The association of HIF-2α expression with stemness and survival genes in human breast cancer stem cells (CD24−/CD44+) exposed to hypoxia

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Abstract. Similar to normal stem cells, breast cancer stem cells (BCSCs) exist in prolonged hypoxia in vivo and are cultured in normoxic conditions (20% O2) in vitro. Our previous study showed that the treatment of in vitro BCSCs with 1% O2 upregulates HIF-1α. This study aimed to investigate HIF-2α expression under prolonged hypoxia and the effects on its downstream gene expression (Oct-4, ALDH1, KLF4, and c-MYC) along with an associated survival gene, survivin. Human BCSCs (CD24−/CD44+) were exposed to hypoxia (1% O2, 5% CO2) at intervals of 0.5 h, 4 h, 6 h, and 24 h. HIF2α, Oct-4, ALDH1, KLF4, c-MYC, and survivin mRNA expression levels were relatively quantified using qRT-PCR. Data were analyzed using the Livak’s formula and one-way ANOVA. In contrast to HIF-1α levels, HIF-2α levels were gradually downregulated under 24 h of hypoxia treatment. In concert, Oct-4, ALDH1, KLF4, and c-MYC expression levels, as a measure of stemness, significantly decreased following hypoxia from 0.5 h to 24 h. The reduced expression of these transcription factors suggests diminished proliferation and a possibility of differentiation of the cells, thus sensitizing them to cell death. In support of this, survivin downregulation was also shown throughout the 0.5–24 h of hypoxia, which confirms our previous result that demonstrated enhanced apoptosis. Hypoxia treatment of in vitro BCSCs, unlike in vivo BCSCs, resulted in decreased HIF-2 levels, indicating acute hypoxia, which may lead to decreases in stemness and cell viability.

1. Introduction
Breast cancer is the second most common type of cancer in women, with a high risk of mortality due to recurrence [1]. As proposed over 40 years ago, a small subset of cancer cells, known as cancer stem cells (CSCs), is responsible for this recurrence [1,2]. CSCs possess characteristics and abilities that resemble those of embryonic stem cells, such as self-renewal, the maintenance of pluripotency, and proliferation [3]. Because they resemble embryonic stem cells in their characteristics, CSCs are also thought to express their pluripotency in hypoxic conditions [4]. In cancer, the abnormal growth of
cells causes a disruption in their arrangement, making it more difficult for the cells to acquire oxygen. Such a structural abnormality explains the phenomenon of hypoxia in solid tumors [5,6]. Following hypoxia, hypoxia-inducible factors (HIFs), which are transcription factors whose existence depends on the absence of oxygen, start to stabilize and activate target genes that are needed to compensate for hypoxia. The HIF family comprises HIF-1α, HIF-2α, and HIF-3α, with HIF-1α and HIF-2α resembling each other in amino acid structure [7]. Despite their similarities, both transcription factors activate different sets of downstream genes in possibly different time sets of hypoxia. In addition, hypoxia, which might cause cell death, is also thought to induce the expression of survivin, which is a member of the inhibitor of apoptotic protein family in CSCs [8].

HIF activation is followed by an upregulation of their downstream targets, including pluripotency genes that may lead to increases in CSC stemness. In breast cancer, pluripotency-related genes, including Oct-4, NANOG, and SOX2, are part of the core pluripotent regulator genes, with KLF4 as their coregulator, along with other transcription factors such as c-Myc and p53 [9]. KLF4 expression in embryonic stem cells increases the expression and activities of the core pluripotency network (Oct-4, NANOG, and SOX2), thus leading to the evasion of apoptotic pathways and promotion of tumorigenesis [10-12]. Our previous study on HIF-1α expression in breast cancer stem cells (BCSCs) showed that hypoxia increased HIF-1α expression, suppressed proliferation, and inhibited apoptosis. Interestingly, ALDH and KLF4 expressions in that study were downregulated, in contrast to the expectation that pluripotent genes should be upregulated under hypoxia [13]. The present study aimed to investigate the effects of varying durations of hypoxia on HIF-2α expression (upregulated under prolonged hypoxia) in BCSCs and the resulting downstream effects on Oct-4, ALDH1, KLF4, c-MYC, and survivin expressions.

2. Materials and Methods

2.1. Hypoxia exposure

Human BCSCs (CD24−/CD44+) were obtained from the cancer stem cell culture laboratory in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. Approximately 1 × 10^5 BCSCs were grown in 12-well plates with DMEM/F12 medium under standard conditions (5% CO2, 37°C, and 20% O2). After 1 day of incubation, cells were exposed to hypoxia (1% O2) for 0, 0.5, 4, 6, and 24 h. Following hypoxia exposure, cells were harvested and total RNA was extracted.

