Biochimica et Biophysica Acta 1863 (2017) 81–91

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Sodium/proton exchanger isoform 1 regulates intracellular pH and cell proliferation in human ovarian cancer

Carlos Sanhueza [a,*], Joaquín Araos [a], Luciano Naranjo [a], Fernando Toledo [a,b], Ana R Beltrán [c,d], Marco A Ramírez [a,d], Jaime Gutiérrez [a,e], Fabián Pardo [a,f], Andrea Leiva [a], Luis Sobrevia [a,g,h,**]

[a] Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynaecology, School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago 8330024, Chile
[b] Department of Basic Sciences, Faculty of Sciences, Universidad del Bío-Bío, Chillán 3780000, Chile
[c] Department of Education, Faculty of Education, Universidad de Antofagasta, Antofagasta 1270300, Chile
[d] Biomedical Department, Faculty of Health Sciences, Universidad de Antofagasta, Antofagasta 1270300, Chile
[e] Cellular Signalling Differentiation and Regeneration Laboratory (CSDRL), Health Sciences Faculty, Universidad San Sebastian, Santiago 7510157, Chile
[f] Metabolic Diseases Research Laboratory, Center of Research, Development and Innovation in Health - Aconcagua Valley, San Felipe Campus, School of Medicine, Faculty of Medicine, Universidad de Valparaíso, San Felipe 2172972, Chile
[g] Department of Physiology, Faculty of Pharmacy, Universidad de Sevilla, Seville E-41012, Spain
[h] University of Queensland Centre for Clinical Research (UQCCR), Faculty of Medicine and Biomedical Sciences, University of Queensland, Herston, QLD 4029, Queensland, Australia

ARTICLE INFO

Article history:
Received 16 August 2016
Received in revised form 5 October 2016
Accepted 18 October 2016
Available online 21 October 2016

Keywords:
NHE1
pHi
Human
Ovarian cancer
Cell proliferation

ABSTRACT

Cancer cells generate protons (H^+) that are extruded to the extracellular medium mainly via the Na^+/H^+ exchanger 1 (NHE1), which regulates intracellular pH (pHi) and cell proliferation. In primary cultures of human ascites-derived ovarian cancer cells (haOC) we assayed whether NHE1 was required for pHi modulation and cell proliferation. Human ovary expresses NHE1, which is higher in haOC and A2780 (ovarian cancer cells) compared with HOSE cells (normal ovarian cells). Basal pHi and pHi recovery (following a NH_4Cl pulse) was higher in haOC and A2780, compared with HOSE cells. Zoniporide (NHE1 inhibitor) caused intracellular acidification and pHi recovery was independent of intracellular buffer capacity, but reduced in NHE1 knockdown A2780 cells. Zoniporide reduced the maximal proliferation capacity, cell number, thymidine incorporation, and ki67 (marker of proliferation) fluorescence in haOC cells. *SLC9A1* (for NHE1) amplification associated with lower overall patient survival. In conclusion, NHE1 is expressed in human ovarian cancer where it has a pro-proliferative role. Increased NHE1 expression and activity constitute an unfavourable prognostic factor in these patients.

1. Introduction

Ovarian cancer is frequently diagnosed at advanced stages [1,2]. Cancer cells generate protons (H^+) [3], which are released by membrane transport systems resulting in low extracellular pH (pHo), i.e., acidity, but high intracellular pH (pHi), i.e., alkalization [4]. The membrane transport systems involved in pHi control include the sodium (Na^+)/H^+ exchangers (NHEs) [5–8]. NHE isoform 1 (NHE1) predominates and plays critical roles in cancer [4,8–10]. NHE1 activity causes intracellular alkalization and extracellular acidification [4,11]. Since NHE1 inactivation reduces proliferation of human tumour gastric [12] and small lung cancer [13] cells, NHE1 may act as a pro-proliferative factor in human tumour cells.

Mechanisms of pHi control in the human ovary or ascites-cancer cells are unknown [8]. Since non-human ovary cells exhibit NHEs activity [14–18], NHE1 may control the pHi in the human ovary. However, there are not reports addressing expression or activity of NHE1 in human ovarian cancer cells [8]. In this study, we show that functional NHE1 is expressed in human ovarian cancer cells playing a critical role in pHi recovery and cell proliferation. Thus, NHE1 expression and activity are factors that could result in pHi-dependent modulation of human ovarian cancer cells proliferation. The latter could be relevant to the reduced survival of patients suffering from this disease.

Abbreviations: NHEs, Na^+/H^+ exchangers; NHE1, NHE isoform 1; haOC, human ascites-derived ovarian cancer cells; K, cell growth rate; D_t, doubling time; K/D_t, maximal proliferation capacity; HMA, 5-N,N-hexamethylene amiloride; V-ATPases, V type ATPases; $1/^{pHi}E$, pHi modulation efficiency; TCGA, The Cancer Genome Atlas; CNA, DNA copy-number alterations.

* Corresponding author.
** Correspondence to: L. Sobrevia Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynaecology, School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago 8330024, Chile.
E-mail addresses: csanhuezamunoz@gmail.com (C. Sanhueza), sobrevia@med.puc.cl (L. Sobrevia).

http://dx.doi.org/10.1016/j.bbadis.2016.10.013
0925-4439/

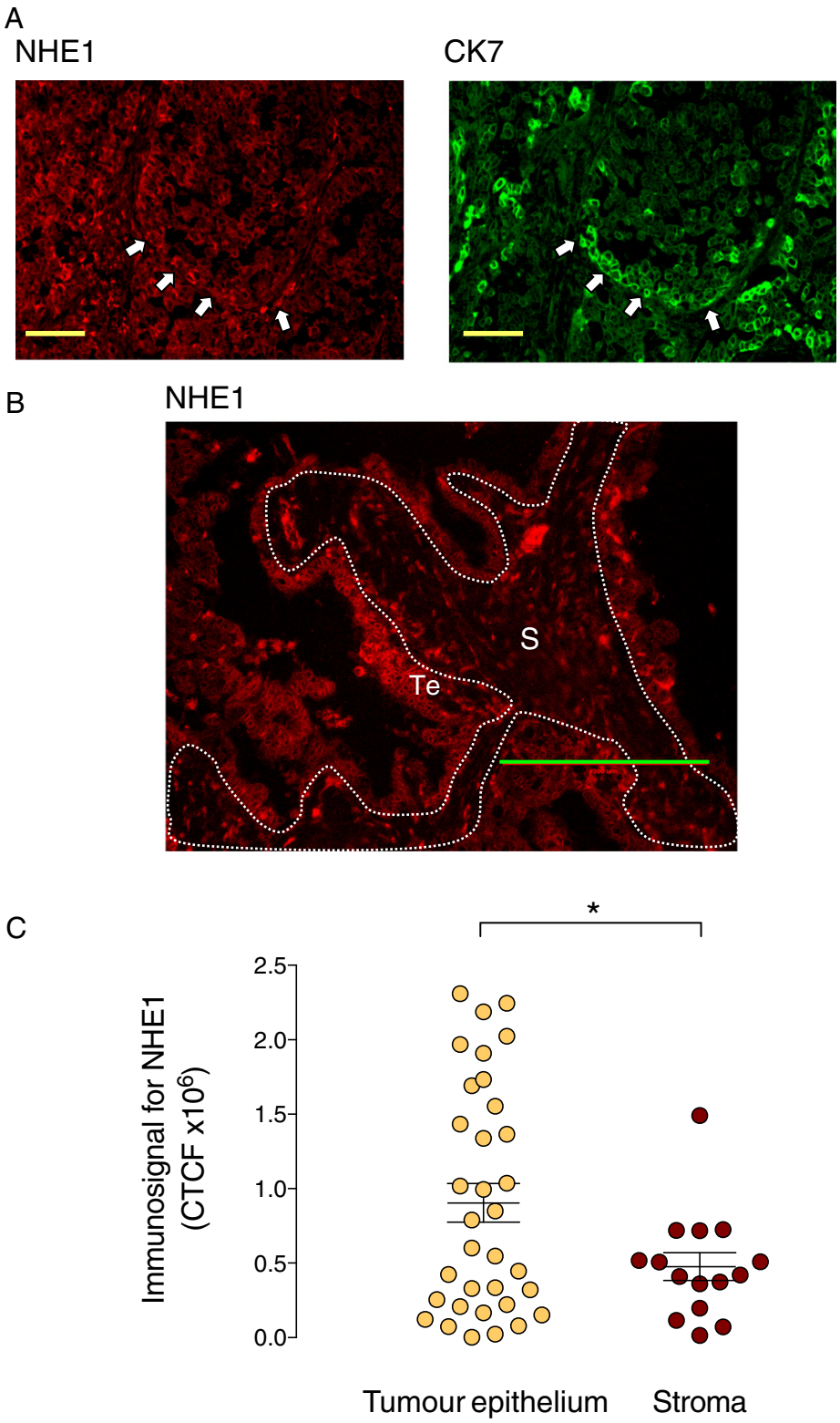

Fig. 1. NHE1 expression in human ovary. A. NHE1 and cytokeratin-7 (CK7) proteins (arrows) detected by immunofluorescence in human ovarian cancer tissue sections. **B.** NHE1 protein at the epithelial origin (Te) and stroma non-epithelial (S) regions (dotted line limits Te and S). **C.** NHE1 immunosignal in Te and S from B. Bars: 50 μm in A and 200 μm in B. *$P<0.05$. Mean ± S.E.M. ($n = 3$).

