学　位　論　文

Tumor endothelial cells survive in lactic acidosis via the activity of pH regulators

（乳酸アシドーシス環境における pH 調節機構を介した腫瘍血管内皮細胞の生存）

２０１８年９月

北海道大学

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List of Publications

1. **Dorcas A. Annan**, Nako Maishi, Tomoyoshi Soga, Randa Dawood, Li Cong, Hiroshi Kikuchi, Takayuki Hojo, Masahiro Morimoto, Nobuo Shinohara, Yasuhiro Hida, Kyoko Hida (2018) Carbonic anhydrase 2 (CAII) supports tumor endothelial cell survival in lactic acidosis. *Cancer Research (Under Review)*

2. Kyoko Hida, Nako Maishi, **Dorcas A. Annan** and Yasuhiro Hida (2018) Contribution of tumor endothelial cells in cancer progression. *International Journal of Molecular Sciences 2018, 19(5), 1272*

3. Goto K., **Annan D. A.**, Morita T., Li W., Muroyama R., Matsubara Y., Ito S., Nakagawa R., Tanoue Y., Jinushi M. and Kato N. (2016) Novel chemoimmunotherapeutic strategy for hepatocellular carcinoma based on a genome-wide association study. *Scientific Reports* 2; 6:38407

4. Hida K., Maishi N., **Annan D. A.**, Kondoh M., Hojo T., Habiba U., Ohga N., Ishikawa K., Sato M., Torii C., Yanagiya M., Morimoto M., Hida Y. and Shindoh M. (2016) Aneuploidy of the murine immortalized endothelial cell line, MS1. *Journal of Oral Biosciences 59(1): 50-54*

5. Komohara Y., Morita T., **Annan D. A.,** Horlad H., Ohnishi K., Yamada S., Nakayama T., Kitada S., Suzu S., Kinoshita I., Dosaka-Akita H., Akashi K., Takeya M. and Jinushi M. (2015) The coordinated actions of TIM-3 on cancer and myeloid cells in the regulation of tumorigenicity and clinical prognosis in clear cell renal cell carcinomas. *Cancer Immunology Research* 3(9):999-1007

List of Presentations

April 2018	**109th Annual Meeting of the American Association for Cancer Research (AACR)** – Tumor endothelial cells survive under lactic acidosis – *Poster Presentation*
September 2017	**97th Hokkaido Medical Congress** – Glutamine metabolism in tumor endothelial cells - *Oral Presentation*

September 2017	**76th Annual Meeting of the Japanese Cancer Association** – Glutamine metabolism in tumor endothelial cells - *Poster Presentation*
July 2017	**5th Cancer and Metabolism Research Meeting in Sapporo** – Tumor endothelial cells tolerate low pH environments by upregulating proton transporters - *Poster Presentation*
September 2016	**75th Annual Meeting of the Japanese Cancer Association** – Tumor endothelial cells are resistant to acidic environments - *Poster Presentation*
September 2015	**95th Hokkaido Medical Congress** - Tumor endothelial cell-secreted protein activates a DNA damage response – *Oral Presentation*

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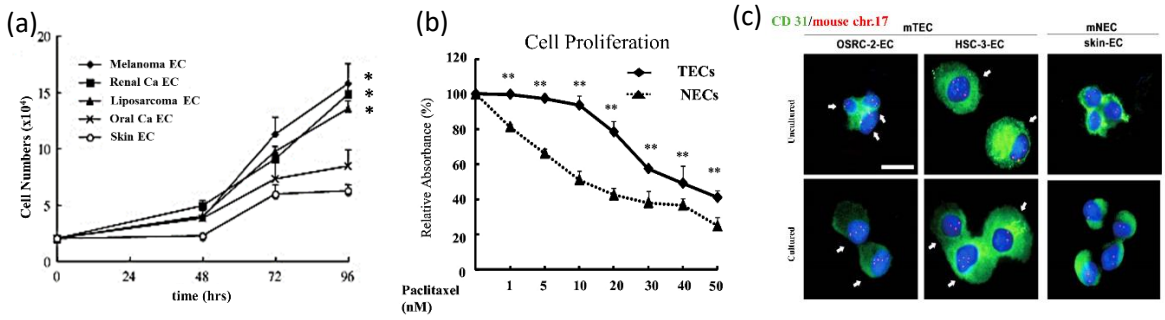
Introduction

Tumor endothelial cells (TECs) are different from normal endothelial Cells (NECs)

Tumor survival and establishment depend on the process of angiogenesis (Folkman, 1990; Verheul et al., 2004). The tumor blood vessels, harbor tumor endothelial cells (TECs) with alterations which makes them different from normal endothelial cells (NECs). A number of the changes in TECs arise from epigenetic modifications (Maishi et al., 2016), influences from tumor-derived factors (Akiyama et al., 2012; Kawamoto et al., 2012), tumor hypoxia (Kondoh et al., 2013), and reactive oxygen species (Hojo et al., 2017). As a consequence, TECs proliferate and migrate at a higher rate than NECs (Matsuda et al., 2010, FigI1a; Ohga et al., 2012). They have chromosomal instabilities, large nuclei and are aneuploid, all of which point to an abnormal karyotype in TECs (Hida et al., 2004; Akino et al., 2009, FigI1c). TECs are also more resistant to anticancer drugs than NECs (Akiyama et al., 2012, FigI1b; Hida et al., 2013; Hida et al., 2017), and recently, high metastatic tumor TECs were shown to promote tumor metastasis (Maishi et al., 2016). In addition to the above mentioned factors, products of tumor metabolism may also influence the function of endothelial cells in the tumor microenvironment.



Source: Matsuda et al., 2010; Akiyama et al., 2012; Akino et al., 2009

Fig.I1 Characteristics of TECs

[Higher migration (a) and proliferation (b) aneuploidy and large nuclei (c)]

Glycolysis in tumor cells

Tumor cells preferentially undergo glycolysis in the presence of oxygen, a process known as Warburg effect (Gatenby and Gillies, 2004), to yield lactic acid as the end-product instead of pyruvate (Vander Heiden, Cantley and Thompson, 2009, Fig. I2). The accumulation of lactic acid creates a condition termed *lactic acidosis – which is characterized by high lactate concentration with an acidic pH* (Hu, Chao and Wu, 2017). Lactic acidosis has been described

as an oncologic emergency (Jabr, 2006) due to its contribution to tumor metastasis and association with poor prognosis and high mortality rates (Munoz et al., 2011; Dhup et al., 2012).

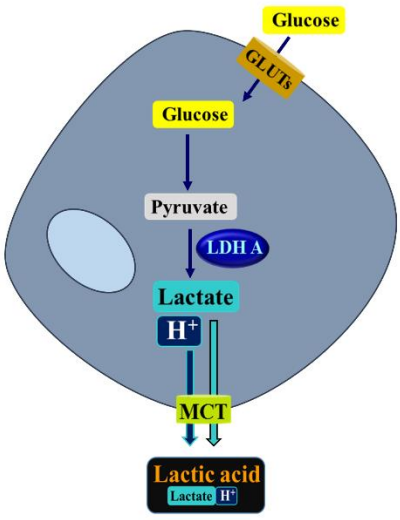


Fig. I2: Tumor cells undergo aerobic glycolysis to yield lactic acid as the end product

The effects of lactic acid on immune and endothelial cells have been demonstrated. In macrophages lactic acid stimulates tumor-associated macrophages toward an M2-like phenotype (Colegio et al., 2014; Ohashi et al., 2017), and it inhibits the proliferation and cytokine production of human cytotoxic T lymphocytes (Fischer et al., 2007) and natural killer cells (Brand et al., 2016). Furthermore, *in vitro* exposure of fibroblasts to lactate increased their hyaluronan deposition and CD44 expression (Stern et al., 2002). Regarding endothelial cells, most reports describe the individual effects of acidity or lactate. For instance, low extracellular pH exerted a reversible inhibition on endothelial cell outgrowth from rat aortic rings (Burbridge et al., 1999). In another study, bovine aortic endothelial cells exposed to acidosis were protected from apoptosis, the acidosis condition further induced the expression of basic fibroblast and vascular endothelial growth factors in the cells (D'Arcangelo et al., 2000). Furthermore, at the molecular level, extracellular acidity decreased the expression of VEGF receptor 2 (VEGFR2) in the normal endothelial cells, HUVECs, and this led to the failure of antiangiogenic therapies that target VEGFR2 (Faes et al., 2016). Alternatively lactate, has been shown to mainly have a proangiogenic effect on endothelial cells. Lactate uptake into endothelial cells drove their migration and tube formation by stimulating the NF-KB/IL-8 (CXCL8) pathway (Vegran et al., 2011); while, the blockade of lactate uptake inhibited HIF-dependent angiogenesis in the endothelial cells (Sonveaux et al., 2012). Notwithstanding, the cumulative effect of both lactate and low extracellular pH on endothelial cells, and especially, TECs is unknown.

Hypothesis

In the tumor microenvironment, TECs may be exposed to high amounts of tumor-derived lactic acid as well as minimal amounts of TEC-derived lactic acid due to their recently discovered hyperglycolytic nature (Cantelmo et al., 2016). Consequently, in this study, it was hypothesized that TECs possess unique properties that promote their survival in a lactic acid-rich environment (Fig.I3). Furthermore, with the quest to find novel therapeutic antiangiogenic targets, it is of much essence to identify and target adaptive mechanisms that support TEC survival under conditions of lactic acidosis within the tumor.

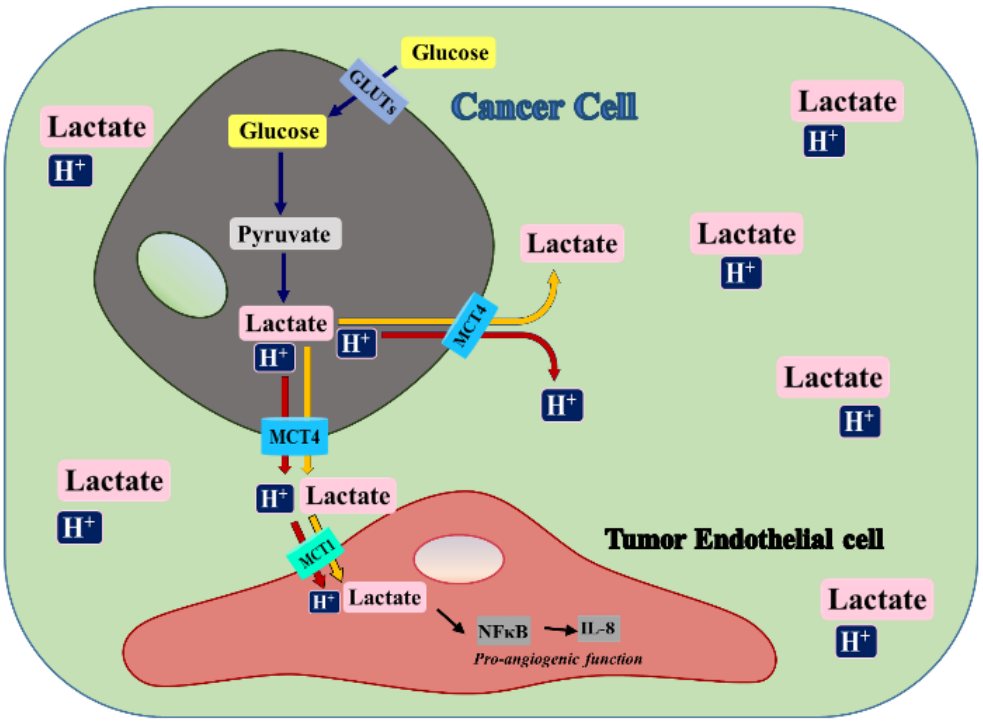


Fig I3. Study Hypothesis – TECs survive in a lactic acid rich environment

Cancer cells have devised many ways of escaping from the harmful effects of the lactic acid that they produce. They show upregulated expression of proton-coupled monocarboxylate transporters (MCTs) such as MCT1 and MCT4, to help regulate intracellular lactate and proton levels (Halestrap and Wilson, 2012b). Moreover, to maintain a stable intracellular acid-base balance they exhibit the upregulated expression of pH regulators including sodium/hydrogen exchanger 1 (NHE1) (Stock and Pedersen, 2017), sodium/bicarbonate (Na^+/HCO_3^-) co-transporters, proton-sensing G protein-coupled receptors (GPCRs) (Justus, Dong and Yang,

2013), vacuolar ATPases (V-ATPase) and carbonic anhydrases (CAs) including CAIX, CAXII and CAII (Damaghi et al., 2013; FigI4).

