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

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Abstract

AIM
To establish a functional and molecular model of the intracellular pH (pH) 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 pH 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 ΔpH induced by perfusing different concentrations of (NH4+):SO4-2. Western blot techniques and immunocytochemistry staining were used to detect the protein expression of pH regulators and pluripotency markers.

RESULTS
In this study, our results indicated that (1) the steady-state pH 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 (pH)2 - 1522.2 (pH) + 5396.9 (correlation coefficient R2 = 0.85), in the estimated pH 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 pH 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 pH 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 proton 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 pH 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 pH regulatory mechanism and demonstrated its strong positive correlation with hiPSC pluripotency.

Key words: Microspectrofluorimetry; Human induced pluripotent stem cells; Na+/H+ exchanger; Na+/HCO3- cotransporter; Cl-/OH- exchanger; Cl-/HCO3- exchanger; V-ATPase; Intracellular buffering power; Intracellular pH; BCECF

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

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INTRODUCTION

The homeostasis of intracellular pH (pH) affects many cellular functions, including cell proliferation, apoptosis, differentiation and epigenetic characteristics[1-7]. The pH in mammalian cells is maintained within an optimal narrow range through the combined operation of transmembrane transporters and the intracellular buffering capacity. Thus far, pH 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 pH, 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 (β) 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 pH 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[12,14,16]. 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 pH has been found to be a commonly adaptive feature in different types of cancer cells[20]. In normally differentiated adult cells, the pH and extracellular pH (pHe) are generally approximately 7.2 and 7.4, respectively[8,9,13,21]. However, a reversed pH gradient of pH ≥ 7.2 and pH ≤ 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[12,20-22]. This dysregulated pH feature further promotes tumor progression, invasion and metastasis[21,24-36]. Indeed, metabolic changes have been reported to be a substantial hallmark of cancer cells[37]. 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 pH homeostasis. The overactivation and/or overexpression of the acid extrusion mechanism results in an increased pH 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[22]. 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 pH regulatory mechanism in hiPSCs is not typical compared to that in most adult cells. Indeed, a few studies have indicated that changes in pH affect the fate of stem cell differentiation. Decreased pH, either by a deficiency or the inhibition of NHE1, has been found to disturb 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)[36]. A recent study has reported that a decreased pH 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 pH 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[6]. Therefore, these recent results implicate that PSCs might share a cancer-like pH 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 pH regulatory mechanism and pluripotency in hiPSCs.

Because of the importance of pH regulation in hiPSCs, the aims of this study are to further investigate the underlying mechanisms of pH regulation in hiPSCs. To determine transporter-mediated membrane fluxes of acid equivalents from measurements of pHe, an accurate knowledge of intracellular buffering power is essential. Therefore, the first aim of this study is to estimate β and βCO₂, and the second aim is to characterize the active pH regulators in hiPSCs to provide the molecular and functional targets of pH regulators for future applications in clinics. Finally, the correlation between the pH 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) from 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% SDS-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 pH 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 Clarity™ Western ECL substrate.

**Measurement of intracellular pH**

The measurement of the pH has been described in detail in our previous reports\textsuperscript{[11].} Briefly, to measure the change in pH, HPS0077 hiPSCs were analyzed by microspectrofluorimetry with a pH-sensitive fluorescent dye, BCECF-AM. BCECF-AM 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 pH. A high potassium/nigericin calibration method was used to convert the emission ratio to the pH value.

When the pH 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\textsuperscript{[22].}