2.2 Primer analysis

Defining the right and suitable pairs of primers is essential for RT-PCR. Therefore, primers were selected using the NCBI primer design. First, accession numbers for Oct-4, ALDH1, HIF-2, c-Myc, KLF4, and survivin were obtained from the NCBI Reference Sequence Database. Next, NCBI primer-BLAST was used to obtain the possible forward and reverse sequences of each gene, and the hairpin, GC content, length, and other indicators were identified using Oligoanalyzer 3.1 (Integrated DNA Technologies). The designed primers are listed in Table 1.

| Gene   | Primer | Sequence               |
|--------|--------|------------------------|
| Oct-4  | Forward| 5'-GAGGAGTCCCAGGACATCAA-3'|
|        | Reverse| 5'-AGCTTCCTCCACCCACTTCT-3'|
| ALDH1  | Forward| 5'-TTGGAAGATAGGGCCCTGCAC-3'|
|        | Reverse| 5'-GGAGGAAACCTGCCTTTTT-3'|

Table 1. Forward and reverse primer sequences of Oct-4, ALDH1, HIF-2, c-MYC, KLF4, survivin, and 18S.
2.3 Isolation and quantification of total RNA
Total RNA was isolated using the TriPure Isolation Reagent (Roche) following the manufacturer’s protocol for a density of 5 × 10^5 cells. RNA concentration and purity were measured using the Varioskan Flash spectrophotometer with 260 and 280 nm wavelengths. Pure RNA extract was indicated by the values of 1.7–2.1 from A260:A280 and 1.8–2.2 from A260:A230.

2.4 One-step qRT-PCR
The mRNA expression of each gene was assessed using one-step qRT-PCR and the Exicycler 96 PCR machine (Bioneer, Korea). In this process, the KAPA SYBR FAST qPCR Master Mix was used as a dye for detecting the amount of amplified DNA, as described in the manufacturer’s protocol. Primer pairs of each gene are shown in Table 1. PCR cycles were as follows: 55°C for c-Myc, 57°C for Oct-4, 60°C for ALDH1, 52°C–58°C for HIF-2, 54.5°C for KLF4, and 65°C for survivin, ending with 72°C for amplification. The results of PCR were in the form of sample cycle threshold (Ct) values and melting curves. The Ct values were then used to calculate the relative expression of each gene. Relative expression was calculated using the Livak formula, with the Ct values for each gene inserted into the formula and those for the 18S gene used for calibration. The formulas used are listed below:

- \( Ct\_\text{calibrator} = \text{Average Ct} \_\text{control} - \text{Average Ct} \_18\text{S} \)
- \( Ct\_\text{sample} = \text{Average Ct} \_\text{hypoxia groups} - \text{Average Ct} \_18\text{S} \)
- \( Ct = \text{Ct} \_\text{sample} - \text{Ct} \_\text{calibrator} \)
- \( \text{Fold change in expression} = 2^{-\text{Ct}} \)

2.5 Statistical analysis
SPSS 23 version program was utilized to assess the normality and homogeneity of the data obtained from calculations. To compare the fold change expressions between the hypoxia and control (before hypoxia) groups, an independent t-test was used. The normality of the data was confirmed using the Shapiro–Wilk test.

3. Results
3.1 HIF-2 expression levels under acute hypoxia in BCSCs CD24−/CD44+
Hypoxia in CSCs has been closely linked to HIF-1 upregulation. Our previous study identified the overexpression of this particular gene in the same cell line used in this study [13]. To further probe BCSC gene expression under hypoxia, the BCSCs CD24−/CD44+ were incubated for different intervals of hypoxia and then measured the relative gene expression. This study found that HIF-2 expression, in
contrast to HIF-1 expression, gradually decreased after 24 h of hypoxia (Figure 1), suggesting a switch between the two inducible factors. However, the expression slowly decreased at 24 h, suggesting a possible extreme decrease in the expression following a longer duration of hypoxia, which might suggest HIF-2 regulation under chronic hypoxia.

![HIF-2α expression](image)

**Figure 1.** HIF-2 is downregulated in BCSCs CD24−/CD44+ after exposure to different intervals of acute hypoxia.

qRT-PCR showing a gradual decrease in HIF-2 gene expression with significant differences at 30 min and 6 h following hypoxia. This steady decrease in HIF-2 expression suggests a possible role of HIF-2 during chronic hypoxia. The amount of HIF2 is expressed as the relative amount of RNA that has been normalized to 18S rRNA. Data are presented as mean ± SD. *, p < 0.05.

