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**Functional and molecular mechanism of intracellular ph regulation in human inducible pluripotent stem cells**

Chao SH *et al*.Characterization of intracellular pH regulation mechanism

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**Abstract**

***AIM***

To establish a functional and molecular model of the intracellular pH (pHi) regulatory mechanism in human induced pluripotent stem cells (hiPSCs).

***METHODS***

hiPSCs (HPS0077) were kindly provided by Dr. Dai from the Tri-Service General Hospital (IRB No. B-106-09). Changes in the pHi were detected either by microspectrofluorimetry or by a multimode reader with a pH-sensitive fluorescent probe, BCECF, and the fluorescent ratio was calibrated by the high K+/nigericin method. NH4Cl and Na-acetate prepulse techniques were used to induce rapid intracellular acidosis and alkalization, respectively. The buffering power (β) was calculated from the ΔpHi induced by perfusing different concentrations of (NH4)2SO4. Western blot techniques and immunocytochemistry staining were used to detect the protein expression of pHi regulators and pluripotency markers.

***RESULTS***

In this study, our results indicated that (1) the steady-state pHi value was found to be 7.5 ± 0.01 (*n =* 20) and 7.68 ± 0.01 (*n =*20) in HEPES and 5% CO2/HCO3--buffered systems, respectively, which were much greater than that in normal adult cells (7.2); (2) in a CO2/HCO3--buffered system, the values of total intracellular buffering power (β) can be described by the following equation: βtot = 107.79 (pHi)2 - 1522.2 (pHi) + 5396.9 (correlation coefficient *R*2 = 0.85), in the estimated pHi range of 7.1-8.0; (3) the Na+/H+ exchanger (NHE) and the Na+/HCO3- cotransporter (NBC) were found to be functionally activated for acid extrusion for pHi values less than 7.5 and 7.68, respectively; (4) V-ATPase and some other unknown Na+-independent acid extruder(s) could only be functionally detected for pHi values less than 7.1; (5) the Cl-/ OH- exchanger (CHE) and the Cl-/HCO3- anion exchanger (AE) were found to be responsible for the weakening of intracellular protein loading; (6) besides the CHE and the AE, a Cl--independent acid loading mechanism was functionally identified; and (7) in hiPSCs, a strong positive correlation was observed between the loss of pluripotency and the weakening of the intracellular acid extrusion mechanism, which included a decrease in the steady-state pHi value and diminished the functional activity and protein expression of the NHE and the NBC.

***CONCLUSION***

For the first time, we established a functional and molecular model of a pHi regulatory mechanism and demonstrated its strong positive correlation with hiPSC pluripotency.

**Key words:** human induced pluripotent stem cells; intracellular pH; Na+/H+ exchanger; Na+/HCO3- cotransporter; Cl-/OH- exchanger; Cl-/HCO3- exchanger; v-ATPase; intracellular buffering power; BCECF; microspectrofluorimetry

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**Core tip:** For the first time, we established a model of the intracellular pH (pHi) regulation mechanism in human induced pluripotent stem cells (hiPSCs). The steady-state pHi value of hiPSCs was 7.50-7.68, which greater than that of normal adult cells. The Na+-H+ exchanger, the Na+-HCO3- cotransporter and vacuolar-ATPase were the main acid extruders, while the Cl--HCO3- anion exchanger and the Cl--OH- exchanger were the main acid loaders. Moreover, the pHi and acid-extruding mechanism were decreased during the loss of pluripotency in hiPSCs. pHi regulators represent an attractive target for differentiation efficiency or culture quality.

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**INTRODUCTION**

The homeostasis of intracellular pH (pHi) affects many cellular functions, including cell proliferation, apoptosis, differentiation and epigenetic characteristics[1-7]. The pHi in mammalian cells is maintained within an optimal narrow range through the combined operation of transmembrane transporters and the intracellular buffering capacity. Thus far, pHi control in mammalian cells has been divided into the following categories: (1) intracellular buffering; (2) acid extrusion systems; (3) acid loading systems; and (4) monocarboxylate-H+ transport[7-11]. Intracellular buffering power (β) minimizes immediate changes in pHi, either in an acidic or alkaline direction. The total intracellular buffering power (βtot) has two components as follows: the intrinsic buffering power of the cell (βi) caused by physicochemical buffers, such as weak acid/base moieties of cytoplasmic proteins, and the buffering capacity caused by intracellular CO2/HCO3- (βCO2)[10]. Furthermore, different ion transporters are involved in the active pHi regulatory mechanism. Acid-equivalent extruders, the Na+-H+ exchanger (NHE), the Na+-HCO3- cotransporter (NBC) and vacuolar-ATPase (V-ATPase) are the main active acid extruders that are activated against intracellular acidification[7-9,12,13]. In contrast, the acid-equivalent loaders, such as the Cl--HCO3- anion exchanger (AE) and the Cl--OH- exchanger (CHE), are activated to prevent intracellular alkalization[12,14,15]. In addition to acid extruders and acid loaders, there is also an H+-monocarboxylate transporter (MCT), which is very important for all mammalian cells because the metabolism and transport of lactate is essential for metabolism and function under physiological or pathological conditions, such as in tumors or hypoxic conditions. The MCT has been demonstrated to play a role either as an acid extruder or an acid loader, depending on the concentration gradient of monocarboxylates, such as lactate acid and pyruvate, between the intracellular and extracellular environments[16,17]. The MCT carrier is stereoselective for L-lactate over D- lactate and has a stoichiometry of 1 H+ with 1 lactate- anion[18,19].

Recently, the dysregulation of pHi has been found to be a commonly adaptive feature in different types of cancer cells[20]. In normally differentiated adult cells, the pHi and extracellular pH (pHe) are generally approximately 7.2 and 7.4, respectively[8,9,13,21]. However, a reversed pH gradient of pHi ≥ 7.2 and pHe ≤ 7.1 has been demonstrated in cancer cells. This reversed pH gradient is caused the overexpression and increased set-point of the acid extrusion mechanism[12,20-23]. This dysregulated pHi feature further promotes tumor progression, invasion and metastasis[21,24-26]. Indeed, metabolic changes have been reported to be a substantial hallmark of cancer cells[27]. Both in the absence or presence of oxygen, cancer cells tend to shift their metabolism from aerobic phosphorylation to aerobic glycolysis, which is known as the Warburg effect. However, the glycolytic byproducts lactate and H+ increase during aerobic glycolysis. Therefore, intracellular acid extruders, such as NHE and MCT, are activated to maintain pHi homeostasis. The overactivation and/or overexpression of the acid extrusion mechanism results in an increased pHi that further promotes proliferation and prevents apoptosis in cancer cells[24,26,28,29]. Furthermore, accompanying extracellular acidification causes restructuring of the extracellular matrix and further promotes malicious metastasis and invasion[26,30,31].