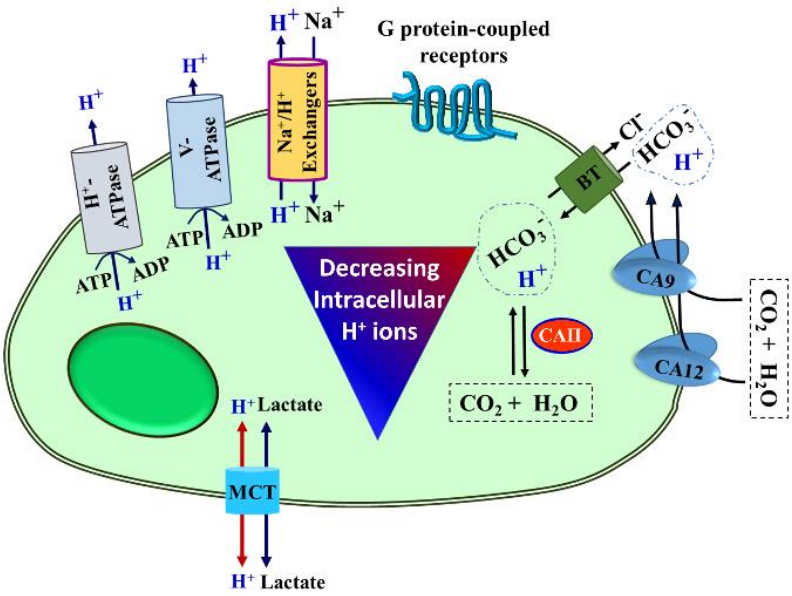


Fig.I4 pH regulators employed by cancer cells

The CAs are zinc metalloenzymes involved in the hydration of carbon dioxide (CO_2) and the dehydration of bicarbonate (HCO_3^-). Some CAs also hydrate small molecules similar to CO_2, such as COS, CS_2, among others (Supuran, 2016). The CAs may be located on the cell membrane (CAIV, CAIX, CAXII), in the cytosol (CAII, CAIII, CAVII, CAVIII, CAX, CA XI) or within certain cytoplasmic organelles e.g. mitochondria (CAVA, CAVB). CAVI is the only CA that is secreted, and it was first isolated from sheep saliva (Sly and Hu, 1995; Fig.I5).

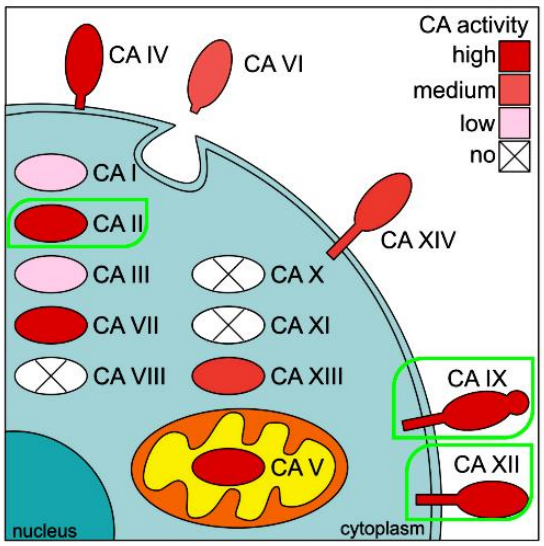


Fig.I5 Cellular location of carbonic anhydrases *(Source: Pastorekova and Zavada, 2000 Cance Ther)*

The CAs have different catalytic rates. CAII is one of the enzymes in nature known to have a very high catalytic activity. CAIII is also a cytosolic CA; however, its catalytic activity is relatively lower than that of CAII (Silverman and Lindskog, 1988). The catalytic activity of CAIV is higher than that of CAIX and CAXII, both of which have moderate activities (Sly and Hu, 1995). CAVIII, CAX, and CAXI lack catalytic activity as a result of the absence of the metal ion in their active sites, and are therefore labelled CA-related proteins (Chegwidden and Carter, 2000).

The expression and contribution of CAs to tumor growth and development has been observed in various types of tumors including melanoma (Yoshiura et al., 2005), brain (Nordfors et al., 2010), colorectal (Saarnio et al., 1998), renal and pancreatic (Kivela et al., 2000) cancers. The CAs are therefore emerging targets of anti-cancer therapies (Pastorekova, Zatovicova, and Pastorek, 2008). CAIX and CAXII are hypoxia-responsive proteins, and are regulated by HIF-1a (Wykoff et al., 2000; Silagi et al., 2018) and not HIF-2a, at least in breast cancer (Jiang et al., 2015). The association of CAIX (Giatromanolaki et al., 2001) and CAXII (Watson et al., 2003; Ilie et al., 2011) to tumors has been well established. However, in some cases, their expression patterns dictate opposing prognosis in a single type of cancer. Functionally, both enzymes are involved in the extracellular regulation of pH by participating in bicarbonate cycling, which involves the conversion of CO_2 to HCO_3^- and H^+; and the HCO_3^- is transported back into cells through Na^+/HCO_3^- co-transporters to maintain a more alkaline pHi, whiles leaving the extracellular environment in an acidic state (Swietach et al., 2007, Silagi et al., 2018). Regardless of their unique expression in the tumor endothelium is not confirmed. Non-small cell lung cancer (NSCLC) tumor sections stained with CD31 and CAIX in one study, showed higher vascularization in CAIX-positive areas than in the negative areas. However, in the tumor, the prognosis defined by CAIX expression was similar to the low and high regions of vascularization. Furthermore, multivariate analysis showed that independent of angiogenesis. CAIX expression is a significant prognostic factor of NSCLC (Giatromanolaki et al., 2001).

The cytosolic CAII, on the contrary, has been reported to be uniquely expressed in the endothelium of melanoma, esophageal, renal, lung (Yoshiura et al., 2005), and brain cancers (Haapasalo et al., 2007). In addition, endothelial CAII expression in meningioma tissues was associated with aggressive tumor characteristics including higher tumor proliferation rates and increasing histological grade (Korhonen et al., 2009). The above findings may suggest a significant role of CAII in either the establishment of the tumor endothelium or the biological

activity of TECs. However, the unique function of CAII in the tumor endothelium or TECs has not been elucidated.

Aim

To investigate the role of CAII and other pH regulators in the survival of TECs under lactic acidosis.

In order to achieve this aim the following objectives were set:

Objectives

1. To investigate the effect of lactic acidosis on the proliferation and angiogenic activity of TECs.

2. To analyze the expression of various classes of pH regulators in TECs and their contribution to the survival of TECs exposed to the lactic acidosis condition.

3. To identify potential therapeutic targets of tumor angiogenesis among the analyzed pH regulators.

List of Abbreviations

4T1	Murine mammary carcinoma cell line
A375-SM	Highly-metastatic A375 cells
CA	Carbonic anhydrase
CAF	Cancer-associated fibroblast
CE-MS	Capillary electrophoresis-mass spectrometry
CHC	α-cyano-4-hydroxycinnamate
CM	Conditioned medium
CO_2	Carbon dioxide
COS	Carbonyl sulfide
CS_2	Carbon disulfide
CSA	Camphor sulfonic acid
DAPI	4′, 6-diamidino-2-phenylindole
DMEM	Dulbecco minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DT	Diphtheria toxin
ECGM-MV2	Endothelial cell growth medium-microvascular
ECGS	Endothelial cell growth supplement
EDTA	Ethylenediaminetetraacetic acid
EGM-2MV	Endothelial cell medium-microvascular
FBS	Fetal bovine serum
FCS	Fetal calf serum
FFPE	Formalin-fixed paraffin-embedded
GPCR	G protein-coupled receptor
H^+	Hydrogen ion (proton)
HCl	Hydrogen chloride
HCO_3^-	Bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMVEC	Human microvascular endothelial cells

HUVEC	Human umbilical vein endothelial cells
iHMVECs	Immortalized HMVECs
MCT	Monocarboxylate transporter
MEM	Minimum essential medium
MES	2-ethanesulfonic acid
mRNA	messenger RNA
Na^+	Sodium
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NEC	Normal endothelial cell
NHA	Sodium hydrogen anti-porter
NHE	Sodium/hydrogen exchanger
NSCLC	Non-small cell lung cancer
pHi	intracellular pH
RCC	Renal cell carcinoma
RNA	ribonucleic acid
RT-qPCR	real-time quantitative polymerase chain reaction
siRNA	small interfering RNA
TEC	Tumor endothelial cell
V- ATPase	Vacuolar ATPase
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor

Materials and Methods

Cells and cell culture

Highly metastatic human A375-SM melanoma cells were kindly provided by Dr. Fidler (M.D. Anderson Cancer Centre, Houston, TX, USA). A375-SM cells were cultured in Minimum Essential Medium (MEM; Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics (Sigma-Aldrich, St. Louis, MO, USA). Where applicable, the pH of the extracellular culture medium was measured using a pH electrode (Mettler Toledo). HMVECs were obtained from Lonza (Tokyo, Japan) and immortalized using the SV40 large T antigen and hTERT. Immortalized HMVECs (iHMVECs) were cultured in ECGM-2MV (PromoCell, Heidelberg, Germany).

Human tissue samples

Tumor tissues were surgically excised from patients clinically diagnosed with renal cell carcinoma (RCC). Normal renal tissues were separated from tumor tissues of the same patients for comparison. The tissues were fixed in 10% formalin and embedded in paraffin (FFPE) to be used for immunohistochemistry. All protocols were approved by the Institutional Ethics Committee of Hokkaido University, and written informed consent was obtained from each patient before surgery.

Mice

Six-week-old female nude mice (BALB/c Ajc1-nu/nu; Clea, Tokyo, Japan) were housed under specific pathogen-free conditions. All procedures for animal care and experimentation adhered to institutional guidelines and were approved by the animal research authorities of Hokkaido University.

Isolation of TECs and NECs

TECs and NECs were isolated following previously established protocols (Hida et al., 2004; Ohga et al., 2009), with some modifications. A375-SM tumor cells were subcutaneously injected into the right flanks of female nude mice. After 4 weeks when the tumors were 1mm^3 or more in sizes, TECs were isolated from the tumor xenografts and NECs were isolated from the dermis of tumor-free counterparts. Due to the presence of CD31 molecule on the surface of the endothelial cells, CD31 microbeads and a magnetic cell sorting device (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for the isolation. This was followed by flow cytometry

to further confirm and purify the CD31-positive cell fraction (FACS, Aria II; BD Biosciences, San Jose, CA, USA). The $CD31^+$ cells were cultured in EGM-2MV (Lonza) containing 15% FBS at 37 °C in a humidified atmosphere with 5% CO_2. The remaining human tumor cells possessing the DT receptor were eliminated using diphtheria toxin (DT, 500 ng/mL; Calbiochem, San Diego, CA, USA). Isolated ECs were further purified using FITC-BS1-B4 lectin (Vector Laboratories, Burlingame, CA, USA). The two TECs used (TEC1 and TEC2) were isolated at different times using the same process as described above. Strict aseptic techniques were used during the isolation process to avoid contamination from microorganisms and other pathogens.

Antibodies

Purified rat anti-mouse CD31 antibody (BD Pharmingen), Alexa 647-conjugated anti-mouse CD31 antibody, APC-conjugated anti-mouse CD45 antibody (BioLegend, San Diego, CA, USA), rabbit polyclonal anti-CAII antibody, rabbit polyclonal anti-CD31 antibody (Abcam, Cambridge, UK), Alexa 647-conjugated anti-rabbit IgG, Alexa 488-conjugated anti-rat IgG antibody (Invitrogen, Tokyo, Japan), and HRP-linked polyclonal goat anti-rabbit IgG (Dako North America Inc., CA, USA).

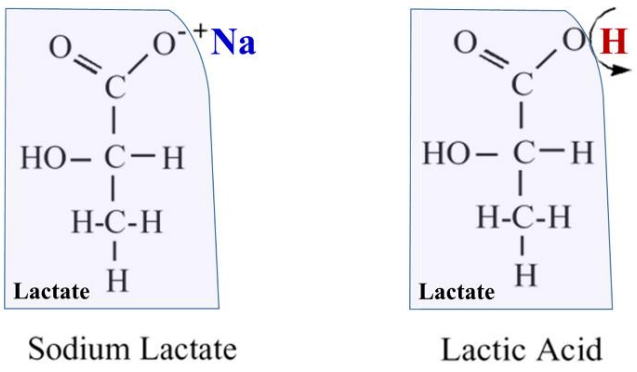


Fig. M1 Lactic acid and the sodium salt (sodium lactate) used in this study

(The presence of the proton ion dictates an acidic media)

***In vitro* cell proliferation assays with lactic acid and sodium lactate**

Nutrient-free DMEM (Sigma-Aldrich) supplemented with 20% FBS and 100 µg/mL endothelial cell growth supplement (ECGS, Corning, Discovery Lab Ware Inc., Bedford, MA, USA) was used as the base medium for this experiment. This DMEM contained sodium bicarbonate as the buffering agent. The media used for the cell culture was prepared by adding increasing concentrations of lactic acid (Sigma-Aldrich, Fig M1) or its sodium salt (sodium

lactate, Fig. M1, Sigma-Aldrich) to the nutrient-free DMEM as metabolic substrates. NECs and TECs were seeded in the base medium at 2×10^3 cells per well, in 96-well plates. The cells were allowed to attach before the addition of the lactate-containing medium. All cells were incubated at 37 °C in 5% CO_2. Cell proliferation was measured by the MTS assay (Fig.M2). For the pH-adjusted experiments with NECs, after addition of lactic acid to the base medium, the pH of the media was adjusted with NaOH (Sigma-Aldrich) to a starting pH of approximately 8. All pH measurements were taken immediately before addition of the media to the cells.

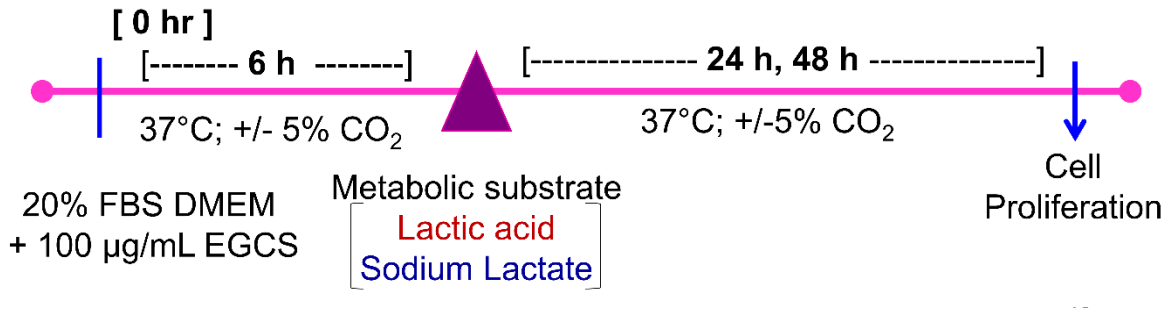


Fig. M2 Experimental plan for preliminary experiments in lactic acid and lactic acidosis

Lactic acidosis and lactosis

A condition of lactic acidosis/lactosis was created by adding sodium lactate (Sigma-Aldrich) to 10% FBS DMEM (Sigma-Aldrich) to a final concentration of 20 mM. The medium also contained 20 mM HEPES (Sigma-Aldrich) and 100 µg/mL ECGS (Corning) The pH of the medium was adjusted with NaOH (Sigma-Aldrich) to pH 6.9 and 7.3 for lactic acidosis and lactosis, respectively (Fig.M3).

