RESEARCH ARTICLE

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Extracellular O_2 Level and pH Modulation Affected the Human Breast Cancer Stem Cells' Survival and Stemness

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Introduction: It has been reported that cancer stem cells could maintain their viability and stemness under certain extracellular changes. Therefore, efforts that modulate tumor hypoxia and acidity should be compelled in order to impede the growth of cancer stem cells. This study was aimed to analyze the effect of extracellular pH and O_2 level modulation on viability, apoptosis, and stemness of the human CD24−/CD44+ breast cancer stem cells (BCSCs). *Methods*: The primary BCSCs (CD24−/CD44+ cells) were cultured under hypoxia (1% O_2) or under supplementation of sodium bicarbonate (100 mM) for various periods. After each incubation time, cell viability was determined by trypan blue exclusion assay and apoptosis was examined using flow cytometry with Annexin V/PI assay. Furthermore, total RNA was isolated for qRT-PCR analysis of HIF1α, ALDH1 and Klf4 mRNA expression. *Results*: This study demonstrated that hypoxia could suppress BCSC proliferation, but inhibit the cell apoptosis. Alkaline pH could also suppress BCSC proliferation and promote early apoptosis at the same time. Interestingly, the expressions of ALDH1 and KLF4 were downregulated in hypoxia-treated BCSCs which might be regulated through the increase of HIF1α. Conversely, ALDH1 and Klf4 expressions were upregulated in sodium bicarbonate-treated BCSCs under alkaline pH. *Conclusion*: The modulation of extracellular pH and the O_2 level has diverse effects on the viability, apoptosis, dan stemness of BCSCs. Therefore, we suggest that targeting tumor hypoxia and acidity may be a prospective therapeutic strategy to eradicate BCSCs.

Keywords: Breast Cancer Stem Cells, Tumor Hypoxia, Extracellular pH, Cell Viability, Stemness.

1. INTRODUCTION

The microenvironment is believed to have a significant role in tumor growth and progression.[1] Likewise, its role in maintaining the unique properties of cancer stem cells (CSCs), the cancer cell subpopulation with stemness phenotype, such as self-renew and the ability to differentiate into multiple cell types comprising the tumor.[2] One major characteristic of tumor microenvironment is hypoxia.[3] Recent studies showed that the capability of the microenvironment to maintain the undifferentiated state of the stem cells, both embryonic, adult, or cancer, required the hypoxic condition.[4,5]

In the hypoxic condition, the cells would perform a various cellular adaptation to survive in the state of oxygen deficiency under the regulation of hypoxia-inducible factor (HIF).[6] One of the dominant phenotype as the cellular response to hypoxia is the glycolytic metabolism.[7] Several studies support the hypothesis that CSCs including the breast CSCs, are more glycolytic, while the differentiated progeny displayed a more oxidative phenotype.[8,9] This metabolic preference, in turn, creates an acidic microenvironment as the results of a high lactate production as the end product of glycolysis.[10] In consequence, the acidic extracellular pH (pHe) is also becoming the other unique characteristic of the tumor microenvironment.[11,12]

In contrast to the normal differentiated adult cells which pHe is generally ∼7.4 and higher than the intracellular pH (pHi) of ∼7.2, cancer cells have a lower pHe of 6.7–7.1 and a higher pHi of >7.4. This specific pH condition has significant roles toward cancer cell's survival dan malignancy.[11,12] Recent evidence suggests that an increased pHi is necessary for cell proliferation, evading apoptosis, and cytoskeletal remodeling for cell migration. While a decreased pHe promotes cell invasion and cell-matrix remodeling, and stimulates the activation of acid-activated proteases to facilitate cancer cell dissemination.[11–13] Seemingly, this cancer pH dysregulation provides a prospective therapeutic target to limit disease progression.

In this study, we cultivated the human breast CSCs in two different extracellular environments. First, the cells were grown in a hypoxic condition with 1% O_2 that was expected to represent the hypoxic intratumoral microenvironment of breast cancer tumors. Second, the cells were cultured in an alkaline pHe supplemented

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with an alkalinizing agent, sodium bicarbonate ($NaHCO_3$), thus forming a reverse condition to the acidic microenvironment of the breast cancer tumors. This study aimed to analyze the role of a low oxygen level and an alkaline pHe in human breast CSC's survival and stemness.

