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PRECLINICAL STUDY

HIF-1 is involved in the negative regulation of AURKA expression in breast cancer cell lines under hypoxic conditions

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Abstract Numerous microarray-based gene expression studies performed on several types of solid tumors revealed significant changes in key genes involved in progression and regulation of the cell cycle, including AURKA that is known to be overexpressed in many types of human malignancies. Tumor hypoxia is associated with poor prognosis in several cancer types, including breast cancer (BC). Since hypoxia is a condition that influences the expression of many genes involved in tumorigenesis, proliferation, and cell cycle regulation, we performed a microarray-based gene expression analysis in order to identify differentially expressed genes in BC cell lines exposed to hypoxia. This analysis showed that hypoxia induces a down-regulation of AURKA expression. Although hypoxia is a tumor feature, the molecular mechanisms that regulate AURKA expression in response to hypoxia in BC are still unknown. For the first time, we demonstrated that HIF-1 activation downstream of hypoxia could drive AURKA down-regulation in BC cells. In fact, we found that siRNA-mediated knockdown of HIF-1 α significantly reduces the AURKA down-regulation in BC cells under hypoxia. The aim of our study was to obtain new insights into AURKA transcriptional regulation in

hypoxic conditions. Luciferase reporter assays showed a reduction of AURKA promoter activity in hypoxia. Unlike the previous findings, we hypothesize a new possible mechanism where HIF-1, rather than inducing transcriptional activation, could promote the AURKA down-regulation via its binding to hypoxia-responsive elements into the proximal region of the AURKA promoter. The present study shows that hypoxia directly links HIF-1 with AURKA expression, suggesting a possible pathophysiological role of this new pathway in BC and confirming HIF-1 as an important player linking an environmental signal to the AURKA promoter. Since AURKA down-regulation overrides the estrogen-mediated growth and chemoresistance in BC cells, these findings could be important for the development of new possible therapies against BC.

Keywords AURKA · Breast cancer · Cell cycle · HIF-1 α · Hypoxia

Introduction

Breast cancer (BC) is one of the most common cancers and a major cause of death among women worldwide [1]. Advances in molecular characterization of tumor by microarray profiling allowed the identification of five distinct molecular subtypes of BC which have led to the identification of a large number of novel targets and, in parallel, to the development of multiple approaches for anticancer therapy [2, 3]. Numerous microarray-based gene expression studies performed on several types of solid tumors revealed significant changes in key genes involved in progression and regulation of the cell cycle [4, 5]. Aberrations in genetic pathways controlling cell cycle cause abnormal cell growth, senescence, and apoptosis,

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inducing malignant transformation of cells [6, 7]. Aurora kinases are a novel family of mitotic serine/threonine kinases involved in different processes of cell cycle control [8]. Aurora kinase A (AURKA), whose expression is cell cycle dependent, is mainly involved in centrosome maturation, spindle assembly, and chromosome segregation, and it is associated with several co-activators such as BORA, TPX2, Ajuba (JUB), TACC1, and GADD45 α that drive the localization, activation, and substrate preference during cell division [9, 10]. AURKA is overexpressed in many types of human malignancies, such as breast, bladder, ovarian, colorectal, pancreatic, and gastric cancers [11–13]. This overexpression correlates with tumorigenesis, metastasis, and chemoresistance, confirming the pro-survival function of AURKA in cancer cells. Aurora-A overexpression may be achieved not only by gene amplification but also by other mechanisms such as transcription activation or suppression of protein degradation. Several studies, carried out on different tumors, showed that *AURKA* transcription may be induced by HIF-1 α , E2F3, E4TF1, TRAP220/MED1, and c-Myc [14–16]. However, the mechanism of transcriptional regulation of *AURKA* remains largely unknown, especially in BC.

In recent years, small molecules with an inhibiting action toward the aurora kinase family were developed. These molecules can act by blocking the protein–protein interaction between the aurora kinase and its substrates or blocking the ATP-binding site of the serine threonine kinase [17].

A typical feature of the microenvironment of several solid tumors is the hypoxia [18] that influences the expression of various proteins involved in proliferation and cell cycle progression [19–21]. The stabilization of the hypoxia-inducible transcription factor, HIF-1, is a crucial event that induces changes in the expression of many genes implicated in the growth and survival of neoplastic cells [22, 23]. HIF-1 is made up of two subunits, α and β [24]. Subunit β is constitutively expressed and its activity is oxygen independent. The expression of the subunit α is induced by cell hypoxia and kept at low levels in a great many cells in normoxia [25, 26]. Under normoxic conditions, HIF-1 α is targeted for degradation through the hydroxylation of its oxygen-dependent degradation domain (ODDD) by prolyl hydroxylases (PHDs) [27]. Von Hippel-Lindau protein (pVHL), which is a component of the E3 ubiquitin ligase complex, recognizes prolyl-hydroxylated HIF-1 α and drives its polyubiquitination and proteasomal degradation. Under hypoxia, HIF-1 α subunit is stabilized and dimerizes with HIF-1 β , translocates to the nucleus, and activates transcription of target genes via binding to hypoxia-responsive elements (HREs) [22, 28]. HIF-1 induces the expression of more than 100 genes [29], including the vascular endothelial growth factor (*VEGF*), glycolytic enzymes [30], and various genes involved in proliferation and

