^- , 1.2 SO_4^{2-} , 1.18 H_2PO_4^- , 10 HEPES, 5.5 glucose, and 0.03 EDTA. All buffers used in functional experiments contained 5 mM probenecid in order to prevent extrusion of BCECF by the organic anion transporter. In $\text{CO}_2/\text{HCO}_3^-$ -free buffers, HCO_3^- was substituted with equimolar amounts of Cl^- ; and in Na^+ -free solutions, Na^+ was substituted with equimolar amounts of *N*-methyl-D-glucammonium. All buffers were adjusted to pH 7.4 at 37°C.

2.3. Statistics. Data are expressed as mean \pm SEM and *n* equals number of patients. To test the effect of two variables on the measured variable, we performed two-way ANOVA followed by Sidak's posttest. We compared cellular net acid extrusion and buffering capacity as function of pH_i between normal and cancer tissue using least-squares linear regression analyses. A probability (*P*) value below 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.03 software (USA).

3. Results

In order to circumvent the risk of phenotypical changes occurring during cell culture, we investigated pH_i in crypt-like structures (Figure 1(a)) freshly isolated from patients with colon cancer.

3.1. Steady-State pH_i is Elevated in Colon Cancer Crypts. Steady-state pH_i was elevated in crypts from human colon cancer tissue compared with normal colon tissue (Figure 1(b)). The difference in intracellular acidity was evident in the presence as well as in the absence of $\text{CO}_2/\text{HCO}_3^-$ (Figure 1(b)). These findings support that enhanced HCO_3^- -independent transport processes increase net acid extrusion in the near-neutral pH_i range.

3.2. Net Acid Extrusion Is Increased in Colon Cancer Crypts. We induced stable intracellular acidification by adding 20 mM extracellular NH_4Cl and after 15 minutes replacing it by Na^+ -free buffer (Figure 1(c)). From the NH_4^+ -prepulse-induced intracellular acidification in absence of $\text{CO}_2/\text{HCO}_3^-$, we calculated the intrinsic intracellular buffering capacity, which was similar in crypts from human colon cancer and normal colon tissue (Figure 1(d)).

Net acid extrusion was predominantly Na^+ -dependent (Figure 1(c)). In the human colon cancer tissue, 95.5 \pm 7.5% and 99.3 \pm 6.0% of the overall pH_i recovery rate were Na^+ -dependent in the presence and absence of $\text{CO}_2/\text{HCO}_3^-$, respectively. Corresponding values for normal colon tissue were 101.1 \pm 12.4% and 99.2 \pm 7.9%.

The cancer cells were able to eliminate intracellular acid at comparatively higher pH_i levels than normal epithelial cells (Figures 1(e) and 1(f)). This finding further emphasizes the upregulated capacity for net acid extrusion in human