2. METHODS

2.1. Patient Population and Characteristics

2.1.1. Culture of Human Breast Cancer Stem Cells (BCSCs)

In our previous study, primary culture of human breast cancer were sorted using magnetic-activated cell sorting (MACS) conjugated with anti CD24 and anti CD44 antibody resulting in CD24−/CD44+ cells for BCSCs and CD24−/CD44-cells for non-BCSCs (Patent registration from the General Directorate of Intellectual Property Right, Ministry of Law and Human Right, Republic of Indonesia; No. P0021300369).

BCSCs were cultured in DMEM-F12 medium containing HEPES buffer, 1% penicillin/streptomycin, 1% amphotericin B (250 ug/ml), 0.2% gentamycin sulfate (50 mg/ml) and 14.5 mM $NaHCO_3$ and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 20% O_2.

2.1.2. Hypoxia Exposure

The BCSCs were initially seeded at 5×105 cells/well in the 6-well plate and cultured in 3 mL/well of DMEM-F12 medium containing HEPES buffer pH = 7.4 at 37 °C, 5% CO_2 and 20% O_2 for 24 hours. Afterwards, the hypoxia was performed by replacing the seeding medium with fresh medium. The cells were then incubated at 37 °C, 5% CO_2 and 20% O_2 (normoxia) as the group control and 1% O_2, 5% CO_2, and 94% N_2 as treated samples for 6-, 24-, and 48-hours, respectively. After each incubation period, pH of cell culture medium (pHe) was immediately measured using pH electrode with Micro Bulb for 96-well plate (Hanna®) connected with pH meter (HI 2210®, Hanna), and the BCSCs were harvested by centrifugation at 1000 rpm for 10 minutes to obtain cell pellet and culture supernatant for various analysis.

2.1.3. Alkalinization of the Culture Medium Using $NaHCO_3$

The BCSCs were initially seeded at 5×105 cells/well in the 6-well plate and cultured in 3 mL/well of DMEM-F12 (pH = 7.4) at 37 °C, 5% CO_2 and 20% O_2 for 24 hours. Afterwards, the extracellular alkalinization was performed by replacing the initial BCSCs medium with DMEM-F12 supplemented with a volume of 8.4% $NaHCO_3$ (Meylon 84®, Otsuka) to generate a final concentration of 100 mM. The cells were then incubated in the alkalinized medium at 37 °C, 5% CO_2 and 20% O_2 for 6-, 24-, and 48-hours, respectively. After each incubation period, the cells were treated as well as the hypoxic cultures.

2.1.4. Cell Viability Assay

To determine cell viability, we applied trypan blue exclusion assay. Viable cells were counted using automated cell counter (Luna). Population doubling time (PDT) was determined using the following formula: $PDT = 1/r$ where $r = 3{,}32$ (log NH–log NI)/(t_2–t_1). Where r = multiplication rate; NI = number of the inoculated cells; NH = number of the harvested cells; t_1 = inoculation time; t_2 = harvest time (hours). Cell viability was also examined using the calculation of percent LDH release obtained from the ratio between LDH activity in supernatant (extracellular) and in cell lysate (intracellular). Extracellular LDH activity represents the presence of this enzyme released by lysed cells, hence this method could be used to determine the cytotoxicity as previously described.[14]

2.1.5. Apoptosis Assay

Apoptosis test was performed using Annexin V-FITC Apoptosis Detection Kit (Abcam). The about 5×105 BCSCs were harvested and rinsed with cold PBS twice. The next steps were carried out following the protocol instructions.

2.1.6. Quantitative Reverse Transcription-PCR

Total RNA was extracted from cell pellets using Tripure® RNA Isolation Kit (Roche) according to the manufacturer's protocol. Total RNA concentration was quantified using spectrophotometer (Varioskan Flash). Samples with an A260/A280 ratio of 1.6–2.0 were considered to be free of DNA and proteins. Quantitative PCR was performed using KAPA SYBR FAST® qPCR (KAPA BIO SYSTEMS) in the ExicyclerTM 96 (Bioneer). The PCR primer sequences used in this study were HIF1α: 5′-GGCGC GAACGACAAGAAAAAG-3′ and 5′-GTGGCAACTGATGAG CAAG-3′; ALDH1: 5′-TTGGAAGATAGGGCCTGCAC-3′ and 5′-GGAGGAAACCCTGCCTCTTTT-3′; KLF4: 5′-CCGCTCCA TTACCAAGAGT-3′ and 5′-TTTCTCACCTGTGTGGGTTC-3′; 18S RNA: 5′-AAACGGCTACCACATCCAAG-3′ and 5′-CC TCCAATGGATCCTCGTTA-3′. *Ct* value for each gene was determined, and $\Delta\Delta Ct$ was normalized to the designated reference sample. Gene expression values were then relatively calculated using the Livak method ($2 - \Delta\Delta Ct$).

