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Effects of Extracellular pH Modulation on HIF-1α, c-Myc, and FOXO1 Expression in Colorectal Cancer Cells

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ABSTRACT

Background: The tumor microenvironment (TME) of colorectal cancer (CRC) is characterized by an inverted pH gradient, with acidic extracellular and alkaline intracellular conditions that promote tumor progression and metabolic reprogramming. This altered pH landscape regulates key transcriptional drivers of glycolysis and proliferation, including hypoxia-inducible factor-1 alpha (HIF-1α), c-Myc, and the tumor suppressor Forkhead Box Protein O1 (FOXO1). Understanding how extracellular pH influences these regulators may provide new insights for pH-targeted cancer therapy. **Methods:** Human colorectal carcinoma HCT116 cells were cultured for 24 hours under six extracellular pH conditions (5.5–9.2). The expression of HIF-1α, c-Myc, and FOXO1 was quantified using quantitative real-time polymerase chain reaction (qPCR), and relative fold changes were analyzed by the 2^-ΔΔCt method. **Results:** Acidic conditions (pH 5.5–6.7) markedly upregulated HIF-1α and c-Myc while strongly suppressing FOXO1 expression. Conversely, mild alkalinity (pH 8.4) reversed this pattern, reducing HIF-1α and c-Myc while restoring FOXO1 expression, suggesting a transcriptional shift from glycolytic to oxidative metabolism. At higher alkalinity (pH 9.2), the expression of all three genes declined, indicating a threshold beyond which excessive pH elevation becomes detrimental to cellular regulation. **Conclusion:** Extracellular pH critically modulates metabolic gene expression in CRC cells. Acidic conditions activate glycolytic and oncogenic pathways via HIF-1α and c-Myc, while mild alkalinity suppresses these signals and reinstates tumor-suppressive FOXO1 activity. Controlled alkalinization of the TME may therefore represent a promising adjunctive approach to disrupt tumor metabolism and limit cancer progression.

Keywords: Colorectal Cancer (CRC), HCT116, pH Modulation, Gene Expression

INTRODUCTION

Colorectal cancer (CRC) remains a significant global health challenge, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related death worldwide. In 2022, there were an estimated 1.9 million new cases and over 900,000 deaths attributed to CRC [1] Standard therapeutic modalities, including surgical resection, chemotherapy, and radiation therapy, form the cornerstone of current treatment strategies. However, their efficacy is often compromised by significant drawbacks such as severe side effects, the development of chemoresistance, and high rates of tumor recurrence [2]. A fundamental contributor to this therapeutic resistance is the distinct metabolic phenotype of cancer cells, notably the HCT116 cell line. These cells exhibit a profound reliance on aerobic glycolysis; a phenomenon termed the Warburg effect. This metabolic reprogramming results in the prolific production and secretion of lactic acid, which acidifies the tumor microenvironment (TME). The acidic TME not only facilitates local invasion and metastasis but also suppresses the

local invasion and metastasis but also suppress the function of anti-tumor immune cells, thereby creating a formidable barrier to effective treatment [3-5] .

In contrast to neoplastic cells, normal differentiated cells primarily generate adenosine triphosphate (ATP) through mitochondrial oxidative phosphorylation (OxPhos), a highly efficient process that completely oxidizes glucose to CO_2 and H_2O. Cancer cells, however, upregulate glycolysis even in the presence of sufficient oxygen, shunting pyruvate away from the mitochondrial TCA cycle and towards lactate production. This metabolic shift, while inefficient in terms of ATP yield per glucose molecule, provides a rapid supply of energy and essential biosynthetic precursors required for accelerated cell proliferation [6]. The resultant accumulation of lactic acid leads to extracellular acidosis, which is a key driver of chronic inflammation, angiogenesis, and immune evasion. This unique metabolic hallmark presents a strategic vulnerability for therapeutic intervention.