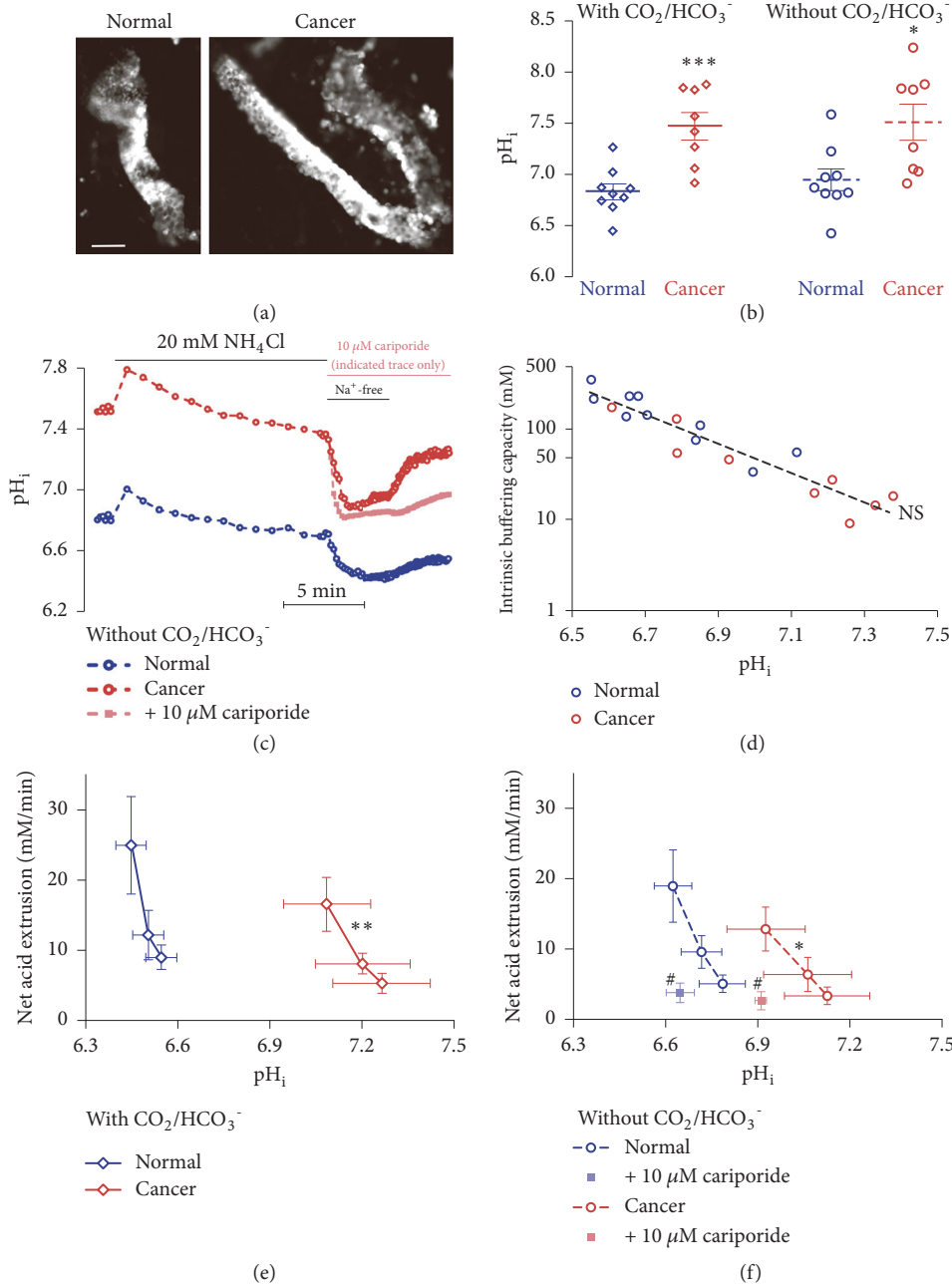


FIGURE 1: Resting steady-state pH_i and the capacity for net acid extrusion are elevated in human colon cancer tissue compared to normal colon tissue due to enhanced Na^+/H^+ -exchange activity. (a) Images of BCECF-loaded colon crypts (495 nm excitation) isolated from normal colon tissue (left panel) and colon cancer tissue (right panel). The size bar represents 100 μm ; both images are shown at the same magnification. (b) Steady-state pH_i —measured at extracellular pH 7.4—is elevated in colon cancer tissue compared with normal colon tissue ($n=8-9$) both with and without $\text{CO}_2/\text{HCO}_3^-$ present. We compared data by repeated-measures two-way ANOVA followed by Sidak's posttests. (c) Original traces of pH_i during NH_4^+ -prepulse experiments performed in the absence of $\text{CO}_2/\text{HCO}_3^-$. In the illustrated experiment performed in presence of 10 μM cariporide, this Na^+/H^+ -exchange inhibitor was added at the time of NH_4Cl washout and maintained in the bath solution for the rest of the experiment. (d) Intrinsic buffering capacity is similar in crypts from human colon cancer tissue and normal colon tissue ($n=9-11$). Based on least-squares linear regression analysis, we compared buffering capacities—plotted on a log-scale—as function of the corresponding pH_i values calculated as the mean of the pH_i before and after NH_4Cl washout. Neither slope ($P=0.80$) nor intercept ($P=0.10$) differed significantly between the groups. (e+f) Rates of net acid extrusion plotted as function of pH_i in crypts from human colon cancer tissue and normal colon tissue ($n=8-11$) in the presence (e) and absence (f) of $\text{CO}_2/\text{HCO}_3^-$. In the experiments ($n=3$) performed in presence of 10 μM cariporide, this Na^+/H^+ -exchange inhibitor was added at the time of NH_4Cl washout and maintained in the bath solution for the rest of the experiment. We compared lines by least-squares regression analyses and the effect of cariporide in normal and cancer tissue by two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. NS: not significantly different vs. normal tissue under similar conditions. # $P < 0.05$ vs. tissue without cariporide evaluated at similar pH_i .

colon cancer tissue compared to normal colon tissue. Notably, the increased capacity for Na^+ -dependent net acid extrusion both in presence (Figure 1(e)) and in absence (Figure 1(f)) of $\text{CO}_2/\text{HCO}_3^-$ supports a key role of Na^+/H^+ -exchange. This conclusion is further reinforced by the observation that $10\ \mu\text{M}$ of the Na^+/H^+ -exchange inhibitor cariporide almost entirely blocked net acid extrusion from both human colon cancer and normal colon crypts (Figures 1(c) and 1(f)).