### 3.2 Downregulation of stemness genes in BCSCs under acute hypoxia

After establishing that HIF-2 was downregulated under acute hypoxia, we measured the stemness genes to observe the effect of acute hypoxia on the pluripotency capacity of BCSCs. The HIF-2 downstream pathway has an effect on the pluripotency of cancer cell lines [13]. qRT-PCR was performed to determine the expression values of the genes that are established indicators for stemness, proliferation, and pluripotency in BCSCs, namely Oct-4, ALDH1, KLF4, c-MYC. ALDH1 gene expression steadily decreased over the period of acute hypoxia, with the lowest relative expression observed at 24 h of hypoxia (p < 0.01). OCT-4 and KLF4 showed a dramatic decrease in their relative gene expressions under acute hypoxia, with values approaching 0 at 24 h of hypoxia (p < 0.05). Interestingly, c-MYC showed relatively fluctuating expression levels throughout the hypoxia induction, whereas it showed a stable downregulation after 24 h of hypoxia. All four genes confirmed the compromised pluripotency of BCSCs when acutely induced with hypoxia (Figure 2), indicating that the cells could no longer continue proliferation and might end up with simultaneous differentiation.

### 3.3 Survivin expression downregulated under acute hypoxia

Because one of the main stem cell features—pluripotency—was compromised in this cell line under acute hypoxia, we tried to explore other features of stem cells, such as their resistance to cell death. To this end, the relative gene expression of survivin, which acts as the survival gene in BCSCs, was measured. Survivin expression indicates survival capacity and resistance to apoptosis. The results showed that survivin was significantly downregulated (p < 0.05) under acute hypoxia (Figure 3), suggesting the possibility of low cell viability and increased BCSC apoptosis under acute hypoxia.
Figure 2. Four representative stemness genes significantly decreased in BCSCs under acute hypoxia.

Oct-4, ALDH1, and KLF4 showing a significant downregulation of expression at all intervals of hypoxia ($p < 0.05$). C-Myc expression fluctuated with a sudden increase at 0.5 h of hypoxia, followed by a general decrease up to 24 h. Low stemness gene expression suggests that cells no longer have the potential to maintain pluripotency. The amount of gene expression is shown as the relative amount of RNA normalized to 18S rRNA with 0 h as the calibrator. Data are presented as mean ± SD. *, $p < 0.05$.

qRT-PCR showing that survivin expression levels were constantly reduced for all intervals of acute hypoxia, suggesting that CSC viability was compromised and indicating a possible increase in apoptosis. The amount of survivin expression is shown as the relative amount of RNA normalized to 18S rRNA. Data are presented as mean ± SD. *, $p < 0.05$.

Figure 3. Acute hypoxia reduced survivin expression in BCSCs.

4. Discussion

HIF-2α and stemness gene expressions were consistently downregulated under hypoxia, along with the expression of a survival marker—survivin. HIF-2α is upregulated after periods of chronic hypoxia (>24 h), whereas HIF-1α is upregulated and overexpressed under acute hypoxia. A decrease in HIF-2α levels
might have influenced Oct-4, KLF4, c-MYC, and ALDH1 expression because they are its downstream targets, and this study found that these were also downregulated. Oct-4 and KLF4 levels are delicate, and minute changes in their levels may lead to differentiation and the loss of stemness capacity [13-18]. c-MYC governs cell proliferation, and its reduced levels might indicate dampened proliferation capacity [19,20]. ALDH1 is a known marker in CSCs whose activity is enhanced in BCSCs, and it is also associated with malignancy [21-23]. Reductions in ALDH1 and KLF4 expressions were consistent with our previous findings. Looking at the perturbed traits of stemness, we postulated that several of the cells would have been dying. This was assessed by examining survivin levels, which was also downregulated. A possible pathway of regulation exists between Oct-4 and survivin, through the STAT3 pathway [24-26], which could also be a reason for surviving downregulation. The results have also shown that viability was decreased because there was increased cell death revealed by the direct apoptosis analysis of the cells [13].

Decreased HIF-2 levels shown in this study might be explained by the culture technique used, which was a 2D monolayer. BCSCs supposedly live in vivo under hypoxia. In our in vitro study, BCSCs were cultured in 20% O2 and exposed to hypoxia for the first time only during the experiment. When in vivo, the structure exists in such a way that groups of cells are confined to the center in hypoxia, and this can be replicated in 3D cell cultures. 3D spherical tumor models can also be adapted, particularly in cases of BCSCs where cells grow in tumor spheres [27]. In this geometry, there would be a subpopulation that has been under chronic exposure to hypoxia and thus could appropriately conform to the treatment as these cells would do in vivo. Another method could also employ step-down exposure to hypoxia, with measurement performed after 24 h of exposure.

5. Conclusion
Exposure of BCSCs to hypoxia in vitro, unlike that in vivo, resulted in decreased HIF-2 levels, indicating acute hypoxia, which can lead to decreases in stemness and cell viability. Further studies are required to analyze the effects of prolonged hypoxia in 3D spherical cultures of BCSCs.

Abbreviations
BCSC, breast cancer stem cell; CSC, cancer stem cell; HIF, hypoxia-inducible factor

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