2. Materials and methods

2.1. Study groups

Patients included in this study (3 patients) coursed with ovarian cancer (high degree of ovarian serous papillary carcinoma stage IIIC or IV, and endometrioid mix type carcinoma with high degree serous differentiation) and were all subjected to primary surgery. Ovarian ascites samples were obtained from the Hospital Clínico UC-CHRISTUS in Santiago de Chile (collected by Dr Mauricio Cuello from Division of Obstetrics and Gynaecology, School of Medicine, Pontificia Universidad Católica de Chile). Ethnicity of patients included in this study was

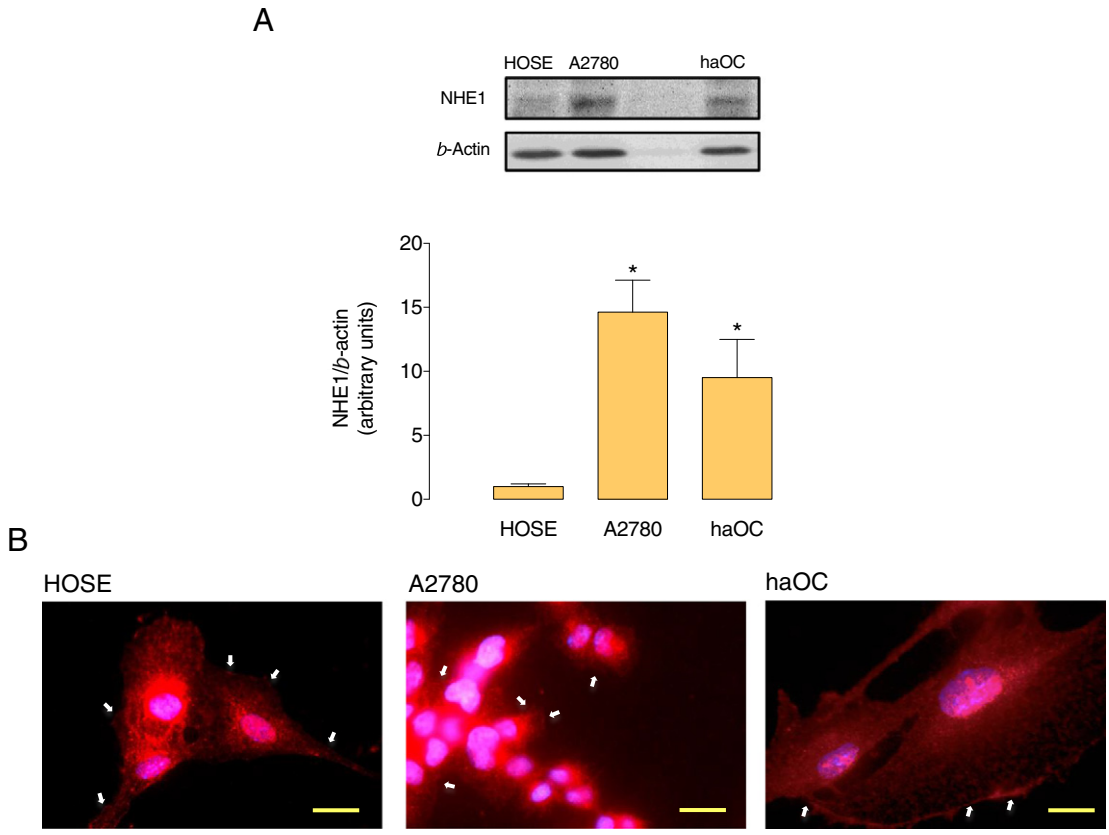

Fig. 2. NHE1 protein abundance in human ovarian tumour cells. **A.** Western blot for NHE1 and β-actin (internal reference) protein abundance in HOSE, A2780, and haOC cells. *Lower panel*: NHE1/β-actin ratio densitometries normalized to 1 in HOSE. **B.** Immunofluorescence for NHE1 (arrows show NHE1). Bars: 10 μm. *$P<0.01$. Mean ± S.E.M. (n = 3-7).

Hispanic. The investigation conforms to the principles outlined in the Declaration of Helsinki. Ethics Committee approval from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and informed consent of patients were obtained.

2.2. Cell culture

Primary cultures of human ascites-derived ovarian cancer cells (haOC) were established by mixing equal amounts of freshly isolated ascites with MCDB 105 (Sigma-Aldrich, St Louis, MO, USA)/medium 199 (M199) (Gibco, Grand Island, NY, USA) (1:1 v/v) culture media containing 10% fetal bovine serum (FBS), 12.5 mmol/L $NaHCO_3$, 4.5 mmol/L D-glucose, 12.5 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 IU/mL penicillin, and 100 μg/mL streptomycin (37°C, pH 7.4) (MCDB 105/M199 culture medium). Cells at 70% confluence were harvested with trypsin/ethylenediamine tetraacetic acid (EDTA) (0.05/0.2%, 3 minutes, 37°C) and grew up to passage 2 [19]. The human ovary cell lines HOSE (normal ovarian surface epithelium) and A2780 (ovary cancer cells) (kindly provided by Dr Carmen Romero from the Hospital Clínico Universidad de Chile, Santiago, Chile) were maintained in Dulbecco's modified Eagle's F12 medium (DMEM-F12) (Gibco) containing high (5 mmol/L) D-glucose and supplemented with 10% FBS, 16 mmol/L $NaHCO_3$, 15 mmol/L HEPES, 100 IU/mL penicillin and 100 μg/mL streptomycin (DMEM-F12 culture medium).

2.3. Cell proliferation assay

HOSE (6.5 x10^3 cells/cm^2), A2780 (2.0 x10^4 cells/cm^2), and primary cultures of haOC (1.0 x10^4 cells/cm^2) were seeded in 24 well plates and cultured for 0-48 hours in MCDB 105/M199 for haOC or DMEM-F12 culture media for HOSE and A2780 in the absence (Control) or presence of 100 nmol/L zoniporide (NHE1 inhibitor) [20]. Cells were resuspended following trypsin/EDTA (0.05/0.2%, 3 minutes, 37°C) digestion and counted in a haemocytometer as described [21]. Cell growth rates (K) were derived from the exponential growth equation:

$$K = \frac{\ln(C_t) - \ln(C_i)}{t \bullet \ln(e)}$$

where t is time in culture, C_t is number of cells at a given time in culture, C_i is number of cells at the beginning of the experiment (i.e., $t = 0$ hour), and e is 2.7182. The K values were expressed as number of cells x10^3 per cm^2 of cell culture surface per hour [21]. Doubling time (D_t) for cell growth was derived from 0.6932/K and expressed in hours. Corrected growth rates by the corresponding doubling times at any given time (i.e., maximal proliferation capacity) in culture was estimated from K/D_t.

2.4. Thymidine incorporation

Cells in the logarithmic phase of cell growth (i.e., 40-45% confluence) were incubated with [^{3}H]thymidine (2 μCi/mL, 6-[^{3}H]thymidine, 6.7 Ci/mmol (NEN, Dreieich, Germany), 48 hours, 37°C) in MCDB105/M199 for haOC or DMEM-F12 culture media for HOSE and A2780 cells in the absence or presence of zoniporide. After this incubation period the cells were rinsed with phosphate-buffered salt (PBS) solution ((mmol/L) NaCl 130, KCl 2.7, Na_2HPO_4 0.8, KH_2PO_4 1.4 (pH 7.4, 4°C)) and exposed to 5% trichloroacetic acid (TCA, 200 mL, 10 minutes). TCA was removed and cells rinsed with 99% methanol (200 mL) and digested with 25 mmol/L formic acid for radioactivity determination as described [21].

Table 1
pHi and pHi recovery rates in human ovary cells.

	HOSE	A2780	haOC
pHi			
Control	7.391 ± 0.034	7.621 ± 0.044 *	7.840 ± 0.053 *
HMA	7.262 ± 0.112 †	7.574 ± 0.145 *†	7.648 ± 0.095 *†
Zoniporide (Z)	7.270 ± 0.079 †	7.383 ± 0.161 †	7.656 ± 0.069 *†
Concanamycin A (CA)	7.601 ± 0.183	7.546 ± 0.031	7.858 ± 0.196
Schering 28080 (Sch)	7.692 ± 0.132	7.607 ± 0.043	7.782 ± 0.163
dpHi/dt (pHi units/minute)			
Control	0.114 ± 0.081	0.152 ± 0.025 *	0.194 ± 0.050 *
HMA	0.018 ± 0.007 *	0.026 ± 0.017 *	0.004 ± 0.001 *
Z	0.051 ± 0.006 *†	0.026 ± 0.008 *	0.049 ± 0.006 *†
CA	0.110 ± 0.010 ‡	0.062 ± 0.018 *‡	0.196 ± 0.086 ‡
Sch	0.106 ± 0.013 ‡	0.080 ± 0.011 *‡	0.157 ± 0.082 ‡
HMA + Z	0.025 ± 0.002 *§	0.025 ± 0.017 *§	0.001 ± 0.001 *§
HMA + CA	0.012 ± 0.015 *§	0.034 ± 0.008 *§	0.001 ± 0.005 *§
HMA + Sch	0.014 ± 0.009 *§	0.037 ± 0.011 *§	0.001 ± 0.030 *§
HMA + Z + CA	0.012 ± 0.029 *§	0.039 ± 0.016 *§	0.009 ± 0.001 *§
HMA + Z + Sch	0.025 ± 0.004 *§	0.033 ± 0.018 *§	0.005 ± 0.014 *§
HMA + CA + Sch	0.011 ± 0.002 *§	0.012 ± 0.007 *§	0.011 ± 0.002 *§
HMA + Z + CA + Sch	0.026 ± 0.016 *§	0.006 ± 0.011 *§	0.001 ± 0.023 *§
Z + CA	0.053 ± 0.032 *	0.031 ± 0.010 *#	0.047 ± 0.011 *
Z + Sch	0.061 ± 0.057 *	0.022 ± 0.015 *#	0.037 ± 0.009 *
Z + CA + Sch	0.051 ± 0.055 *	0.011 ± 0.015 *#	0.037 ± 0.009 *
CA + Sch	0.112 ± 0.013 $	0.033 ± 0.025 *£	0.120 ± 0.051 $

The intracellular pH (pHi) was measured in BCECF-AM–preloaded HOSE, A2780 and human ascites ovarian cancer cells (haOC) in the absence (Control) or presence of 5 μmol/L 5-N,N-hexamethylene amiloride (HMA) (NHEs general inhibitor), 100 nmol/L zoniporide (Z) (NHE1 inhibitor), 0.1 μmol/L concanamycin A (CA) (V type ATPases inhibitor), or 10 μmol/L Schering 28080 (Sch) (H^+/K^+ ATPase inhibitor) as described in Materials and Methods. Initial rates of pH_i recovery (*dpHi/dt*) were determined in cells subjected to an acid pulse (NH_4Cl assay) as above. In *pHi* section: *$P<0.05$ versus Control in HOSE. †$P<0.05$ versus corresponding Control values. In *dpHi/dt* section: *$P<0.05$ versus Control in HOSE or in the corresponding cell type. †$P<0.05$ versus all other corresponding values except Z + CA, Z + Sch, and Z + Ca + Sch. ‡$P<0.05$ versus corresponding HMA and Z. §$P<0.05$ versus corresponding values in Z, CA, or Sch. ¶$P<0.05$ versus corresponding values in HMA (alone or in mix with other inhibitors), CA, and Sch. #$P<0.05$ versus corresponding values in CA or Sch. $$P<0.05$ versus corresponding values in HMA and Z (alone or in mix with other inhibitors), CA or Sch. £$P<0.05$ versus all other corresponding values except CA and Sch. Values are mean ± S.E.M. (n = 3-22).

