

FUT3 EXPRESSION IN HUMAN BREAST CANCER CELLS UNDER HYPOXIA AND SERUM DEPRIVATION

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This study aimed to investigate the effects of hypoxia and serum deprivation on regulation of fucosyltransferase-3 (FUT3) expression in breast cancer cells. **Materials and Methods:** FUT3 expression was evaluated in T47D and MCF7 cells. Transcriptional and protein analysis was performed under hypoxia and serum deprivation conditions after 6 and 24 hours; and after 24 and 48 hours, respectively. **Results:** In T47D cells, experimental conditions induced a significant decrease in FUT3 expression at both, transcriptional and protein levels, while in MCF7 cells the same conditions induced a significant increase of FUT3 expression. **Conclusions:** Regulation of FUT3 expression under hypoxic and serum deprivation conditions may be involved in the acquisition of advantages related to apoptosis resistance and metastasis promotion, according to the intrinsic differences presented by T47D and MCF7 cells. **Key Words:** breast cancer, FUT3, hypoxic microenvironment, serum deprivation.

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In tumor microenvironment, cancer cells are under extremely stressful conditions, especially nutrient starvation and hypoxic conditions, because of their uncontrolled growth and proliferation [1]. In response to this scenario, cancer cells activate adaptive mechanisms at different molecular levels to suppress cell death and promote tumor progression [2–4]. Of note, the hypoxic regulation of glycosylation was suggested to be involved in tumor progression and chemoresistance mechanisms [5, 6].

During malignant transformation the increase of fucosylation, one of the most important glycosylation modifications associated with tumor progression, often results from the fucosyltransferases (FUTs) altered activity (reviewed in [7]). Among α 1,3/4-FUTs involved in tumorigenesis, fucosyltransferase-3 (FUT3) expression acts in the epithelial-mesenchymal transition [8, 9], in sialylated Lewis antigens synthesis [8, 10] and in the induction or resistance to apoptosis mediated by C-type lectin and TNF-related apoptosis-inducing ligand (TRAIL) receptors [11, 12].

In breast cancer, FUT3 expression is associated with molecular subtype, poor prognosis and survival [13]. FUT3 acts an effector of metastasis in hormone receptor dependent manner [14] and in the sialylated Lewis antigens synthesis [15] and possibly plays role in the apoptosis resistance [16]. In several studies, FUT3 was indicated as the most suitable gene for the detection of circulated breast cancer cells [17].

However, although its functions in breast cancer biology are known, its role in breast cancer hypoxic and serum deprivation microenvironment remains unclear.

In an effort to investigate the hypoxia and serum deprivation effects on FUT3 regulation in breast cancer, this study evaluates its expression at the transcriptional and protein levels, in two breast cancer cell lines with differences in their metastatic potential and apoptosis resistance [18, 19].

MATERIALS AND METHODS

Cell lines and culture conditions. T47D and MCF7 breast cancer cell lines were obtained from the Banco de Células do Rio de Janeiro (BCRJ, BRA) and routinely grown in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂.

Hypoxia and serum deprivation assays. To establish the hypoxic and serum deprived microenvironment, T47D and MCF7 cells were cultured under normoxic conditions and then incubated in RPMI 1640 medium supplemented with 10% or 1% FBS in a modular incubator chamber (Billups-Rothenberg, USA) for 6, 24 and 48 h. Thus, four conditions were analyzed: normoxia (21% O₂/94.7% N₂/5% CO₂) in RPMI 10% FBS — control group (N10); normoxia (21% O₂/94.7% N₂/5% CO₂) in RPMI 1% FBS — N1 group; hypoxia (1% O₂/94.7% N₂/5% CO₂) in RPMI 10% FBS — H10 group; and hypoxia (1% O₂/94.7% N₂/5% CO₂) in RPMI 1% FBS — H1 group.

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Six and twenty-four hours after incubation under N10, N1, H10 and H1 conditions, total RNA was extracted from T47D and MCF7 cells using TRIzol Reagent (Invitrogen, USA) according to manufacturer's instructions. Complementary DNA was synthesized from 1 µg of RNA using the SuperScript® II Reverse

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Abbreviations used: FBS — fetal bovine serum; FUT3 — fucosyltransferase-3; RT-qPCR — real-time quantitative polymerase chain reaction; TNF — tumor necrosis factor; TRAIL — TNF-related apoptosis-inducing ligand; XIAP — X-linked inhibitor of apoptosis protein.