Human induced pluripotent stem cells (hiPSCs), which are reprogrammed from somatic cells by expressing pluripotent transcription factors, are defined by their ability for self-renewal and differentiation into the three germ layers[32]. Pluripotent stem cells (PSCs) shared many similar properties with cancer cells, such as increased glycolysis, proliferation and adaptation to hypoxia[33-35]. Therefore, it has been proposed that the pHi regulatory mechanism in hiPSCs is not typical compared to that in most adult cells. Indeed, a few studies have indicated that changes in pHi affect the fate of stem cell differentiation. Decreased pHi, either by a deficiency or the inhibition of NHE1, has been found in retinoic acid-induced neuronal differentiation in mouse embryonal carcinoma cells. A similar phenomenon has been claimed to contribute to osteogenesis in human umbilical cord-derived mesenchymal stem cells[1,36]. Furthermore, overexpressed NHE1 has been shown to increase cardiomyocyte differentiation in mouse embryonic stem cells (mESCs)[6]. A recent study has reported that a decreased pHi by knocking out or inhibiting NHE obstructed drosophila follicle stem cell differentiation and delayed the loss of pluripotency during spontaneous differentiation induced by the removal of LIF/2i[6]. Therefore, an elevated pHi is considered necessary for PSCs to differentiate. Furthermore, another study has shown that acidic culture medium, caused by the accumulation of lactic acid from glycolysis, promotes pluripotency in both mESCs and hESCs through several mechanisms. However, studies that have optimized the culture environment showed that although acidic culture medium (pH < 7.0) promotes the retention of OCT-4 and pluripotency, it also causes significant growth arrest and an apoptotic effect in mESCs[37]. Notably, although decreasing pHi has been shown to retain pluripotency during differentiation, the resting pHi level in the pluripotent state is maintained at pHi about 7.4 and is greater than that in differentiated adult cells[6]. Therefore, these recent results implicate that PSCs might share a cancer-like pHi regulatory mechanism and consequently create a reversed pH gradient to promote pluripotent properties. However, there is a lack of reports on the correlation between the pHi regulatory mechanism and pluripotency in hiPSCs.

Because of the importance of pHi regulation in hiPSCs, the aims of this study are to further investigate the underlying mechanisms of pHi regulation in hiPSCs. To determine transporter-mediated membrane fluxes of acid equivalents from measurements of pHi, an accurate knowledge of intracellular buffering power is essential. Therefore, the first aim of this study is to estimate βi and βCO2, and the second aim is to characterize the active pHi regulators in hiPSCs to provide the molecular and functional targets of pHi regulators for future applications in clinics. Finally, the correlation between the pHi regulatory mechanism and hiPSC pluripotency was examined in this study.

**MATERIALS AND METHODS**

***Cell culture***

The hiPSCs (HPS0077) were a kind gift from Dr. N.Z. Dai (TSGH-IRB No: 100-05-251) form the Tri-Service General Hospital, Taipei, Taiwan. In this study, vitronectin was used to support the growth and adhesion of HPS0077 cells. To prepare the vitronectin-coated culture plate, 100 μL vitronectin (500 μg/mL) was directly added and mixed into cold DPBS. The vitronectin-DPBS solution was then added into the culture plate at a final concentration of 0.5 μg/cm2 and incubated at room temperature for at least 2 h. This vitronectin-coated culture plate could be used immediately or stored at 4 °C for later use within 2 wk. To maintain pluripotency, HPS0077 cells were continuously cultured with mTeSR1 or mTeSR-E8 medium. When the cell colonies were grown to a sufficient size, Accutase was added to the cells at 37 °C for 3 min to suspend the cells. The cell suspension was centrifuged at 1000 rpm for 3 min, and the collected cell pellet was resuspended in fresh medium. The vitronectin solution was aspirated, and the cells were seeded in a suitable ratio with mTeSR1 or mTeSR-E8 medium containing 10 μmol/L Y-27632. The Y-27632-containing culture medium was replaced with Y-27632-free medium after 24 h, and the medium was subsequently changed every day. To induce the loss of pluripotency, the mTeSR1 or mTeSR-E8 medium was replaced by mTeSR-E6 medium for 1 to 4 d, and the medium was changed once every two days.

***Immunocytochemistry staining and immunoblotting***

For immunocytochemistry staining, a pluripotent stem cell 4-marker immunocytochemistry kit (Invitrogen), including primary antibodies against OCT4, SSEA4, SOX2 and TRA-1-60, was used to evaluate the pluripotency. Briefly, the experimental procedure was performed according to the manufacturer’s instructions. For immunoblotting, whole cell lysates were prepared using RIPA lysis buffer containing 1% protease, 1% phosphatase, and 0.1% Triton X. The supernatant was collected after centrifugation at 12000 rpm for 30 min at 4 °C. A total of 40 μg of total protein per sample was subjected to 10% PAGE and transferred to a PVDF membrane and subsequently blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membranes were then incubated overnight with primary antibodies of different pHi regulators and an internal control at 4 °C. Then, the membranes were washed three times in TBST to remove the unbound primary antibodies and the secondary antibody was then added and incubated for 60 min at room temperature. The membranes were washed three times in TBST, and chemiluminescence was detected using a ClarityTM Western ECL substrate.

***Measurement of intracellular pH***

The measurement of the pHi has been described in detail in our previous reports[12]. Briefly, to measure the change in pHi, HPS0077 hiPSCs were analyzed by microspectrofluorimetry with a pH-sensitive fluorescent dye, BCECF-AM. When cell colonies (on a 24 mm round coverslip) were grown to a sufficient size, cells were then incubated with BCECF-AM (diluted to 6.25 μg/mL with standard HEPES solution) for 1 hour at room temperature. Then, the coverslip containing the cells was moved to an inverted fluorescence microscope and excited with light at wavelengths of 490 and 440 nm. The change in the BCECF emission ratio of the 530 nm wavelength emission at a 490 and 440 nm excitation (490/440) was detected and indicated the change in pHi. A high potassium/nigericin calibration method was used to convert the emission ratio to the pHi value.

When the pHi was measured using a Synergy 2 Multi-Mode Reader, the cells were seeded on 24-well culture plates. The solution was replaced with a pipette instead of a perfusion system (including a peristaltic pump and suction). The experimental procedure is similar to microspectrofluorimetry, and the details are described in our previous study[23].