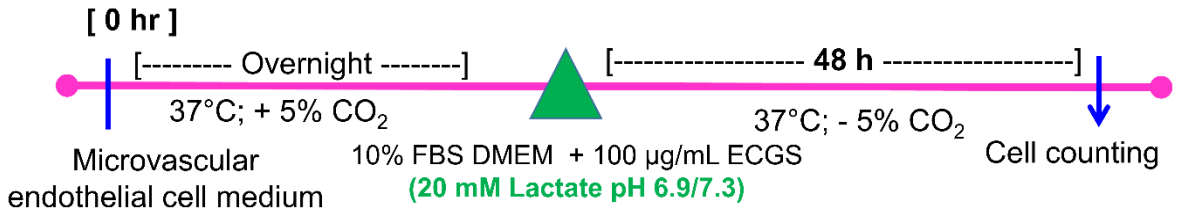


Fig. M3 Experimental plan for cell proliferation in lactic acidosis and lactosis

***In vitro* cell proliferation assay (lactic acidosis and lactosis)**

To analyze cell proliferation under conditions of lactic acidosis and lactosis, 1×10^5 cells were seeded into 12-well plates. The cells were cultured in EGM-2MV for 12 h, after which the

medium was changed to a condition of lactic acidosis or lactosis. The cells were counted using a hemocytometer after 48 h of incubation at 37 °C without 5% CO_2. Each condition was prepared in triplicate and the experiment was repeated three times.

***In vitro* cell proliferation assay (post-small interfering RNA (siRNA) transfection)**

Cells were treated with 20 nM siRNA and seeded at 2×10^3 cells in transfection medium directly into 96-well plates. The medium was changed after 6 h to the complete-medium, ECGM-MV2 (PromoCell), which is fully supplemented with glucose and glutamine to create a nutrient-replete environment. The cells were incubated at 37 °C in 5% CO_2 for 72 h. Cell proliferation was measured by the MTS assay (Promega; Fig.M4).

To monitor cell growth under conditions of lactic acidosis and lactosis, cells were cultured in ECGM-MV2 (PromoCell) for 24 h after siRNA transfection. The conditions of the medium were changed to either lactic acidosis or lactosis. Cell proliferation was measured by the MTS assay after 48 h (Fig.M5).

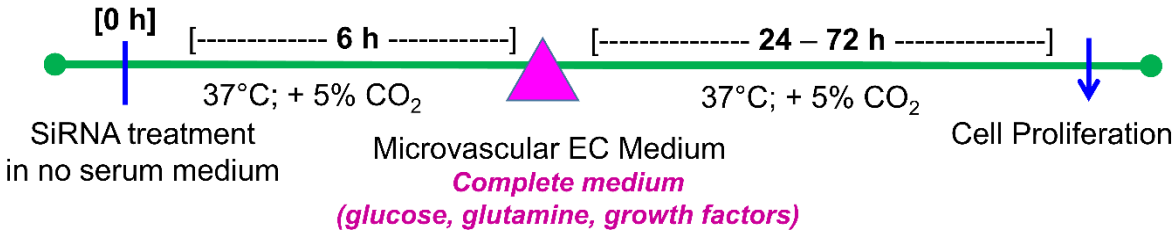


Fig. M4 Experimental plan for analyzing the effect of siRNA knockdown on cell proliferation

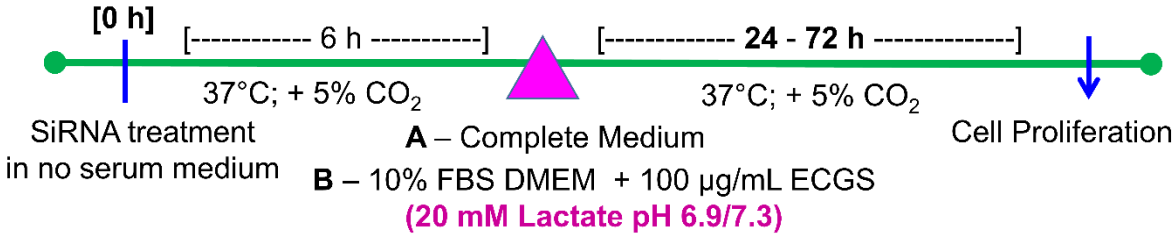


Fig. M5 Experimental plan for analyzing the effect of siRNA knockdown on cell proliferation in lactosis and lactic acidosis

***In vitro* cell proliferation assay (CHC inhibitor)**

NECs and TECs were seeded at 2×10^3 cells into 96-well plates overnight. The inhibitor, α-cyano-4-hydroxycinnamate (CHC, Sigma-Aldrich) was dissolved in DMSO (Sigma) and

diluted in the culture medium. CHC was added to the wells at a final concentration of 5 mM. The cells were incubated at 37 °C in 5% CO_2 for 72 h. Cell proliferation was measured by the MTS assay (Fig. M6).

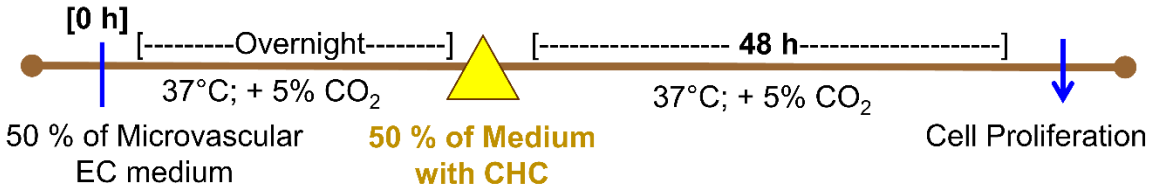


Fig. M6 Experimental plan for analyzing the effect of CHC on cell proliferation

Isolation of RNA and real-time quantitative reverse-transcription PCR (RT-qPCR)

Total RNA was isolated using the ReliaPrep™ RNA Cell Miniprep System (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions. The cDNA was synthesized using ReverTra-Plus (Toyobo Co., Japan) and amplified by PCR. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using KAPA SYBR® Fast qPCR Kit (KAPA Biosystems Pty (Ltd.), Cape Town, South Africa). Cycling conditions were set according to the CFX manager recommendations (Bio-Rad, Hercules, VA, USA). The expression of all messenger RNA (mRNA) was normalized to that of Rps13 for murine cells (except for the Slc2a10 and Slc2a3 mRNA expression) and RSP13 for human cells. The mouse primers used were as follows:

Actb: FW: 5′-TTTGCACATGCCGGAGCCGTTG-3′,

RV: 5'-TTTGCAGCTCCTTCGTTGCCGG-3′;

Slc2a3 FW: 5′-AGAGGTCACTGAATTCCTGGGG-3′,

RV: 5′-ATGTTCTCGGCAGCAAGTGTTC-3′;

Slc2a10 FW: 5′- CACTCCAGGGAAGGGAGACAAG-3′,

RV: 5′-ACGGAGCGAAAGATGGTAGAGG-3′;

Rps13: FW: 5′-TGCGGCTTGATTTCCTGTGCCG-3′,

RV: 5'-GCGTGCATGCGACCCATGATGATG-3′;

Slc16a1 (MCT1): FW: 5′-ACACCAAGTGGATCAGACCTCG-3′,

RV: 5′-GGTTGTAGACAAAGGGGCAAGC-3′;

Slc16a3 (MCT4): FW: 5′-CAAGGCGGACAGAGGCAGATAC-3′,

RV: 5′-CAGTCCAGCCTACTCGTCTCTC-3′;

Car2 (CAII): FW: 5′-TGCGTCCAAGAGCATTGTCAAC-3′,

RV: 5′-GTCACTGAGGGGTCCTCCTTTC-3’;
Car3 (CAIII): FW: 5′-CTCTTCGGGCAAGAAACTCTGC-3′,
RV: 5′-GGTTGCATGTGACTGCTTCTCC-3′;
Car4 (CAIV): FW: 5′-TTGGTGATTGACCCTAGGCTGG-3′,
RV: 5′-AGTCTGGGGTTCACCTTTGTCC-3′;
Car9 (CAIX): FW: 5′-TTCCTGCTGCTCCAAGTGTCTG-3′,
RV: 5′-TCAGGTGCATCCTCTTCACTGG-3′;
Car12 (CAXII): FW: 5′-ATCTCCTTCCGACAAGGACTGC-3′,
RV: 5′-CTGCCAGAACAATGGAGATGCC-3′;
Slc9a1 (NHE1): FW: 5′-TGGTGAACGAGGAGTTGAAGGG-3′,
RV: 5′-ACTTGATCCAGGGGTGAAGACG-3′;
Slc9b2 (NHA2): FW: 5′-TGGTGAACGAGGAGTTGAAGGG -3′,
RV: 5′-ACTTGATCCAGGGGTGAAGACG -3′;
Gpr4: FW: 5′-AACTGTCATCCTGCACCCTTCC-3′,
RV: 5′-CGCCCATGATGACAAACTCCTG-3′;
Gpr65: FW: 5′-ATGCGTATCCTTTCTGCAAGCG-3′,
RV: 5′-ACTGCTAAATAGCGGTCCAGGG-3′.

The human primers used were as follows:
RPS13: FW: 5′-TCTCCTTTCGTTGCCTGATCGCC-3′,
RV: 5′-ACTTCAACCAAGTGGGGACGCTGC-3’;
CAR2 (CAII): FW: 5′-TCCCCTGTTGACATCGACACTC-3′,
RV: 5′-ACCAAGTGAAGCTGCTTTGTCC-3’.

Wound healing assay

To analyze endothelial cell motility, the cells were seeded into 12-well plates at 2.5×10^5 and 3×10^5 cells for TECs and NECs, respectively. The cells were exposed to 24 h of lactic acidosis or lactosis after being allowed to attach overnight. The center of the cell monolayer was scratched and cell motility was monitored every 4 h for 12 h, under conditions of lactic acidosis or lactosis.

Metabolomic analysis

TECs and NECs were seeded at 1×10^6 cells and cultured overnight. The old medium was aspirated and the cells were washed with 5% mannitol (Wako, Osaka, Japan). Metabolites were

extracted with methanol containing 25 μM each of L-methionine sulfone (Alfa Aesar, Heysham, UK), MES (Dojindo, Kumamoto, Japan), and CSA (Wako) at 10,000g for 3 min at 4 °C. The aqueous layer was collected and concentrated at 9,100g for 2 h at 20 °C. The centrifugal concentrate was stored at −80°C until the time of analysis. Prior to metabolite analysis by the capillary electrophoresis-mass spectrometry (CE-MS) method, the concentrates were diluted with water containing 3-aminopyrrolidine (Sigma-Aldrich) and Trimesate (Wako). The metabolome analysis was performed at the IAB metabolomics lab, Keio University. The metabolites were analyzed using an Agilent CE-TOFMS system, as described previously (Soga et al., 2009).

Lactate measurement

The lactate level was determined based on the oxidation of lactate to pyruvate by the lactate dehydrogenase enzyme in the presence of nicotinamide adenine dinucleotide (NAD^+). Lactate concentrations in supernatants of cells cultured for 48 h were measured enzymatically using an L-lactate assay kit (Abcam, Cambridge, UK), in accordance with the manufacturer's protocol. The lactate contents of equal amounts of A375-SM tumor, normal skin, and kidney tissue were measured. All samples were deproteinized using Amicon® Centrifugal Filters Ultracel®-10K (Merck Millipore Ltd., Ireland), prior to measurement.

Knockdown by siRNA

To silence MCT1, MCT4, and CAII mRNAs, TECs were transfected with specific siRNAs. The siRNAs were introduced into TECs using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). A non-targeting control siRNA (Invitrogen, Carlsbad, CA, USA) was used as a negative control. The siRNAs targeted the following sequences:
MCT1 si 5′-GGCUUGAUCGCAGCUUCUUUCUGUA-3′,
5′-UACAGAAAGAAGCUGCGAUCAAGCC-3′;
MCT4 si 5′-GGUAAUGGGUGUACCCGACAAAG-3′,
5′-CUUUGUGUCGGGUACACCCAUAUCC-3′;
Car2 si 5′-CCAUUACUGUCAGCAGCGAGCAGAU-3′,
5′-AUCUGCUCGCUGCUGACAGUAAUGG-3′;

Immunohistochemistry

Frozen sections of A375-SM tumors were prepared as previously described (Ohga et al., 2012). For immunofluorescent analysis, double staining with anti-CAII and anti-CD31 antibodies was

performed, followed by counterstaining with DAPI. Images were obtained with the FV10-ASM Viewer Software (Olympus). Serial sections (4 µm thick) were obtained from FFPE blocks of human RCC tumor and normal counterparts. The sections were deparaffinized in xylene (Sigma-Aldrich, Tokyo, Japan), and antigen retrieval was performed by heating the samples in Tris-EDTA buffer at 95 °C for 30 min. The sections were stained individually with anti-CAII and anti-CD31. Immunoreactivity was visualized with HRP-linked secondary antibody (Dako). The sections were counterstained with hematoxylin (Wako). Images were captured with a NanoZoomer 2.0-HT Slide Scanner (NanoZoomer 2.0-HT, version 2.3.27, Hamamatsu, Japan) and observed with the NanoZoomer Digital Pathology software.