Transcriptase Kit (Invitrogen, USA) following the manufacturer's instructions. RT-qPCR was performed with 2 μ l of diluted cDNA, 10 μ M of each primer, 5 μ l SYBR® Green Master Mix (1X) (Thermo Fischer Scientific, USA) and ultrapure water to a final volume of 10 μ l using the ABI 7500 (Applied Biosystems, USA). The primers used were the following: FUT3 for 5'-CCTGCTGGAGTCCTTTGTGGCC-3'; rev 5'-GCAGGCAAGTCTTCTGGAGGGG-3'; ACTB for 5'-AGAAAATCTGGCACCACACC-3'; rev 5'-GAGGCGTACAGGGATAGCA-3; and HPRT1 for 5'-GGACCCACGAAGTGTG-3'; rev 5'-GGCGATGTCAATAGGACTCC-3'. Normalization of target gene abundance was carried out with ACTB/HPRT1. Two independent experiments and three technical replicates per condition were performed.

Immunoblotting. Total cellular proteins were extracted with lysis buffer (50 mM Tris-HCl, pH 8.0; NaCl 150 mM; 1% NP-40 and Protease Inhibitor Cocktail) and centrifuged at 15,000 rpm at 4 °C for 30 min. Protein quantification was performed by Bradford Method, 40–60 μ g were electrophoresed in SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked with 5% milk solution in TBS-T/0.05% (Tris Buffered Saline with Tween 20 to 0.05%) at 25 °C for 2 h, FUT3 antibody (SAB1401146, Sigma-Aldrich) was incubated overnight at 4 °C in TBS-T/0.05%. After washing, secondary antibody incubation was performed at 25 °C for 2 h followed by TBST/0.05% washes. Western blot signal was detected using Enhanced Chemiluminescent substrate (GE Healthcare Life Sciences).

Statistical analysis. Two-way ANOVA was performed to determinate statistical significance using GraphPad Prism (version 6) software. All error bars represent the mean standard error $p \leq 0.05$ values were considered significant (*), $p \leq 0.05$ (**), $p \leq 0.001$ and (***) $p \leq 0.0001$.

RESULTS

FUT3 transcription analysis of breast cancer cells under hypoxia and serum deprivation. Our

results evidenced that hypoxia and serum deprivation induced different FUT3 transcriptional regulation in T47D and MCF7 cells after 6 h and 24 h of exposure. In T47D cells, hypoxia with or without serum deprivation induced a significant FUT3 downregulation in all times, except in H1 after 6 h of exposure where FUT3 expression was similar to the control (Fig. 1, a). On the other hand, exposure of MCF7 cells to hypoxia with or without serum deprivation promoted significant FUT3 upregulation of in all times analyzed (Fig. 1, b).

Hypoxia and serum deprivation induces different regulation of FUT3 expression in T47D and MCF7 cells.

The FUT3 expression in T47D and MCF7 cells was studied to address whether the changes found at transcription level were translated into protein. For this purpose, cells were cultured 24 h and 48 h under different conditions. Transcriptional analysis showed that FUT3 expression in T47D cells decreased significantly in all conditions analyzed except in H1 after 24 h of exposure where the expression was similar to the control (Fig. 2, a). Hypoxia with or without serum deprivation induced a significant decrease of FUT3 expression after 24 h (Fig. 2, b) in MCF7 cells. Moreover, a significant increase in FUT3 expression was observed under hypoxia with serum deprivation after 48 h (Fig. 2, b).

DISCUSSION

Breast cancer still is responsible for a high number of women deaths worldwide [20]. In this cancer type, the hypoxic microenvironment is associated with poor prognosis [21], regulation of numerous genes related to tumor initiation, progression, metastasis, apoptosis (reviewed in [4]) and possibly promoting the aggressiveness and resistance by regulation of glycosylation-related genes [5]. In addition, although the increase of α 1,3/4-fucosylation in breast cancer has been correlated to tumor invasive status [22] and the FUT3 expression has been associated with poor prognostic and survival [13], its function in breast cancer hypoxic and serum deprivation microenvironment is unknown.