***Weak acid/base prepulse technique***

NH4Cl and Na-acetate prepulse techniques were used to induce intracellular acidification and alkalization, respectively, and the subsequent recovery from induced acidification and alkalization represent the activity of the acid extruder(s) and acid loader(s), respectively[12]. Taking NH4Cl prepulse as an example, it can be described by 4 phases, as shown in Figure 2A. Cells were first perfused with 20 mmol/L NH4Cl for 5 min, which caused an initial rapid alkalization. This mechanism is simply caused by the small molecular weight and nonpolar [NH3]e easily crossing the cell membrane and acquiring hydrogen in the cytosol to produce NH4+ (phase 1: rapid alkalization, NH3 + H+→NH4+). Then, the pHi slowly recovered and stabilized through the activation of acid loaders, such as AE and CHE (phase 2: slow recovery). The removal of NH4Cl caused rapid intracellular acidification because [NH3]i rapidly effluxed and further produced hydrogen from [NH4+]i in the cytosol (phase 3: rapid acidification, NH4+→NH3 + H+). The subsequent pHi recovery following NH4Cl-induced intracellular acidification is due to the activation of acid extruders, such as NHE and NBC, and this recovery slope represents the function of acid extruders (phase 4: pHi recovery). To accurately quantify the H+ flux through pHi regulators, all pHi recovery rate data was converted to the JH (pHi recovery rate multiplied by buffering power)[10].

***Measurement of intracellular buffering power to derive the net influx or the net efflux***

After the loading of BCECF-AM, cells were sequentially perfused with Na+/Cl--free HEPES or 5% CO2/HCO3--buffered solution (the details of the composition of the solutions are listed in the *Solution* section below) containing different concentrations of (NH4)2SO4 (40, 20, 10, 5, 2.5 and 0 mmol/L). Perfusion with (NH4)2SO4 induced an initial intracellular alkalization, and the subsequent removal of (NH4)2SO4 or decrease in (NH4)2SO4 concentration caused acidification. The buffering power is defined as the ability to resist the change in pHi induced by the impact of hydrogen, *i.e.*, (NH4)2SO4. Therefore, if the buffering power is stronger, the change in pHi will be smaller. The buffering power can be defined by the following equation[38]:

β(mM) = [H+]i/∆pHi, (e.1)

where [H+]i is the change in the concentration of intracellular protons, and ∆pHi is the resulting change in pHi.

For experiments with the NH4Cl prepulse technique, the application of (NH4)2SO4 externally induces intracellular alkalosis. This is due to the rapid diffusion of NH3 into the cell and its subsequent hydrogenation to form NH4+. Upon the removal of extracellular (NH4)2SO4, NH4+ exits the cell as uncharged NH3, leaving behind an equal concentration of H+ and causing intracellular acidosis. If [H+]i is assumed to equal the intracellular concentration of NH4+ at the moment of their removal from the external solution, then equation 1 can be expressed as follows:

β(mM) = [NH4+]i/∆pHi. (e.2)

According to the Henderson-Hasselbalch equation, the relationship between internal and external NH4+ concentration is as follows:

pHo-pHi = log([NH4+]i / [NH4+]o). (e.3)

Equation 3 can then be rearranged as follows:

[NH4+]i = [NH4+]o × 10(pHo-pHi). (e.4)

In the extracellular solution, pHo = pKa + log ([NH3]o/[NH4+]o) (Henderson-Hasselbalch equation). Therefore, this equation can be rearranged as follows:

[NH4+]o = C/(10(pHo-pK) +1), (e.5)

where C is the total extracellular concentration of NH4+ and pK is the dissociation constant of (NH4)2SO4. Combining equations 4 and 5, we can derive [NH4+]i at a given pHi as follows:

[NH4+]i = [C/(10(pHo-pK) +1)] × 10(pHo-pHi). (e.6)

In an open system, the theoretical βCO2 can be calculated as follows:

βCO2 = 2.3 × [HCO3-]i. (e.7)

Similar to the calculation procedures outlined above for NH4+, [HCO3-]i can then be calculated as follows:

[HCO3-]i = [C/(10(pK- pHo) +1)] × 10(pHi-pHo). (e.8)

***Solutions and chemicals***

Nigericin calibration solution was composed of 140 mmol/L KCl, 1 mmol/L MgCl2, 0.01 mmol/L nigericin and 10 mmol/L buffer (MES, HEPES or CAPSO), and the pH was adjusted to 5.5, 6.5, 7.0, 7.5, 8.5 or 9.5 with 6 mol/L NaOH. The buffers used in the calibration solution were in accordance with the pKa of the buffers and the pH of the solution (MES was used for pH = 5.5 and 6.5; HEPES was used for pH = 7.0, 7.5 and 8.5; and CAPSO was used for pH = 9.5).

Standard HEPES-buffered solution was composed of 140 mmol/L NaCl, 4.5 mmol/L KCl, 1 mmol/L MgCl2, 2.5 mmol/L CaCl2, 11 mmol/L glucose, and 20 mmol/L HEPES. Standard bicarbonate-buffered Tyrode’s solution (equilibrated with 5% CO2/22 mmol/L HCO3-) was the same as above, except that the NaCl concentration was reduced to 117 mmol/L, and 22 mmol/L NaHCO3 was added instead of HEPES (pH 7.40 at 37 °C).

**Ion-substituted solutions:** For Na+-free HEPES-buffered Tyrode’s solution, NaCl was replaced with 140 mmol/L N-methyl-D-glucamine (NMDG), and the pH was adjusted to 7.4 with HCl. For Cl--free CO2/HCO3--buffered Tyrode’s solution contained 117 mmol/L sodium gluconate, 4.5 mmol/L potassium gluconate, 12 mmol/L calcium gluconate, 22 mmol/L NaHCO3, 1 mmol/L MgSO4, and 11 mmol/L glucose. The Na+/Cl--free solution (for the buffering power experiment) was composed of 140 mmol/L NMDG, 4.5 mmol/L K-gluconate, 1 mmol/L Mg-gluconate, 2.5 mmol/L Ca-gluconate, 11 mmol/L glucose and 20 mmol/L HEPES (for 5% CO2/HCO3--free system) or bubbled with 5% CO2 (for 5% CO2/HCO3- system). The pH was adjusted to 7.4 with 6 mol/L NaOH, HCl or H2SO4 at 37 °C for all solutions. NH4Cl, Na-acetate and (NH4)2SO4 were directly added as solids to the buffered solutions before use. HOE 694 (HOE, a NHE1 specific inhibitor), S0859 (an NBC-specific inhibitor), bafilomycin A1 (Ba, a V-type ATPase-specific inhibitor) and SCH-28080 (SCH, a KHE-specific inhibitor) were added as stocks to solutions shortly before use. All drugs mentioned above were obtained from Sigma-Aldrich.

***Statistical analysis***

The data were expressed as the mean ± SE of *n* preparations. The statistical significance was analyzed using one-way or two-way ANOVA followed by Tukey's or Dunnett's multiple comparisons with GraphPad Prism 6 software, respectively. A *p*-value less than 0.05 were regarded as statistically significant.