**Weak acid/base prepulse technique**

NHCl 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\textsuperscript{[12].} Taking NHCl prepulse as an example, it can be described by 4 phases, as shown in Figure 1A. Cells were first perfused with 20 mmol/L NHCl for 5 min, which caused an initial rapid alkalization. This mechanism is simply caused by the small molecular weight and nonpolar [NH₄⁺] easily crossing the cell membrane and acquiring hydrogen in the cytosol to produce NH₄⁺ (phase 1: rapid alkalization, NH₄⁺ + H⁺→NH₃⁺). Then, the pH slowly recovered and stabilized through the activation of acid loaders, such as AE and CHE (phase 2: slow recovery). The removal of NHCl caused rapid intracellular acidification because [NH₃⁺] rapidly effluxed and further produced hydrogen from [NH₄⁺] in the cytosol (phase 3: rapid acidification, NH₄⁺→NH₃⁺ + H⁺). The subsequent pH recovery following NHCl-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: pH recovery). To accurately quantify the H⁺ flux through pH regulators, all pH recovery rate data was converted to the J⁺ (pH recovery rate multiplied by buffering power)\textsuperscript{[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 pH induced by the impact of hydrogen, i.e., (NH₃⁺)₂SO₄. Therefore, if the buffering power is stronger, the change in pH will be smaller. The buffering power can be
Figure 1  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 pH 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 (H₂O) and Na⁺-free + 30 μmol/L bafilomycin A1 (Ba₃0) on the pH recovery slope. E: The curve of the pH recovery rates for Na⁺-free, H₂O 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 (SCH₃0) and Na⁺/Cl⁻-free + Na⁺-free + Ba₃0 in HEPES-buffered solution, and the change in pH was detected by a multimode reader. Error bars represent the mean ± SE.

defined by the following equation:\(^{38}\):
\[
\beta(mM) = \frac{[H^+]}{\Delta pH_i} \tag{e.1}
\]
Where \([H^+]:\) is the change in the concentration of intracellular protons, and \(\Delta pH\) is the resulting change in pH.

For experiments with the NH₄Cl prepulse technique, the application of (NH₄)₂SO₄ externally induces intracellular alkalization. This is due to the rapid diffusion of NH₄ into the cell and its subsequent hydrogenation to form NH₃. Upon the removal of extracellular (NH₄)₂SO₄, NH₃ exits the cell as uncharged NH₃, leaving behind an equal concentration of H⁺ and causing intracellular acidification. If \([H^+]:\) 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:
\[
\beta(mM) = [NH_4^+] / \Delta pH_i \tag{e.2}
\]
According to the Henderson-Hasselbolch equation, the relationship between internal and external NH₄⁺ concentration is as follows:
\[
pH_i - pH = \log([NH_4^+] / [NH_4^+]_o) \tag{e.3}
\]
Equation 3 can then be rearranged as follows:
\[
[\text{NH}_4^+] = [\text{NH}_4^+]_o \times 10^{(\text{pH}_i - \text{pH})} \tag{e.4}
\]
In the extracellular solution, pHₖ = pKa + log ([NH₃]/[NH₄⁺]) (Henderson-Hasselbolch equation). Therefore, this equation can be rearranged as follows:
\[
[\text{NH}_4^+]_o = C / (10^{(\text{pH}_o - \text{pK}_a)} + 1) \tag{e.5}
\]
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⁺] at a given pH as follows:

\[ [\text{NH}_4^+] = \frac{C}{(10^{(\text{pK}-\text{pH})} + 1)} \times 10^{(\text{pH}-\text{pK})}. \quad (e.6) \]

In an open system, the theoretical \( \kappa \) can be calculated as follows:

\[ \kappa = 2.3 \times [\text{HCO}_3^-]. \quad (e.7) \]

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

\[ [\text{HCO}_3^-] = \frac{C}{(10^{(\text{pK}-\text{pH})} + 1)} \times 10^{(\text{pK}-\text{pH})}. \quad (e.8) \]

### 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). 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, HCI or H₂SO₄ at 37 °C for all solutions. NH₄Cl, Na-acetate and (NH₄⁺):SO₄ were directly added as solids to the buffered solutions before use. HOE 694 (HOE, a NHE1 specific inhibitor), S0859 (an NBC-specific inhibitor), baflomycin 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 pH 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 pH to equal the pHᵢ, as shown in Figure 2A. The calibration equation was obtained from ten similar experiments and a nonlinear BCECF fluorescence-pH curve of function, as shown below and in Figure 2B. The following equation was used to convert the fluorescence ratio into pHᵢ:

\[ \text{pHᵢ} = \text{pKᵢ} + \log([R_{\text{max}} - R]/[R - R_{\text{min}}]) + \log(F_{440\text{max}}/F_{440\text{min}}). \]