Targeting the regulators of intracellular pH (pHi) and extracellular pH (pHe), such as monocarboxylate transporters (MCTs) and vacuolar-type H^+-ATPases (V-ATPases), or modulating the core transcriptional regulators of this metabolic switch, offers a promising avenue for novel anti-cancer therapies [6-8]. Previous investigations have provided compelling evidence that manipulating the pH of the TME can disrupt cancer cell pathophysiology. The use of alkaline agents, such as sodium bicarbonate or other buffering systems, has been shown to neutralize the acidic TME, thereby attenuating cancer cell viability and metastatic potential [9,10]. Mechanistically, this alkalization is hypothesized to reverse the pH gradient across the plasma membrane, inducing a state of intracellular alkalosis that can trigger apoptosis and inhibit proliferation. Crucially, these effects are linked to the modulation of key metabolic signaling pathways. Transcriptional regulators such as Hypoxia-Inducible Factor 1-alpha (HIF-1α) and the oncogene c-Myc are master drivers of the glycolytic phenotype, upregulating the expression of glucose transporters and glycolytic enzymes. Conversely, transcription factors like Forkhead Box Protein O1 (FOXO1) often act as tumor suppressors by promoting metabolic homeostasis and inhibiting unrestrained growth [11,12].

Studies suggest that alkaline therapy may exert its anti-neoplastic effects by downregulating the expression of HIF-1α and c-Myc while potentially restoring the tumor-suppressive functions of FOXO1 [13]. While the phenomenon of pH inversion has been extensively characterized across various tumor types, its implications in colorectal cancer remain relatively underexplored. Colorectal carcinoma exhibits distinct metabolic dependencies and microenvironmental dynamics compared to other solid tumors, including differences in oxygen utilization, lactate handling, and ion transporter expression. These unique features may confer a distinct transcriptional sensitivity to extracellular pH modulation. Understanding this specificity is critical to delineate how metabolic and pH-regulatory pathways converge to sustain tumor progression in colorectal malignancies.

CRC cells exhibit extensive metabolic plasticity, enabling survival and progression in the fluctuating intestinal niche. This adaptability is driven by intrinsic metabolic rewiring, microenvironmental cues, and interactions with the gut microbiota, highlighting multiple potential therapeutic targets [14-16]. Yet, the extent to which pH alterations drive transcriptional responses in CRC particularly involving HIF-1α, c-Myc, and FOXO1 has not been clearly elucidated. Despite the promising premise of pH-targeted therapy, the precise molecular effects following treatment with an alkaline agent in CRC cells remain to be fully elucidated. It is imperative to systematically investigate the direct impact of extracellular alkalization on the transcriptional machinery that regulates the Warburg effect. Therefore, in this study, we sought to determine how changes in extracellular pH modulate the expression of key metabolic regulators, HIF-1α, c-Myc, and FOXO1 in HCT116 colorectal cancer cells. This approach allows us to assess how extracellular pH influences colorectal cancer cell metabolism within the tumor microenvironment

MATERIALS AND METHODS

Research Design

Human colorectal carcinoma cells (HCT cell line) were cultured in vitro under six different extracellular pH conditions to investigate the influence of pH modulation on metabolic gene expression. Cells were maintained in McCoy's 5A medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (Pen-Strep), and fungizone (amphotericin B), referred to as the complete medium.To create different extracellular pH environments, the complete medium was adjusted to pH 5.5, 6.0, 6.7, 7.0, 8.0, and 9.4 using hydrochloric acid (HCl) for acidification and sodium bicarbonate ($NaHCO_3$) for alkalinization. The pH values were verified using a calibrated pH meter prior to cell seeding to ensure stability.

Cell Line and Culture Conditions

HCT cells were seeded at a density of 1×10^5 cells per well in 24-well culture plates (Corning, USA). Each pH condition was prepared in triplicate wells. The cells were incubated in the corresponding pH-adjusted complete medium for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. No medium change was performed during the incubation to preserve the adjusted extracellular pH environment.