**RESULTS**

***In situ calibration of BCECF and the detection of hiPSC pluripotency markers***

To monitor the change in pHi, an *in situ* calibration was conducted in hiPSCs. A high potassium/nigericin calibration method was used to convert the emission ratio to the pHi value. Briefly, BCECF-loaded cells were perfused with six different nigericin calibration solutions with different pH levels (5.5-9.5) (the details of the composition of the six nigericin calibration solutions are listed above in the solutions section) that caused the pHi to equal the pHe, as shown in Figure 1A. The calibration equation was obtained from ten similar experiments and a nonlinear BCECF fluorescence-pHi curve of function, as shown below and in Figure 1B. The following equation was used to convert the fluorescence ratio into pHi:

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where R is the ratio of the 530 nm fluorescence emission at 490 nm and 440 nm excitation (490/400), and F is the fluorescence value at 490 nm and 440 nm excitation. The maximum and minimum ratios (Rmax and Rmin) of 530/490 and 530/440 (Em/Ex) were obtained from perfusion with pH 9.5 and 5.5 calibration solutions, respectively.

Because the HPS0077 cell line was used as a representative example of hiPSCs in this study, we first examined whether pluripotency markers, such as OCT4, SOX2, SSEA-4 and TRA-1-60, are present in HPS0077 cells. As shown in Figure 1C, the four pluripotency markers were clearly identified by immunofluorescence staining and labeling. Our results support the hypothesis that the HPS0077 cell line possesses the characteristics of hiPSCs and is suitable as the subject for this study.

***Functional characterization of acid extruders in HEPES buffered system***

To investigate whether there is an acid extrusion mechanism in the cultured hiPSCs, the cells were first perfused in HEPES-buffered solution (CO2/HCO3--free). As shown in Figure 2A, a pHi recovery slope following NH4Cl prepulse-induced intracellular acidification was a typical trace for the control (*n =* 3). Either removal of the extracellular Na+ (*n =* 3) or application of 30 μmol/L HOE 694 (H30, *n =* 4) significantly inhibited the pHi recovery rate, as shown in Figures 2B and 2C, respectively, which demonstrates the presence of Na+-dependent acid extruder(s) and NHE1 in HPS0077 cells.

However, besides Na+-dependent acid extruders, there is another acid extrusion mechanism responsible for the remaining acid extrusion in HEPES solution. Therefore, to further investigate whether the remaining Na+-independent pHi recovery (*i.e.*, could not be inhibited by Na+-free solution) is caused by the vacuolar-type ATPase (V-ATPase), HPS0077 cells were perfused with an Na+-free solution pulse with 30 μmol/L bafilomycin A1 (Ba30; V-ATPase-specific inhibitor, *n =* 3), as shown in Figure 2D. However, either no significant inhibition or slight inhibition of pHi recovery was observed between the Na+-free solution group and the Na+-free solution + Ba30 group (Figures 2B and 2D, respectively). These results suggest that V-ATPase does not play a role in acid extrusion to the cytosol in hiPSCs. Experimental data similar to those shown in Figures 2A-D were summarized and plotted as a function of the pHi recovery rate *vs* pHi in Figure 2E. As shown in Figure 2E, in HEPES solution (*i.e.*, when HCO3--dependent acid extruder(s) were not activated), the acid extrusion mechanism was mainly attributed to NHE1 (the difference between the trace of the Na+-free group and the trace of the H30 group), apart from other Na+-dependent acid extruder(s) (the difference between the trace of H30 and the trace of Na+-free). Moreover, the other Na+-independent acid extruder(s) were activated when the pHi was less than 7.1 ± 0.01 (see the trace of Na+-free + Ba30).

To further examine whether the Na+-independent acid extruders shown in Figure 2E are KHE or Cl--dependent acid extruder(s), HPS0077 cells were either perfused by adding 40 μmol/L SCH-28080 (SCH40, a KHE-specific inhibitor) or removing [Cl-]o. The change in pHi in this series of experiments was detected using a Synergy 2 Multi-Mode Reader with BCECF-AM dye. The data for this series of experiments were summarized and plotted as a function of the pHi recovery rate *vs* pHi in Figure 2F. As shown in Figure 2F, the pHi recovery rate between the trace before and after adding SCH40 (*n =* 3, solid circles and squares, respectively) was not significantly different. Moreover, the removal of [Cl-] (*n =* 3, solid triangles) surprisingly caused a dramatic increase in the pHi recovery rate instead of inhibition. This phenomenon is most likely caused by the inhibition of the activity of the Cl--dependent acid loader. In summary, these results provide clear pharmacological evidence that the NHE is mainly responsible for acid extrusion and functionally coexists with other Na+-dependent and -independent acid extrusion mechanisms in HPS0077 cells. Moreover, the Na+-independent acid extrusion mechanism is neither a KHE nor a Cl--dependent acid extruder(s).

***Functional characterization of acid extruders in a 5% CO2/HCO3--buffered system***

To quantify the [Hi]+ flux through pHi regulators in 5% CO2/HCO3--buffered conditions, we first quantified intracellular buffering (β). The experimental details are shown in the materials and methods section, and we found that β increased as pHi increased at pHi = 7.0 to 8.0 (*n =* 35, data not shown). The equation can be expressed as β = 107.79 (pHi)2 - 1522.2 (pHi) + 5396.9 (correlation coefficient R2= 0.85). The obtained β can be used to calculate the [Hi]+ flux through pHi regulators by the following equation: JH = β × pHi recovery rate (pHi value/minutes). To further investigate whether the NBC is functionally involved in the 5% CO2/HCO3- condition, we used a protocol similar to the previously mentioned experiments except for the replacement of HEPES-buffered solution with 5% CO2/HCO3--buffered solution. The pHi recovery slope following NH4Cl prepulse-induced intracellular acidification in 5% CO2/HCO3--buffered solution was a typical trace for the control (*n =* 7), as shown in Figure 3A. As shown in Figures 3B-E, the pHi recovery rate was significantly inhibited under four different conditions as follows: removal of [Na+] (*n =* 3, addition of H30 (*n =* 4), addition of 50 μmol/L S0859 (S90; an inhibitor of NBC, *n =* 3), and addition of H30 and S90 (H30 + S50, *n =* 3). Experimental data similar to those shown in Figures 3A-E were summarized and plotted as a function of JH *vs* pHi in Figure 3F. As shown in Figure 3F, a similar pHi recovery rate between Na+-free and H30 + S50 conditions indicated that the NHE1 and the NBC were both involved in the Na+-dependent acid extrusion mechanism in the 5% CO2/HCO3- condition in HPS0077 cells.

