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Intracellular pH Regulates Cancer and Stem Cell Behaviors: A Protein Dynamics Perspective

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Abstract

The International Society of Cancer Metabolism (ISCaM) meeting on Cancer Metabolic
Rewiring, held in Braga Portugal in October 2019, provided an outstanding forum for
investigators to present current findings and views, and discuss ideas and future directions on
fundamental biology as well as clinical translations. The first session on *Cancer pH Dynamics*
was preceded by the opening keynote presentation from our group entitled *Intracellular pH
Regulation of Protein Dynamics: From Cancer to Stem Cell Behaviors*. In this review we
introduce a brief background on intracellular pH (pHi) dynamics, including how it is regulated as
well as functional consequences, summarize key findings included in our presentation, and
conclude with perspectives on how understanding the role of pHi dynamics in stem cells can be
relevant for understanding how pHi dynamics enables cancer progression.

Introduction

Intracellular pH (pHi) was previously thought to be mostly constant for cellular homeostasis and
possibly dysregulated in diseases. We now know, however, that pHi is dynamic in normal cells
and clearly dysregulated in a number of diseases. In normal cells, pHi changes during cell cycle
progression, increasing ~ 0.3-0.4 pH units at the end of S phase and if this increase is blocked,
G2/M is delayed with increased inhibitory phosphorylation of Cdk1-Tyr15 and suppressed cyclin
B1 expression (1-3). Additionally, pHi dynamics regulates cell-substrate adhesion remodeling
and migration, with increased pHi enabling both behaviors (4-7). Emerging evidence also
indicates a critical role for increased pHi in epithelial plasticity, including epithelial to
mesenchymal transition (EMT) (8) and stem cell differentiation (9-12). Moreover, it is now well
established that dysregulated pHi is seen with many diseases, most notably cancers, which often
have a constitutively increased pHi (13-18), and neurodegenerative disorders, which are
associated with a constitutively decreased pHi (19, 20). Our review focuses on dysregulated pHi
dynamics in cancer; however, another feature of cancers is a dysregulated extracellular pH that is
lower (~ 7.0) compared with normal tissues (~ 7.4).

Although many factors contribute to pHi dynamics, the major regulators in most
mammalian cells are plasma membrane ion exchangers, including the Na_+-H_+ exchanger NHE1,
the Na_+-$HCO3_-$ transporter NBC, and the Na_+-dependent Cl_--$HCO3_-$ transporter NDCBE, which
are acid-extruders, and Cl_--$HCO3_-$ exchangers of the anion exchanger (AE) family, which are
acid loaders (21-23). The BioParadigms Solute Carrier tables[3] are an excellent resource on the
classification, expression, and transport characteristics of these ion exchangers. Additional
plasma membrane ion transport proteins that contribute to pHi dynamics, albeit to less of an
extent, include V-ATPases and monocarboxylate transporters of the MCT family. The broad
range of ion transport proteins regulate pHi dynamics through changes in their expression and
activity, the latter mostly mediated by posttranslational modifications as many are substrates of
key signaling kinases, including for NHE1, p90rsk (24), Akt (25, 26), the Rho kinase ROCK
(27), and the Ste20 kinase MAP4K4 (28), previously termed NIK. Experimentally, these
exchangers can be pharmacologically or genetically targeted to understand how they contribute
to pHi dynamics and how pHi dynamics regulates cell behaviors.

We have a relatively strong understanding of how changes in pHi are generated and the
effects of pHi changes on myriad cell functions. However, a mechanistic understanding of how
pHi changes regulate cell behaviors remains understudied, particularly effects on signaling
networks and protein functions. At the ISCaM meeting we presented our work on how changes

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[3] http://slc.bioparadigms.org/