cell cycle progression, to insure the cancer cell survival [20, 31, 32]. Recently, the HIF-1 role as a repressor in response to hypoxia has been shown in several tumors. In 2005, Chen et al. [33] showed that the suppression of the *CAD* expression under hypoxia is HIF-1 α dependent in human colon cancer cells, inducing cell proliferation arrest. Furthermore, Feige et al. [34] reported that HIF-1 represses the expression of the *MITF* oncogene through induction of the transcriptional repressor DEC1 in melanoma and clear cell sarcoma cells. In addition, in 2011, Ryu et al. [35] described the HIF-1 role as transcriptional repressor of the estrogen receptor alpha (ER α) gene in MCF-7 and T47D human BC cells under hypoxia. The authors showed that hypoxia induces HIF-1 α -mediated repression of the *ESR1* gene at the transcriptional level, promoting hormone insensitivity in the tumor microenvironment.

Although hypoxia is a typical tumor condition, the molecular mechanisms that regulate *AURKA* expression in response to hypoxic condition in BC are still unknown. Therefore, the aim of our study was to obtain new insights on *AURKA* regulation in BC cell lines under hypoxia, by evaluating the possible HIF-1 role in transcriptional control of *AURKA* expression. In this work, we hypothesize that hypoxia down-regulates *AURKA* expression in a HIF-1 α -dependent manner, suggesting, for the first time, a novel potential role of HIF-1 as transcriptional repressor of *AURKA* in BC cells.

Methods

Cell culture

Human BC cell lines, MCF-7, MDA-MB-231, and SK-Br3, purchased from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's Modified Eagle Medium (DMEM:F12) supplemented with 10 % fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 50 mg/mL streptomycin) (Invitrogen, Carlsbad, CA, USA).

Cell lines were cultured at 80 % confluence in a normoxic atmosphere of 16 % O₂, 79 % N₂, and 5 % CO₂ (by volume) for 24 h. Then, medium was renewed and cells were further cultured under normoxia or hypoxia (3 % O₂, 87 % N₂, 5 % CO₂, by volume) at two different time points, 24 and 48 h.

Microarray analysis

Microarray analysis was performed as previously described [36]. Total RNA was extracted according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Fragmented cRNA was hybridized using a human oligonucleotide array U133 Plus 2.0 (Genechip Affymetrix, Santa Clara, CA).

Washing and staining were performed through Affymetrix GeneChip Fluidic Station 450. Probe arrays were scanned using Affymetrix GeneChip Scanner3000 G7 enabled for High-Resolution Scanning. Two biological replicates were performed for each experimental condition. Images were extracted with the GeneChip Operating Software (Affymetrix GCOS v1.4). Quality control of the arrays was performed using the AffyQCReport software [37].

Statistical analysis

For statistical analysis, the background subtraction and normalization of probe set intensities were performed using the method of Robust Multiarray Analysis (RMA) described by Irizarry et al. [38]. To identify differentially expressed genes (DEGs), gene expression intensity was compared using a moderated *t* test and a Bayes smoothing approach developed for a low number of replicates [39]. To correct for the effect of multiple testing, the false discovery rate was estimated from *P* values derived from the moderated *t* test statistics [40]. The analysis was performed using the affyGUI Graphical User Interface for the Limma Microarray Package (Bioconductor Software) [41].

Significant differences were determined by Student's *t* test. *P* < 0.05 was considered to be statistically significant.

Microarray data analysis

Hierarchical cluster (HCA) and heat map analyses were performed using the MultiExperiment Viewer (MeV v4.8) program of TM4 Microarray Software Suite.

The Gene Set Analysis Toolkit was used to investigate the biological significance of a set of genes represented by the specific expression pattern during the progression of cell cycle. DEGs were analyzed according to predefined pathways annotated by KEGG [42] and Biocarta bioinformatic resources. For an over-represented KEGG or Biocarta pathway, a cutoff *P* value of 0.01 was selected. All shown values are in logarithm scale.