2.5. Measurement of pHi

Cell monolayers in 96 well plates (70% confluence) were incubated for 10 minutes at 37°C with the fluorescent pH sensitive probe 2,7-bicarboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM, 12 μmol/L) (Sigma-Aldrich), as described [22]. Some experiments were also performed in cells incubated for 1 minute with BCECF-AM, and the results for pHi were not significantly different from 10 minutes incubation (not shown). The excess of probe was removed by rinse three times with control solution (CS) ((mmol/L) NaCl 145, KCl 5, NaH_2PO_4 1 Na_2SO_4 1, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 30, D-glucose 5 (pH 7.4, 37°C). The fluorescence ratios was registered in a fluorimeter Tecan M200Pro (Untersbergstr, Austria) after alternate cell excitation at 440 and 490 nm, and emission at 530 nm registered every 2 seconds interval for a period of 150 seconds. pHi was estimated by interpolation of emission ratios using standard calibration curves in cells incubated with 10 μmol/L nigericin in a calibrating solution ((mmol/L) KCl 130, NaCl 20, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 5 (pH 6.2, 7.2, or 8.2)) as described [21,22]. Cells were incubated without or with 5 μmol/L 5-N,N-hexamethylene amiloride (HMA, NHEs general inhibitor) [23], 100 nmol/L zoniporide [20], 0.1 μmol/L concanamycin A (V type ATPases (V-ATPases) inhibitor) [24], and 10 μmol/L Schering 28080 (H^+/K^+ ATPase inhibitor) [25].

2.6. Recovery of pH_i

The pH_i recovery was examined by applying the NH_4Cl pulse technique [21,22]. In brief, BCECF-AM loaded cells were incubated in CS until the basal pH_i was stabilized (~3 minutes). Cells were exposed (2 minutes) to CS supplemented with NH_4Cl (NH_4Cl/CS solution) ((mmol/L) NaCl 121, KCl 5.4, $CaCl_2$ 1, KH_2PO_4 0.4, $MgCl_2$ 0.5, $MgSO_4$ 0.4, Na_2HPO_4 0.3, HEPES 10, D-glucose 0.6, NH_4Cl 20 (pH 7.4, 37°C)). After this incubation period the NH_4Cl/CS solution was replaced by rinsing the cells with CS free of NH_4Cl ($_0Na^+$/CS) ((mmol/L) *N*-methyl-D-glucamine (NMDG) 120, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 30, D-glucose 5 (pH 7.4, 37°C)), without or with HMA, zoniporide, concanamycin A and/or Schering 28080 as above.

Recovery rates of pH_i (*dpHi/dt*) were calculated from data collected for the first 60 seconds of the recovery (i.e., after removing the NH_4Cl load) and fitted by a first order lineal regression as described [21,22]. The results were expressed in pH_i units/minute. The NHEs-mediated component of *dpHi/dt* (^{NHEs}f) was estimated from:

$$^{NHEs}f = \frac{^{HMA}\alpha - \delta}{^{HMA+CA+Sch}\alpha - \delta} \cdot 100$$

where $^{HMA}\alpha$ is *dpHi/dt* inhibited by HMA, $^{HMA+CA+Sch}\alpha$ is *dpHi/dt* inhibited by HMA + concanamycin A + Schering, and δ represents the corresponding remaining *dpHi/dt* (background) on each condition. The involvement of NHE1 (^{NHE1}f) in the ^{NHEs}f component was estimated by $^{NHE1}f = {}^{NHEs}f - {}^{Z}\alpha$, where $^{Z}\alpha$ is *dpHi/dt* inhibited by zoniporide. The involvement of V-ATPase (^{V}f) and H^+/K^+ ATPase ($^{H/K}f$) components were estimated as for ^{NHEs}f considering $^{CA}\alpha$ (*dpHi/dt* inhibited by concanamycin A) or $^{Sch}\alpha$ (*dpHi/dt* inhibited by Schering) and $^{HMA+Z+Sch}\alpha$ (*dpHi/dt* inhibited by HMA + zoniporide + Schering) or $^{HMA+Z+CA}\alpha$ (*dpHi/dt* inhibited by HMA + zoniporide + concanamycin A), respectively.

Initial velocity for pHi recovery was derived from slope of linear phase of *dpHi/dt* adjusted to the one phase exponential association equation considering the least squares fit [26]:

$$v_i = \frac{dpHi}{dt} \cdot \left(1 - e^{-(k \cdot t)}\right)$$

where v_i is initial velocity, *dpHi/dt* is mayor recovery rate at a given time (t) (in this study $t = 0.03$ seconds) and e and k are constants. The capacity of cells to change the pHi value in certain units of pHi as a result in the *dpHi/dt* modifications (i.e., efficiency of pHi modulation ($1/^{pHi}E$)) was estimated from:

$$1/^{pHi}E = \frac{(\Delta dpHi/dt)}{\Delta pHi} \cdot \alpha$$

where $\Delta dpHi/dt$ is the change in *dpHi/dt* from control, ΔpHi is the change in pHi from control, and α is a given pHi unit.

2.7. Intrinsic buffering capacity

The ability of intrinsic cellular components to buffer changes in pHi, i.e., intracellular buffer capacity (βi), was measured as described [21]. After determining the basal pHi the cells were incubated in a 0.5 mmol/L KCl-containing $_0Na^+$/CS plus Schering 28080 + concanamycin A (pH 7.4, 37°C) until the pHi was stabilized under this experimental condition (~3 minutes). Cells were then incubated in the latter solution containing decreasing concentrations of NH_4Cl (50, 20, 10, 5, 2.5 or 1 mmol/L). To assay the effect of each of the concentrations of NH_4Cl the cells were rinsed three times with the corresponding lower NH_4Cl concentration used in this study. The βi was calculated from the expression:

$$\beta i = \frac{\Delta [NH_4^+]_i}{\Delta pHi}$$

where the intracellular NH_4^+ concentration ($[NH_4^+]_i$) was obtained from the Henderson-Hasselbalch equation on the assumption that $[NH_3]_i$

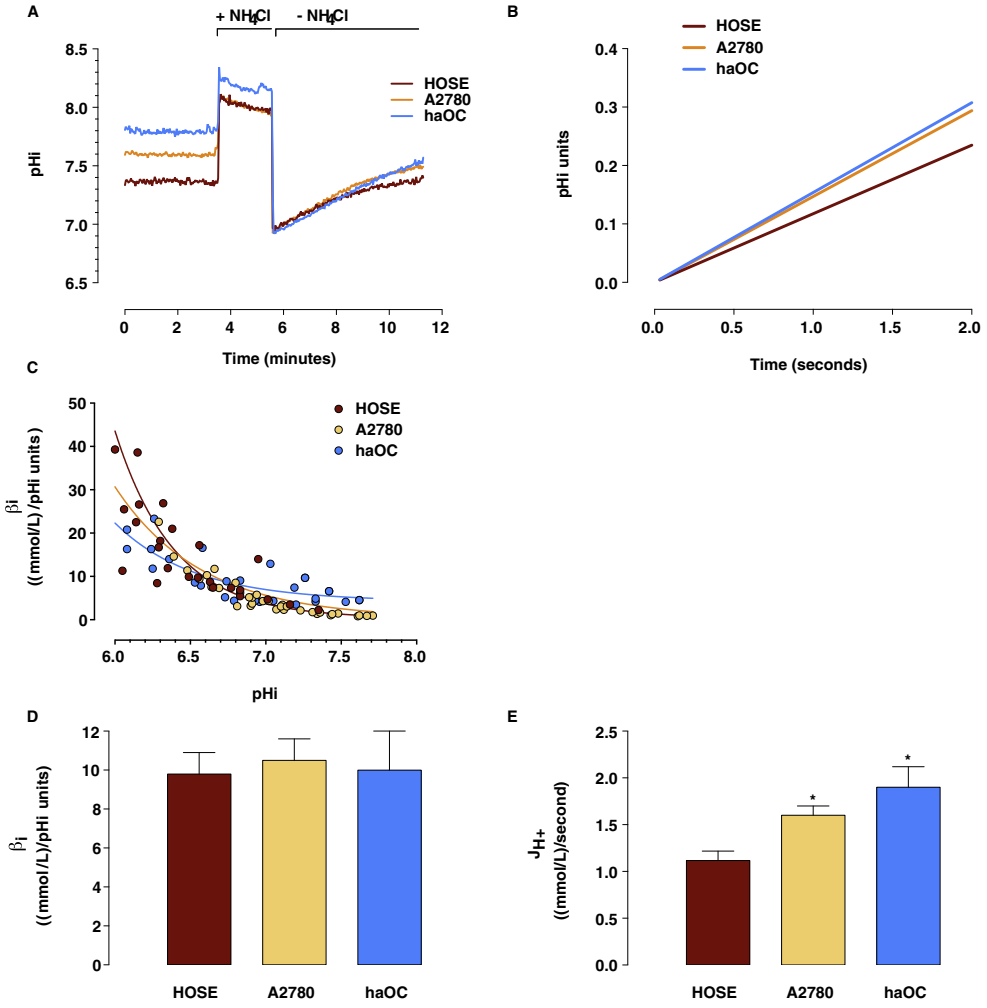

Fig. 3. pHi recovery. **A**. HOSE, A2780, and haOC cells were exposed to 20 mmol/L NH_4Cl (+NH_4Cl). NH_4Cl was removed (–NH_4Cl) and initial rates of pHi recovery were calculated (see Methods). **B**. Initial velocity for pHi recovery as in A. **C**. Intracellular buffering capacity (βi) determined as described in Methods. **D**. βi for corresponding pHi from data in C. **E**. H^+ flux rates (J_{H+}) (see Methods). *$P<0.05$ versus all other values. Values are mean ± S.E.M. (n = 3-10).