2.1.7. HIF1α Protein Level

Total protein was extracted from 5.0×105 cells. HIF1α protein level was assayed using HIF1A Human ELISA kit (Abcam) according to the manufacturer's protocol. Data were presented per total protein.

2.1.8. Statistical Analysis

We present all data as mean ± standard error means (SEM) of at least triplicates. A p-value of at least < 0.05 in the independent t-test was statistically considered to be significant.

3. RESULT

3.1. Hypoxia Lowered While Alkalinization Increased the pHe

After 6-hour and the following incubation periods, the pHe of hypoxic BCSCs rapidly decreased compared to normoxia. Meanwhile, the alkalinization increased the pHe to 8,86 ± 0,03. After 6-hour and the following incubation periods, the pHe gradually decreased but still in the alkaline pHe range (Fig. 1).

3.2. Hypoxia and Alkaline pHe Induce HIF1α Expression of Breast CSCs

HIF1α mRNA expression and protein level were measured to evaluate the response of breast CSCs to low (1%) O_2

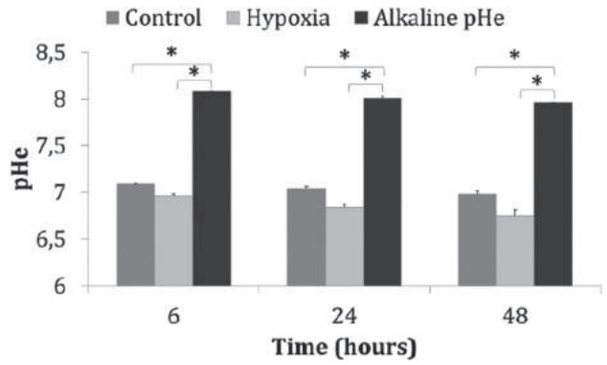

Fig. 1. The extracellular pH (pHe) of control, hypoxic-, and alkalinized-BCSCs after 6-, 24-, and 48-hour incubation. The pHe was determined by measuring the culture medium immediately after achieving each incubation period using pH electrode with micro-bulb. $^{*}p < 0,05$.

concentration exposure. The measurement also conducted to the sodium bicarbonate-treated cells (alkalinized breast CSCs) to know whether a low pHe could be a non-hypoxic activator of HIF1α. Breast CSCs gave a positive response to the hypoxia that showed a significant increase ($p < 0,01$) of HIF1α mRNA expression from 6- and 24-hour incubation compared to control and alkalinized breast CSCs. After 48-hour incubation, there was no significant difference in the expression between control, hypoxic-, and alkalized-breast CSCs. Surprisingly, HIF1α mRNA expression significantly ($p < 0,01$) increased after 24-hour alkalinization compared to control (Fig. 2(A)). At the protein level, HIF1α expressions of hypoxic breast CSCs were significantly higher ($p < 0,01$) than control and alkalized breast CSCs at all incubation periods. There was also an increase of alkalinized-breast CSCs' HIF1α protein level after 24-hour incubation (Fig. 2(B)).

3.3. The Alkaline pHe Reduces Breast CSCs Survival But Not the Hypoxia

To evaluate the impact of hypoxia exposure and alkalinization on breast CSCs survival, the viability assay was conducted with the measurement of %LDH release that equal to the number of lysis cells. The population doubling time (PDT) was also counted to analyze the ability of breast CSC to resume proliferation after the exposures. Apoptosis was determined using Annexin V/PI flow cytometry method. Alkalinization induced breast CSCs lysis where the %LDH releases were significantly ($p < 0,01$) higher than control and hypoxic-breast CSCs at all incubation periods. Meanwhile, %LDH releases from the hypoxic-breast CSCs were lower ($p < 0,05$) compared to control and alkalinized-breast