Notably, the acid extrusion mechanism in the 5% CO2/HCO3- condition was regulated mainly by the NBC in the pHi range of 7.50-7.68 because the pHi recovery rate could be completely inhibited by S50 (Figure 3D). Moreover, the addition of S50 did not affect pHi recovery when the pHi was less than 6.9 ± 0.01 (*n =* 3, see the trace of S50), which indicated that the NBC was not responsible for acid extrusion in the relatively acidic cytoplasm (Figure 3D). In summary, NHE1, NBC and Na+-independent acid extruder(s) were mainly functionally activated in the pHi ranges of < 7.5, 6.9-7.68 and < 7.1, respectively.

***Functional characterization of acid loaders***

The homeostasis of pHi is coregulated by both acid extruders and acid loaders. The CHE and the AE are two known acid loaders in mammalian cells. Unlike the NHE and the NBC, the acid loading mechanism depends on [Cl-]o and further exchange of [OH-]i or [HCO3-]i into the cytoplasm to neutralize intracellular alkalization. To estimate the function of acid loaders, an Na-acetate prepulse was used to induce intracellular alkalization in this study. The subsequent pHi recovery slope was expressed as the acid loading activity of acid loaders. Figures 4A and 4C show the typical pHi recovery slope following the Na-acetate prepulse either in HEPES or HCO3--buffered solution, respectively (*n =* 3). Removal of [Cl-]o in the 5% CO2/HCO3--buffered solution completely inhibited the pHi recovery (*n =* 3), as shown in Figure 4D, which indicated that the acid loading mechanism is completely Cl--dependent in HPS0077 cells. However, interestingly, a rapid acid loading phenomenon was observed before the total inhibition at pHi = 7.9 ± 0.01 (*n =* 3) in HEPES solution, as shown in Figure 4B. These results indicated that CO2 or HCO3- may inhibit this unknown Cl--dependent acid loader(s), but the characterization requires further studies. Due to the lack of specific inhibitors of the CHE and the AE, according to previous studies on the acid loading mechanism in mammalian cells conducted by Vaughan-Jones *et al*[39], we speculate that the Cl--dependent acid loading mechanism is mainly attributed to the CHE and the AE[39]. Notably, as shown in Figure 4E, the pHi recovery rate is nearly identical between the 5% CO2/HCO3- system and the HEPES system, which indicates that the CHE (solid circles) plays a more important role than the AE (solid squares) in the acid loading mechanism in HPS0077 cells.

***Decrease in pHi during the loss of pluripotency: molecular and functional evidence***

Our previously mentioned results showed that the acid extruders NHE and NBC mainly functionally coexist in hiPSCs.

We further investigated the dynamic changes in pHi during the loss of pluripotency in hiPSCs. In the pluripotent state, the resting pHi observed from the pHi completely recovered after NH4Cl prepulse-induced intracellular acidification was found to be 7.5 ± 0.01 (*n =* 20) and 7.68 ± 0.01 (*n =* 20) in HEPES and 5% CO2/HCO3- conditions, respectively, as shown in Figures 5A and 5B. Moreover, in 5% CO2/HCO3--buffered solution, as expected, the resting pHi shifted to 7.46 ± 0.02 (*n =* 5) and 7.66 ± 0.02 (*n =* 5) after adding S0859 (S50) and HOE694 (H30), respectively (Figure 5B, the data were collected from the data shown in Figures 3C and 3D). Notably, there was no significant difference between the resting pHi in HEPES and in the 5% CO2/HCO3- plus S90 conditions, which indicates that the set-point of NHE activation is pHi = 7.5. In the 5% CO2/HCO3- condition, the resting pHi showed no significant difference between the untreated and H30-treated conditions, which indicates that the pHi is regulated by the NBC instead of the NHE in the pHi range of 7.50-7.68.

To further induce the loss of pluripotency, HPS0077 cells were first transferred from mTeSR1 media (designed for maintaining long-term pluripotency) to mTeSR-E8 medium (containing fibroblast growth factor 2, FGF2, and transforming growth factor β1, TGFβ1) and then subsequently replaced with mTeSR-E6 medium (without FGF2 and TGFβ1) for 1 to 4 days (E6-1d to 4d) to induce the loss of pluripotency. Notably, the expression of the pluripotency marker OCT4 was significantly decreased after culture in mTeSR-E6 medium, as shown in Figure 5C. We also found that the expression of NHE1, NHE3, V-ATPase, NBCe1 and NBCe2 decreased during the loss of pluripotency, while the expression of NBCn1 did not decrease, as shown in Figure 5C.

To further investigate the role of the NHE and the NBC on the loss of pluripotency, we detected the pHi recovery rate following NH4Cl prepulse-induced intracellular acidification. The pHi recovery traces in different culture mediums, *i.e.*, E8, E6-1d, E6-2d, E6-3d and E6-4d in HEPES and 5% CO2/HCO3--buffered solution are shown in Figures 6A and 6D, respectively. The graphs in Figure 6B show the pHi recovery rate in E6-1d to E6-4d normalized from the E8 condition (% of E8) in HEPES, estimated at pHi = 6.9 and 7.2, respectively, and averaged for 3 experiments similar to that shown in Figure 6A. The NHE is mainly responsible for acid extrusion in the HEPES condition. When the pHi recovery rate was measured at pHi = 6.9, E6-1d showed no significant change, while E6-2d, E6-3d and E6-4d significantly decreased by 76.3%, 60.6% and 51.7%, respectively (*n =* 3). When the pHi recovery rate was measured at pHi = 7.2, the pHi recovery rates of E6-1d, E6-2d, E6-3d and E6-4d significantly decreased by 82.7%, 67.4%, 47.6% and 16.3%, respectively (*n =* 3). The max/min charts in Figure 6C show the resting pHi in E8, E6-1d, E6-2d, E6-3d and E6-4d, respectively, averaged from similar experiments as shown in Figure 6A (*n* = 5-20). The resting pHi decreased from 7.5 to 7.49, 7.4, 7.28 and 7.21 in E6-1d, E6-2d, E6-3d and E6-4d, respectively (*n* = 5 to 20).