4. Discussion

We successfully isolated crypt-like structures from biopsies of human colonic adenocarcinomas and matched normal colon tissue (Figure 1(a)). Based on these freshly isolated tissue preparations, we show that steady-state pH_i is dramatically elevated (by ~ 0.5) in colon cancer tissue compared to normal colon tissue and that this difference in pH_i does not depend on $\text{CO}_2/\text{HCO}_3^-$ (Figure 1(b)). We also show that Na^+ -dependent net acid extrusion is increased in colon cancer crypts compared with normal colon crypts in the presence as well as in the absence of $\text{CO}_2/\text{HCO}_3^-$ (Figures 1(e) and 1(f)). These findings and the efficient inhibition of net acid extrusion achieved upon addition of $10\ \mu\text{M}$ cariporide (Figures 1(c) and 1(f)) support a predominant role of Na^+/H^+ -exchange for pH_i regulation in colon cancer tissue. Working with freshly isolated colonic crypts—rather than cultured cell lines—reduces the risk of changes in cell function and protein expression caused by cell culture and has the advantage of maintained cellular interactions and overall architecture. The polarization of the colonic epithelium is increasingly compromised in dedifferentiated cancers that also gradually lose crypt structure [13]. The relatively well-maintained crypt-like structure of the investigated preparations (Figure 1(a)) supports that upregulation of net acid extrusion is an early carcinogenic event that occurs already in well-differentiated cancer cells.

In congruence with the current study, the capacity for net acid extrusion is much greater in human breast cancer tissue compared to normal breast tissue [14, 15]. However, the molecular mechanism for enhanced acid extrusion in breast carcinomas depends on $\text{Na}^+,\text{HCO}_3^-$ -cotransport rather than Na^+/H^+ -exchange [15–17]. Together, these findings highlight that cancer cells rely on enhanced net acid extrusion in order to develop and progress in the acidic tumor microenvironment but the molecular machinery allowing them to do so and hence the most promising targets for anticancer therapy vary between different types of cancers.

It is notable from the current study that the relationship between net acid extrusion and pH_i for the normal colon tissue shifts to the left upon addition of $\text{CO}_2/\text{HCO}_3^-$ to the bath solution whereas this is not the case for the colon cancer tissue (compare Figures 1(e) and 1(f)). The reason for this phenomenon is not yet clear but it may reflect a much more substantial $\text{Cl}^-/\text{HCO}_3^-$ -exchange activity in the normal colon tissue compared to the colon cancer tissue. This hypothesis is consistent with earlier reports that the expression of the anion-exchanger SLC26A3 (Down-Regulated in Adenoma, DRA) decreases during colon carcinogenesis [18, 19].

The intrinsic intracellular buffering capacities of human colon cancer tissue (Figure 1(d)) and human breast cancer tissue [15] are similar to those of equivalent normal tissue. Protonatable groups on proteins and peptides are important for intracellular buffering, and the intrinsic buffering capacity will therefore depend on the protein expression patterns of the cells, which may again be determined by the degree of cellular dedifferentiation.

5. Conclusions

In conclusion, our experiments—based on freshly isolated crypts from human colon tissue—demonstrate a dramatic increase in Na^+/H^+ -exchange activity and steady-state pH_i in colon cancer tissue compared to normal colon tissue.

List of Abbreviations

pH_i : Intracellular pH.

Data Availability

Anonymized data generated and analyzed during this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ebbe Boedtkjer and Henrik Kold-Petersen conceived the project. Ninna C. S. Voss, Mikkel B. Henningsen, and Casper Homilius performed the experiments. Ninna C. S. Voss and Ebbe Boedtkjer analyzed the data. Ninna C. S. Voss and Ebbe Boedtkjer interpreted the results. Ninna C. S. Voss and Ebbe Boedtkjer wrote the manuscript. All authors revised the manuscript and approved the final version.

Acknowledgments

The authors would like to thank the doctors—particularly Ida Elisabeth Holm and Jens Christian Christiansen—and nurses and technicians at Regional Hospital Randers for assistance with tissue sampling. This work was supported by the Danish Cancer Society [Grants no. R127-A8262 to Mikkel B. Henningsen and R72-A4273 and R146-A9461 to Ebbe Boedtkjer].

References

- [1] M. Ždravlečić, I. Marchiq, M. M. C. de Padua, S. K. Parks, and J. Pouyssegur, “Metabolic plasticity in cancers—distinct role of glycolytic enzymes GPI, LDHs or membrane transporters MCTs,” *Frontiers in Oncology*, vol. 7, no. 313, 2017.