(intracellular NH_3) was equivalent to $[NH_3]_o$ (extracellular NH_3), and Δ *pHi* is the fraction of change in units of pHi value.

Knowing the *dpHi/dt* and the βi values (at similar pHi values in each cell type), the rate of overall transmembrane H^+ flux (J_{H+}) was calculated from the following expression:

$$J_{H^+} = \frac{dpHi}{dt} \bullet \beta i$$

2.8. Western blotting

Total protein was obtained from confluent cells rinsed (x2) with ice-cold PBS and harvested in 100 μL of lysis buffer (100 mmol/L NaCl, 10 mmol/L Na_4P2O_7 x10-H_2O, 10 mmol/L NaF, 1% Triton X100, 1 mmol/L sodium orthovanadate, 50 mg/mL leupeptin, 20 mmol/L HEPES (pH 7.4, 4°C) as described [22]. Cells were sonicated (6 cycles, 5 seconds, 100 W, 4°C) and total protein was separated by centrifugation (13500 *g*, 15 minutes, 4°C). Proteins (70 μg) were separated by polyacrylamide gel (8%) electrophoresis, transferred to Immobilon-P polyvinylidene difluoride membranes (BioRad Laboratories, Hertfordshire, UK) and probed with primary monoclonal rabbit *anti*-NHE1 (1:500 dilution, 12 hours, 4°C) or monoclonal mouse *anti*-β-actin (1:2500 dilution, internal reference) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. The membranes were rinsed in PBS/0.1% Tween 20 (PBS-T) and further incubated (1 hour) in PBS-T/0.2% bovine serum albumin (BSA) containing secondary horseradish peroxidase-conjugated goat *anti*-rabbit or *anti*-mouse antibodies (Thermo Scientific, Rockford, IL, USA). Proteins were detected by enhanced chemiluminescence in a ChemiDoc-It 510 Imagen System (UVP, LCC Upland, CA, USA) and quantified by densitometry [22].

2.9. Immunofluorescence microscopy

Paraformaldehyde-fixed, paraffin-embedded ovarian cancer serial sections (4 μm) were dewaxed and subsequently rehydrated with serial incubations using 100, 95, or 70% ethanol solutions. Histological slices were permeabilized (3% Tween 20, 10 minutes) and blocked with defatted-cow milk (5% in PBS, 1 hour, 22°C). NHE1, cytokeratin-7 (CK-7) (marker for epithelial cells) and (marker for proliferation) were immunolocalized following incubation (overnight at 4°C) with primary policlonal rabbit *anti*-NHE1 (1:200 dilution) (Santa Cruz Biotechnology), monoclonal mouse *anti*-CK-7 (1:200 dilution) (Dako, Glostrup, Denmark) or *anti*-ki67 (1:50 dilution) (Thermo Scientific, Rockford, IL, USA) in PBS containing 2% BSA (PBS/2% BSA) and followed by incubation (1 hour, 22°C) with the secondary antibody Alexa Fluor 568 goat *anti*-rabbit or *anti*-mouse IgG (H + L, λexc/λem: 578/603 nm, 1:750) (Life Technologies).

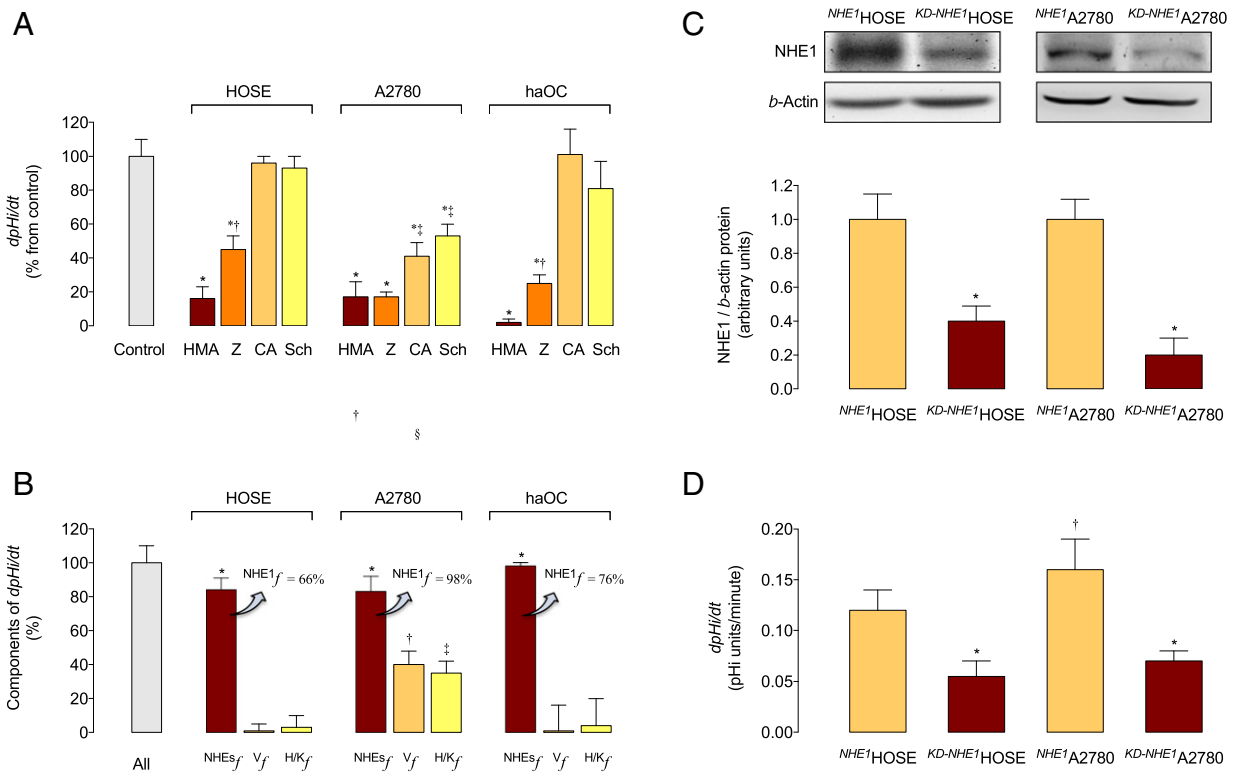

Fig. 4. NHE1 involvement in pHi recovery. **A**. pHi recovery rates (*dpHi/dt*) in HOSE, A2780, and haOC cells without (Control, 100%) or with 5 μmol/L 5-N,N-hexamethylene amiloride (HMA), 100 nmol/L zoniporide (Z), 0.1 μmol/L concanamycin A (CA), and/or 10 μmol/L Schering 28080 (Sch). **B**. *dpHi/dt* fraction mediated by NHEs (^{NHEs}f), V type ATPases (^{V}f), or H^+/K^+ ATPase ($^{H/K}f$) components. Arrows indicate the *dpHi/dt* fraction mediated by NHE1 (^{NHE1}f) of ^{NHEs}f component (see Methods). 'All' is all components of *dpH/dt*. **C**. Western blot for NHE1 and β-actin (internal reference) protein abundance in HOSE and A2780 expressing NHE1 (^{NHE1}HOSE, ^{NHE1}A2780) or NHE1 knockdown ($^{KD\text{-}NHE1}$HOSE, $^{KD\text{-}NHE1}$A2780) cells. *Lower panel*: NHE1/β-actin ratio densitometries normalized to 1 in ^{NHE1}HOSE cells. **D**. *dpH/dt* in cells as in C. In A: **P*<0.04 versus Control, †*P*<0.05 versus all other corresponding values, ‡*P*<0.05 versus all other values except for Z in HOSE cells. In B: **P*<0.05 versus all other values, †*P*<0.04 and ‡*P*<0.05 versus corresponding values in HOSE and haOC cells. In C and D: **P*<0.05 versus corresponding values in ^{NHE1}HOSE or ^{NHE1}A2780. In D: †*P*<0.05 versus all other values. Values are mean ± S.E.M. (n = 3-10).

Cells were grown on microscope cover glasses (6 x10^4 cells per slide) (Sail Brand, Shangai, China) in corresponding culture media to 70% confluence as described [27]. In brief, cells were fixed (4% paraformaldehyde, 15 minutes), permeabilized (0.1% Triton X-100, 10 minutes) and blocked (PBS/2% BSA, 1 hour, 22°C). NHE1 and ki67 were immunolocalized following incubation (overnight at 4°C) with primary policlonal rabbit *anti*-NHE1 (1:200 dilution) or monoclonal mouse *anti*-ki67 (1:50 dilution) in PBS/2% BSA and followed by incubation (1 hour, 22°C) with the secondary antibody Alexa Fluor 488 goat *anti*-mouse IgG (H + L, λexc/λem: 488/519 nm, 1:750 dilution) or Alexa Fluor 568 goat *anti*-rabbit IgG (H + L, λexc/λem: 578/603 nm, 1:750 dilution) (Life Technologies). Cell nuclei were stained with Hoescht 33342 (4 μmol/L, 10 minutes, 22°C) (Thermo Scientific). Images were obtained under an EVOS FL Imaging System (AMF 4300) (Life Technologies). Fluorescence quantification was performed by the method of Corrected Total Fluorescence Intensity (CTCF) as described [28] in regions of optical interest (ROI) in serial slices. ROI was defined as a section of the tissue that was positive (epithelial) or negative (stroma) for the epithelial marker CK-7 where NHE1 and ki67 staining was determined. Images were processed with Image J version 1.48v (Wayne Rusband NIH, USA).