92   in pHi regulate protein dynamics to enable cancer and stem cell behaviors, which we summarize
93   in this review. Key to pH-regulated protein structure and function is considering protonation and
94   deprotonation as a protein posttranslational modification, analogous to posttranslational
95   modification by phosphorylation, acetylation, and methylation as we previously described (29).
96   However, studying protonation and deprotonation as a posttranslational modification is more
97   difficult compared with other posttranslational modifications because it is not catalyzed by an
98   enzyme and cannot be detected by mass spectrometry or antibodies. Furthermore, many
99   endogenous "pH sensors" or proteins that are regulated by pH dynamics within the cellular range
100   are coincidence (AND-gate) detectors with their structural conformations, activities, or binding
101   affinities dependent on multiple posttranslational modifications, most commonly
102   phosphorylation or dephosphorylation and protonation or deprotonation.
103
104   **Intracellular pH and cancer cell behaviors: From the protein view**
105   Most cancer cells have a higher pHi compared with untransformed cells, regardless of the
106   mutational landscape or tissue origin. This higher pHi enables many cancer behaviors, including
107   increased proliferation, directional migration, tumorigenesis, and most recently recognized, the
108   oncogenic and tumor-suppressor functions of proteins with charge-changing mutations (Fig. 1).
109   At the ISCaM meeting we presented our findings on pH sensors regulating cell migration and
110   tumorigenesis as well as how pHi dynamics in cancer cells affect the functions of proteins with
111   somatic mutations encoding arginine to histidine substitutions.
112   *Cell migration* is confirmed to be regulated by pHi in many cell types and species (6, 30-
113   34). An increased pHi of ~ 0.3-0.4 units is seen in migrating cells and preventing the increased
114   pHi inhibits migratory rate and directionality, and impairs cell polarity. Our presentation
115   described several pH sensors we identified in atomistic detail that collectively regulate different
116   aspects of migration. These include guanine nucleotide exchange factors for the low molecular
117   weight GTPase Cdc42 involved in cell polarity (35), talin binding to actin filaments (36) and
118   focal adhesion kinase (FAK) activity for cell-substrate adhesion dynamics (5) as well as cofilin
119   for actin polymerization (37). The single histidine in cofilin, His133 (human), has an upshifted
120   pKa to ~ 7.2 and must be neutral for increased cofilin activity (Fig. 1A). However, cofilin is a
121   coincidence detector and full activity also requires dephosphorylation of Ser3 (Fig. 1A) by one
122   of several phosphatases, which releases an autoinhibited interaction between phosphorylated
123   serine and lysine 126 and 127 to allow binding to actin filaments. This AND-gate regulation
124   enables signaling mechanisms to increase cofilin activity in time (with migratory cues) and space
125   (at the leading edge of a migrating cell), and highlights that for many pH sensors a change in
126   protonation state does not function as a binary switch.
127   *Tumorigenesis and dysplasia* are enabled by increased pHi regulated by NHE1, NBCs
128   and MCTs, including tumor cell proliferation, growth, and survival (38-40). Our presentation
129   included two of our recent key findings on pHi and tumorigenesis. First, that increased pHi from
130   ~ 7.30 to ~ 7.65 in Drosophila eye epithelia by overexpressing Drosophila *dnhe2,* an ortholog of
131   mammalian NHE1, is sufficient to induce dysplasia in the absence of an activated oncogene (41).
132   Second, that β-catenin, an adherens junction and Wnt pathway protein is a pH sensor, with pHi
133   not regulating its activity but rather its stability, which decreases at pHi > 7.5 (42). Using a
134   phenotype screen, we found that overexpressing β-catenin suppresses dysplasia in Drosophila
135   eye epithelia with constitutively increased pHi induced by overexpression of *dnhe2*. These data
136   suggested a lower abundance of β-catenin at higher pHi, which we confirmed in mammalian
137   cells. We also resolved the pH sensing mechanism of His36 (human) in the N-terminus of β-