Quantitative Real-Time PCR (qPCR)

Following the 24-hour treatment period, total RNA was extracted from the harvested cells using the TRIzol™ Reagent according to the manufacturer's protocol. Briefly, cells were lysed directly in the wells, and the lysate was processed for phase separation using chloroform. RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol, and resuspended in nuclease-free water. The concentration and purity of the extracted RNA were determined using a NanoDrop™ spectrophotometer, with A260/A280 ratios between 1.8 and 2.0 considered acceptable. For first-strand cDNA synthesis, 1 µg of total RNA from each sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit with random primers according to the manufacturer's instructions. Quantitative PCR was conducted using SYBR™ Green PCR Master Mix on a real-time PCR system. Primers for HIF-1α, c-Myc, FOXO1, and GAPDH were designed using Primer-BLAST. Each 20 µL reaction contained SYBR Green Master Mix, specific primers, cDNA template, and nuclease-free water. The thermal profile included initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt curve analysis confirmed amplification specificity.

Data Analysis

The relative quantification of gene expression was performed using the comparative Ct (ΔΔCt) method. The cycle threshold (Ct) values of the target genes (HIF-1α, c-Myc, FOXO1) were normalized to the Ct value of the endogenous control gene (β-actin) for each sample to obtain the ΔCt value. The ΔΔCt was then calculated by subtracting the ΔCt of the control group (pH 7.4) from the ΔCt of each treatment group. The fold change in gene expression relative to the control was determined by the formula $2^{-\Delta\Delta Ct}$.

RESULTS

The relative mRNA expression levels of three key metabolic transcription factors HIF-1α (Hypoxia-Inducible Factor 1α), c-Myc, and FOXO1 were quantified in colorectal cancer HCT cells

cultured under varying extracellular pH conditions (5.5, 6.0, 6.7, 7.4, 8.4, and 9.2). All data were compared to the control group at physiological pH (7.4) and presented as fold changes (Figure 1). Under acidic conditions (pH 5.5–6.7), the expression of glycolytic transcription factors markedly increased. HIF-1α reached its highest induction at pH 6.0 (4.24-fold), followed by pH 5.5 (3-fold), compared with the control. Similarly, c-Myc expression rose significantly under acidic stress, peaking at pH 5.5 (2-fold) relative to the control (0.72-fold). In contrast, FOXO1, a tumor suppressor involved in oxidative metabolism and apoptosis, was strongly repressed under the same conditions, showing only 0.23-fold expression at pH 6.0 and 0.02-fold at pH 6.7. On the contrary, under alkaline conditions (pH 8.4–9.2), the transcriptional trend was reversed. Both HIF-1α and c-Myc expression decreased progressively with increasing alkalinity, reaching their lowest values at pH 9.2 (0.52-fold for HIF-1α and 0.47-fold for c-Myc). Interestingly, FOXO1 exhibited a biphasic response: it was upregulated at pH 8.4 (1.43-fold) but sharply declined again at pH 9.2 (0.03-fold).