2.10. NHE1 suppression

Suppression of NHE1 expression was done using the commercially available short interference RNAs (siRNA) NHE-1 siRNA (h) or control sequence (siC) (Santa Cruz Biotechnology) following manufacturer's instructions (http://datasheets.scbt.com/siRNA_protocol.pdf). HOSE and A2780 cells knockdown for NHE1 ($^{KD\text{-}NHE1}$HOSE and $^{KD\text{-}NHE1}$A2780, respectively) were generated. haOC cells were not viable *in vitro* under this approach, thus $^{KD\text{-}NHE1}$haOC cells was not possible.

2.11. TCGA data analysis

Genomic, mRNA expression and clinical information of high-grade serous cancer were obtained *in silico* from The Cancer Genome Atlas Ovarian Cancer (https://confluence.broadinstitute.org/display/GDAC/Home). Analyses of patient's survival and NHE1 signalling pathway were performed in cBioPortal platform for Cancer Genomics (http://www.cbioportal.org/study.do?cancer_study_id=ov_tcga) according to previously published protocols [29,30].

2.12. Statistical analysis

The values are mean ± S.E.M., where *n* indicates the number of different biological samples (3 ascites from patients with ovarian cancer) and corresponding cell cultures with 3-4 replicates per experiment. Comparisons between two groups were performed by means of Student's unpaired *t*-test. The difference between more than two groups was performed by ANOVA (one or two ways). If the ANOVA demonstrated a significant interaction between variables, *post hoc* analyses were performed by the multiple-comparison Bonferroni test. To estimate the correlation between NHE1 and ki67 expression in tissue samples, the correlation analysis was performed using Pearson's correlation coefficient (r). Patient survival analyses were addressed by Kaplan Meier methodology using log-rank, Wilcoxon parameters.

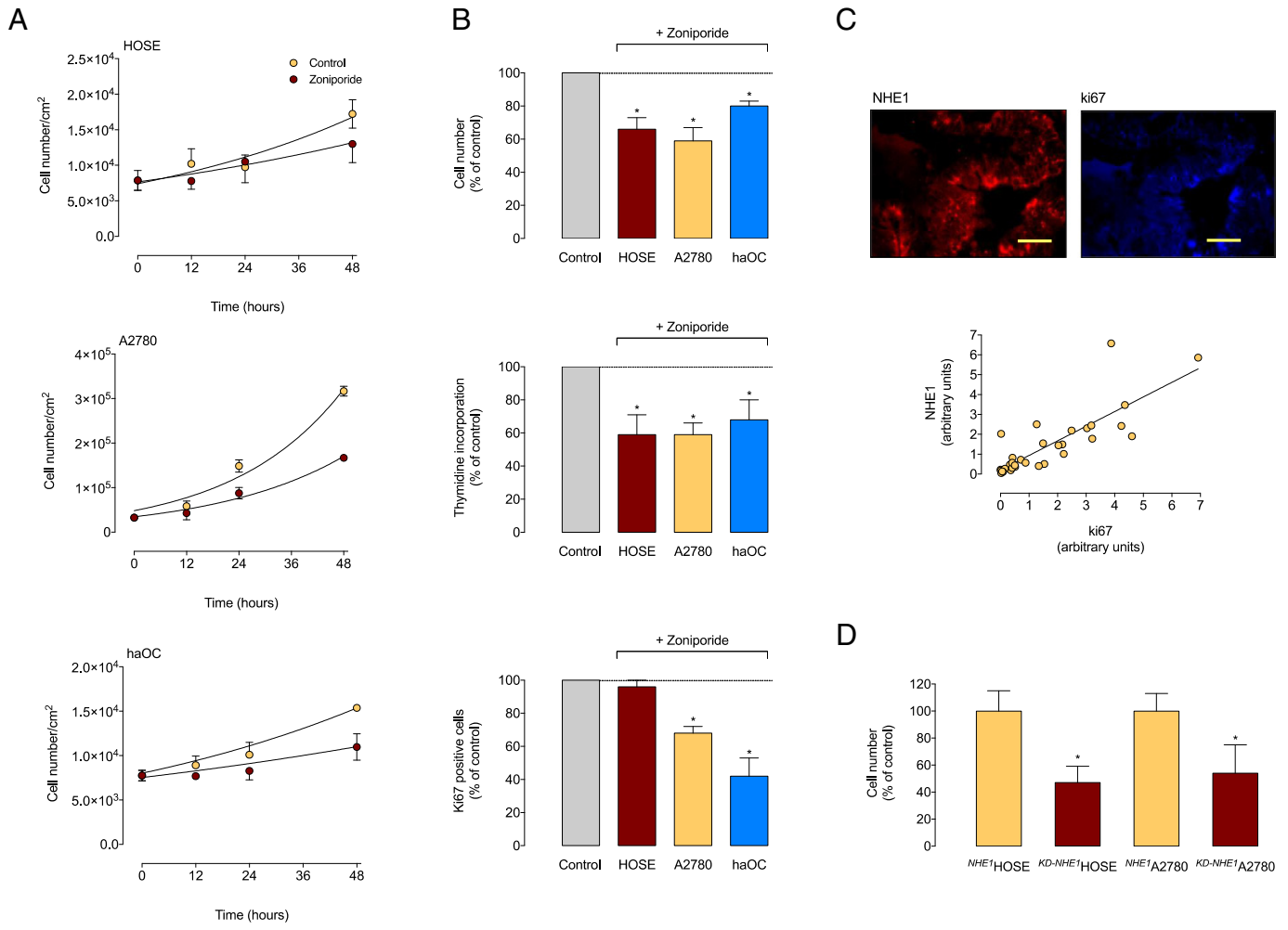

Fig. 5. NHE1 involvement in cell proliferation. **A**. HOSE, A2780, and haOC cells cultured in the absence (Control) or presence of 100 nmol/L zoniporide. Cell number was determined in a haemocytometer (see Methods). **B**. Cell number, thymidine incorporation, and ki67 positive cells in the absence (Control) or presence of zoniporide (+Zoniporide) as in A. **C**. Immunofluorescence for NHE1 and ki67 protein in human ovarian cancer tissue sections (bars: 50 μm). The graph represents NHE1 versus ki67 immunosignal adjusted to a first-order equation (Pearson's correlation coefficient (r) = 0.83, goodness-of-fit R^2 = 0.672, P<0.0001, n = 32 ROIs in 3 patients). (D) Cell number in HOSE and A2780 expressing NHE1 (^{NHE1}HOSE, ^{NHE1}A2780) or NHE1 knockdown cells ($^{KD\text{-}NHE1}$HOSE, $^{KD\text{-}NHE1}$A2780). Values are normalized to 1 in ^{NHE1}HOSE or ^{NHE1}A2780 cells. In B: *P<0.05 versus Control. In D, *P<0.04 versus corresponding ^{NHE1}HOSE or ^{NHE1}A2780. Values are mean ± S.E.M. (n = 3-10).

P<0.05 value was considered statistically different. The statistical software GraphPad InStat 3.0b and GraphPad Prism 7.0b (GraphPad Software Inc., San Diego, CA, USA) were used for data analysis. P<0.05 was considered statistically significant.

3. Results

3.1. NHE1 expression in human ovary

The first goal of this study was to determine whether NHE1 was expressed in human ovarian cancer tissue. Ovarian cancer tissue was positive for NHE1 and CK7 (Fig. 1A). NHE1 was detected in tumour epithelium and stroma (Fig. 1B), with higher fluorescence at the epithelium, compared with stroma (Fig. 1C). We then assayed whether NHE1 was expressed in cultured human ovarian cancer cells. NHE1 protein was detected in all cell types (Fig. 2A) and was higher in haOC and A2780 compared with HOSE cells. NHE1 protein was detected in the cells and in the cell periphery (Fig. 2B).

3.2. Basal pHi and dpHi/dt

Since NHE1 expression associates with pHi modulation in several tumour cells [4–10], we determined basal pHi and the potential functional involvement of this and other membrane transport mechanisms to regulate intracellular H^+ content. Basal pHi in haOC and A2780 was higher than in HOSE cells (Table 1). Zoniporide

Table 2
Effect of zoniporide on the parameters for human ovary cells proliferation.

	K (1/hour)	D_t (hour)	K/D_t (1/(hour)^2)
HOSE			
Control	0.01702 ± 0.00477	41 ± 11	0.00042 ± 0.00011
Zoniporide	0.01131 ± 0.00402	61 ± 22	0.00019 ± 0.00007 *
A2780			
Control	0.03949 ± 0.00304 †	17 ± 1 †	0.00232 ± 0.00016 †
Zoniporide	0.03328 ± 0.00401 †	21 ± 3 †	0.00158 ± 0.00023 *†
haOC			
Control	0.01357 ± 0.00131	51 ± 1	0.00027 ± 0.00003
Zoniporide	0.00791 ± 0.00074 *	88 ± 1 *	0.00009 ± 0.00001 *

HOSE, A2780, and human ascites ovarian cancer cells (haOC) cells were seeded in 24-well plates (2.05 cm^2 surface) and cultured for 48 hours in primary culture medium without (Control) or with 100 nmol/L zoniporide. Cell number was estimated by counting at different periods of time and cell growth rates (K) and doubling time (D_t) for cell growth was defined as described in Materials and Methods. K/D_t represents corrected cell growth rate by doubling times (maximal proliferation capacity) of cells in culture. *P<0.05 versus corresponding Control values. †P<0.05 versus corresponding values in HOSE and haOC cells. Values are mean ± S.E.M. (n = 3-22).