The graphs shown in Figure 6E show the pHi recovery rate in E6-1d to E6-4d normalized to E8 (control) in 5% CO2/HCO3-buffered solution, which was estimated at pHi = 6.9, 7.2 and 7.5, respectively, and averaged for 3 experiments similar to that shown in Figure 6D. As shown in Figure 6E, in the 5% CO2/HCO3- condition (*i.e.*, where the NHE and the NBC were both involved in the acid extrusion mechanism), the pHi recovery rate measured at pHi = 6.9 and 7.2 showed no significant difference between E8 and E6-1d, but it was significantly decreased by 85.2% when measured at pHi = 7.5. The pHi recovery rate for E6-2d, E6-3d and E6-4d was significantly decreased by 88.7, 74.9 and 61%, respectively, when measured at pHi = 6.9, decreased by 82%, 77.2% and 51.5%, respectively, when measured at pHi = 7.2, and decreased by 53.4%, 44.8% and 22.3%, respectively, when measured at pHi = 7.5 (*n* = 3). The max/min charts shown in Figure 6F show the resting pHi in E8, E6-1d, E6-2d, E6-3d and E6-4d, averaged from similar experiments as those shown in Figure 6D (*n =* 5-20). We found that the resting pHi decreased from 7.68 to 7.64, 7.61, 7.56 and 7.48 in E6-1d, E6-2d, E6-3d and E6-4d, respectively (*n =* 5, Figure 6F). In summary, our results provide clear evidence that the loss of hiPSC pluripotency decreased the activity and expression of acid extruders (NHE and NBC), further resulting in a decrease in the pHi recovery rate and resting pHi.

**DISCUSSION**

***The functional and molecular evidence of active transmembrane acid extruders and acid loaders in hiPSCs***

In this study, we have clearly demonstrated that transmembrane active pHi regulators, such as NHE1, NBC, AE and CHE, functionally coexisted in hiPSCs (Figures 3 and 4). Moreover, we successfully quantified the net acid efflux of each functional acid transporter, as shown in Figures 3 and 7, by considering intracellular buffering. From Figure 3F, we can clearly observe that the active efflux was mainly dependent on the activity of the NBC in hiPSCs in the pHi range less than 7.35 because the S90 group (*i.e.*, inhibiting NBC activity) substantially decreased the activity compared to other groups (inhibiting NHE1 or other Na-independent acid extruders). Moreover, the role of NHE1 on acid extrusion decreased as the pHi increased (Figures 2, 3 and 7). Notably, the activity of NHE1 was nominally undetectable when the pHi was greater than 7.50, as shown in Figures 2, 3 and 7.

Relevant molecular candidates for the NBC include at least five members of the slc4 family, including 2 electrogenic Na+-HCO3- cotransporters (NBCe1/SLC4A4 and NBCe2/SLC4A5), 1 electroneutral Na+-HCO3- cotransporter (NBCn1/SLC4A7) and 2 Na+-dependent Cl--HCO3- exchangers (NCBE/SLC4A10 and NDCBE/SLC4A8)[7,40,41]. In this study, we found that three isoforms of the NBC, NBCn1, NBCe1 and NBCe2, coexist in hiPSCs, which is similar to our previously reported results in cultured human renal artery smooth muscle cells[7]. However, the Aalkjaer group has demonstrated that the NBC is NBCn1, *i.e.*, it is electroneutral, in rat and mouse smooth muscle cells[42], which is similar to the results reported in guinea pig myocytes by the Vaughan-Jones group[10]. In other words, the coexistence of 3 types of NBCs in hiPSCs is different from the results in mouse and rat models (c.f. Aalkjaer’s group) and guinea pig models (c.f. Vaughan-Jones’s group, which is likely due to differences in species/organs.

Moreover, in contrast to the results reported in our previous studies in cardiovascular cells, we found that Na+-independent acid extruder(s) and Cl--independent acid loader(s) were substantially present for acid extrusion (pHi < 7.1) and acid loading (pHi > 7.9) in hiPSCs (see Figures 2, 4 and 7). We further demonstrated that the unknown Na+-independent acid extruder(s) is not the V-ATPase, KHE[43] or Cl--dependent acid extruder (localized on lysosome and gastric cell membranes)[44,45] (Figures 2D and 2F). Therefore, we hypothesize that this unknown Na+-independent mechanism is most likely an ATP-dependent transporter instead of a concentration gradient-driven transporter. For example, ATP deficiency, induced by the addition of oligomycin, combined with the addition of bafilomycin A1 during the perfusion experiments would allow us to observe whether it inhibits Na+/V-ATPase-independent acid extrusion in hiPSCs[46]. However, functional and molecular characterization requires further studies in the future.

In addition to being an acid extruder, NBCe1 has been reported to be responsible for the acid loading mechanism during the process of changing from the HEPES-buffered solution to the 5% CO2/HCO3--buffered solution in mouse astrocytes[47]. However, in our findings, the addition of 50 μM S0859 still failed to inhibit the Cl--independent acid extrusion mechanism in the HEPES-buffered condition (data not shown). This result suggested that the Cl--independent acid extruder(s) was not NBCe1 in hiPSCs. Due to this unknown Cl--independent acid extrusion mechanism being completely inhibited in the CO2/HCO3--buffered system and the lack of related studies, future works should further characterize the possible existence of a CO2-related pHi acid loading mechanism.

***The implication of the existence of extra acid extrusion/loading mechanisms in hiPSCs***

The existence of an unknown acid extrusion mechanism, *i.e.*, Na+-independent acid extruder(s) (see Figure 3F) and acid loading mechanisms, *i.e.*, Cl--independent acid loader(s), in hiPSCs might imply that the ability to resist the acid/base impact is very important for the pluripotency of hiPSCs[37,48,49]. It has been reported that hiPSCs share many cellular properties with cancer cells, such as increased cell proliferation and dependence on glycolysis for metabolism[24,26,50,51]. Many studies showed that a lower pHi decreased proliferation and energy production in either normal or cancer cells[15,26]. Indeed, in this study, we found that the acid extrusion mechanism was fully activated at an acidic pHi (< 7.2), including the NHE, the NBC and an unknown Na+-independent acid extruder(s), in hiPSCs. As expected, the resting pHi in hiPSCs was found to be 7.5 and 7.68 in the HEPES and 5% CO2/HCO3- conditions, respectively, and was relatively higher than that of normal differentiated adult cells (resting pHi = 6.9-7.2), such as cardiovascular cells and tissues demonstrated in our previous studies[7,13,52,53]. In cancer, the reversal of the intracellular/extracellular pH (pHi/pHe) gradient (alkaline pHi and acidic pHe) is a common feature and further promotes carcinogenesis. The reason for the gradient reversal is that cancer cells overexpress and upregulate the set-point of acid extruders[24-26]. Therefore, it is likely that hPSCs may upregulate the acid extrusion mechanism to adapt to cancer-like cellular properties. Some studies showed that, in addition to hiPSC growth being inhibited by an acidic culture environment, the alkalization of culture medium significantly decreases the cell growth rate and expression of pluripotency markers at a minimum pHe = 7.8[37,48,49]. The proliferative ability and pluripotency in hPSCs are critical for development[54]. Therefore, the expression of additional unknown Na--independent and Cl--independent acid-regulating extruder(s) in hiPSCs implicates the function of resisting the potential impact of intracellular proton changes in hPSCs. However, further study on characterizing the mechanisms should be conducted in the future.