138 catenin, which when neutral (at higher pHi) increases binding affinity for the E3 ligase β-TrCP1.
139 However, like cofilin described above, β-catenin is a coincidence detector requiring both a
140 neutral His36 and phosphorylated flanking Ser33 and Ser37 for binding β-TrCP1 (Fig. 1B). The
141 role of phosphorylated serines in enabling proteasome-mediated degradation of β-catenin has
142 long been recognized (43). The importance of a neutral His36 for binding β-TrCP1 is evident in
143 the crystal structure of β-TrCP1in complex with an N-terminal β-catenin peptide (44) (PDB:
144 1P22), which shows the proximity of β-catenin-His36 and β-TrCP1-Lys365 (Fig. 1B). This
145 suggests that binding would be electrostatically unfavorable with a protonated His36 at lower
146 pHi. Importantly, the DSxxHS motif is conserved in all species of β-catenin and occurs in a
147 number of other β-TrCP1 target proteins (45), including the transmembrane protein polycystin 2,
148 the tumor suppressor tensin 2, the centrosomal protein Cep97, the hedgehog pathway protein
149 Gli3, and myosin-XVIIIa, suggesting these substrates may have similar pH sensitive binding to
150 β-TrCP1 and regulated protein stability. We also described that a cancer-associated somatic
151 mutation, β-catenin-H36R, is insensitive to pHi-regulated degradation and, when expressed in
152 Drosophila eye epithelia, enhances Wnt pathway activity, causes tissue overgrowth growth, and
153 induces ectopic tumors. With this mutation, β-catenin stability could be retained at the higher
154 pHi of a cancer cell and enable tumorigenesis. As described in the section below, this is an
155 example of a charge-changing mutation that confers a loss of pH sensing.
156   *Charge-changing somatic mutations* can confer a change in pH sensing and enable cancer
157 behaviors specifically at increased pHi. We recently showed that recurrent arginine to histidine
158 mutations in p53 and EGFR can confer a gain in pH sensing to the mutant proteins. Arginine,
159 with a pKa of 12, will be protonated regardless of pHi while histidine, with a pKa near neutral,
160 can titrate with cellular changes in pHi. We found that a highly recurrent arginine to histidine
161 mutation in the tumor suppressor p53 (p53-R273H) could confer pH-dependent DNA binding
162 and transcription of p53 target genes, with decreased transcription at a higher pHi of 7.6
163 compared with 7.2 (46). The crystal structure of wild-type p53 (47) (PDB: 4HJE) and mutant
164 p53-R273H (48) (PDB: 4IBW) in complex with DNA suggests that wild-type Arg273 forms an
165 electrostatic interaction with the negatively charged phosphate-backbone of DNA (Fig 1C). At
166 the lower pHi of a non-transformed cell, His273 is likely protonated and retains some binding to
167 the negatively-charged DNA but, at the higher pHi of a cancer cell, His273 is likely
168 deprotonated, reducing DNA binding and expression of p53 target genes (Fig 1D). Importantly,
169 lowering pHi in cancer cells expressing p53-R273H recovered p53 transcriptional activity and
170 p53-dependent cell death in response to double-strand breaks (46). We also showed that a
171 cancer-associated arginine to histidine substitution in the epidermal growth factor receptor
172 (EGFR-R776H) that is recurrent in lung cancers confers pH sensing to the mutant protein.
173 Increasing pHi from 7.2 to 7.6 increases activity of EGFR-R776H but not wild-type receptor,
174 and increases cell proliferation and cellular transformation in cells expressing the mutant but not
175 wild-type receptor (46). These results suggest that charge-changing mutations can confer a gain
176 in pH-sensing not seen with the wild-type protein. This work also indicates that charge-changing
177 somatic mutations can confer dynamic function to mutant proteins, specifically inactivating a
178 tumor suppressor and specifically activating an oncogene at the increased pHi of cancer.
179
180 **Intracellular pH and epithelial plasticity: Focus on stem cell differentiation**
181 Recent findings indicate that pHi dynamics is a key regulator of epithelial plasticity, with
182 increased pHi enabling EMT (8) and epithelial branching morphogenesis (49) as well as
183 differentiation of melanocytes (50), embryonic and adult stem cells (9, 11), and mesenchymal