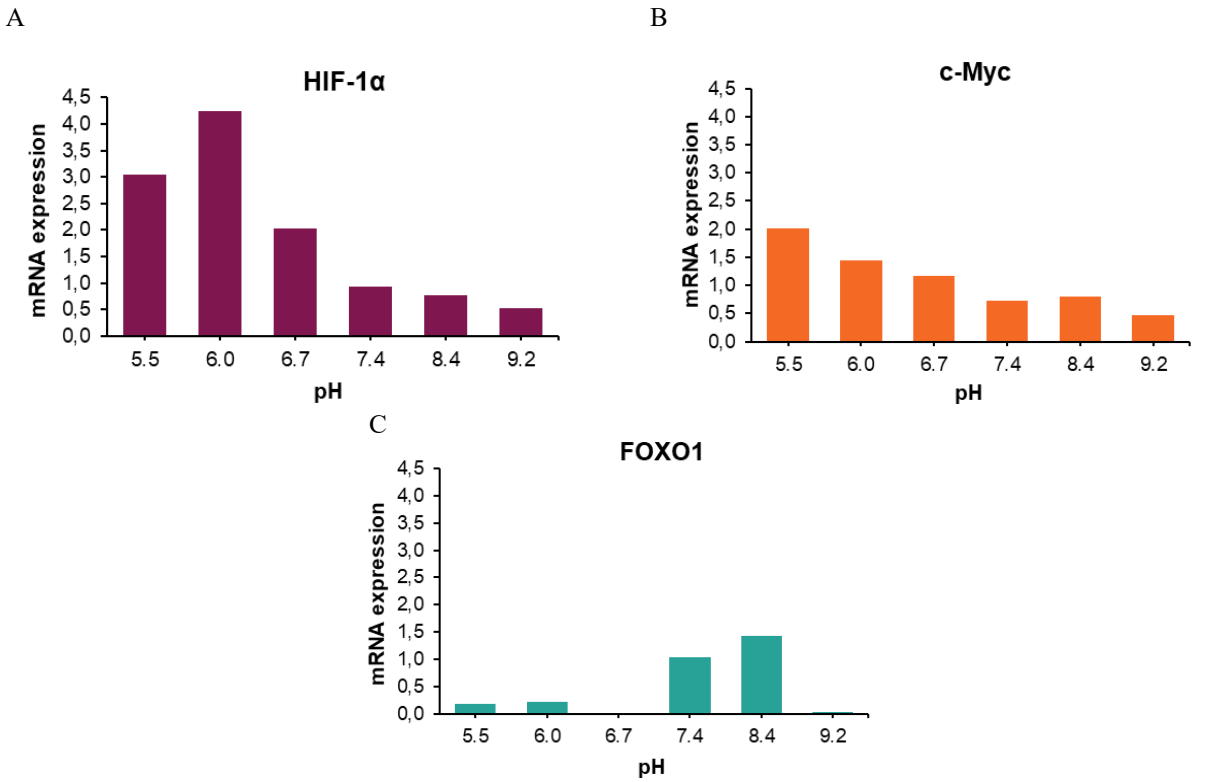

Figure 1. Relative Expression of HIF-1α, c-Myc, and FOXO1 mRNA under Different Extracellular pH Conditions.

DISCUSSION

The observed mRNA expression in this study align with the pH inversion concept in the tumor microenvironment (TME). Cancer cells maintain an abnormally alkaline intracellular pH (pHi 7.4–7.6) and an acidic extracellular pH (pHe 6.5–6.9) compared to normal tissues (pHi 7.2; pHe 7.4) [17,18]. This inversion is sustained by upregulation of proton transporters such as Na^+ /H^+ exchanger 1 (NHE1), Carbonic Anhydrase IX (CAIX), monocarboxylate transporters (MCTs), and bicarbonate exchangers that facilitate active H^+ and lactate efflux, preserving intracellular alkalinity essential for proliferation and biosynthesis. The acidic pHe, in turn, promotes invasion, immune evasion, and therapy resistance[19,20]. In this study, exposure to low pH significantly enhanced the expression of HIF-1α and c-Myc, two central transcriptional regulators of metabolic reprogramming[21].

Under normal oxygen (normoxic) conditions, HIF-1α levels are tightly regulated through hydroxylation by prolyl hydroxylase domain (PHD) enzymes, which target it for ubiquitin-mediated degradation via the von Hippel–Lindau (VHL) pathway[22-24]. Under hypoxic or metabolically stressed conditions where oxygen limitation, reactive oxygen species, or acidic stress inhibit PHD activity, HIF-1α escapes degradation, accumulates, and translocates to the nucleus to activate hypoxia-responsive genes[25,26]. HIF-1α enhances glycolytic flux by upregulating glucose transporters and glycolytic enzymes, leading to lactate accumulation. Supporting this, studies in cervical cancer have shown that hypoxia-induced OIP5-AS1 promotes the Warburg effect through the miR-124-5p/IDH2/HIF-1α signaling axis[26]. Although HIF-1 traditionally antagonizes MYC activity under normoxia, many tumors co-opt these pathways such that HIF-1 and c-Myc act cooperatively to maximize glycolysis. Together, they upregulate hexokinase 2 (HK2)—which catalyzes the first glycolytic step—and pyruvate dehydrogenase kinase 1 (PDK1), which inhibits mitochondrial respiration by inactivating pyruvate dehydrogenase[27].