Tumor conditioned medium (CM) preparation and heat inactivation

A375-SM tumor cells were seeded at 1 x 10^6 cells in 4 mL of 10% FBS MEM for 48 h. Tumor-conditioned medium was collected from A375-SM cultures and filtered with a 0.22-µm filter (Millipore). The conditioned medium (CM) was mixed with equal portions of 5% FBS EBM2 to support EC growth. The CM from iHMVEC culture was prepared in a similar way and was used as the control CM (Fig.M7).

Heat inactivation of the CM was performed by heating for 60 min at 95 °C. iHMVECs were treated with the tumor CM or control CM for 24 h after cooling down to room temperature. RNA was isolated and CAII expression was determined by RT-qPCR.

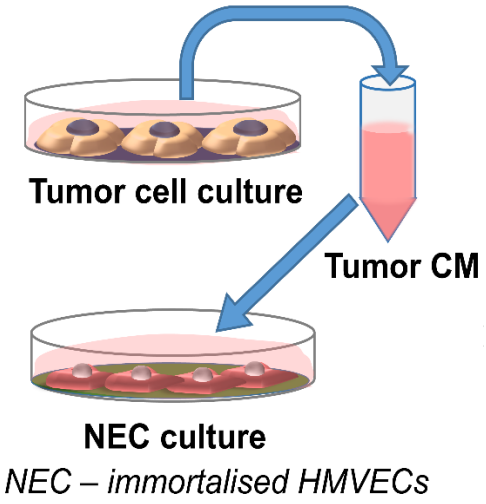


Fig. M7 Tumor-conditioned medium treatment to iHMVECs

VEGF stimulation and VEGFR2 kinase inhibitor assay

iHMVECs were cultured in ECGM-2MV overnight. The cells were serum-starved for 24 h in 5% FBS EBM2 prior to VEGF stimulation. The cells were stimulated with 20 ng/mL recombinant human vascular endothelial growth factor (VEGF) $_{165}$ (PeproTech, Rocky Hill, NJ, USA) for 24 h.

For the treatments with the vascular endothelial growth factor receptor 2 (VEGFR2) kinase inhibitor Ki8751 (Calbiochem, UK) and iHMVECs were pre-treated with 10 µM Ki8751 for 2 h. This was followed by treatment with Ki8751-containing CM for 24 h. CAII mRNA expression was determined by RT-qPCR.

Pharmacological inhibition of TEC proliferation with Acetazolamide

TECs were cultured at 2 x 10^3 cells overnight in ECGM-MV (Promega). The cells were treated with only 2.5 mM acetazolamide (Sigma-Aldrich) pre-dissolved in DMSO (Sigma-Aldrich) and diluted with culture media, or a combination of acetazolamide and Ki8751 (10 µM). Cell proliferation was measured after 72 h by the MTS assay (Fig. M8).

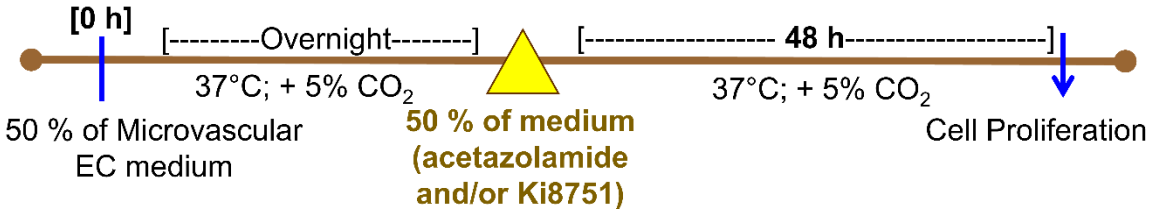


Fig. M8 Effect of acetazolamide and Ki8751inhibitors on cell proliferation

***In vivo* pharmacological inhibition of tumor angiogenesis with acetazolamide**

A375-SM tumor cells (1 x 10^6) were subcutaneously injected into the right flanks of female nude mice. After 14 days when the tumors were visible, the mice were divided into vehicle-control and acetazolamide-experimental groups. Each experimental group had five mice each. Acetazolamide was prepared by dissolving the powder in 1M NaOH and neutralizing with 1 M HCl to produce a fine suspension. The suspension was administered daily (80mg/kg) by intraperitoneal injection. The tumor volume was measured and calculated using the formula:$Tumor\ volume = (\pi/6) \times (L \times W \times H)$ where L was the length, W was the width and H was the height of the tumors respectively. All measurements were taken with a pair of calipers. The tumors were excised after 10 days of treatment (Fig M9).

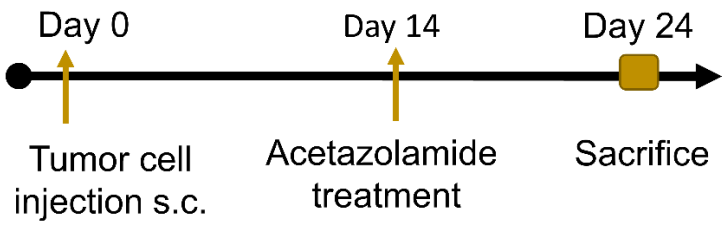


Fig. M9 In vivo pharmacological inhibition of tumor angiogenesis with acetazolamide

Statistical analysis

Unless otherwise stated, all data are presented as mean ± standard deviation. A two-way Student's *t*-test was used for comparison between two groups. A *P*-value < 0.05 was considered to be significant and a *P*-value < 0.0001 was considered to be very significant.

Results

Characteristics of the TECs and NECs used in the study

TECs and NECs were isolated from xenografted tumors and the dermis of normal nude mice respectively. Prior to use in the experiments, the cells were characterized as previously reported, with some modifications (Hida et al., 2004; Matsuda et al., 2010; Akiyama et al., 2012). The TECs (both TEC1 and TEC2) and NECs expressed known endothelial cell markers such as CD31, CD105, CD144 and BS1-B4. They were negative for CD11b and the hematopoietic marker, CD45. TEC1 and TEC2 also expressed higher levels of VEGFR1 and VEGFR2 as compared to NECs. Furthermore, the proliferation and migration rates of the two TECs were higher than that of the NECs. However, TEC1 and TEC2 proliferation rates were slightly different.

Glycolysis is more activated in TECs than in NECs

In addition to the established differences in TEC and NEC proliferation rates (Matsuda et al., 2010), it was observed that after culturing NECs and TECs the total confluency in media containing a pH indicator, the TEC culture medium showed a more acidic milieu (yellow color) than the NEC culture medium. The pH ranges of TEC and NEC media were 6.6–7.1 and 7.02–7.16 respectively (Fig. 1a), confirming that the TEC culture medium was indeed more acidic. Since an acidic culture medium may suggest a difference in cellular metabolic activity, the metabolomes between TECs and NECs were compared. The data revealed a more glycolytic metabolome in TECs than in NECs, characterized by higher levels of glycolytic metabolites and lactate in TECs. The expression of the glucose transporters Slc2a10 (Fig. 1c) and Slc2a3 (Fig. 1d) were upregulated in TECs than in NECs. Congruently, TECs produced more lactate than NECs, as analyzed in their culture medium (Fig. 1e). To show that TECs were exposed to a lactate-rich environment *in vivo*, the lactate content of whole tumors was compared with that in equal quantities of skin and kidney tissues. There was a significantly higher level of lactate in the tumors than in the skin or kidney (Fig. 1f). These results suggested that TECs may survive in an acidic environment that has a rich lactate supply from the tumor and TEC glycolysis.

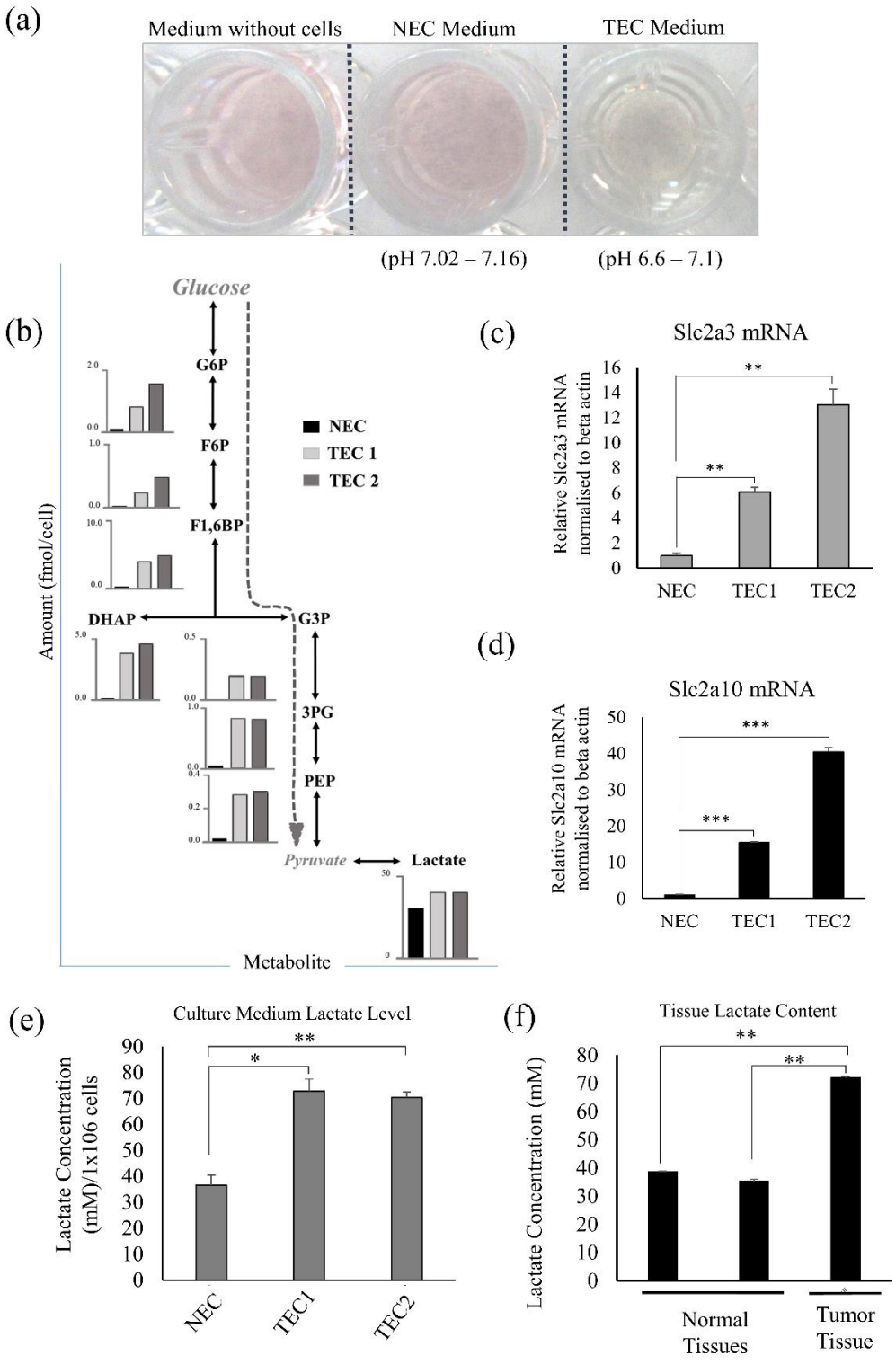


Figure 1 – **Glycolysis is more activated in TECs than in NECs**

(**a**) Images of medium without cells, and medium of TEC and NEC cultures after cells reached confluence; the extracellular pH of media was measured with a pH probe.
(**b**) Comparison of the metabolomes of NECs and TECs cultured in complete-medium.

(**c**) (**d**) Slc2a3 and Slc2a10 mRNA expression in TECs and NECs was evaluated by RT-qPCR (mean ± S.D., **P < 0.001, ***P < 0.0001).

(**e**) Lactate levels determined enzymatically in media of cells cultured for 48 h (mean ± S.D., *P < 0.05, **P < 0.001).

(**f**) Enzymatically determined lactate levels in supernatants of minced normal skin, kidney, and A375-SM tumor tissues (mean ± S.D., **P < 0.001) prepared immediately after resection.

TECs proliferation was not inhibited by the acidic extracellular environment in the presence of lactate

TECs showed a dose-dependent proliferation pattern in the presence of sodium lactate (Fig. 2b) and lactate acid regardless of the decreasing extracellular pH in the lactic acid-containing medium (Fig. 2a). NECs on the other hand proliferated in a dose-dependent manner only in the sodium lactate-containing medium which had an initial pH of 8 (Fig. 2b). The addition of lactic acid-containing medium with an initial pH below 8 (10 mM and 20 mM) stunted the growth of NECs (Fig. 2a). To verify whether the inhibition of NEC proliferation was due to the low pH of the medium, NECs were treated with medium, containing 20 mM lactic acid with pH adjusted to approximately 8.0. The pH adjustment resulted in NECs proliferating in media with 20 mM lactic acid (Fig. 2c), which was not observed previously (Fig. 2a). These observations suggested that TECs can withstand slight decreases in the extracellular environment more than NECs.