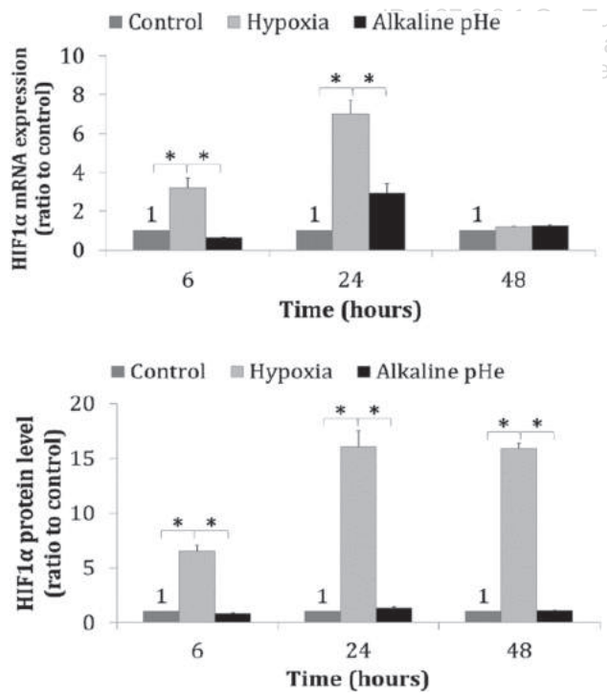

Fig. 2. HIF1α mRNA expression (A) and protein level (B) of control, hypoxic-, and alkalinized-BCSCs after 6-, 24-, and 48-hour incubation, respectively. mRNA expressions were determined using real-time qRT-PCR and presented as a relative expression to untreated control based on the Livak method calculation ($2 - \Delta\Delta Ct$). While protein levels were measured by ELISA and presented as the ratio of HIF1α protein level of the sample to control $^{*}p < 0,05$.

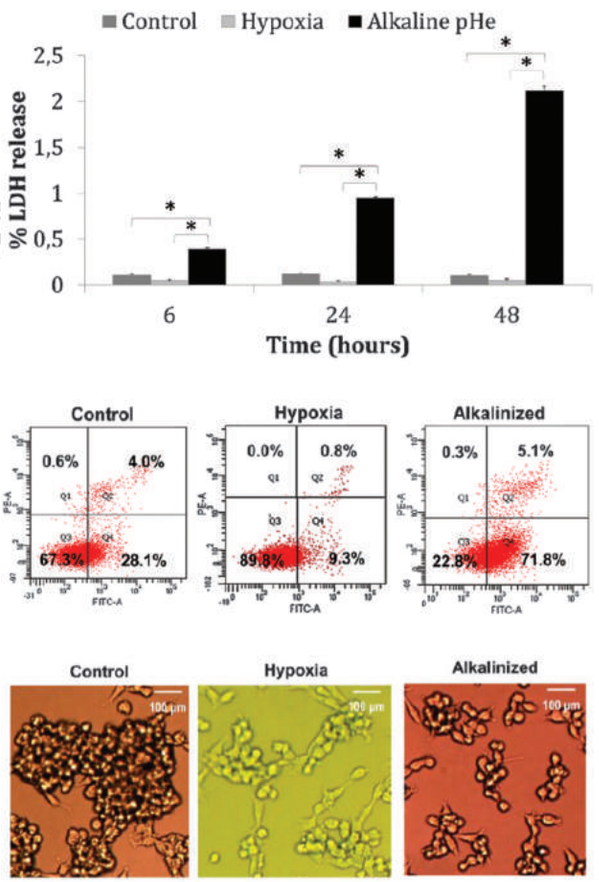

Fig. 3. The viability of control, hypoxic-, and alkalinized-BCSCs was determined using the measurement of % LDH release that is equal to the number of lysed cells (A) while the apoptosis was detected by flow cytometry using FITC Annexin V/PI protocol (B). There were different appearances between control, hypoxic-, and alkalinized-BCSCs after 24-hour incubation that were observed under an inverted microscope with 10× magnification (C) $^{*}p < 0,05$.

Table I. Population dobling time (PDT) of control, hypoxic, and alkalinized BCSCs in hours.