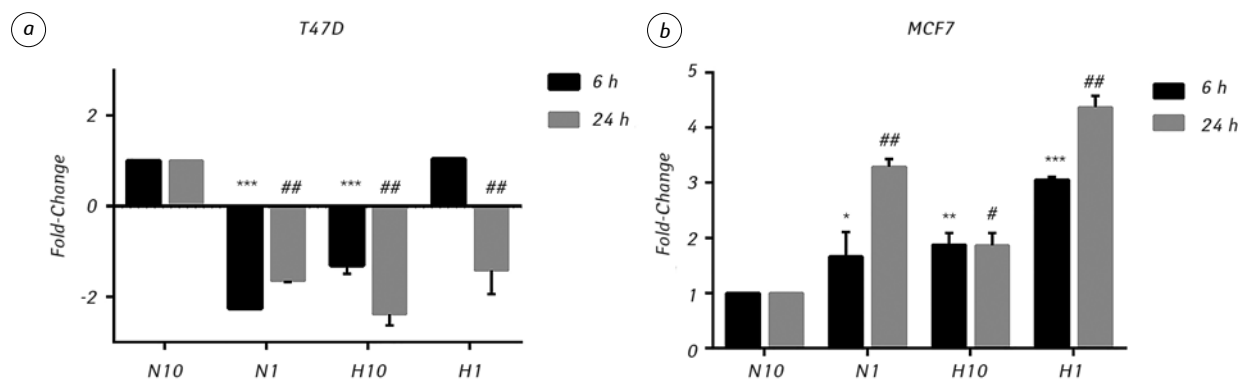


Fig. 1. FUT3 transcription analysis in breast cancer cells under hypoxia and serum deprivation. (a) RT-qPCR showing levels of expression of FUT3 in T47D cells relative to the N10 condition. (b) RT-qPCR showing levels of FUT3 expression in MCF7 cells relative to the N10 condition. The ACTB/HPRT1 housekeeping genes analysis was used to normalize the expression. RNA was collected after 6 h and 24 h of exposure to normoxia with serum supplementation (21% O₂ 10% FBS — N10), hypoxia with serum supplementation (1% O₂ 10% FBS — H10) and normoxia and hypoxia with 1% FBS supplementation, N1 and H1, respectively. Graphs represent the average value of two independent experiments with three technical replicates. Significant values are as follows: * $p < 0.05$, ** $p < 0.01$; *** $p < 0.0001$ in 6 h and # $p < 0.05$; ## $p < 0.01$ in 24 h

FUT3 activity in tumor progression pathways involves the promotion of metastasis through sialylated Lewis antigens synthesis [8, 15, 30] as well as its involvement in apoptosis regulation mediated by C-type lectin receptors, such as CD94, and TRAIL receptors, such as DR4 and DR5 [11, 12].

Although MCF7 and T47D are breast cancer cell models with the same molecular subtype, luminal-A (estrogen receptor positive, progesterone receptor positive and human epidermal growth factor receptor 2 negative) [18], studies reveal intrinsic differences related to: (1) metastatic potential — MCF7 cells are derived from a metastatic adenocarcinoma and T47D cells from invasive ductal carcinoma [23]; (2) caspase-3 expression — MCF-7 cells lack caspase-3 expression as a result of a functional deletion mutation while T47D expresses all caspases [24], the presence or absence of caspase-3 may determine the sensitivity or resistance to apoptosis [25, 26]; and (3) TRAIL-mediated apoptosis resistance — MCF7 cells presented natural resistance to TRAIL-induced apoptosis when compared to T47D cells [24].

In hypoxic microenvironment, the pro-apoptotic TRAIL signaling, recognized by the capacity to selectively induce the death of cancer cells [27], and C-type lectin-mediated apoptosis are severely impaired through X-linked inhibitor of apoptosis protein (XIAP) stabilization and modulation of the pro-apoptotic activity of the CD95 death receptor [28]. In addition, in breast cancer cells the XIAP stabilization, the most potent caspase inhibitor [29] was crucial to block autophagy, necroptosis and apoptosis induced by hypoxic and serum deprivation conditions [2, 3].

Our results revealed that FUT3 expression was significantly upregulated by hypoxia and serum de-

privation conditions, both at the transcriptional and protein levels. In T47D cells, the FUT3 expression was negatively regulated while in MCF7 was positively regulated. Based on the highest sensitivity of T47D cells to TRAIL-induced apoptosis, as well as in the FUT3 expression relevance for apoptosis regulation and metastatic phenotypes, we suggest that FUT3 expression regulation under hypoxic and serum deprivation conditions may be involved in the acquisition of advantages related to apoptosis resistance and metastasis promotion, according to the intrinsic differences presented by T47D and MCF7 cells.

In this context, we infer that negative regulation of FUT3 induced by hypoxia and serum deprivation in T47D cells may be an adaptive strategy since the FUT3 expression could increase sensitivity to C-type lectin or TRAIL receptors-induced apoptosis. This hypothesis is in agreement with Nascimento *et al.* (2015) where the FUT3 negative expression was suggested as an immunoresistance strategy in biopsies of invasive ductal carcinoma. Furthermore, studies showed that when apoptosis is blocked, TRAIL death receptors and CD95 can promote tumor progression by elicit pro-inflammatory signaling pathways [31, 32]. Nevertheless, further research is needed to determine how FUT3 expression can regulate the apoptosis resistance and tumor progression mechanisms in breast cancer in hypoxic and serum-deprived microenvironment.