184 (12) and cardiomyocyte (10) stem cells. These findings raise questions on the role of pHi
185 dynamics in morphogenesis and animal development, which remain largely unresolved. New
186 genetically-encoded tools to measure pHi (51) and genetic and pharmacological approaches to
187 selectively change pHi temporally and spatially will enable new studies necessary to resolve
188 pHi-regulated developmental processes with promise for new approaches to correct impaired
189 morphogenesis.
190   Toward a goal of resolving the role of pHi dynamics in cell fate decisions, at the ISCaM
191 meeting we discussed our findings on pHi-regulated embryonic and adult stem cell
192 differentiation. As we previously described (11), with differentiation of naïve clonal mouse
193 embryonic stem cells (mESC) to primed epiblast-like cells there is an NHE1-dependent transient
194 increase in pHi of ~ 0.3 units (Fig. 2A). Preventing this increase in pHi blocks differentiation, as
195 indicated by sustained expression of the mESC markers Rex1, Stra8, and Nanog, and attenuated
196 expression of the epiblast markers Brachyury, fibroblast growth factor 5, and Pax6. An increase
197 in pHi is also necessary for differentiation of adult follicle stem cells in the Drosophila ovary to
198 prefollicle cells and follicle cells (9, 11) (Fig. 2B), the later necessary for germ cell maturation.
199 Consistent with germ cells requiring enrichment from differentiated follicle cells, preventing the
200 increase in pHi along the follicle stem cell lineage impairs ovary morphology and adult
201 oogenesis and substantially decreases fertility (9). These findings were obtained by genetically
202 silencing Drosophila *dnhe2*, an acid extruder, or overexpressing a newly identified Drosophila
203 *ae2*, an ortholog of the mammalian acid loader AE2.
204   There are several important questions to resolve on the role of pHi dynamics in stem cell
205 differentiation. First is whether pHi is a conserved regulator of stem cell differentiation in
206 different tissues, perhaps using established and well characterized models for intestinal epithelial
207 (52) and skin epidermal (53) stem cell lineages. Second is how pHi dynamics regulates activity
208 of pathways and functions of proteins with established roles in stem cell behaviors. One
209 possibility is a role for pH sensing by β-catenin (as described above) in Wnt signaling, because
210 high Wnt pathway activity (54) at low pHi may retain self-renewal of stem cells and inhibit
211 differentiation. Third is whether pHi-regulated stem cell differentiation can inform regenerative
212 medicine approaches to correct or restore impaired cell and tissue functions.
213
214 **Integrating pHi dynamics in cancer and stem cells**
215 To consider how pHi dynamics in stem cells and cancer might be linked, we concluded our
216 presentation by showing new data on pHi heterogeneity in spheroids of clonal human lung
217 cancer cells (Fig. 2C). Using H1299 cells expressing the previously described (41) genetically
218 encoded and ratiometric pH biosensor mCherry-pHluorin, we observe distinct intercellular
219 differences in pHi when grown in 3D (Fig. 2C). Distinct pH heterogeneity (including
220 intracellular and extracellular pH) is seen in cancer spheroids (55-58) and a mouse model of
221 breast ductal carcinoma (59); however, whether this heterogeneity reflects differences in
222 mutational signatures, cell identity, phenotypes, or epithelial or metabolic plasticity remains
223 unresolved. For example, might cells with a lower pHi be stem-like tumor initiating cells? Could
224 cells with a higher pHi have increased glycolysis to fuel rapid proliferation or be undergoing
225 EMT for metastasis? The possibility that a lower pHi could enable tumor initiating cells raises
226 caution on the idea of lowering pHi to limit cancer progression. Tumor heterogeneity, whether
227 genetic, epigenetic, or phenotypic, is increasingly being recognized as a challenge for cancer
228 therapies (60, 61), and improved understanding of the determinants and consequences of pHi
229 heterogeneity could contribute to resolving these therapeutic challenges.