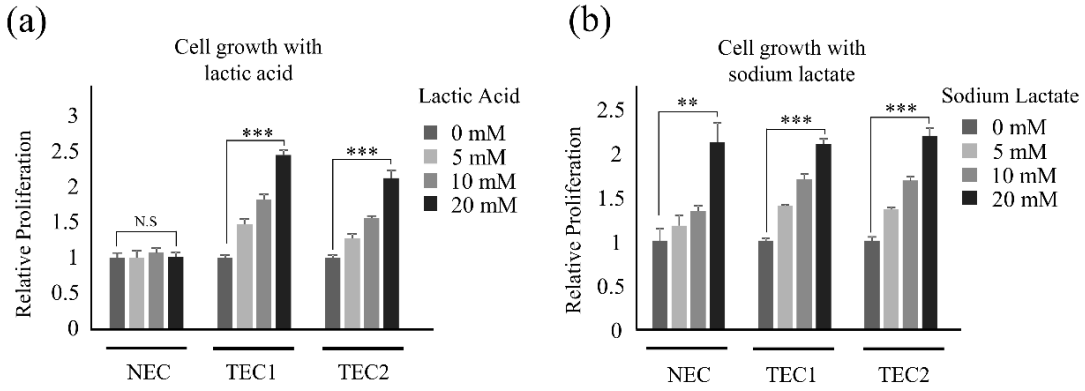


Table 1a Medium pH after application

pH of media (Temperature °C)	8.2 (28.1)	7.9 (28.0)	7.7 (27.9)	7.3 (27.6)
Lactic Acid (mM)	0	5	10	20

Decreasing media pH

Table 1b Medium pH after application

pH of media (Temperature °C)	8.00 (29.0)	8.07 (29.9)	8.00 (29.4)	7.99 (30.1)
Sodium Lactate (mM)	0	5	10	20

Alkaline media pH

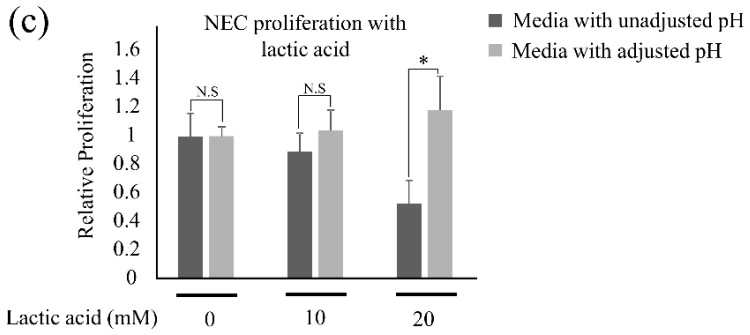


Table 1c Medium pH before and after pH adjustmen

Lactic Acid Concentration	Media with unadjusted pH	Media with pH adjustment
0 mM	8.17 (30.6)	8.05 (30.3)
10 mM	7.73 (30.3)	8.03 (30.5)
20 mM	7.32 (29.6)	8.08 (30.2)

Figure 2 – **TEC proliferation was not inhibited by the acidic extracellular environment in the presence of lactate**

(**a**) TEC and NEC proliferation in lactic acid-supplemented medium at indicated concentrations; the pH of the media (Table 1a) were measured immediately before the plates were placed in the incubator; cell proliferation was measured by the MTS assay after 72 h (mean ± S.D., $^{**}P < 0.001$, $^{***}P < 0.0001$, N.S., not significant)

(**b**) TEC and NEC proliferation in sodium lactate-supplemented medium at indicated concentrations; the pH of the media (Table 1b) were measured immediately before the plates were placed in the incubator; cell proliferation was measured by the MTS assay after 72 h (mean ± S.D., $^{**}P < 0.001$, $^{***}P < 0.0001$)

(**c**) NEC proliferation in pH-adjusted, 20 mM lactic acid-supplemented medium; the pH of the media (Table 1c) was adjusted before applying to the cells; cell proliferation was measured by the MTS assay after 72 h (mean ± S.D., $^{*}P < 0.01$, N.S., not significant)

TECs proliferate in lactic acidosis

Various studies have explored the effects of exogenous lactate (Kennedy et al., 2013), acidity, or both on tumors (Wu et al., 2012; Chen et al., 2008) and stromal cells (Colegio et al., 2014; Vegran et al., 2011; Beckert et al., 2005). In this study, we sought to determine the effects of both high lactate levels and low pH on TEC function. TECs and NECs were exposed to 20 mM exogenous lactate at pH 7.3 for lactosis and pH 6.9 for lactic acidosis. It was observed that TECs survived under lactic acidosis with accompanying increases in cell number, whereas NEC survival and growth were not supported by this condition (Fig. 3a). Despite the harsh conditions of lactic acidosis in the tumor microenvironment, tumor angiogenesis has not been reported to be inhibited but rather to be enhanced. However, TECs exposed to lactic acidosis maintained a slower pace of motility than those exposed to lactosis (Fig. 3c). The motility rates of NECs under the two conditions were comparable (Fig. 3b). The above observations may imply that TEC motility is affected more by a condition of lactic acidosis.

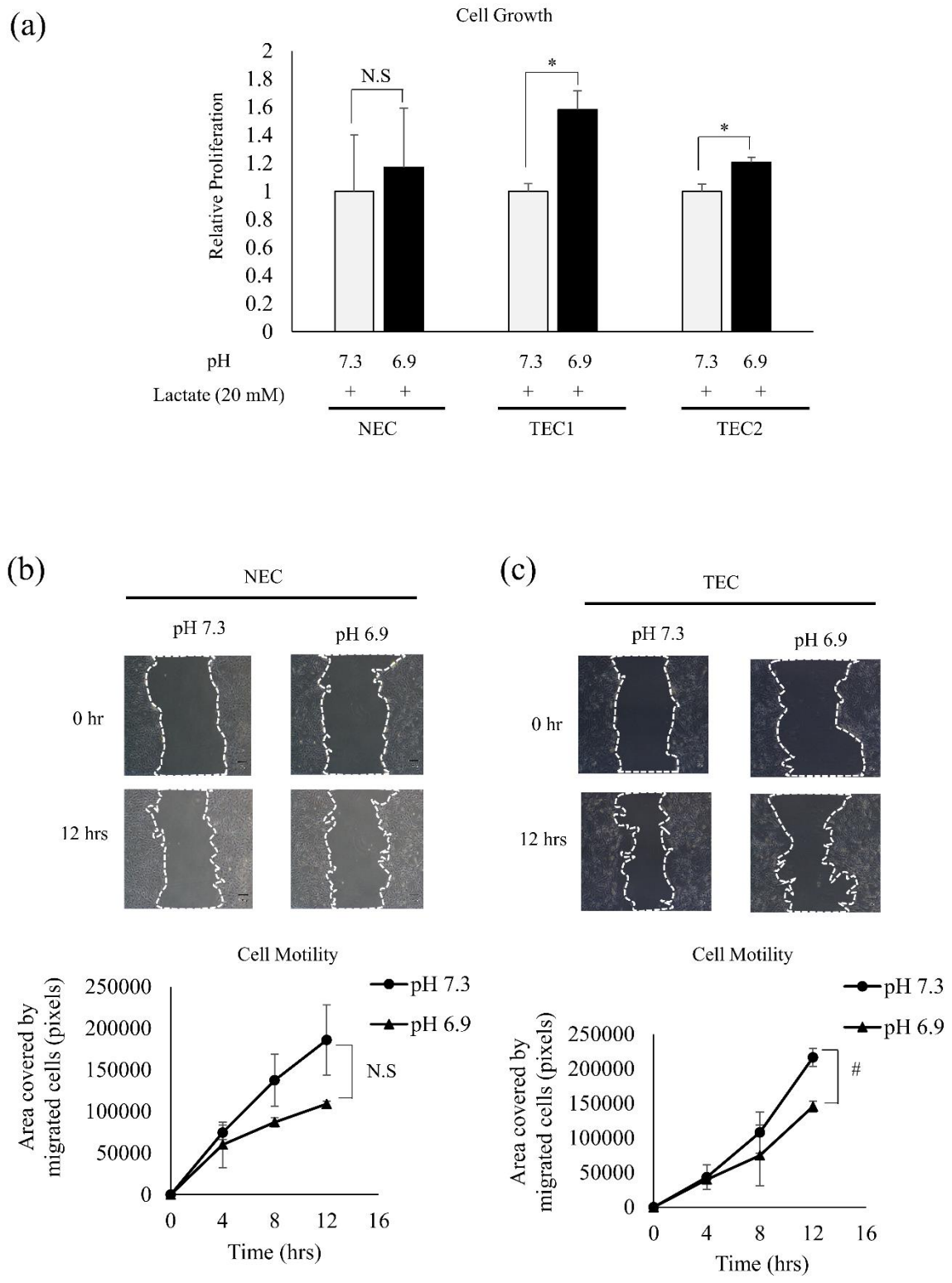


Figure 3 – **TECs proliferate in lactic acidosis**

(**a)** TEC and NEC proliferation in lactosis (20 mM sodium lactate, pH 7.3) and lactic acidosis (20 mM sodium lactate, pH 6.9) after 48 h of culture; cell proliferation was measured by counting cells (mean ± S.D., **P* < 0.01, N.S., not significant)

(**b**), (**c**) Representative images of NEC and TEC migration in lactosis and lactic acidosis: graphs represent the area remaining after cell migration at the indicated time ($^{\#}P < 0.05$, N.S., not significant)

Inhibition of MCT1 and MCT4 decreased TEC proliferation in lactic acidosis

Lactate can enter endothelial cells via MCT1 to support EC migration and tube formation (Sonveaux et al., 2012; Vegran et al., 2011). MCT4, which functions in lactate extrusion in glycolytic muscle fibers (Halestrap and Price, 1999) and tumors (Sonveaux et al., 2008), has not been studied in endothelial cells. However, since the process of lactate transport by MCT1 and MCT4 is associated with proton transfer across the cell membrane (Halestrap and Price, 1999; Sonveaux et al., 2008), their roles in TEC proliferation and survival in lactic acidosis was studied. Both MCT1 and MCT4 were expressed in TECs at the mRNA level, but MCT4 expression was significantly upregulated in TECs compared with that in NECs (Fig. 4a, 4b). Employing the MCT inhibitor CHC to inhibit lactate transport (Manning et al., 2000) and subsequently proton transfer, it was observed that TECs were sensitive to MCT1 inhibition in the complete-medium, which is fully supplemented and contains several endothelial growth factors (Fig. 4c). In the same complete-medium, targeting both transporters with siRNAs (Fig.4d, 4e) showed that TEC proliferation was decreased significantly by MCT1 knockdown and not MCT4 knockdown (Fig. 4f). In lactic acidosis, MCT1 and MCT4 inhibition led to a decrease in cell proliferation. However, in lactosis, only MCT4 knockdown minimally affected TEC proliferation (Fig. 4g), suggesting that both MCT1 and MCT4 may play significant roles in TEC survival in lactic acidosis.

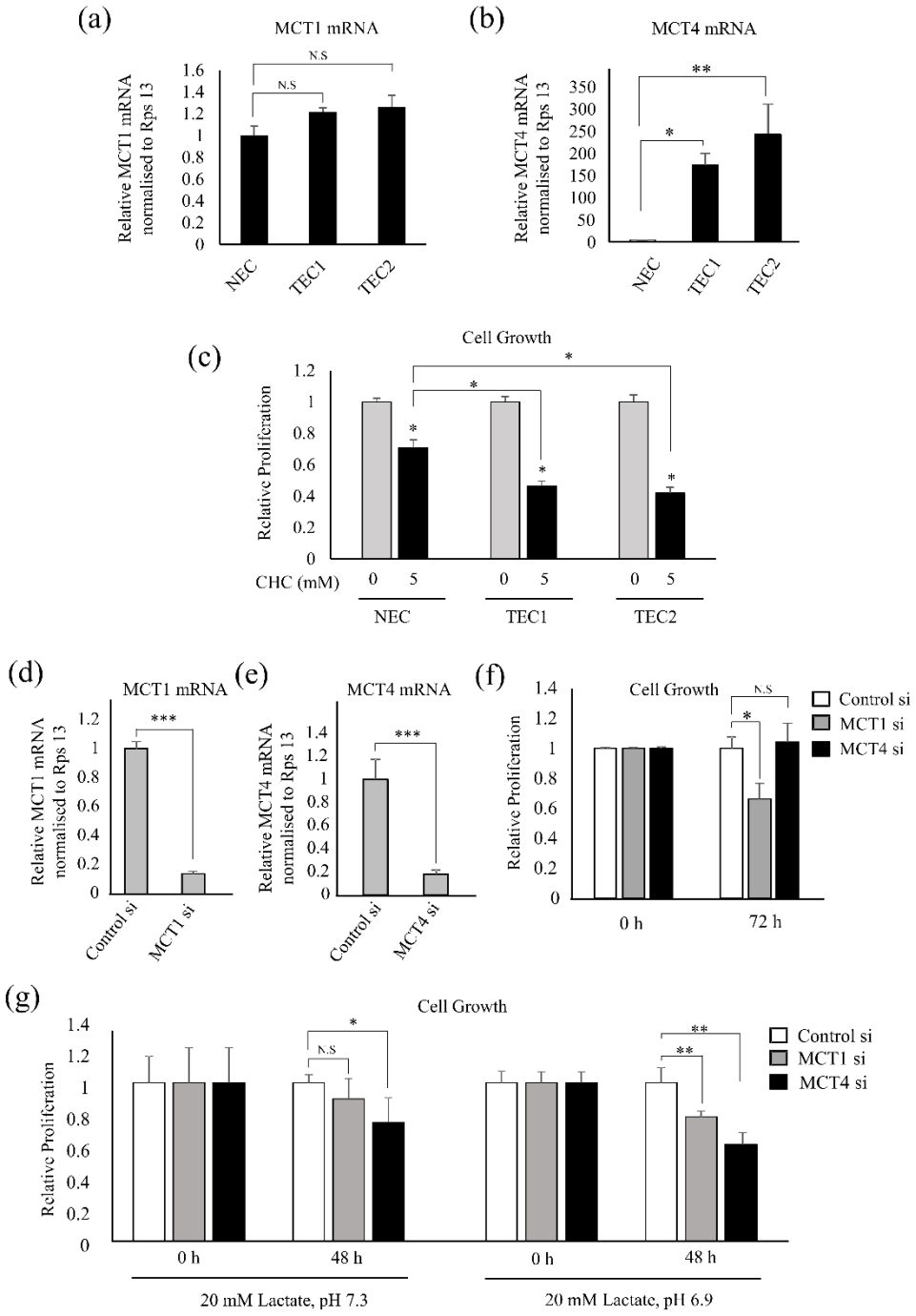


Figure 4 – **Inhibition of MCT1 and MCT4 decreased TEC proliferation in lactic acidosis**

(**a**), (**b**) MCT1 and MCT4 mRNA expression in TECs and NECs was evaluated by RT-qPCR (mean ± S.D., **P* < 0.01).