Under hypoxic and acidic conditions, HIF-1α also interacts with Smad3, reprogramming the TGF-β signaling pathway toward a pro-glycolytic profile. This occurs through displacement of p107 and E2F4/5, releasing repression of c-Myc and thereby enhancing its transcriptional activity. Elevated c-Myc subsequently induces pyruvate kinase M2 (PKM2), consolidating the glycolytic phenotype typical of rapidly proliferating cancer cells [28]. Therefore, the concurrent upregulation of HIF-1α and c-Myc under acidic conditions observed here supports a metabolic shift toward glycolysis and anabolic growth, consistent with the Warburg effect.

Conversely, FOXO1, a member of the Forkhead box O (FOXO) transcription factors, was markedly repressed under acidic conditions. FOXO proteins (FOXO1, FOXO3, FOXO4, and FOXO6) regulate genes involved in cell cycle arrest, apoptosis, DNA repair, and oxidative stress resistance. Their inactivation through gene deletion, cytoplasmic sequestration, or microRNA-mediated silencing, removes essential anti-tumor barriers, facilitating malignant progression. FOXO downregulation has been documented across various cancers including breast, liver, colon, prostate, bladder, gastric, and hematological malignancies[29,30]. Our findings, therefore, indicate that extracellular acidity promotes oncogenic activation while suppressing tumor-suppressive transcriptional programs. At mildly alkaline conditions (pH 8.4), an opposite pattern emerged characterized by downregulation of HIF-1α and c-Myc and upregulation of FOXO1. This transcriptional profile suggests a potential metabolic reversion from glycolysis toward oxidative phosphorylation. Consistent with previous findings in MDA-MB-231 breast cancer cells, mild alkalinity reduces proliferation, elevates reactive oxygen species, and induces apoptosis[31,32].

Our findings demonstrate that extracellular pH critically regulates metabolic gene expression and determines cancer cell phenotype. Acidic conditions activate HIF-1α and c-Myc to promote oncogenic metabolism, whereas mild alkalinity suppresses these factors and reactivates tumor-suppressive FOXO1 signaling. This pH-dependent regulation underscores the metabolic plasticity of cancer cells and highlights pH modulation as a potential therapeutic strategy. Previous studies support this concept, showing that most tumors develop an acidic extracellular environment (pH 6.5–6.8) that fosters proliferation, migration, and immune evasion through activation of PI3K/AKT/mTOR signaling and upregulation of HIF-1α[33-35]. The acidic milieu arises from enhanced glycolytic flux and proton/lactate export mediated by NHE1, MCT1/4, and CAIX [36,37].

Targeting pH regulation in the tumor microenvironment represents a promising therapeutic avenue. Pharmacological inhibition of NHE1, MCT1/4, and CAIX or buffering strategies that slightly elevate extracellular pH may disrupt the metabolic adaptability of cancer cells. However, excessive alkalinization (e.g., pH 9.2) appears detrimental even to tumor-suppressive mechanisms, as shown by the concurrent reduction of HIF-1α, c-Myc, and FOXO1 in our data. Hence, maintaining

extracellular pH within a mildly alkaline range may represent an optimal therapeutic condition sufficient to impair tumor metabolism while preserving normal tissue viability.

CONCLUSION

This study demonstrates that extracellular pH serves as a key regulator of metabolic gene expression in cancer cells. Acidic conditions (pHe 6.5–6.9) upregulate HIF-1α and c-Myc while suppressing FOXO1, collectively driving glycolytic reprogramming and anabolic growth consistent with the Warburg effect. In contrast, mildly alkaline conditions (pHe 8.4) downregulate HIF-1α and c-Myc and restore FOXO1 expression, suggesting a metabolic shift toward oxidative metabolism and reduced proliferative capacity. These findings show that cancer cells are highly sensitive to extracellular pH, and that mild alkalinization within physiological limits may disrupt tumor metabolism and suppress progression.

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Competing Interests

The authors declare that there is no conflict of interest.

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