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 2C, 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 1A, a pHᵢ recovery slope following NHCl 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 (H₅₀, n = 4) significantly inhibited the pHᵢ recovery rate, as shown in Figures 1B and 1C, 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 remai-
ning Na+-independent pH 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 1D. However, either no significant inhibition or slight inhibition of pH recovery was observed between the Na+-free solution group and the Na+-free solution + Ba30 group (Figures 1B and 1D, 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 1A-D were summarized and plotted as a function of the pH recovery rate vs pH in Figure 1E. As shown in Figure 1E, 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 pH 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 1E are KHE or Cl−-dependent acid extruder(s), HPS0077 cells were either performed by adding 40 μmol/L SCH-28080 (SCH40, a KHE-specific inhibitor) or removing [Cl−]. The change in pH 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 pH recovery rate vs pH in Figure 1F. As shown in Figure 1F, the pH 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 pH 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% CO\(_2\)/HCO\(_3\)-buffered system**

To quantify the [H\(^{+}\)]\(^+\) flux through pH\(_i\) regulators in 5% CO\(_2\)/HCO\(_3\)-buffered conditions, we first quantified intracellular buffering (\(\beta\)). The experimental details are shown in the materials and methods section, and we found that \(\beta\) increased as pH increased at pH = 7.0 to 8.0 (\(n = 35\), data not shown). The equation can be expressed as \(\beta = 107.79 (pH)^{-1} - 1522.2 (pH) + 5396.9\) (correlation coefficient \(R^{2} = 0.85\)). The obtained \(\beta\) can be used to calculate the [H\(^{+}\)]\(^+\) flux through pH\(_i\) regulators by the following equation: \(j_{\beta} = \beta \times pH\) recovery rate (pH\(_i\) value/minutes). To further investigate whether the NBC is functionally involved in the 5% CO\(_2\)/HCO\(_3\)-condition, we used a protocol similar to the previously mentioned experiments except for the replacement of HEPES-buffered solution with 5% CO\(_2\)/HCO\(_3\)-buffered solution. The pH\(_i\) recovery slope following NH\(_4\)Cl prepulse-induced intracellular acidification in 5% CO\(_2\)/HCO\(_3\)-buffered solution was a typical trace for the control (\(n = 7\)), as shown in Figure 3A. As shown in Figures 3B-E, the pH\(_i\) recovery rate was significantly inhibited under four different conditions as follows: removal of [Na\(^{+}\)] (\(n = 3\), addition of H\(_{2}O\) (\(n = 4\)), addition of 90 μmol/L S0859 (S\(_{90}\); an inhibitor of NBC, \(n = 3\)), and addition of H\(_{2}O\) and S\(_{90}\) (H\(_{2}O\) + S\(_{90}\), \(n = 3\)). Experimental data similar to those shown in Figures 3A-E were summarized and plotted as a function of \(J_{\beta}\) vs pH in Figure 3F. As shown in Figure 3F, a similar pH\(_i\) recovery rate between Na\(^{+}\)-free and H\(_{2}O\) + S\(_{90}\) conditions indicated that the NHE1 and the NBC were both involved in the Na\(^{+}\)-dependent acid extrusion mechanism in the 5% CO\(_2\)/HCO\(_3\)-condition in HPS0077 cells.

Notably, the acid extrusion mechanism in the 5% CO\(_2\)/HCO\(_3\)-condition was regulated mainly by the NBC in the pH\(_i\) range of 7.50-7.68 because the pH\(_i\) recovery rate could be completely inhibited by S\(_{90}\) (Figure 3D). Moreover, the addition of S\(_{90}\) did not affect pH\(_i\) recovery when the pH was less than 6.9 ± 0.01 (\(n = 3\), see the trace of S\(_{90}\)), 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 pH\(_i\) ranges of < 7.5, 6.9-7.68 and < 7.1, respectively.