(**c**) Proliferation of NECs and TECs exposed to CHC (5 mM), was measured by the MTS assay (mean ± S.D., ***P* < 0.001).

(**d**), (**e**) MCT1 and MCT4 knockdown in TECs was confirmed by RT-qPCR (mean ± S.D., ***$P < 0.0001$). (**f**) Proliferation of MCT1 or MCT4 knockdown TECs in complete-medium was measured by the MTS assay after 72 h of culture (mean ± S.D., **$P < 0.001$).

(**g**) Proliferation of MCT1 and MCT4 knockdown TECs after exposure to lactosis (20 mM lactate, pH 7.3) and lactic acidosis for 48 h (20 mM lactate, pH 6.9) was determined by the MTS assay (mean ± S.D., *$P < 0.01$, **$P < 0.001$, N.S., not significant).

Targeting the pH regulator, CAII significantly decreased TEC proliferation in lactic acidosis

Owing to the fact that maintaining intracellular pH at physiological levels is a basic requirement for cell survival (Aoi and Marunak, 2014), the contribution of pH regulators to TEC survival in lactic acidosis was also investigated. TECs showed different expressions for various classes of pH regulators. The CAs, CAII and CAIII were upregulated in TECs, whereas CAIV, CAIX, and CAXII expression levels were comparable between TECs and NECs (Fig. 5a, 5b). The mRNA expression of the sodium/hydrogen antiporter 2 (NHA2) was more upregulated in TECs than in NECs (Fig. 5c), whiles the expression levels of NHE1 and the proton-sensing GPCRs Gpr4 and Gpr65 were similar between TECs and NECs (Fig. 5c, 5d). Further work focused on the role of CAII in TECs because of its high catalytic activity among the CAs (Sly and Hu, 1995; Kida et al., 2006), in addition to being a tumor endothelium-associated CA (Yoshiura et al., 2005; Haapasalo et al., 2007). Also, because of the upregulated expression of NHA2 in TECs and the fact that sodium/hydrogen antiporters like NHE1 are very effective in tumor pH regulation (Damaghi et al., 2013), NHA2 was recruited for further studies to define its novel function in TECs. Upon successful knockdown of CAII and NHA2 with siRNA (Fig. 5e, 5f) it was observed that TEC proliferation was significantly decreased by NHA2 only under nutrient-replete conditions (Fig. 5g). CAII knockdown on the other hand decreased TEC proliferation at very significant levels in nutrient-replete conditions (Fig. 5g) as well as in lactic acidosis and lactosis (Fig. 5h). These results suggested that NHA2 affects TEC proliferation when nutrient supply is not limiting; whereas CAII plays a very important role in TEC proliferation irrespective of nutrient availability.

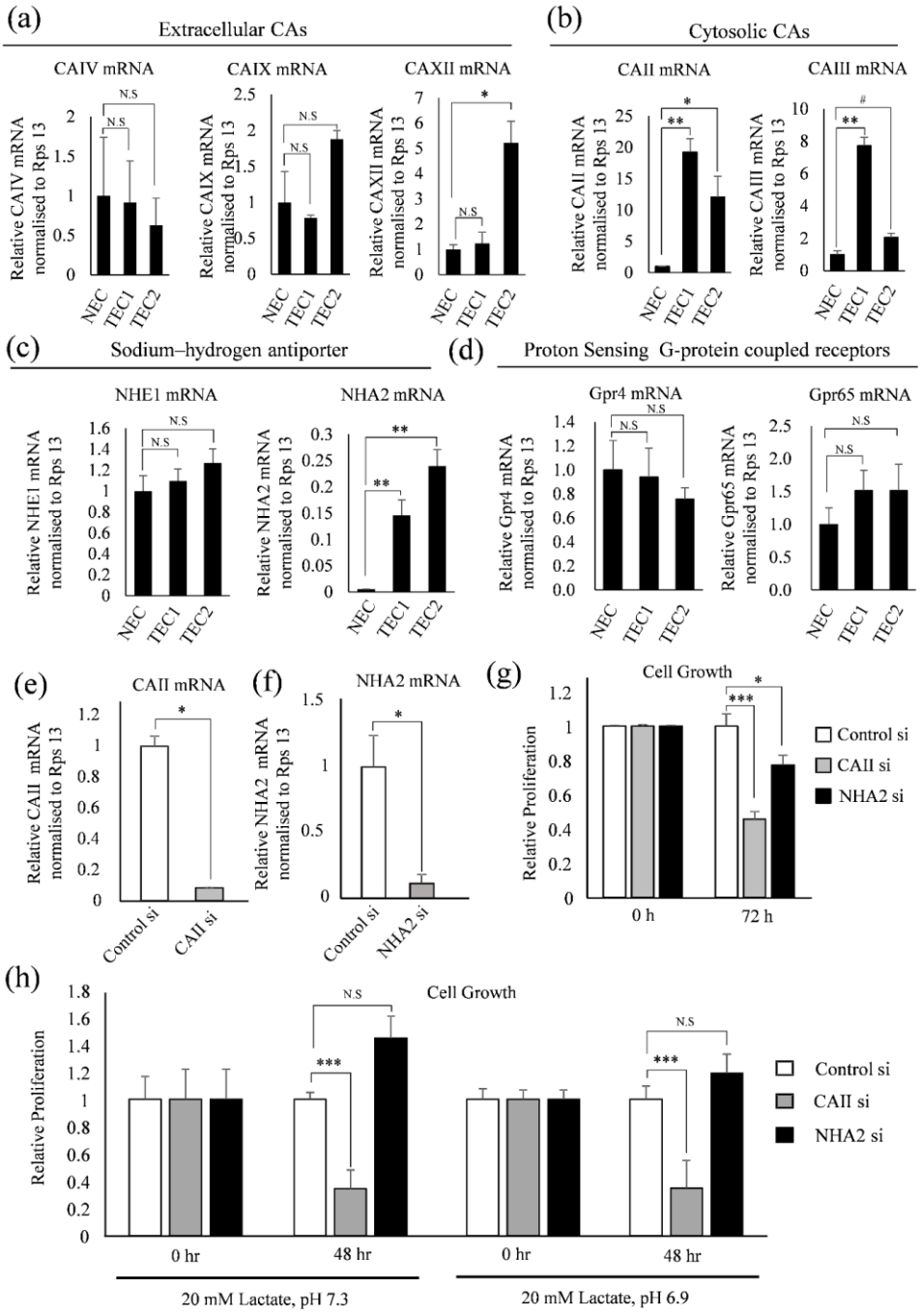


Figure 5 – **Targeting the pH regulator CAII significantly decreased TEC proliferation in lactic acidosis**

(**a**), (**b**), (**c**), (**d**) mRNA expression of the pH regulators CAIV, CAIX, CAXII, CAII, CAIII, NHE1, NHA2, Gpr4, and Gpr65 was evaluated by RT-qPCR (mean ± S.D., *P < 0.01, ***P* < 0.001).

(**e**) (**f**) CAII and NHA2 knockdown in TECs was confirmed by RT-qPCR (mean ± S.D., **P* < 0.01).

(**g**) Proliferation of CAII and NHA2 knockdown TECs was measured by the MTS assay after 72 h of culture (mean ± S.D., ****P* < 0.0001).

(**h**) Proliferation of TECs exposed to lactosis (20 mM lactate, pH 7.3) and lactic acidosis (20 mM lactate, pH 6.9) for 48 h after CAII or NHA2 knockdown was determined by the MTS assay (mean ± S.D., ****P* < 0.0001, N.S., not significant)

CAII is expressed in tumor endothelium

To verify the expression of CAII *in vivo*, tissue sections of normal murine kidneys and A375-SM tumor xenografts were stained with anti-CAII and anti-CD31 antibodies. As a positive control, CAII expression was observed only in the tubules of the normal kidney and not in the glomerulus as expected (Yoshiura et al., 2005) (Fig. 6a). Distinct CAII expression was observed in the blood vessels and tumor cells of the A375-SM tumor xenografts (Fig. 6a). In the human RCC cases studied, CAII was highly expressed in the tumor endothelium of a stage III clear cell RCC patient, but was absent in the blood vessels of the normal renal tissues of the same patient (Fig. 6b). In another case of a stage I papillary RCC patient, CAII expression was absent in both the tumor and the normal renal tissues (Fig. 6c). These observations suggested that CAII is indeed expressed in the tumor endothelium. However, the stage of the cancer may also dictate CAII expression in the tumor endothelium.

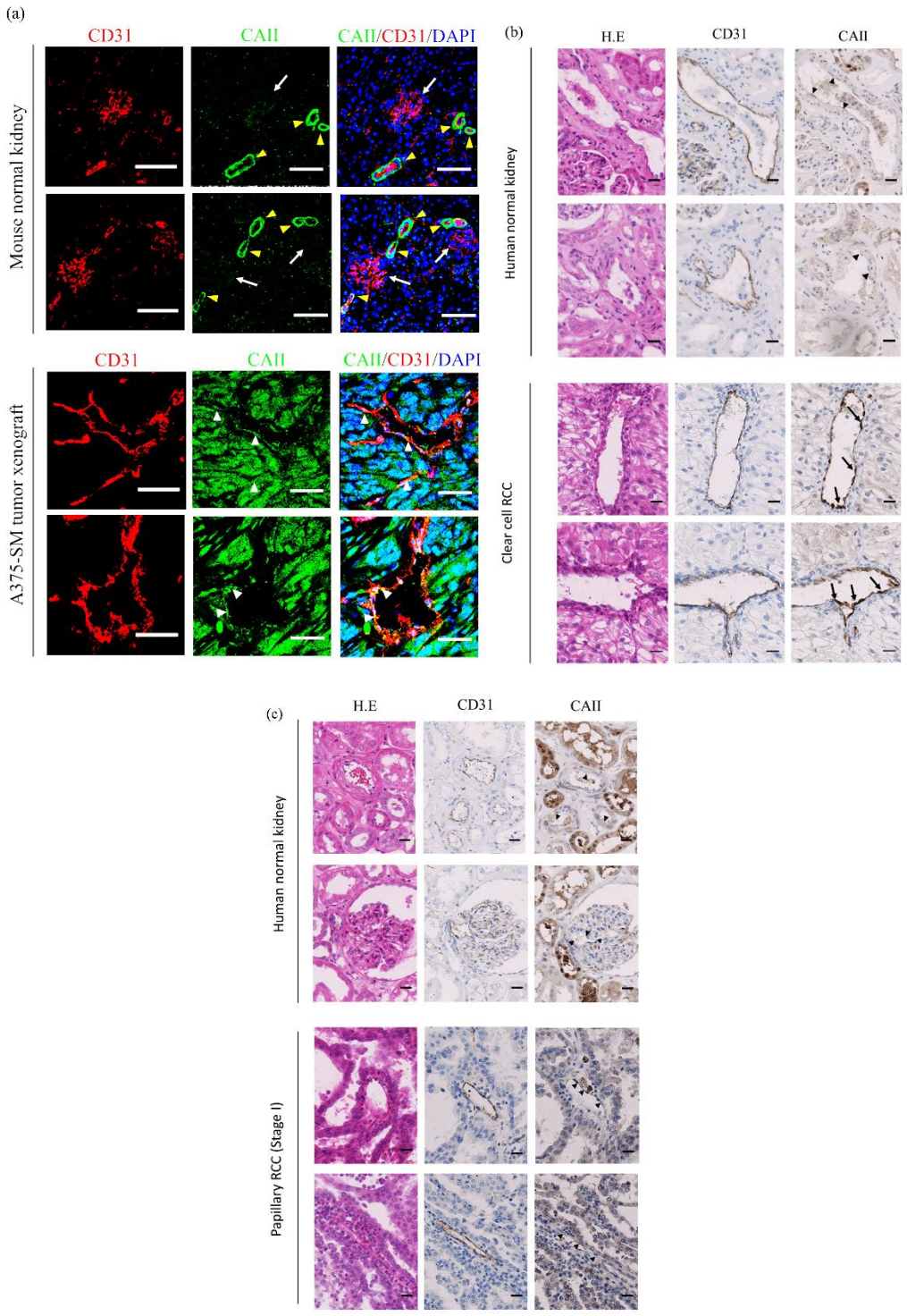


Figure 6 – **CAII is expressed in tumor endothelium**

(**a**) Double immunofluorescent staining for CD31 (red) and CAII (green) in normal mouse tissue (kidney) and A375-SM tumor xenografts, arrows point to CAII-negative glomerulus and yellow arrowheads to CAII-positive tubules. White arrowheads indicate CAII-positive blood vessels in the A375-SM tumor. Merged image DAPI (blue) shows colocalization of CAII (green) and CD31 (red) (scale bar = 50 µm).