	Control	Hypoxia	Alkaline pHe
Population doubling time (hours)	36.4 ± 4.0	102.7 ± 0.4	51.2 ± 2.02

CSCs (Fig. 3(A)). These results indicated that hypoxic condition protected the cells to lyse. Both hypoxia and alkalinization could cause the extension of PDT (Table I). This result showed that there was a delayed proliferation rate in this two conditions. It also was observed under inverted microscope that the cell counts of hypoxic and alkalinized BCSCs from one view were lesser than control (Fig. 3(B)). Moreover, the ability of BCSCs to form cell aggregates was inhibited under hypoxia and alkalinization. Apoptosis assay seemed in line with the %LDH release. The alkalinization promoted the breast CSCs entering the early apoptosis (68,1%) and late apoptosis (14,5%) state, but in contrast, the hypoxia did not, in fact, the percentage of viable cells (89,8%) was higher compared to control (59,1%) after 24-hour exposure (Fig. 3(C)).

3.4. ALDH1 and KLF4 mRNA Expression After Hypoxia Exposure and Alkalinization

The impact of hypoxia exposure and alkalinization on breast CSCs' stemness was determined with the measurement of ALDH1 and KLF4 mRNA expression level by real-time qRT-PCR (Figs. 4(A and B)). After hypoxia exposure, ALDH1 mRNA expressions were significantly lower than control at all incubation periods. Unexpectedly, mRNA expressions of the gene encoding stemness marker were significantly higher than control. Almost same results were obtained from the mRNA KLF4 expression. Although after 6-hour hypoxia exposure, the mRNA KLF4 expression was significantly higher compared to control, the expression after 24- and 48-hour was lower. Meanwhile, at all incubation periods of alkalinization, the expression of this gene that plays a role in maintaining self-renewal were actually higher than controls.

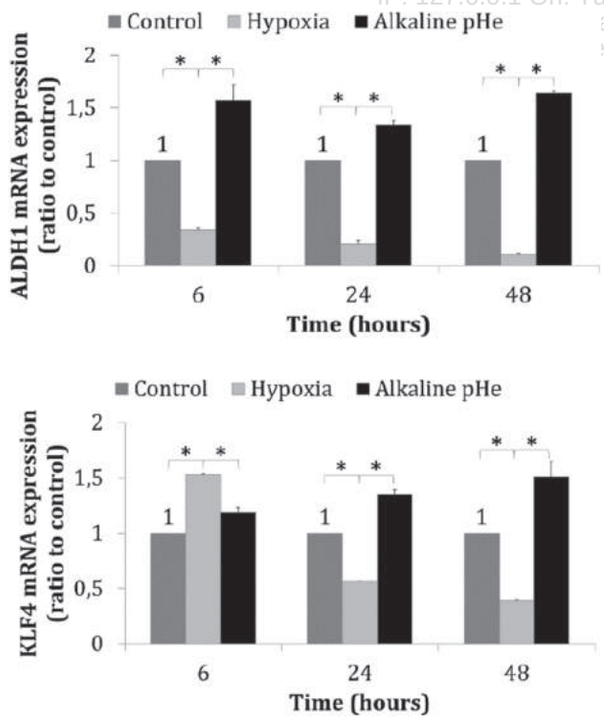

Fig. 4. ALDH1 (A) and KLF4 (B) mRNA expression of control, hypoxic-, and alkalinized-BCSCs after 6-, 24-, and 48-hour incubation, respectively. mRNA expressions were determined using real-time qRT-PCR and presented as a relative expression to untreated control based on the Livak method calculation $(2 - \Delta\Delta Ct)$ $^{*}p < 0,05$.

4. DISCUSSION

The results presented here demonstrated that BCSCs responded the alteration of O_2 level and pH toward their microenvironment. The hypoxia increased the HIF1α mRNA and protein expression of BCSCs. As widely recognized, HIF1α is the oxygen-responsive subunit of HIFs, the transcription factors that sense and coordinate cellular response to hypoxia.[6] Recent studies showed that multiple normal stem cells depend on HIF activity to maintain their undifferentiated phenotype.[5] The roles of hypoxia and HIF in cancer stem cells were largely unknown. However, several studies demonstrated that hypoxia, mediated by HIFs, also played a critical role in regulating the self-renewal of BCSCs, and induced tumor cells de-differentiation by maintaining the stem cell properties such as Oct4, cMyc, and Nanog.[15,16]