Quantitative real-time PCR (qRT-PCR) and RT-PCR

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Then, RNA was controlled through 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified through the spectrophotometer NanoDrop ND-1000 (CELBIO). For *AURKB*, *AURKA*, and *HIF-1 α* mRNA detection, 2 ng of total RNA was reverse transcribed into single-stranded cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the vendor's instructions. Gene primers for *AURKA*, *AURKB*, and *HIF-1 α* were purchased from Applied Biosystems (TaqMan

gene expression assay). Quantitative real-time PCR (qRT-PCR) was performed with the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SDS software version 2.1. The reactions were performed in triplicate and the results were normalized using Human β -actin Pre-Developed TaqMan assay reagents (Applied Biosystems). Changes in the target mRNA content were determined using a comparative CT method (ABI User Bulletin No. 2). An average CT value for each RNA was obtained for triplicate reactions. A reverse transcription polymerase chain reaction (RT-PCR) was also performed in order to confirm the results previously obtained with qRT-PCR. The data were evaluated in 2 % agarose gel.

Western blotting (WB)

Cells were lysed using the complete Lysis-M reagent set (Roche, Mannheim Germany). Protein concentration was measured using Quick Start Bradford (Bio-Rad Laboratories, Hercules, CA). 80–100 μ g of total protein lysate was separated on 10 % polyacrylamide gel under denaturing conditions and immunoblotted into nitrocellulose membrane. The following antibodies (Abs) were used: anti-HIF-1 α rabbit (Bethyl Laboratories, Montgomery, USA); anti-phospho-aurora A (Thr288) (CD39D8) (Cell Signaling Technology, Boston, MA, USA); anti-VEGF (147); anti-ARK1(N-20); and anti-GAPDH(6C5) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA interference and stabilization of HIF-1 α

The small-interfering RNA (siRNA) oligonucleotides specific for human HIF-1 α were purchased from Thermo Scientific Dharmacon[®] (ON-TARGETplus SMARTpool L-004018-00-0005, Human HIF1A, NM_181054, 5 nmol). ON-TARGETplus SMARTpool siRNA is a mixture of 4 siRNAs (provided as a single reagent) that target the following HIF-1 α sequences: GAACAAAUACAUGGGA UUA, AGAAUGAAGUGUACCCUAA, GAUGGAAGCA CUAGACAAA, and CAAGUAGCCUCUUUGACAA. The cells were cultured in six-well culture dishes, maintained in 2 mL of DMEM medium, and transfected by adding 5 μ M siRNA (siHIF-1 α) and DharmaFECT[®] four according to manufacturer's instructions. After 24–48 h (for mRNA analysis) or 48–96 h (for protein analysis) of incubation with siRNA and transfection reagent, the cells were exposed to hypoxia for 24 and 48 h. ON-TARGETplus Non-targeting Control Pool (Thermo Scientific Dharmacon[®]) was used as a siRNA negative control (non-specific scrambled siRNA) (data not shown).

Furthermore, cells were incubated in the absence or the presence of HIF hydroxylase inhibitor, dimethylxylglycine (DMOG) (Sigma-Aldrich, St. Louis, MO), for 24

and 48 h, at a final concentration of 1 mM. Subsequently, cells were lysed and protein extracts were analyzed by Western blot.

Chromatin immunoprecipitation (ChIP)

Immunoprecipitation was performed using the ChIP Assay Kit (Millipore, Temecula, CA) according to the manufacturer's protocol. 1×10^6 cells were cultured on a 10-cm culture dish under normoxia and hypoxia (24 and 48 h). The cells then were treated with a solution of PBS and 1 % formaldehyde, and incubated for 8 min at 37 °C in order to cross-link histones to DNA. Cells were collected (4 min; 4 °C; 4,000 rpm) and sonicated in order to shear DNA to lengths between 200 and 1,000 bp. Soluble chromatin was immunoprecipitated with 5 mg of HIF-1 α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. DNA–protein immune complexes were eluted, reverse cross-linked, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The binding of HIF-1 α to the *AURKA* promoter region –336/–236 containing the three putative HREs (HRE-1: –336/–332; HRE-2: –323/–319; HRE-3: –240/–236) was determined by PCR using the following primers: forward 5'-AGTC GTTTCTGTGGTTTTCTC-3' and reverse 5'-GAGATAA AGTCCAAGGAGGTGAAC-3'. Furthermore, an *AURKA* promoter region without HREs was used as a control using the following primers: forward 5'-CTGTTCTATCCGGT CTCTTCACTT-3' and reverse 5'-TTGGGGATAATTAG GCTTCTGTT-3'. The PCR conditions were as follows: 5 min at 95 °C; 30 s at 95 °C, 30 s at 60 °C, 40 s at 72 °C for 35 cycles; 7 min at 72 °C.