(**b**) CAII expression in CD31-positive blood vessels in human RCCs (arrows) and absence in normal renal tissues (arrowheads) (scale bar = 50 μm)
(**c**) CAII expression in CD31-positive blood vessels in human RCCs (arrows) and absence in normal renal tissues (arrowheads) (scale bar = 50 μm).

Tumor-derived factors induced the upregulation of CAII in endothelial cells

To investigate the factors responsible for CAII upregulation in TECs and the human tumor endothelium, iHMVECs were treated with CM from A375-SM tumor cells for 24 h. RT-qPCR analysis revealed that CAII expression was significantly upregulated in the cells exposed to the tumor CM. This upregulated expression was canceled upon heat inactivation of the CM (Fig. 7a). CAII expression was slightly increased by the control CM as well (Fig. 7a). The above results indicate that CAII upregulation in TECs may be induced by proteins released from tumor cells, which may also be present in the iHMVECs culture medium. Our previous reports showed that A375-SM CM contains significant amounts of VEGF (Akiyama et al., 2012). It was therefore investigated whether VEGF was one of the factors responsible for CAII upregulation in TECs. VEGF treatment indeed increased CAII mRNA expression (Fig. 7b). Furthermore, a decrease in CAII expression was observed after treating iHMVECs with tumor CM containing Ki8751 (Fig. 7c). This implies that CAII upregulation by the tumor CM involves VEGF signaling via VEGFR2 receptor phosphorylation.

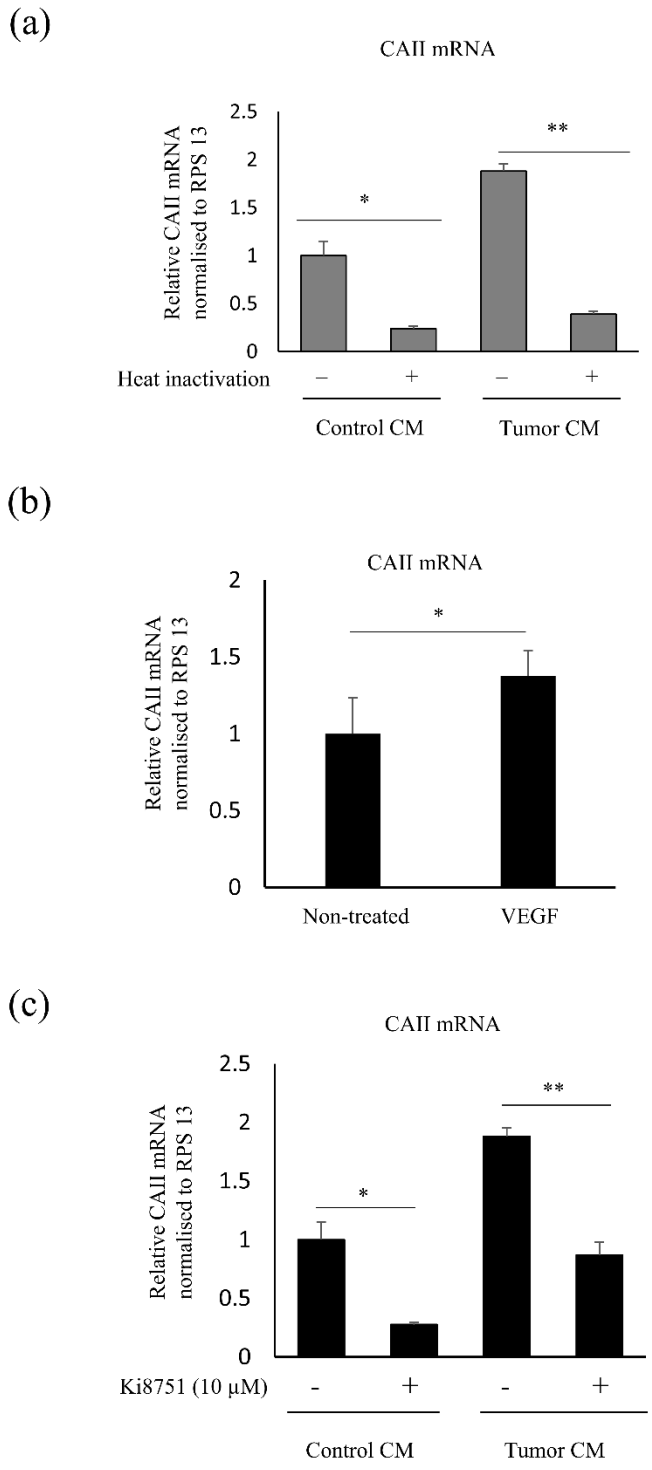


Figure 7 – **Tumor-derived factors induced the upregulation of CAII in endothelial cells**

(**a**) iHMVECs were treated with tumor CM or control CM for 24 h. CAII expression was determined by RT-qPCR (mean ± S.D., **P* < 0.01, ***P* < 0.001).

(**b**) iHMVECs were stimulated with 20 ng/mL VEGF for 24 h. CAII expression was determined by RT-qPCR (mean ± S.D., **P* < 0.01).

(**c**) iHMVECs were exposed to tumor or control CM containing 10 μM Ki8751 (VEGFR2 inhibitor) for 24 h. CAII expression was determined by RT-qPCR (mean ± S.D., *$P < 0.01$, **$P < 0.001$).

Pharmacological inhibition of CAII decreased TEC proliferation *in vitro*

Acetazolamide (broad range CA inhibitor) was used to test the effect of CA inhibition on TECs. *In vitro* application of acetazolamide led to a significant decrease in TEC proliferation (Fig. 8a). This decrease in TEC proliferation was augmented after combining CA inhibition with VEGFR2 inhibition with Ki8751 (Fig.8a). This suggested that CA targeting may improve VEGFR2 antiangiogenic therapies. However, injecting tumor-bearing mice with acetazolamide did not cause a significant reduction in tumor angiogenesis (Fig. 8c) as compared to the control group. Also the tumor volume was not significantly different between the groups (Fig. 8b). The results of the *in vivo* pharmacological inhibition with acetazolamide implied that acetazolamide treatment alone might not be sufficient to decrease *in vivo* tumor growth or angiogenesis.

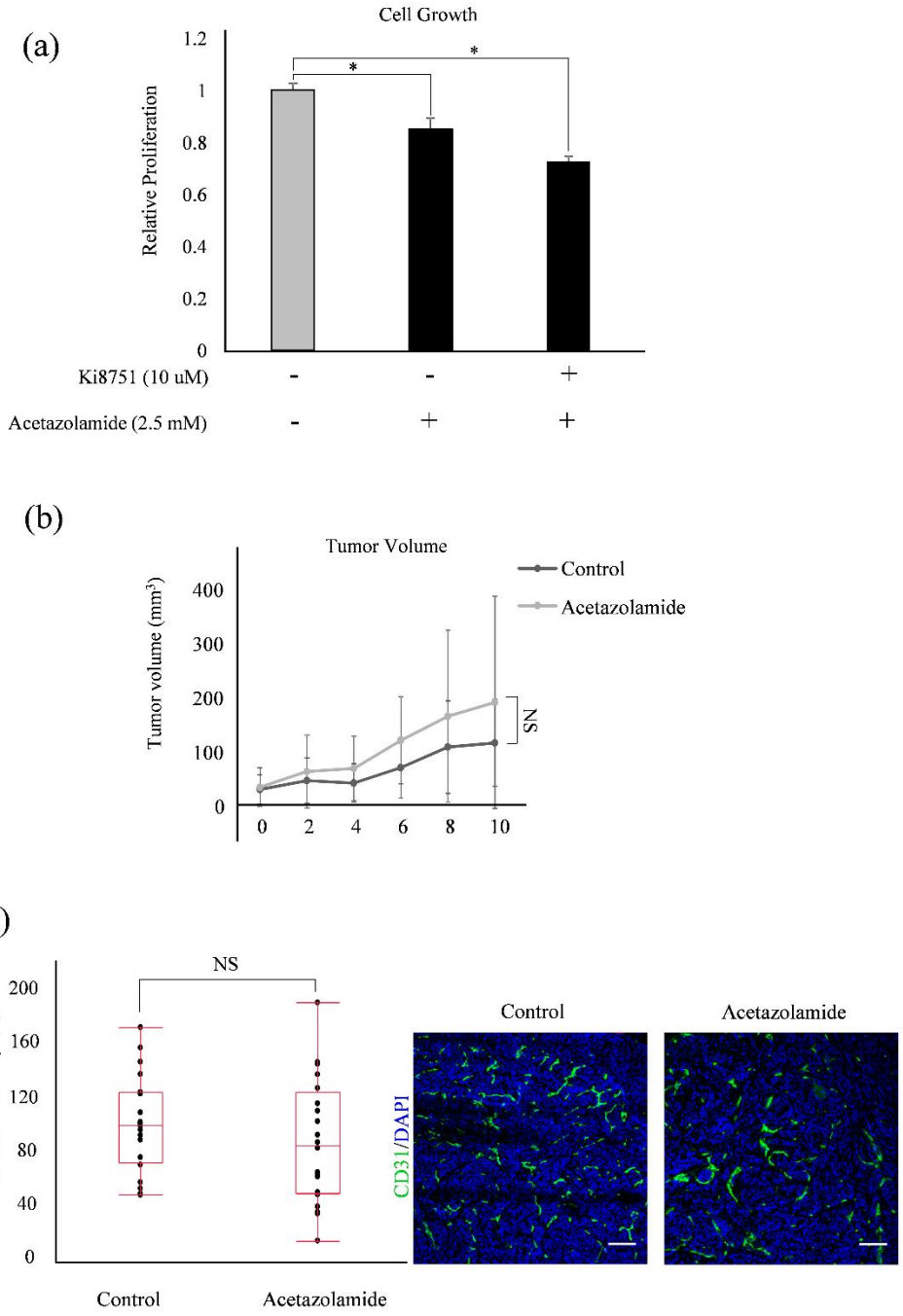


Figure 8 – **Pharmacological Inhibition of CAII decreased TEC proliferation *in vitro***

(**a**) *In vitro* proliferation of TECs exposed to acetazolamide (CA inhibitor) or acetazolamide and Ki8751 (VEGFR2 inhibitor) was determined by the MTS assay (mean ± S.D., **P* < 0.01). (**b**) Tumor volume was calculated after obtaining length, width and height of tumors from the tumor-bearing mice. The measurements were taken on the indicated days (*P* = 0.4, n = 5)

(**c**) Tumor sections were stained with anti-CD31 antibody (green) and counterstained with DAPI (blue). The CD31-positive blood vessel density was analyzed in both groups by image J (scale bar = 50 μm, N.S., not significant, $P = 0.31$, n = 21 fields)

Discussion

The findings from this study indicate that TECs express various pH regulators. Among them, the upregulated expression of CAII in the tumor endothelial cells is very essential for their survival in lactic acidosis as well as in nutrient-replete conditions. Furthermore, CAII expression in the tumor endothelium may partially be a consequence of tumor-derived factors.

Accelerated glucose metabolism leading to elevated lactate production in the presence of oxygen is a unique metabolic signature of most cancers cells (Gatenby and Gillies, 2004). Recently, however, it has been shown that cancer-associated fibroblasts (CAFs) isolated from melanoma, and colon cancer also undergo increased aerobic glycolysis and lactate production (Zhang et al., 2015). Similarly, the TECs used in this study were found to be highly glycolytic, and produced more lactate than NECs. This observation further confirms other reports describing tumor endothelial cells as being hyperglycolytic (Cantelmo et al., 2016; Zhang et al., 2018). The TECs in this study may have developed a hyperglycolytic character through the upregulated expression of glucose transporters. Such transporters would facilitate rapid uptake of glucose into the TECs to be metabolized. Moreover, the upregulated expression of glucose transporters in TECs would be useful in the tumor microenvironment, where they have to compete with the glucose-loving cancer cells for the limited glucose reserves.

The end product of the rapid glycolysis in tumors – lactic acid is reported to be responsible for the acidic extracellular environments in tumors (Gatenby and Gillies, 2004). Therefore, considering the hyperglycolytic nature of the TECs in this study as well as the lactate content of TEC culture medium, it can be suggested that the export of TEC-derived lactic acid to the extracellular space is the most likely cause of acidification of TEC culture medium. At this point, it may be proposed that acidification of TEC culture medium may have no detrimental effects on TECs. As indicated by the results of this study, decreases in the pH of the lactic acid-containing culture medium did not inhibit TEC proliferation. On the contrary, NECs could not survive in acidic extracellular environments. Similar to this observation, it was reported that acidosis (pH 7.0) inhibited the proliferation of bovine aortic endothelial cells (a type of NECs) in 10% FCS-containing medium (D'Arcangelo et al., 2000).

In tumors, lactic acid performs a tumor-promoting function whether it acts on tumor cells directly, or on other stromal cells, to achieve similar effects. Lactic acid exerts stimulatory and inhibitory effects on macrophages (Colegio et al., 2014) and T-cells respectively (Fischer et al.,

2007; Brand et al., 2016); and both effects culminated in supporting tumor growth. Furthermore, it has been reported that the direct exposure of cancer cells to lactic acidosis conferred on the cells a resistance to glucose deprivation and survival instead of apoptosis (Wu et al., 2017). A Xie and others demonstrated that lactic acidosis supports the efficient usage of glycolytic products to maintain 4T1 proliferation. In the study it was shown that 4T1 breast cancer cells exposed to lactic acidosis with low glucose (0.5 mM) exhibited progressive increases in cell proliferation, whiles 4T1 cells cultured without lactic acidosis, but with 6 mM glucose rapidly died after glucose depletion (Xie et al., 2014). In the current study, the metabolome of TECs revealed that TECs contained more glycolytic metabolites than NECs. Therefore, although TECs were not supplied with glucose during exposure to lactic acidosis, it can be suggested that under lactic acidosis, these glycolytic metabolites were managed to support TEC survival and proliferation. The absence of these glycolytic metabolite stores may justify the non-proliferative phenotype of NECs under lactic acidosis.