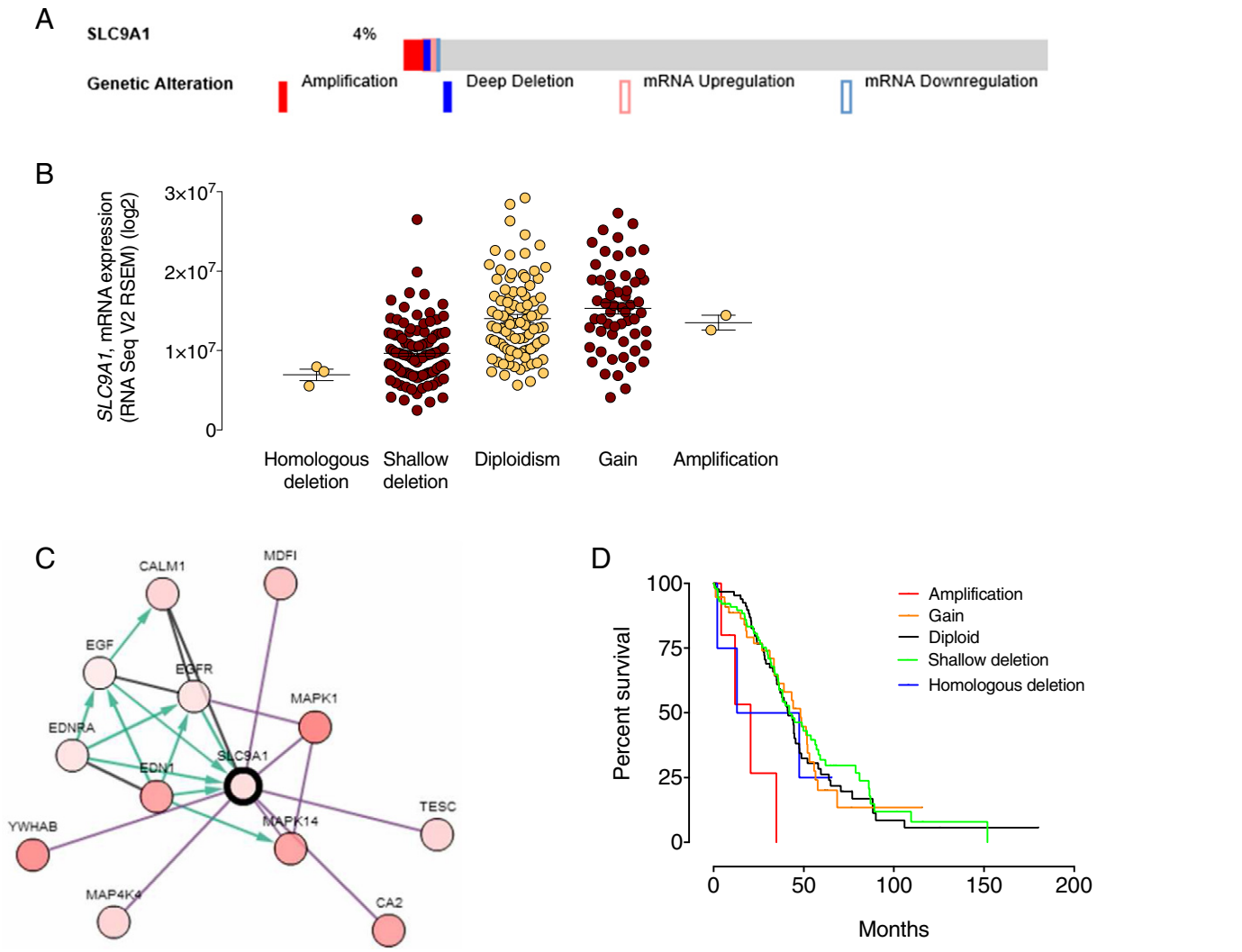

Fig. 6. NHE1 expression and survival in patients with ovarian cancer. **A**. Analysis of the DNA copy-number alterations (CNA) for *SLC9A1* in human ovarian cancer (The Cancer Genome Atlas Ovarian Cancer (TCGA) database) (the number of cases with the indicated genetic alterations (Genetic Alteration) is given). **B**. Relation between mRNA number of copies and alterations in CNA of *SLC9A1* in patients with high-grade serous ovarian cancer. **C**. Representation of NHE1 pathway as in C. Green arrows depicts direct-modulatory pathway of *SLC9A1* by endothelin-1 (END1)/endothelin receptor A (ENDRA) and epidermal growth factor (EGF)/EGF receptor (EGFR). **D**. Overall survival of patients with ovarian cancer according to the indicated CNA alterations (by cBioPortal platform for Cancer Genomics).

and HMA, but not concanamycin A or Schering 28080 caused intracellular acidification in all cell types. To estimate the capacity of cells to recover from an acidic pHi cells were subjected to the NH_4Cl pulse assay [21–23]. The acidic pHi caused by the NH_4Cl pulse was fully restored after ~3 minutes (Fig. 3A). Basal *dpHi/dt* (Table 1) and v_i for *dpHi/dt* (Fig. 3B) in haOC ($v_i = 10.2 \pm 0.001$ pHi units/minute) and A2780 cells ($v_i = 9.8 \pm 0.002$ pHi units/minute) were higher compared with HOSE cells ($v_i = 7.8 \pm 0.001$ pHi units/minute).

3.3. ßi and J_{H+}

Since a change in basal pHi and *dpHi/dt* depends on the *ßi* capacity of cells [21], this parameter was estimated and used to determine J_{H+}. The results show that *ßi* increased as pHi value decreased (Fig. 3C) and *ßi* at equal pHi after a 20 mmol/L NH_4Cl pulse (pHi HOSE = 6.95, pHi haOC = 6.93, pHi A2780 = 6.95) were similar in all cell types (Fig. 3D). In addition, J_H^+ was higher in haOC and A2780 compared with HOSE cells (Fig. 3E).

3.4. NHE1, V-ATPase, and H^+/K^+ ATPase involvement on dpHi/dt

To know the relative contribution of the membrane transport mechanisms involved in the regulation of pHi, the *dpH/dt* was measured in the absence or presence of specific inhibitors for NHE1, V-ATPase, and H^+/K^+ ATPase. The results show that HMA reduced *dpHi/dt* in all cell types (Table 1). Partial inhibition was detected in HOSE and A2780 cells; however, HMA abolished *dpHi/dt* in haOC cells (Fig. 4A). Zoniporide caused a partial reduction of *dpHi/dt* in HOSE and haOC cells, which was similar to HMA effect in A2780 cells. Concanamycin A and Schering 28080 caused partial reduction (47-59%) of *dpHi/dt* only in A2780 cells. The ^{NHEs}f component accounted for a fraction of *dpHi/dt* similar in HOSE and haOC cells (Fig. 4B). However, ^{NHEs}f accounted for almost all *dpHi/dt* in A2780 cells. Involvement of ^{NHE1}f component in ^{NHEs}f resulted in a fraction of *dpHi/dt* that was similar in HOSE and haOC cells; however, in A2780 cells accounted for all the ^{NHEs}f component. The ^{V}f and $^{H/K}f$ components were detected only in A2780 cells.

With the aim to corroborate that NHE1 expression is required for a pHi recovery, cells knockdown for this protein were used to

estimate *dpHi/dt*. The NHE1 protein abundance was lower in $^{KD\text{-}NHE1}$HOSE and $^{KD\text{-}NHE1}$A2780 cells compared with cells expressing NHE1 (Fig. 4C). The $^{KD\text{-}NHE1}$HOSE and $^{KD\text{-}NHE1}$A2780 cells also show lower *dpHi/dt* compared with cells expressing NHE1 (Fig. 4D).

3.5. Cell proliferation and NHE1 and ki67 correlation

Since an acidic pHo due to increased activity of NHE1 is associated with increased tumour cells proliferation [12,13], cell proliferation was assayed in the presence of a NHE1 inhibitor. The results show that zoniporide reduced proliferation (Fig. 5A) and the K/D_t in all cell types (Table 2). The reduction in the K/D_t was less pronounced in A2780 compared with HOSE or haOC cells. Cell growth rate was higher, but doubling times were lower in A2780 cells compared with HOSE or haOC cells. Zoniporide decreased growth rate and increased the doubling time only in haOC cells. Results in the kinetics of cell growth were complemented with an estimation of the changes in the actual cell number changes and nucleic acid turnover. The cell number and [^{3}H]thymidine incorporation were reduced by zoniporide (Fig. 5B). Additionally, the number of ki67 positive cells (marker for cell proliferation) in the presence of zoniporide was lower in A2780 and haOC, but unaltered in HOSE cells.

To confirm a parallel increase in NHE1 and ki67 protein expression in human ovarian tissue, a potential correlation between these proteins expression was analysed. The results show that NHE1 and ki67 proteins were detected in the same regions in ovarian cancer tissues, with a positive correlation for NHE1 and ki67 protein abundances (Fig. 5C). Additionally, the cell number counted in $^{KD\text{-}NHE1}$HOSE and $^{KD\text{-}NHE1}$A2780 cells was lower compared with these cells expressing NHE1 (Fig. 5D).

3.6. SLC9A1 and overall survival

In the aim of knowing whether NHE1 overexpression associates with adverse prognostic in patients with high-grade serous ovarian cancer, we analysed *in silico* the DNA copy-number alterations (CAN) for *SLC9A1*. Analysis of the CNA for this gene in human ovarian cancer reveals that 4% of cases will present with genetic changes including amplification (nine patients), deep deletions (one patients), and upregulation (six patients) or downregulation (two patients) of mRNA expression (Fig. 6A). *SLC9A1* mRNA expression increases according to the type of CNA alteration in patients with high-serous ovarian cancer (Fig. 6B). The analysis *in silico* reveals that *SLC9A1* is in direct-pathway with endothelin-1 (END1)/endothelin receptor A (ENDRA) and with epidermal growth factor (EGF)/EGF receptor (EGFR) in patients with ovarian cancer (Fig. 6C). *SLC9A1* amplification associated with reduced overall survival (~50%) compared with patients without changes in *SLC9A1* (diploid (98 patients) (41.4 versus 20.5 months in nine patients with *SLC9A1* amplification, respectively) (Log-rank = 0.0005, Wilcoxon = 0.0013) or with mild CNA alteration (shallow deletion (108 patients) or gain (63 patients)) (Fig. 6D). There was not a difference between amplification and homologous deletions (5 patients, Log-rank = 0.36, Wilcoxon = 0.68), or homologous deletion and overall survival when compared with unaltered or mild CNA alteration.