Surprisingly, our study showed that the alkalized BCSCs' HIF1α mRNA expression increased after 24-hour incubation followed by a slight increase of HIF1α protein level. This results gave rise a question, and further studies are required to understand whether an alkaline pHe could be an O_2-independent activator for HIF1α. Several factors were proved as the non-hypoxic stimulators/activators for HIF1α such as some growth factors, cytokines, vascular hormones, and viral proteins.[17]

Our results indicated that BCSCs were able to survive in the hypoxic condition shown by a low% LDH release and a high% viable cells compared to control (normoxia). Hypoxia has a dual role in determining the cell survival, both as a pro-apoptotic or an anti-apoptotic factor. A severe chronic hypoxia may initiate apoptosis. Meanwhile, the cells may adapt and survive in a moderate acute hypoxia.[18] Apoptosis regulation toward hypoxia was mediated by HIF. This transcription factor could initiate the apoptosis via several mechanisms such as by upregulating the expression of anti-apoptotic binding protein like BNIP3 and NIX; inducing anti-apoptotic protein, Bcl-2 and IAP-2; and down-regulating the expression of pro-apoptotic proteins Bid and Bax.[19,20] In this study, 24-hour hypoxia exposure to BCSCs might be categorized as an acute hypoxia that seemed to induce the anti-apoptotic factors. However, the hypoxic BCSCs also showed a PDT extension until 101 hours that might indicate a halt of cell proliferation or quiescence. Previous studies revealed that hypoxic condition is necessary for maintaining dormancy phenotype (quiescence) both normal stem cells or CSCs in solid tumors such as neuroblastoma, breast, and cervical cancer.[20]

Conversely, alkalinization seemed to increase the lysis BCSCs and the percentage of cells entering both the early and late apoptosis phase. Our unpublished data showed that alkalinization could promote a significant increase of anaerobic glycolysis phenotypes in BCSCs as a compensation mechanism toward the alkaline (high) pHe. The main consequence of the anaerobic glycolysis is the high lactate production, and the accumulation of intracellular lactate might be the basic mechanism of

the apoptosis by reducing the intracellular pH (pHi). pHi was known to induce apoptosis by altering the membrane potential of mitochondria, promoting DNA fragmentation, and activating the caspase-3.[21] Another study demonstrated that alkaline culture could induce the mitochondrial instability due to subsequent $Ca2^{+}$ overloads that lead to cell death.[22] The alkalinized BCSCs also had an extension of the PDT (52 hours). It seemed that alkaline culture not only promoting apoptosis but also suppressing proliferation.

Unexpectedly, ALDH1 and KLF4 mRNA expressions in the hypoxic BCSCs were lower than their expressions in normoxia. Hypoxia did not seem to induce the increase expression of ALDH1 but suppressed it instead. The previous study revealed that the high ALDH activity through the activation of HIF2α enhanced stemness in the breast cancer cells.[23] The inconsistency of our result might arise from the different type of cells. However, it is necessary to answer whether the low ALDH1 mRNA expression of hypoxic BCSCs correlates with its role as proliferation modulator whereas CSCs are likely in a dormant state under the hypoxic condition.[24,25] Hypoxia seemed to up-regulate the self-renewal properties of the BCSCs that represented here by the increase of KLF4 mRNA expression compared to the normoxia after 6-hour incubation. Further studies are necessary to confirm this result due to the decrease expression of this gene at the following incubation periods. In contrast to hypoxia, ALDH1 and KLF4 mRNA expressions of the alkalinized BCSCs were significantly higher than control at all incubation periods. These also need further studies especially for the protein level analysis to confirm the impact of alkaline culture on stemness properties of BCSCs.

5. CONCLUSION

In conclusion, extracellular modulation on the O_2 level and pH could alter the BCSC survival dan stemness. Low O_2 level seemed to enhance BCSCs' survival. Conversely, alkaline culture decreases it. Further studies are required for a deeper understanding of the roles of hypoxia, and especially the pH on stemness since this study indicated potential therapeutic targets from these two parameters of the microenvironment. Hence, cancer therapy is not only focused on the cancer cells per se but also in conjunction with the microenvironment modulation which is expected to reduce the cancer cell survival, in particular, the cancer stem cells.

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