Lactate (Vegran et al., 2011; Beckert et al., 2005) and acidity (Burbridge et al., 1999; D'Arcangelo et al., 2000) have opposing effects on endothelial cell migration or motility. In the current study, it was observed that despite the availability of lactate, low extracellular pH caused a delay in TEC motility. This observation may be partly due to the ability of extracellular acidity to delay the onset of endothelial cell migration (Burbridge et al., 1999; D'Arcangelo et al., 2000).

Monocarboxylate transport by MCTs also carries a proton transfer across the cell membrane (Doherty and Cleveland, 2013). In cancers, MCT1 and MCT4 are overexpressed to maintain intracellular pH and lactate at physiological levels (Damaghi et al., 2013): MCT4 exports lactate (Wilson et al., 1998; Manning et al., 2000; Dimmer et al., 2000), while MCT1 is used by oxidative tumor cells for lactate uptake (Sonveaux et al., 2008; Halestrap, 2012a). Thus, within a single tumor, there is a symbiotic relationship, where hypoxic tumor cells expressing MCT4 export glycolysis-derived lactate and the oxidative cells expressing MCT1 take up the released lactate (Sonveaux et al., 2008; Nakajima and Van Houten, 2012). Similarly, TECs may express both MCT1 and MCT4 to facilitate opposing processes. In this study, even though TECs express both MCT1 and MCT4, they exhibited a dependency on MCT1 for survival under regular conditions. TECs were more sensitive to CHC, which selectively inhibits MCT1 rather than MCT4 (Manning et al., 2000). This importance of MCT1 to TECs was further confirmed through MCTI knockdown leading to a significant decrease in TEC proliferation. Reported

pharmacological inhibition or siRNA knockdown of MCT1 further demonstrates the importance of MCT1 to tumor angiogenesis (Sonveaux et al., 2012; Vegran et al., 2011). Despite all the above points, the role of MCT1 in TEC proliferation under lactic acidosis cannot currently be explained. Additionally, MCT4 knockdown did not affect TEC proliferation under nutrient-replete conditions; however, its upregulated expression and a targeted decrease in TEC proliferation in lactic acidosis suggest a role in the export of lactate and protons out of the TECs into the surrounding medium to help maintain TEC function under unfavorable conditions of lactosis or lactic acidosis.

Apart from MCTs, an alkaline intracellular pH can be achieved through the activities of other pH regulators (Damaghi et al., 2013). NHE1, the most common isoform of the NHEs, is key to intracellular pH (pHi) homeostasis. In tumors or transformed cells, this transporter is hyperactivated through an increased affinity of the allosteric intracellular proton-binding site, leading to increased pHi and acidification in the extracellular spaces (Cardone et al., 2005). In the present study, however, due to the similarity in NHE1 expression between NECs and TECs its pH regulatory property was not explored further. Instead, the emerging NHA2, which is a Na^+ selective-NHE and is ubiquitously expressed in multiple mammalian organs and cultured cells was studied (Xiang et al, 2007; Chintapalli et al, 2015). Although the NHA2 knockdown led to a reduction in TEC proliferation in nutrient-replete conditions, its role here, may not involve a pH regulatory function since the medium was buffered with sodium bicarbonate. Moreover, NHA2 knockdown in TECs did not affect their proliferation in lactic acidosis. These observations imply that TECs use other pH regulators to sustain their function and survival in lactic acidosis.

Proton-sensing GPCRs, including Gpr4, Gpr65, Gpr68, and Gpr132 may be activated by the acidic tumor extracellular environment to exert their effects in tumor development, metastasis and angiogenesis (Justus et al., 2013). For further studies, Grp4 was selected due to its function in blood vessel growth, pH sensing, integrity, response to VEGF, and support of tumor growth (Yang et al., 2007; Wyder et al., 2011). Gpr65 was chosen as a regular tumor-associated proton-sensing GPCR. Gpr65 plays roles which either promote or suppress tumor progression (Justus et al., 2013). On the one hand, Gpr65 over-expression in Lewis lung carcinoma (LLC) induced in the cells resistance to acidic environments and increased tumor development in mice (Ihara et al., 2010); and on the other hand, its knockdown decreased the protective effect of acidosis

on mouse lymphoma cells exposed to glutamine starvation (Ryder et al. 2012). However, the absence of a unique expression of Gpr4 and Gpr65 in TECs precluded their investigations.

Analysis of the expression of the CO_2 hydrating CAs (Supuran, 2016) at the mRNA level showed that the expression of three membrane-associated extracellular CAs (CAIV, CAIX, and CAXII) was not specific to TECs. However, the two cytosolic CAs (CAII and CAIII) were upregulated in TECs, implying that cytosolic CAs may be more essential to TECs. Furthermore, CAII exhibits versatility in its function and it associates with other proteins involved in intracellular pH control such as NHE1 (Kida et al., 2006) and MCTs (Becker et al., 2010; Klier et al., 2014; Jamali et al., 2015). CAII was also postulated to be involved in endothelial cell proliferation via its pH regulatory role in proliferating and differentiating endothelial cells in the microvessels of the developing human brain (Kida et al., 2006). Similarly in the current study, it was shown that CAII is very important for TEC proliferation. CAII knockdown decreased TEC proliferation in fully supplemented medium, lactic acidosis, and lactosis. In lactic acidosis, CAII may perform its pH regulatory actions by interacting with MCT4 to enhance proton-coupled lactate export after import into the TECs (by a transporter such as MCT1), or by activating NHE1 to increase H^+ extrusion (Kida et al., 2006; Sweitach et al., 2007; Damaghi et al., 2013). The inhibitory effects of CAII knockdown on TEC proliferation in fully supplemented medium and lactosis may, however, show that CAII functions to support TEC proliferation, regardless of the available metabolic substrates.

Further analysis revealed that protein products in tumor CM and VEGFR2 signaling were partially responsible for CAII upregulation. To the best of my knowledge there are no reports regarding the role of VEGFR2 signaling in CAII expression or function. However, TECs have been shown to upregulate VEGFR2 (Ohga et al., 2012), and subsequent activation of this receptor may induce novel downstream activities such as MDR1 upregulation in support of TEC drug resistance (Akiyama et al., 2012), or in this case, CAII upregulation to facilitate TEC proliferation in the tumor microenvironment. Furthermore, a study demonstrating the effects of acidosis, hypoxia, and both conditions on CAII expression in HUVECs showed that CAII mRNA is upregulated by both acidity and hypoxia (Yoshiura et al., 2005). These observations further show that conditions in the tumor microenvironment may effectively stimulate CAII upregulation in TECs and possibly in the tumor endothelium to enhance the survival of TECs and stimulate their proangiogenic activity.

In this study, it was observed that CAII is expressed in the tumor endothelium of A375-SM tumors *ex vivo* and this correlated with the upregulated CAII mRNA expression in the isolated TECs. The endothelium in both murine and non-cancerous human tissues lacked CAII expression as reported (Yoshiura et al., 2005). Additionally, analysis of RCC specimen showed a distinctive CAII expression in tumor endothelium of the stage III clear cell RCC patient, but not in the tumor endothelium of the stage I papillary RCC patient. CAII expression has been associated with the malignancy grade (grade III) of meningioma (Korhonen et al., 2009). These results, first of all, confirm the reports that CAII is a tumor endothelium-associated CA (Yoshiura et al., 2005; Haapasalo et al., 2007), and secondly, indicate that CAII expression may be associated with the malignant progression of cancers such as RCC.

Current targets of tumor angiogenesis have not been successful at eliminating cancer or decreasing angiogenesis effectively due to various mechanisms of acquired resistance after drug treatment. Some of the mechanisms reported, include the upregulation of proangiogenic growth factors by tumor cells to induce new vessel formation (Motzer and Bukowski, 2006), recruitment of vascular progenitor cells (Azab et al., 2014), increased pericyte coverage (Pinto et al., 2016), vessel co-option (Leenders et al., 2004), vasculogenic mimicry (Xu et al., 2012), and many others (Zarrin et al., 2017; Bergers and Hanahan, 2008). Although acetazolamide treatment alone did not decrease tumor growth *in vivo*, there was a little but insignificant tendency towards angiogenesis reduction in the treated tumors as compared to the control group. This suggests that targeting TEC-specific intracellular pH control and not only pH regulation in the tumor cells may lead to a reduction in tumor angiogenesis. Furthermore, combining pH control with the administration of established antiangiogenic drugs may be more efficacious in inhibiting tumor angiogenesis and ultimately the growth of tumors.

Summary and Conclusion

TECs can survive in lactate-rich environments and are not inhibited by low extracellular pH like NECs; therefore, they are able to proliferate in lactic acidosis. This property gives TECs a survival advantage in the tumor microenvironment loaded with tumor-derived lactic acid. Furthermore, TECs showed an upregulated expression of the pH regulators – MCT4, NHA2, CAIII and CAII. The abundance of such pH regulators in TECs suggests that TECs can manage pH changes in their extracellular environment to maintain their normal biological activities. In lactic acidosis, MCT1 and MCT4 were required to sustain TEC survival. Consequently, these two transporters cannot be overlooked in TEC-pH regulation, especially in the lactic acid laden tumor microenvironment. The role of CAII in *in vitro* TEC proliferation in lactic acidosis and nutrient available conditions was shown. The results indicated that regardless of the metabolic substrates in play TEC proliferation depended on the presence of CAII enzyme. These findings may suggest a significant contribution of TEC-specific CAII to the *in vivo* angiogenesis process in tumors. The overall pH regulation in TECs, either in lactic acidosis or nutrient abundant conditions, may not only depend on CAII, but also involve an interplay between CAII and other pH regulators like MCT4, NHE1 or even the extracellular CAs (see Fig. S1 for summary). Owing to the observation that CAII expression was present in the tumor endothelium of a late-stage RCC patient as compared to the early-stage RCC patient, it can be suggested that CAII contributes to tumor malignancy in some types of cancer including RCC. Furthermore, on account of the observation that targeting CAs with both acetazolamide and Ki8751 led to a more significant decrease in TEC proliferation as compared to acetazolamide only, it would be important to investigate further the effects of targeting both CAs and VEGFR2 signaling on tumor angiogenesis, tumor growth, and metastasis. On a whole, the findings of this study point to a potential role of pH regulation in facilitating angiogenesis *in vivo*, making CAII and intracellular pH control prospective targets of tumor anti-angiogenic therapy.

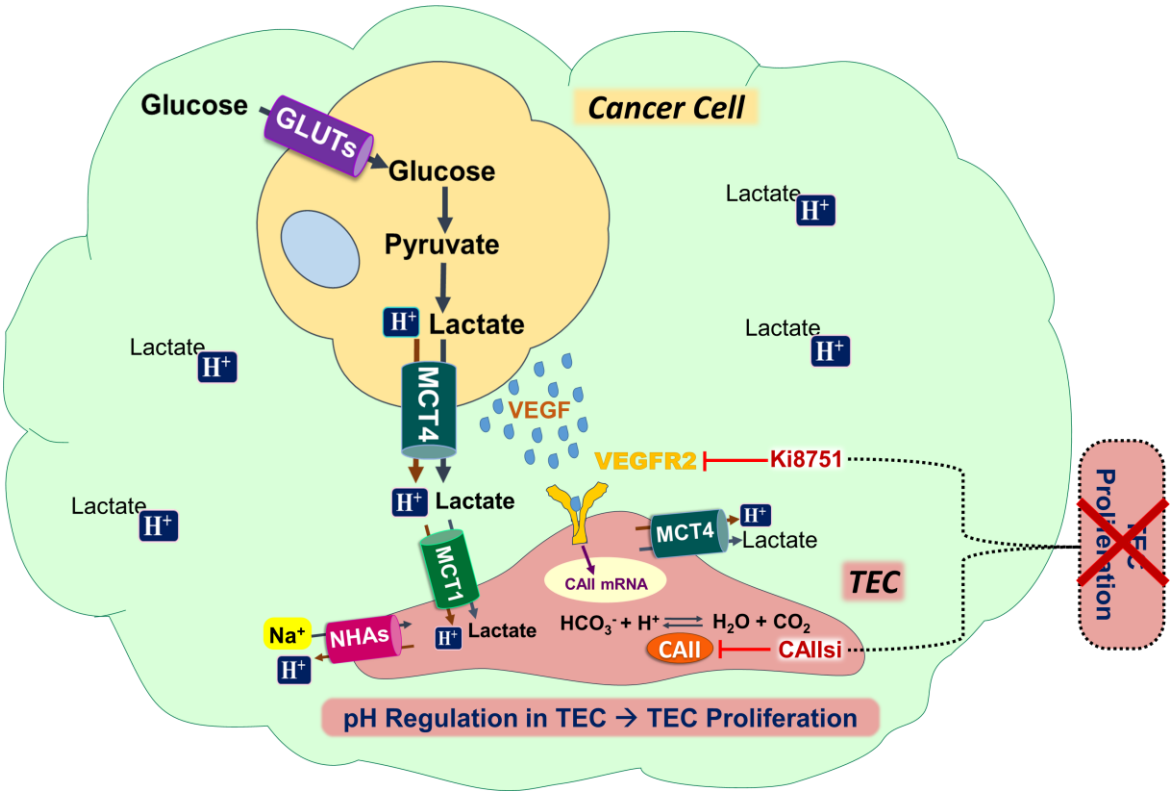


Fig. S1 CAII inhibition decreases endothelial cell survival in lactic acidosis

(Summary of the study)

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