4. Discussion

This study shows that NHE1 is expressed in the human ovarian cancer and primary cultures of human ascites ovarian cancer cells (haOC). Tumour cells show intracellular alkalization compared with normal tissues [4,31,32]. This phenomenon depends on the expression of membrane transport systems for H^+ removal [8,33]. haOC cells show pHi values higher (~0.35 pHi units) than in non-tumour cells, suggesting that pHi regulation is altered by this pathology. This condition may be responsible for cell malignancy since an increase in 0.2-0.4 pHi units associates with higher cancer cell proliferation [4,21,33]. NHE1, 2, 3, and 4 are critical regulators of pHi in tumour tissues [8,34]. Our results show that NHE1 protein is expressed in CK7 positive tumour epithelium in the human ovary. Additionally, haOC cells show increased NHE1 expression (~12 fold) compared with non-tumour cells, suggesting its potential role in this type of cancer. haOC cells also show increased *dpHi/dt* and pHi that was comparable to that in non-tumour cells, suggesting that intracellular alkalization may result from a higher *dpHi/dt*. Indeed, increased v_i of *dpHi/dt* in haOC agrees with other cancer cells [4,8,31,32]. Since haOC and non-tumour cells intrinsic buffer capacity (βi) were similar, increased *dpHi/dt* and J_H^+ are not due to abnormal cell capacity to buffer a change in pHi. Interestingly, the βi values determined in HOSE, haOC, and A2780 cells are in the same range as for other tumour cells such as T_{84} cells (~31 (mmol/L)/pHi units) [22,35], and lower than in non-tumour MDCK cells (~110 (mmol/L)/pHi units) [21]. The differences in the βi values could be due to specie differences (human versus dog) since the experimental conditions to determine this parameter were similar in these studies.

HMA reduced the *dpHi/dt* and pHi, suggesting a role for NHEs in these phenomena. Since HMA-reduced pHi was higher (~1.5 fold) in haOC than non-tumour cells, haOC cells are more sensitive to NHEs activity-dependent pHi regulation. Same results were found in cells treated with zoniporide. Thus, haOC cells require NHE1 activity by a larger fraction (^{NHE1}f / ^{NHEs}f ~0.8), in this phenomenon. ^{NHE1}f involvement to increase pHi is supported by the lower *dpHi/dt* detected in NHE1-knockdown ovarian tumour cells.

The maximal proliferative capacity (K/D_t) of haOC was similar to non-tumour cells, suggesting that ovarian cancer does not alter ovarian cells proliferation. NHE1's activity plays a preferential role in haOC cells proliferation since zoniporide-reduced K/D_t was ~1.3 fold higher than non-tumour cells. Since a positive correlation between NHE1 and ki67 protein abundance was found (i.e., NHE1-expression dependent proliferation), and zoniporide reduces K/D_t (i.e., NHE1-activity dependent proliferation), NHE1 expression and activity are required for haOC cells proliferation. The latter is supported by results showing zoniporide-reduced haOC cell number and DNA turnover.

haOC cells show pHi modulation efficiency ($1/^{pHi}E$ ~4.74 pHi units/minute) lower than in non-tumour cells. Thus, haOC cells may count with a less efficient mechanism protecting for intracellular acidification than in non-tumour cells. Zoniporide-reduced K/D_t in haOC cells may depend on a lower $1/^{pHi}E$ in haOC ($1/^{pHi}E$ ~0.17 pHi units/minute in the presence of zoniporide) compared with non-tumour cells ($1/^{pHi}E$ ~0.11 pHi units/minute in the presence of zoniporide) [$(K/D_t)/1/^{pHi}E$ ~0.31 in haOC and ~0.11 in HOSE cells]. This mechanism will favour tumour cells survival since intracellular acidification causes cell death [36–38]. Interestingly, a high pHi favours the transition from phase S to G2/M in the cell cycle and DNA/RNA synthesis [6,39]. Our results show that zoniporide reduced [^{3}H]thymidine incorporation. Thus, blocking NHE1 activity leads to acidic pHi-dependent reduced proliferation likely due to reduced cell cycle progression of haOC cells. These findings are supported by the reduced *dpHi/dt* detected in A2780 and non-tumour NHE knockdown cells. Thus, NHE1 expression and activity are pro-proliferative factors in human ovarian cells.

Parallel analysis of CNA in *SLC9A1* (for NHE1) revealed that a fraction of patients showed with CNA including higher amplification. Since *SLC9A1* mRNA expression is increased depending on CNA alterations, this change in *SLC9A1* could determine human ovarian cancer stage. CNA alterations may modulate the *SLC9A1* expression by endothelin-1 and epidermal growth factor, complementing reports involving endothelin-1 and endothelin receptor A in this type of cancer [40] and endothelin-1 in combination with β-arrestin-1 activation increasing chemoresistance [41]. Equally, activation of epidermal growth factor/epidermal growth factor receptor signalling is involved in higher angiogenesis [42] and resistance to paclitaxel [43] in human ovarian cancer. Higher serum epidermal growth factor [44] and endothelin-1 in the peritoneal fluid in patients with malignant ascites [45,46] are reported in human ovarian cancer. Thus, epidermal growth factor and

endothelin-1 modulation of *SLC9A1* expression and their involvement in ovarian cancer are suggested. Certainly future experiments should be considered to confirm the potential role of endothelin-1 and epidermal growth factor in the alterations in pHi modulation and cell proliferation in haOC and other ovarian cancer cells. *SLC9A1* amplification results in lower patients overall survival. Thus, NHE1 overexpression is an adverse prognostic factor in patients with high-grade serous ovarian cancer presenting alterations in these signalling pathways.

In summary, human ovary expresses NHE1 membrane transporters whose expression and activity are increased in human ascites cells from ovary cancer, suggesting a role for this protein in this disease. NHE1 contributes to pHi recovery by a higher expression in tumour cells acting as a pro-proliferative factor in human ovary tumour cells. Additionally, higher *SLC9A1* expression may constitute worse survival prognostic factor in patients with ovarian cancer. Since antineoplastic drugs treatment of patients with ovary cancer has various and serious adverse effect sounds reasonable to propose the use of a lower concentration of these drugs together with NHE1 inhibitors to obtain a similar inhibition of cancer growth in future therapy. Supporting this possibility are the findings showing that inhibition of NHE1 activity results in a higher sensitivity to paclitaxel in breath cancer [47]. Thus, strategies to control NHE1 expression and activity in patients with ovary cancer could be of benefit reducing the adverse patient's outcome of this disease.

Sources of funding

This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 3140516, 1150377, 1150344, 11150083, 3160194), Dirección de Investigación, Universidad San Sebastián (USS 2015-0032-I), and Semillero Dirección de Investigación, Universidad de Antofagasta (5309, 5313), Chile.

Disclosures

None.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

Authors thank Mrs Amparo Pacheco from CMPL, Pontificia Universidad Católica de Chile (PUC), for excellent technical and secretarial assistance, respectively.