**Functional characterization of acid loaders**

The homeostasis of pH\(_i\) 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\(^{-}\)]\(^+\) and further exchange of [OH\(^{-}\)] or [HCO\(_3\)]\(^-\) out of 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 pH\(_i\) recovery slope was expressed as the acid loading activity of acid loaders. Figures 4A and 4C show the typical pH\(_i\) recovery slope following the Na-acetate prepulse either in HEPES or HCO\(_3\)-buffered solution, respectively (\(n = 3\)). Removal of [Cl\(^{-}\)] in the 5% CO\(_2\)/HCO\(_3\)-buffered solution completely inhibited the pH\(_i\) recovery (\(n = 3\)), as shown in Figure 4D, which indicated that the acid loading mechanism is completely Cl\(^{-}\)-independent in HPS0077 cells. However, interestingly, a rapid acid loading phenomenon was observed before the total inhibition at pH\(_i\) = 7.9 ± 0.01 (\(n = 3\)) in HEPES solution, as shown in Figure 4B. These results indicated that CO\(_2\) or HCO\(_3\)\(^-\) 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 Leem et al\(^{[39]}\), we speculate that the Cl\(^{-}\)-independent acid loading mechanism is mainly attributed to the CHE and the AE\(^{[39]}\). Notably, as shown in Figure 4E, the pH\(_i\) recovery rate is nearly identical between the 5% CO\(_2\)/HCO\(_3\)-system (solid circles) and the HEPES system (solid squares), which indicates that the CHE plays a more important role than the AE in the acid loading mechanism in HPS0077 cells.

**Decrease in pH 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 pH during the loss of pluripotency in hiPSCs. In the pluripotent state, the resting pH\(_i\) observed from the pH completely recovered after NH\(_4\)Cl 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% CO\(_2\)/HCO\(_3\)-conditions, respectively, as shown in Figures 5A and 5B. Moreover, in 5% CO\(_2\)/HCO\(_3\)-buffered solution, as expected, the resting pH\(_i\) shifted to 7.46 ± 0.02 (\(n = 5\)) and 7.66 ± 0.02 (\(n = 5\)) after adding S0859 (S\(_{90}\) and HOE694 (H\(_{2}O\)), 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 pH\(_i\) in HEPES and in the 5% CO\(_2\)/HCO\(_3\)-plus S\(_{90}\) conditions, which indicates that the set-point of NHE activation is pH \(\geq 7.5\). In the 5% CO\(_2\)/HCO\(_3\)-condition, the resting pH\(_i\) showed no significant difference between the untreated and H\(_{2}O\)-treated conditions, which indicates that the pH is regulated by the NBC instead of the NHE in the pH\(_i\) range of 7.50-7.68.
To further induce the loss of pluripotency, HPS0077 cells were first transferred from mTeSR1 medium (designated for maintaining long-term pluripotency) to mTeSR-E6 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 E6-4d) 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 recovery of pluripotency, we determined the pH recovery rate following NH4Cl prepulse-induced intracellular acidification. The pH 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 pH recovery rate in E6-1d to E6-4d normalized from the E8 condition (% of E8) in HEPES and 5% CO2/HCO3- buffered solution, estimated at pH = 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 pH recovery rate was measured at pH = 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 pH recovery rate was measured at pH = 7.2, the pH 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 pH 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 pH 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 pH recovery rate in E6-1d to E6-4d normalized to E8 (control) in 5% CO2/HCO3- buffered solution, which was estimated at pH = 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 pH recovery rate measured at pH = 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 pH = 7.5. The pH recovery rate for E6-2d, E6-3d and E6-4d was significantly decreased by 88.7, 74.9 and 61%, respectively, when measured at pH = 6.9, decreased by 82%, 77.2% and 51.5%, respectively, when measured at pH = 7.2, and decreased by 53.4%, 44.8% and 22.3%, respectively, when measured at pH = 7.5 (n = 3). The max/min charts shown in Figure 6F show the resting pH 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 pH 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 pH recovery rate and resting pH.

**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 pH 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 pH range less than 7.35 because the S\textsuperscript{hi} group (i.e., inhibiting NBC activity) substantially decreased the activity compared to other groups (inhibiting NHE1 or other Na\textsuperscript{-}independent acid extruders). Moreover, the role of NHE1 on acid extrusion decreased as the pH increased (Figures 1, 3 and 7). Notably, the activity of NHE1 was nominally undetectable when the pH was greater than 7.50, as shown in Figures 1, 3 and 7.