On the other hand, we believe that the natural resistance to TRAIL-induced apoptosis presented by the MCF7 cells allows the significant induction of FUT3 expression under hypoxia and serum deprivation conditions as a possible strategy for the acquisition of metastatic phenotype, since the increased expression of this FUT in breast cancer is directly as-

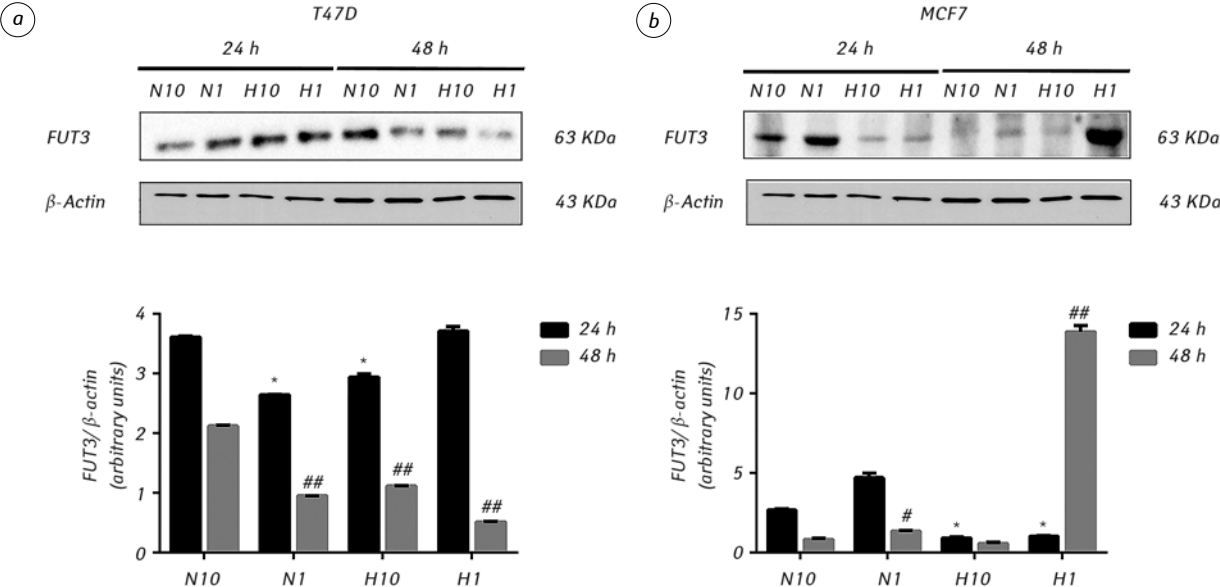


Fig. 2. FUT3 protein regulation under hypoxic and serum deprivation conditions. (a) Immunoblotting and densitometry of FUT3 expression in T47D cells. (b) Immunoblotting and densitometry of FUT3 expression in MCF7 cells. β -actin was used as a loading control. Protein was collected after 24 h and 48 h of exposure to normoxia with serum supplementation (21% O₂ 10% FBS — N10), hypoxia with serum supplementation (1% O₂ 10% FBS — H10) and normoxia and hypoxia with 1% FBS supplementation, N1 and H1, respectively. Graphs represent the average value of two independent experiments with three technical replicates. Significant values are as follows: * $p < 0.05$ in 24 h and # $p < 0.05$; ## $p < 0.01$ in 48 h

sociated with expression of sialylated Lewis antigens and promotion of metastasis [15]. Moreover, it is well known that hypoxia condition induces the increase in expression of sialylated Lewis antigens [33].

The differences in glycan expression signatures observed between molecular subtypes of breast cancer and normal breast tissue [34, 35] suggest that glycosylation alterations may be used to track disease progression, treatment response and/or the development of chemoresistance and can provide predictive biomarkers for diagnostic, prognostic and therapy. In this scenario, the effects of hypoxic microenvironment under glycosylation might have therapeutic relevance in breast cancer.

In summary, FUT3 expression in breast cancer cells under hypoxic and serum deprivation stressful environments may be involved in the survival and metastatic regulatory control system, functioning differently by apoptosis inhibition or promoting metastasis, although this possibility should be better elucidated in further research.

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CONFLICT OF INTERESTS

The authors declared no conflict of interests.

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