References

[1] I. Romero, R.C. Bast Jr., Minireview: human ovarian cancer: biology, current management, and paths to personalizing therapy, Endocrinology 153 (2012) 1593–1602, http://dx.doi.org/10.1210/en.2011-2123.
[2] E. Lengyel, J.E. Burdette, H.A. Kenny, D. Matei, J. Pilrose, P. Haluska, et al., Epithelial ovarian cancer experimental models, Oncogene 33 (2014) 3619–3633, http://dx.doi.org/10.1038/onc.2013.321.
[3] E.P. Spugnini, P. Sonveaux, C. Stock, M. Perez-Sayans, A. De Milito, S. Avnet, et al., Proton channels and exchangers in cancer, Biochim. Biophys. Acta 2015 (1848) 2715–2726, http://dx.doi.org/10.1016/j.bbamem.2014.10.015.
[4] S. Harguindey, J.L. Arranz, J.D. Polo Orozco, C. Rauch, S. Fais, R.A. Cardone, et al., Cariporide and other new and powerful NHE1 inhibitors as potentially selective anticancer drugs–an integral molecular/biochemical/metabolic/clinical approach after one hundred years of cancer research, J. Transl. Med. 11 (2013) 282, http://dx.doi.org/10.1186/1479-5876-11-282.
[5] J. Orlowski, S. Grinstein, Na^+/H^+ exchangers, Compr. Physiol. 1 (2011) 2083–2100, http://dx.doi.org/10.1002/cphy.c110020.
[6] X. Yang, D. Wang, W. Dong, Z. Song, K. Dou, Suppression of Na+/H + exchanger 1 by RNA interference or amiloride inhibits human hepatoma cell line SMMC-7721 cell invasion, Med. Oncol. 28 (2011) 385–390, http://dx.doi.org/10.1186/1477-7819-10-270.
[7] S.J. Reshkin, M.R. Greco, R.A. Cardone, Role of pHi, and proton transporters in oncogene-driven neoplastic transformation, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369 (2014) 20130100, http://dx.doi.org/10.1098/rstb.2013.0100.
[8] C. Sanhueza, J. Araos, L. Naranjo, R. Villalobos, F. Westermeier, C. Salomon, et al., Modulation of intracellular pH in human ovarian cancer, Curr. Mol. Med. 16 (2016) 23–32, http://dx.doi.org/10.2174/1566524016666151222143437.
[9] S.Y. Loo, M.K. Chang, C.S. Chua, A.P. Kumar, S. Pervaiz, M.V. Clement, NHE-1: a promising target for novel anti-cancer therapeutics, Curr. Pharm. Des. 18 (2012) 1372–1382, http://dx.doi.org/10.2174/138161212799504885.
[10] S.J. Reshkin, R.A. Cardone, S. Harguindey, Na^+-H^+ exchanger, pH regulation and cancer, Recent Pat. Anticancer Drug Discov. 8 (2013) 85–99, http://dx.doi.org/10.2174/1574892811308010085.
[11] S. Wakabayashi, M. Shigekawa, J. Pouyssegur, Molecular physiology of vertebrate Na+/H+ exchangers, Physiol. Rev. 77 (1997) 51–74.
[12] S. Hosogi, H. Miyazaki, K. Nakajima, E. Ashihara, N. Niisato, K. Kusuzaki, et al., An inhibitor of Na(+)/H(+) exchanger (NHE), ethyl-isopropyl amiloride (EIPA), diminishes proliferation of MKN28 human gastric cancer cells by decreasing the cytosolic Cl(-) concentration via DIDS-sensitive pathways, Cell. Physiol. Biochem. 30 (2012) 1241–1253, http://dx.doi.org/10.1159/000343315.
[13] S. Li, P. Bao, Z. Li, H. Ouyang, C. Wu, G. Qian, Inhibition of proliferation and apoptosis induced by a Na+/H+ exchanger-1 (NHE-1) antisense gene on drug-resistant human small cell lung cancer cells, Oncol. Rep. 21 (2009) 1243–1249, http://dx.doi.org/10.3892/or_00000347.
[14] C.S. Owen, P.M. Pooler, M.L. Wahl, R.A. Coss, D.B. Leeper, Altered proton extrusion in cells adapted to growth at low extracellular pH, J. Cell. Physiol. 173 (1997) 397–405, http://dx.doi.org/10.1002/(SICI)1097-4652(199712)173:3<397::AID-JCP12>3.0.CO;2-9.
[15] T. Kawamoto, H. Kimura, K. Kusumoto, S. Fukumoto, M. Shiraishi, T. Watanabe, et al., Potent and selective inhibition of the human Na+/H+ exchanger isoform NHE1 by a novel aminoguanidine derivative T-162559, Eur. J. Pharmacol. 420 (2001) 1–8, http://dx.doi.org/10.1016/S0014-2999(01)00991-8.
[16] B. Kramhøft, I.H. Lambert, E.K. Hoffmann, Na+/H+ exchange in Ehrlich ascites tumor cells: activation by cytoplasmic acidification and by treatment with cupric sulphate, J. Membr. Biol. 102 (1988) 35–48, http://dx.doi.org/10.1007/BF01869096.
[17] D. Rotin, D. Steele-Norwood, S. Grinstein, I. Tannock, Requirement of the Na+/H+ exchanger for tumor growth, Cancer Res. 49 (1989) 205–211.
[18] A. Livne, E.K. Hoffmann, Cytoplasmic acidification and activation of Na+/H+ exchange during regulatory volume decrease in Ehrlich ascites tumor cells, J. Membr. Biol. 114 (1990) 153–157, http://dx.doi.org/10.1007/BF01869096.
[19] T.G. Shepherd, B.L. Thériault, E.J. Campbell, M.W. Nachtigal, Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients, Nat. Protoc. 1 (2006) 2643–2649, http://dx.doi.org/10.1038/nprot.2006.328.
[20] R.B. Marala, J.A. Brown, J.X. Kong, W.R. Tracey, D.R. Knight, R.T. Wester, et al., Zoniporide: a potent and highly selective inhibitor of human Na+/H+ exchanger-1, Eur. J. Pharmacol. 451 (2002) 37–41, http://dx.doi.org/10.1016/S0014-2999(02)02193-3.
[21] C. Aravena, A.R. Beltrán, M. Cornejo, V. Torres, E.S. Díaz, E. Guzmán-Gutiérrez, et al., Potential role of sodium-proton exchangers in the low concentration arsenic trioxide-increased intracellular pH and cell proliferation, PLoS ONE 7 (2012) e51451, http://dx.doi.org/10.1371/journal.pone.0051451.
[22] A.R. Beltrán, L.R. Carraro-Lacroix, C.N.A. Bezerra, M. Cornejo, K. Norambuena, F. Toledo, et al., Escherichia Coli heat-stable enterotoxin mediates Na+/H+ exchanger 4 inhibition involving cAMP in T84 human intestinal epithelial cells, PLoS ONE 10 (2015) e0146042, http://dx.doi.org/10.1371/journal.pone.0146042.
[23] M.A. Ramírez, R. Toriano, M. Parisi, G. Malnic, Control of cell pH in the T84 colon cell line, J. Membr. Biol. 177 (2000) 149–157, http://dx.doi.org/10.1007/s002320001108.
[24] T. Kataoka, K. Takaku, J. Magae, N. Shinohara, H. Takayama, S. Kondo, et al., Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of concanamycin A, an inhibitor of vacuolar type H (+)-ATPase, on CTL-mediated cytotoxicity, J. Immunol. 153 (1994) 3938–3947.
[25] C.K. Scott, E. Sundell, Inhibition of H + K + ATPase by SCH 28080 and SCH 32651, Eur. J. Pharmacol. 112 (1985) 268–270, http://dx.doi.org/10.1016/0014-2999(85)90508-4.
[26] R. Salsoso, E. Guzmán-Gutiérrez, T. Sáez, K. Bugueño, M.A. Ramírez, M. Farías, et al., Insulin restores L-arginine transport requiring adenosine receptors activation in umbilical vein endothelium from late-onset preeclampsia, Placenta 36 (2015) 287–296, http://dx.doi.org/10.1016/j.placenta.2014.12.007.
[27] F. Pardo, L. Silva, T. Sáez, R. Salsoso, J. Gutiérrez, C. Sanhueza, et al., Human supraphysiological gestational weight gain and fetoplacental vascular dysfunction, Int. J. Obes. 39 (2015) 1264–1273, http://dx.doi.org/10.1038/ijo.2015.57.
[28] T.A. Potapova, S. Sivakumar, J.N. Flynn, R. Li, G.J. Gorbsky, Mitotic progression becomes irreversible in prometaphase and collapses when Wee1 and Cdc25 are inhibited, Mol. Biol. Cell 22 (2011) 1191–1206, http://dx.doi.org/10.1091/mbc.E10-07-0599.
[29] E. Cerami, J. Gao, U. Dogrusoz, B.E. Gross, S.O. Sumer, B.A. Aksoy, et al., The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data, Cancer Discov. 2 (2012) 401–404, http://dx.doi.org/10.1158/2159-8290.CD-12-0095.
[30] J. Gao, B.A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S.O. Sumer, et al., Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal, Sci. Signal 6 (2013), http://dx.doi.org/10.1126/scisignal.2004088 (pl1).
[31] Y.D. Bhutia, E. Babu, V. Ganapathy, Re-programming tumour cell metabolism to treat cancer: no lone target for lonidamine, Biochem. J. 473 (2016) 1503–1506.

[32] L. Stransky, K. Cotter, M. Forgac, The Function of V-ATPases in Cancer, Physiol. Rev. 96 (2016) 1071–1091, http://dx.doi.org/10.1042/BCJ20160068.
[33] S. Wu, T. Song, S. Zhou, Y. Liu, G. Chen, N. Huang, et al., Involvement of Na+/H+ exchanger 1 in advanced glycation end products-induced proliferation of vascular smooth muscle cell, Biochem. Biophys. Res. Commun. 375 (2008) 384–389, http://dx.doi.org/10.1016/j.bbrc.2008.08.008.
[34] D.G. Fuster, R.T. Alexander, Traditional and emerging roles for the SLC9 Na+/H+ exchangers, Pflugers Arch. 466 (2014) 61–76, http://dx.doi.org/10.1007/s00424-013-1408-8.
[35] A.R. Beltrán, M.A. Ramírez, L.R. Carraro-Lacroix, Y. Hiraki, N.A. Reboucas, G. Malnic, NHE1, NHE2, and NHE4 contribute to regulation of cell pH in T84 colon cancer cells, Pflugers Arch. 455 (2008) 799–810, http://dx.doi.org/10.1007/s00424-007-0333-0.
[36] D. Rotin, P. Wan, S. Grinstein, I. Tannock, Cytotoxicity of compounds that interfere with the regulation of intracellular pH: a potential new class of anticancer drugs, Cancer Res. 47 (1987) 1497–1504.
[37] L.K. Putney, S.P. Denker, D.L. Barber, The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 527–552, http://dx.doi.org/10.1146/annurev.pharmtox.42.092001.143801.
[38] S.K. Parks, J. Chiche, J. Pouysségur, Disrupting proton dynamics and energy metabolism for cancer therapy, Nat. Rev. Cancer 13 (2013) 611–623, http://dx.doi.org/10.1038/nrc3579.
[39] S.F. Pedersen, The Na+/H+ exchanger NHE1 in stress-induced signal transduction: implications for cell proliferation and cell death, Pflugers Arch. 452 (2006) 249–259, http://dx.doi.org/10.1007/s00424-006-0044-y.
[40] L. Rosanò, F. Spinella, A. Bagnato, The importance of endothelin axis in initiation, progression, and therapy of ovarian cancer, Am. J. Phys. 299 (2010) R395–R404, http://dx.doi.org/10.1152/ajpregu.00304.2010.
[41] L. Rosanò, R. Cianfrocca, P. Tocci, F. Spinella, V. Di Castro, V. Caprara, et al., Endothelin A receptor/β-arrestin signaling to the Wnt pathway renders ovarian cancer cells resistant to chemotherapy, Cancer Res. 74 (2014) 7453–7464, http://dx.doi.org/10.1158/0008-5472.CAN-13-3133.
[42] H.J. Choi, G.N.A. Pena, S. Pradeep, M.S. Cho, R.L. Coleman, A.K. Sood, (2015). Anti-vascular therapies in ovarian cancer: moving beyond anti-VEGF approaches, Cancer Metastasis Rev. 34 (2015) 19–40, http://dx.doi.org/10.1007/s10555-014-9538-9.
[43] N. Tang, J. Guo, Q. Zhang, Y. Wang, Z. Wang, Greater efficacy of chemotherapy plus bevacizumab compared to chemo-and targeted therapy alone on non-small cell lung cancer patients with brain metastasis, Oncotarget 7 (2016) 3635, http://dx.doi.org/10.18632/oncotarget.6184.
[44] E. Balcan, F. Demirkiran, Y. Aydin, C. Sanioglu, T. Bese, M. Arvas, et al., Serum levels of epidermal growth factor, transforming growth factor, and c-erbB2 in ovarian cancer, Int. J. Gynecol. Cancer 22 (2012) 1138–1142, http://dx.doi.org/10.1097/IGC.0b013e31825b7dcc.
[45] A. Bagnato, F. Spinella, L. Rosano, Emerging role of the endothelin axis in ovarian tumor progression, Endocr. Relat. Cancer 12 (2005) 761–772, http://dx.doi.org/10.1677/erc.1.01077.
[46] N. Said, D. Theodorescu, Permissive role of endothelin receptors in tumor metastasis, Life Sci. 91 (2012) 522–527, http://dx.doi.org/10.1016/j.lfs.2012.03.040.
[47] S.R. Amith, J.M. Wilkinson, S. Baksh, L. Fliegel, The Na^+/H^+ exchanger (NHE1) as a novel co-adjuvant target in paclitaxel therapy of triple-negative breast cancer cells, Oncotarget 6 (2015) 1262–1275.