230   The field has taken a first important step in identifying a number of normal and
231 pathological cell behaviors regulated by pHi dynamics. A second step in understanding how pHi
232 regulates the signaling pathways mediating these behaviors is now emerging. A third step of
233 improved mechanistic understanding is an important future direction to resolve design principles
234 and functions of pH sensitive proteins mediating pHi-regulated cell behaviors. This third step is
235 experimentally challenging and remains largely unexplored, but holds promise for identifying
236 new therapeutic targets and informing the design of therapeutics for regenerative medicine and
237 treating diseases with dysregulated pHi dynamics, including cancer.
238
239

246 **Author Contributions**
247 All authors contributed to obtaining data included in the figures, including data on pHi and
248 cancer (KAW, DLB) and pHi and stem cell differentiation (YL, DLB). All authors contributed to
249 writing and editing the manuscript.
250

251 **Conflict of Interest Statement**
252 The authors declare no direct or perceived conflict of interest.
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256 **References**
257

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448

449 **Figure Legends**
450

451 **Figure 1.** The higher pHi of cancer cells enables many behaviors, including directional
452 migration and tumorigenesis as well as the tumorigenic functions of proteins with charge-
453 changing arginine to histidine mutations. **(A)** Cell migration is in part dependent on increased
454 activity of cofilin with increased pHi. Cofilin is a coincidence-regulated pH sensor that is
455 activated by deprotonation of His133 (cyan) and dephosphorylation of Ser3 (magenta) for actin
456 polymerization enabling cell migration. **(B)** Dysplasia is associated with increased pHi, which
457 decreases β-catenin stability. β-catenin is a coincidence-regulated pH sensor with deprotonation
458 of His36 (cyan) and phosphorylation of Ser33/37 by GSK3β enabling binding to the E3 ligase β-

459 TrCP1 for targeting to the proteasome for degradation. Crystal structure data show that β-
460 catenin-His36 is in close proximity to β-TrCP1-Lys365, which suggest that binding would be
461 electrostatically unfavorable with a protonated His36 at lower pHi. **(C)** Charge changing somatic
462 mutations can confer pH-regulated protein activity. Structure of wild-type p53 (top) and mutant
463 p53-R273H (bottom) in complex DNA indicating an electrostatic interaction of Arg273 with the
464 negatively charged phosphate-backbone of DNA that could be partially enabled by protonated,
465 but not neutral, His273.
466
467 **Figure 2. (A)** Schematic showing that clonal self-renewing mESC (Naïve), derived from the
468 inner cell mass of the early blastocyst, have a lower pHi than differentiated primed epiblast-like
469 stem cells (EpiSC), which are analogous to cells in the late epiblast stage. **(B)** Schematic of
470 Drosophila germarium showing an increase in pHi from self-renewing follicle stem cell (Follicle
471 SC) to differentiated prefollicle and follicle cell. **(C)** Image of lung cancer H1299 cells
472 expressing the pHi biosensor mCherry-pHluorin and grown in Matrigel as 3D spheroids shows
473 intracellular pHi heterogeneity that might reflect phenotypic heterogeneity, such as cells with a
474 higher pHi undergoing EMT and cells with a lower pHi being self-renewing tumor initiating
475 stem-like cells.
476

A Cell migration

pSer3
His133⁺

Ser3
His133

pHi

Cofilin activity

B Dysplasia

β-catenin-His36   β–TrCP1-Lys365

pHi

β-catenin-His36+ → His36 neutral

β-catenin stability

DpSxxH36pS β-catenin

E3 ligase β-TrCP1

Proteosome degradation

C Function of charge-changing somatic mutations

HN
H₂N   NH₂
Arginine
pKa > 12

Arg > His mutation can confer a gain in pH sensing

Histidine
pKa ~ 6.5

DNA   DNA
p53-Arg273   p53-His273

WT p53   Mutant p53

pHi-independent

pHi-dependent activity decreased at higher pHi of cancer cell

Tumor suppressor activity

Fig. 1. Liu et al.

A

Late Blastocyst

Early Blastocyst

EpiSC
pHi 7.65

Naïve
pHi 7.35

B

pHi

Follicle cell
pHi 7.45

Prefollicle
pHi 7.2

Follicle SC
pHi 6.9

C

pHi
7.8

7.2

EMT?

Stem-like?

Liu et al., Fig. 2