Relevant molecular candidates for the NBC include at least five members of the slc4 family, including 2 electrogenic Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransporters (NBCe1/SLC4A4 and NBCe2/SLC4A5), 1 electroneutral Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransporter (NBCn1/SLC4A7) and 2 Na\textsuperscript{+}-dependent Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchangers (NCBE/SLC4A10 and NDCBE/SLC4A8\textsuperscript{[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\textsuperscript{[7]}.

However, the Aalkjaer group has demonstrated that the NBC is NBCn1, i.e., it is electroneutral, in rat and mouse smooth muscle cells\textsuperscript{[42]}. 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\textsuperscript{+}-independent acid extruder(s) and Cl\textsuperscript{-}-independent acid loader(s) were substantially present for acid extrusion (pH < 7.1) and acid loading (pH > 7.9) in hiPSCs (Figures 1, 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 1D and 1F). 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 pH 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 pH 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 pH (< 7.2), including the NHE, the NBC
and an unknown Na\(^{+}\)-independent acid extruder(s), in hiPSCs. As expected, the resting pH in hiPSCs was found to be 7.5 and 7.68 in the HEPES and 5% CO\(_2\)/HCO\(_3\)- conditions, respectively, and was relatively higher than that of normal differentiated adult cells (resting pH = 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 (pH\(_i\)/pH\(_e\)) gradient (alkaline pH\(_i\) and acidic pH\(_e\)) 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

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**Figure 6** The change in the activity of the Na\(^{+}\)/H\(^{+}\) exchanger and the Na\(^{+}\)/HCO\(_3\)-cotransporter and the resting pH during the loss of pluripotency in human induced pluripotent stem cells. A: The traces showed the changes in pH recovery after NH\(_4\)Cl 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 pH recovery rate in E6-1d to -4d normalized to the rate in E8 (% of E8) in HEPES-buffered solution, which was estimated at pH\(_i\) = 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 pH 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 pH recovery after NH\(_4\)Cl prepulse-induced intracellular acidification in E8 and E6-1d to E6-4d media in 5% CO\(_2\)/HCO\(_3\)-buffered solution; E: The graphs show the pH recovery rate in E6-1d to E6-4d normalized to the rate in E8 (control) in 5% CO\(_2\)/HCO\(_3\)-buffered solution, which was estimated at pH\(_i\) = 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 pH 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.
expression of pluripotency markers at a minimum pH = 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 pH significantly increased at 48 and 72 h and returned to baseline at 96 h, and this increase was dependent on the loss of NHE1 function[55]. However, in this study, the decrease in resting pH 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 the pH at 48 and 72 hours during early differentiation in mESCs. This result implies that increasing resting pH occurred during the naïve to primed pluripotency states[6]. Subsequently, the resting pH 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 pH in cancer cells. 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. This switch between OXPHOS and glycolysis supports the dynamic changes in the resting pH observed during the loss of pluripotency in mESCs and the decrease in the resting pH 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. To maintain pluripotency in hiPSCs, FGF2 has been added to the culture medium to activate PI3K/AKT signaling. The activation of PI3K/AKT signaling further promotes the relative gene expression of pluripotency markers and inhibits differentiation by suppressing MEK/ERK signaling. Therefore, the removal of FGF2 decreases the ratio of AKT activity to ERK and further causes cell differentiation. ERK is a well-known activator of NHE1, 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, AKT is stimulated by insulin and growth factors and further activates NHE1 in cancer cells and fibroblasts. Therefore, this study implies 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 pH regulatory model in hiPSCs, as shown in Figure 7. In this model, we demonstrated that the steady-state pH 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 pH regulatory mechanism, and each is activated in different pH 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 pH (pH) affects many cellular functions, such as cell proliferation and differentiation. However, the knowledge of pH 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 pH regulation mechanism model and investigate the relationship of pH regulation and pluripotency in hiPSCs.

Research methods
In the pluripotent state and during the loss of pluripotency in hiPSCs, we observed the activity of pH 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 vacular-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 pH 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 pH 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 pH regulation mechanism in hiPSCs. The higher resting pH 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 pH regulation mechanism and its functional/expressional roles in maintenance of pluripotency of hiPSCs. We proposed that targeting either pH 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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