***Decreases in acid extrusion activity during the loss of pluripotency in hiPSCs***

A previous study showed that during the early spontaneous differentiation of mESCs, the resting pHi significantly decreased at 48 and 72 h and returned to baseline at 96 h, and this decrease was dependent on the loss of NHE1 function[6]. However, in this study, the decrease in resting pHi and the downregulation of the acid extrusion mechanism were demonstrated during the early loss of pluripotency in hiPSCs either in HEPES-buffered conditions or in 5% CO2/HCO3--buffered conditions. These contradictory results may be due to the different pluripotent states between mESCs and hiPSCs, *i.e.*, naïve and primed pluripotency, respectively[54, 55]. As expected, the cells in the preprimed (naïve) and primed states significantly increased at 48 and 72 hours during early differentiation in mESCs. This result implies that increasing resting pHi occurred during the naïve to primed pluripotency states[6]. Subsequently, the resting pHi returned to baseline at 72-96 h, which may indicate that the primed state is further differentiated. Furthermore, to adapt to the intracellular acidification caused by increased glycolysis, *i.e.*, the Warburg effect, the acid extrusion mechanism is upregulated and further alkalizes the resting pHi in cancer cells[24,26]. During the processes of PSC development, metabolism has been found to rely on different metabolic pathways, *i.e.*, oxidative phosphorylation (OXPHOS), glycolysis and OXPHOS in naive, primed and early differentiation states, respectively[51,56,57]. This switch between OXPHOS and glycolysis supports the dynamic changes in the resting pHi observed during the loss of pluripotency in mESCs and the decrease in the resting pHi and acid extrusion in hiPSCs demonstrated in this study.

The possible underlying mechanism for the observed decrease in the acid extrusion mechanism during the process of the loss of pluripotency in hiPSCs may be due to the crosstalk between the PI3K/AKT and MEK/ERK signaling pathways, which plays a curial role in pluripotency[58]. To maintain pluripotency in hPSCs, FGF2 has been added to the culture medium to activate PI3K/AKT signaling[58,59]. The activation of PI3K/AKT signaling further promotes the relative gene expression of pluripotency markers and inhibits differentiation by suppressing MEK/ERK signaling[58]. Therefore, the removal of FGF2 decreases the ratio of AKT activity to ERK and further causes cell differentiation[58,60]. ERK is a well-known activator of NHE1[61,62], but we did not find that removal of FGF2 (in E6 medium) resulted in an increase of the NHE1-dependent acid extrusion rate in this study. Although AKT has been shown to inhibit NHE1 activity in cardiovascular cells[63], AKT is stimulated by insulin and growth factors and further activates NHE1 in cancer cells and fibroblasts[64,65]. Therefore, this study implicates that the removal of FGF2 causes the loss of AKT activity and thus decreases the acid extrusion rate in hiPSCs.

In conclusion, for the first time, we established a functional pHi regulatory model in hiPSCs, as shown in Figure 7. In this model, we demonstrated that the steady-state pHi value is approximately 7.50-7.68 in hiPSCs. Additionally, we showed that at least four types of acid extruders [NHE, NBC, V-ATPase and Na+-independent acid extruder(s)] and three types of acid loaders (CHE, AE and Cl--independent acid loader(s)) coexist and are responsible for the pHi regulatory mechanism, and each is activated in different pHi ranges in hiPSCs. Moreover, the activity of the acid extrusion mechanism decreased by changing both the expression and activity of acid extruders during the process of the loss of pluripotency in hiPSCs.

**ARTICLE HIGHLIGHTS**

***Research background***

Homeostasis of intracellular pHi (pHi) affects many cellular functions, such as cell proliferation and differentiation. However, the knowledge of pHi regulation mechanism in human pluripotent stem cells still unknown.

***Research motivation***

The changes of acid-base kinetic were observed during the loss of pluripotency in mouse embryonic stem cells. Moreover, the balance of intracellular and extracellular pH significantly affected the reprogramming efficiency and culture quality of human induced pluripotent stem cells (hiPSCs).

***Research objectives***

We aimed to establish the pHi regulation mechanism model and investigate the relationship of pHi regulation and pluripotency in hiPSCs.

***Research methods***

In the pluripotent state and during the loss of pluripotency in hiPSCs, we observed the activity of pHi regulation mechanism by acutely induced intracellular acidification and alkalization in the physiological buffered solution.

***Research results***

In hiPSCs, the Na+-H+ exchanger (NHE), the Na+-HCO3- cotransporter (NBC) and vacuolar-ATPase (V-ATPase) were the main active acid extruders that were activated against intracellular acidification. In contrast, the acid-equivalent loaders, such as the Cl--HCO3- anion exchanger (AE) and the Cl--OH- exchanger (CHE), were activated to prevent intracellular alkalization. In addition to the classic pHi regulators NHE, NBC, V-ATPase, AE and CHE, we also demonstrated the functional existence of unknown acid-extruder(s) and –loader(s) in hiPSCs. Moreover, the pHi and acid-extruding mechanism were decreased during the loss of pluripotency in hiPSCs.

***Research conclusions***

For the first time, we established a model of the pHi regulation mechanism in hiPSCs. The higher resting pHi and acid-extruding mechanism might be the specific feature to adaptive the cancer-like cellular function and pluripotency in hiPSCs.

***Research perspectives***

In summary, we characterized the pHi regulation mechanism and its functional/expressional roles in maintenance of pluripotency of hiPSCs. We proposed that targeting either pHi regulators or pH environments of culture medium could be an effective way to modify the pluripotency state of hiPSCs, which may contribute the differentiation efficiency or culture quality.

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**Figure 1 Calibration of the BCECF fluorescence ratio and pluripotency characterization.** A: The trace showed the protocol of BCECF fluorescence ratio (510 nm emission at 490 nm and 440 nm excitations) calibration in HPS0077 cells. The top bars represent the application of different conditions; B: The plots of pHi *vs* the BCECF fluorescence ratio were collected from 6 similar experiments shown in A; C: Immunofluorescence analysis showed the expression of pluripotency markers, OCT4, SSEA4, TRA-1-60 and SOX2, in HPS0077 cells.

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**Figure 2 Functional characterization of acid extruders in the HEPES-buffered system.** A-D: The top bar shows the buffer system used in perfusion experiments. The application of NH4Cl and different conditions were respectively shown with the solid and dotted lines above the trace. The trace shown in A showed a typical pHi recovery slope after NH4Cl prepulse-induced intracellular acidosis in HEPES-buffered solution as a control. The traces shown in B-D showed the effect of the removal of extracellular Na+ (Na+-free), addition of 30 μmol/L HOE 694 (H30) and Na+-free + 30 μmol/L bafilomycin A1 (Ba30) on the pHi recovery slope. E: The curve of the pHi recovery rates for Na+-free, H30 and Na+-free with Ba30 were collected from 3-6 similar experiments shown in A-D. F: After pre-treatment with NH4Cl for 5 min, HPS0077 cells were treated with Na+-free + Ba30, Na+-free + Ba30 + 40 μmol/L SCH-28080 (SCH40) and Na+/Cl--free + Na+-free + Ba30 in HEPES-buffered solution, and the change in pHi was detected by a multimode reader. Error bars represent the mean ± SE.