- [2] R. A. Cardone, V. Casavola, and S. J. Reshkin, "The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis," *Nature Reviews Cancer*, vol. 5, no. 10, pp. 786–795, 2005.
- [3] M. Damaghi, J. W. Wojtkowiak, and R. J. Gillies, "pH sensing and regulation in cancer," *Frontiers in Physiology*, vol. 4, article 370, 2013.
- [4] P. Vaupel, F. Kallinowski, and P. Okunieff, "Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review," *Cancer Research*, vol. 49, no. 23, pp. 6449–6465, 1989.
- [5] G. Busco, R. A. Cardone, M. R. Greco et al., "NHE1 promotes invadopodial ECM proteolysis through acidification of the perinvadopodial space," *The FASEB Journal*, vol. 24, no. 10, pp. 3903–3915, 2010.
- [6] A. Schwab, A. Fabian, P. J. Hanley, and C. Stock, "Role of ion channels and transporters in cell migration," *Physiological Reviews*, vol. 92, no. 4, pp. 1865–1913, 2012.
- [7] N. C. Voss, H. Kold-Petersen, and E. Boedtkjer, "Enhanced nitric oxide signaling amplifies vasorelaxation of human colon cancer feed arteries," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 316, no. 1, pp. H245–H254, 2019.
- [8] M. V. Sørensen, A. B. Strandsby, C. K. Larsen, H. A. Praetorius, and J. Leipziger, "The secretory KCa1.1 channel localises to crypts of distal mouse colon: Functional and molecular evidence," *Pflügers Archiv - European Journal of Physiology*, vol. 462, no. 5, pp. 745–752, 2011.
- [9] C. Aalkjaer and E. J. Cragoe, "Intracellular pH regulation in resting and contracting segments of rat mesenteric resistance vessels," *The Journal of Physiology*, vol. 402, no. 1, pp. 391–410, 1988.
- [10] W. F. Boron and P. De Weer, "Intracellular pH transients in squid giant axons caused by CO_2 , NH_3 , and metabolic inhibitors," *The Journal of General Physiology*, vol. 67, no. 1, pp. 91–112, 1976.
- [11] E. Boedtkjer, J. Praetorius, and C. Aalkjaer, "NBCn1 (slc4a7) mediates the Na^+ -dependent bicarbonate transport important for regulation of intracellular pH in mouse vascular smooth muscle cells," *Circulation Research*, vol. 98, no. 4, pp. 515–523, 2006.
- [12] A. Roos and W. F. Boron, "Intracellular pH," *Physiological Reviews*, vol. 61, no. 2, pp. 296–434, 1981.
- [13] A. Humphries and N. A. Wright, "Colonic crypt organization and tumorigenesis," *Nature Reviews Cancer*, vol. 8, no. 6, pp. 415–424, 2008.
- [14] E. Boedtkjer, J. M. A. Moreira, M. Mele et al., "Contribution of Na^+ , HCO_3^- -cotransport to cellular pH control in human breast cancer: A role for the breast cancer susceptibility locus NBCn1 (SLC4A7)," *International Journal of Cancer*, vol. 132, no. 6, pp. 1288–1299, 2013.
- [15] S. Lee, M. Mele, P. Vahl, P. M. Christiansen, V. E. Jensen, and E. Boedtkjer, " Na^+ , HCO_3^- -cotransport is functionally upregulated during human breast carcinogenesis and required for the inverted pH gradient across the plasma membrane," *Pflügers Archiv - European Journal of Physiology*, vol. 467, no. 2, pp. 367–377, 2015.
- [16] S. Lee, T. V. Axelsen, N. Jessen, S. F. Pedersen, P. Vahl, and E. Boedtkjer, " Na^+ , HCO_3^- -cotransporter NBCn1 (Slc4a7) accelerates ErbB2-induced breast cancer development and tumor growth in mice," *Oncogene*, vol. 37, no. 41, pp. 5569–5584, 2018.
- [17] S. Lee, T. V. Axelsen, A. P. Andersen, P. Vahl, S. F. Pedersen, and E. Boedtkjer, "Disrupting Na^+ , HCO_3^- -cotransporter NBCn1 (Slc4a7) delays murine breast cancer development," *Oncogene*, vol. 35, no. 16, pp. 2112–2122, 2016.
- [18] C. W. Schweinfest, K. W. Henderson, S. Suster, N. Kondoh, and T. S. Papas, "Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 9, pp. 4166–4170, 1993.
- [19] T. M. Antalis, J. A. Reeder, D. C. Gotley et al., "Down-regulation of the down-regulated in adenoma (DRA) gene correlates with colon tumor progression," *Clinical Cancer Research*, vol. 4, no. 8, pp. 1857–1863, 1998.