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**Figure 3 Functional characterization of acid extruders in the 5% CO2/HCO3--buffered system.** A-E: The trace shown in A showed a typical pHi recovery slope after NH4Cl prepulse-induced intracellular acidosis in HEPES-buffered solution as a control. The traces shown in B-E showed the effect of the removal of extracellular Na+ (Na+-free), addition of 90 μmol/L S0859 (S90), addition of 30 μmol/L HOE 694 (H30) and addition of S90 + H30 on the pHi recovery slope; F: The curve of the pHi recovery rates after the addition of Na+-free, S90, H30 and S90 + H30 were collected from 2-10 similar experiments shown in A-E. Error bars represent the mean ± SE.



**Figure 4 Functional characterization of the acid loader.** A-D: The traces shown in A and C showed typical pHi recovery slopes after Na-acetateprepulse-induced intracellular alkalization in HEPES and 5% CO2/HCO3--buffered solution as a control. The traces shown in B and D showed the effect of the removal of extracellular Cl- (Cl--free) on the pHi recovery slope in HEPES and 5% CO2/HCO3--buffered solution; E: The curve of the pHi recovery rates in HEPES and 5% CO2/HCO3--buffered solution were collected from 3-4 similar experiments shown in A and C. Error bars represent the mean ± SE.



**Figure 5 Steady-state pHi in HEPES and 5% CO2/HCO3--buffered solution and the change in the expression of pHi regulators during the loss of pluripotency in human induced pluripotent stem cells.** A: The resting pHi was a steady-state taken from the completely recovered pHi after intracellular acidification or alkalization. The dotted line indicates the value of the resting pHi; B: The max/min chart of the resting pHi in hiPSCs was collected from A (*n =* 20) and Figures 4C and D (*n =* 5). The means of the resting pHi in HEPES and 5% CO2/HCO3--buffered solution were found to be 7.50 ± 0.01 and 7.68 ± 0.01, respectively. After treatment with H30 and S90 in 5% CO2/HCO3--buffered solution, the resting pHi shifted to 7.66 ± 0.02 and 7.46 ± 0.02, respectively; C: Immunoblot analysis of the expression of NHE1, NHE3, V-ATPase, NBCe1, NBCe2, NBCn1 and OCT4 in hiPSCs in different culture media for different days (E8 and E6-1d to E6-4d). The histograms in B display the mean and the min to max values. hiPSCs: human induced pluripotent stem cells; NHE: the Na+/H+ exchanger; NBC: the Na+/HCO3- cotransporter; V-ATPase: vacuolar-ATPase.

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*P* < 0.01

*P* < 0.0001

*P* < 0.0001

*P* < 0.001

*P* < 0.05

*P* < 0.0001

*P* < 0.0001

*P* < 0.0001

*P* < 0.001

*P* < 0.01

*P* < 0.05

**Figure 6 The change in the activity of the Na+/H+ exchanger and the Na+/HCO3- cotransporter and the resting pHi during the loss of pluripotency in human induced pluripotent stem cells.** A: The traces showed the changes in pHi recovery after NH4Cl prepulse-induced intracellular acidification in E8 medium (containing fibroblast growth factor 2, FGF2, and transforming growth factor β1, TGFβ1) and E6 medium (without FGF2 and TGFβ1) for 1 to 4 d (E6-1d to E6-4d) in HEPES-buffered solution; B: The charts showed the pHi recovery rate in E6-1d to -4d normalized to the rate in E8 (% of E8) in HEPES-buffered solution, which was estimated at pHi = 6.9 and 7.2, respectively, and averaged for 3 experiments similar to that shown in A; C: The max/min plots showed the resting pHi in E8, E6-1d, E6-2d, E6-3d and E6-4d media that were averaged from similar experiments shown in A (*n =* 5-20); D: The traces showed the changes in pHi recovery after NH4Cl prepulse-induced intracellular acidification in E8 and E6-1d to E6-4d media in 5% CO2/HCO3--buffered solution; E: The graphs show the pHi recovery rate in E6-1d to E6-4d normalized to the rate in E8 (control) in 5% CO2/HCO3--buffered solution, which was estimated at pHi = 6.9, 7.2 and 7.5, respectively, and averaged for 3 experiments similar to that shown in D; F: The max/min plots showed the resting pHi in E8 E6-1d, E6-2d, E6-3d and E6-4d media, averaged from similar experiments shown in D (*n =* 5-20). Error bars represent the mean ± SE. The histograms in C and F show the mean and min to max values. ns: no significant difference; hiPSCs: human induced pluripotent stem cells.



**Figure 7 Kinetic model of the pHi regulatory mechanism in human induced pluripotent stem cells.** A: A kinetic model illustrating the pHi regulatory mechanism in HPS0077 cell, including acid extrusion, acid loading and passive buffering power. For the first time, we demonstrated that the active membrane pHi regulators NHE1, NHE3, V-ATPase, NBCe1, NBCe2, NBCn1, AE and CHE functionally coexisted in hiPSCs, and in addition, unknown Na+-independent acid extruder(s) and Cl--independent acid loader(s) were also observed. The length of the triangle indicates the pHi range of pHi regulator activation, and the height indicates the magnitude of the pHi regulatory activity. For example, the NHE, NBC, AE and CHE were activated at pHi ≤ 7.5, between 6.9 and 7.68, ≥ 7.4 and between 7.6 and 8.1, respectively. The non-NHE acid extruders [V-ATPase, unknown Na+-independent acid extruder(s)] and unknown Cl--independent acid loader(s) were activated during extreme intracellular acidification, *i.e.*, pHi < 7.1, and alkalization, *i.e.*, pHi > 7.9, respectively. Moreover, the intracellular passive buffering capacity (β) increased as the pHi shifted to the alkalization direction; B: In the process of the loss of pluripotency, the activity of the acid extrusion mechanism gradually decreased, including the participation of at least the NHE, the NBC and V-ATPase, and resulted in the resting pHi shifting from 7.68 to 7.48. hiPSCs: human induced pluripotent stem cells; NHE: the Na+/H+ exchanger; NBC: the Na+/HCO3- cotransporter; V-ATPase: vacuolar-ATPase; AE: anion exchanger; CHE: Cl--OH- exchanger.