Luciferase activity assay

Luciferase assay was performed as previously described by Klein et al. [14] using the luciferase reporter plasmidic construct pGL3-*AURKA*-Luc. Firefly luciferase reporter plasmid containing the human *AURKA* promoter (from –986 to +15) was generated using the pGL3 basic plasmid (Promega Italia, Milano, Italy). The human *AURKA* promoter 5'-flanking region containing three putative HREs was amplified by PCR from genomic DNA and the corresponding PCR product was cloned into pGL3 vector at the KpnI and XhoI sites. BC cells were transfected with firefly luciferase vector (pGL3-*AURKA*-Luc) or with pGL3-basic-Luc empty vector used as a negative control, using Lipofectamine 2000 (Life Technologies). A *Renilla* luciferase reporter, pRL-TK (Promega), was co-transfected as a control for normalization. After 24 h of incubation, cells were cultured under normoxia or hypoxia (for 24 and 48 h) and then lysed in passive lysis buffer (Promega). Luciferase activity of cell lysates was measured using the

Dual-Luciferase Reporter Assay System Kit (Promega) and the GloMax[®] 96 Microplate Luminometer (Promega) according to the manufacturer's protocol. Normalized data were represented as the ratio of Firefly/*Renilla* luciferase activities. The shown data represent the mean \pm SD from three independent experiments, each performed in duplicate.

Results

Gene expression profiling in BC cells under hypoxia

Considering that hypoxia is a condition that during tumor growth influences the expression of several genes involved in proliferation and cell cycle progression, we first performed a microarray analysis, using Affymetrix platform, in order to compare differential gene expression profiles in MCF-7, MDA-MB-231, and SKBr3 human BC cell lines in response to hypoxic exposure for 24 and 48 h. This work was carried out in order to evaluate the involvement of some genes in molecular pathways related to tumor pathogenesis. This analysis allowed us to obtain, for each examined BC cell line, two lists of DEGs in hypoxia at 24 and 48 h with respect to normoxia (control). In particular, for this study, the lists were screened considering as significant only the genes with fold change (M) > |1| (logarithm scale) and $P < 0.01$. Thus, we obtained six lists of DEGs with respect to the normoxic condition, including after 24 h of hypoxia 4,019 genes (2,482 down-regulated and 1,537 up-regulated) for MCF-7; 4,742 genes (3,032 down-regulated and 1,710 up-regulated) for MDA-MB-231; and 6,970 genes (4,615 down-regulated and 2,355 up-regulated) for SKBr3; and after 48 h of hypoxia 4,229 genes (2,362 down-regulated and 1,867 up-regulated) for MCF-7; 4,830 genes (1,832 down-regulated and 2,998 up-regulated) for MDA-MB-231; and 9,575 genes (6,287 down-regulated and 3,288 up-regulated) for SKBr3.

Then, six lists of genes were compared with each other and analyzed in order to identify a set of DEGs common to three BC cell lines (Supplementary Table S1). Using Venn diagrams, we found 270 shared genes (81 down-regulated and 189 up-regulated) that showed a gene expression variation in hypoxia (24 and 48 h) with respect to normoxia. Furthermore, 725 genes showed an altered expression only in MCF-7, 511 genes in MDA-MB-231, and 2513 genes in SKBr3 (Fig. 1).

Molecular pathways altered in hypoxia

A further series of studies was conducted on the microarray analysis data in order to identify any genes of particular interest. Our attention was focused mainly on the genes involved in proliferation, cell cycle progression, mitosis,

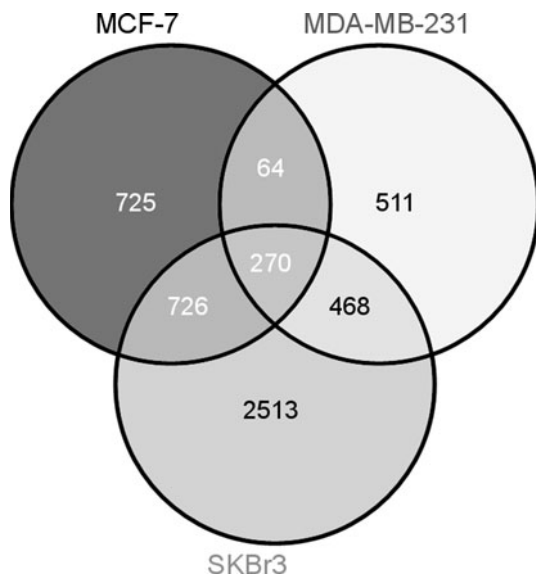


Fig. 1 Venn diagrams summarizing DEGs in breast cancer cell lines in response to hypoxia. The number of total, unique, and common DEGs ($M > 11$ and $P < 0.01$) is shown. Each Venn diagram includes for each cell line the number of genes (down- and up-regulated) whose expression is altered in hypoxic conditions (for 24 and 48 h) with respect to normoxia: 1,785 genes for MCF-7; 1,313 genes for MDA-MB-231; and 3,977 genes for SKBr3. The intersection among three Venn diagrams has allowed the identification of 270 DEGs shared by all three cell lines in response to hypoxia

and response to hypoxia. The integrated analysis resulting from KEGG and Biocarta databases allowed us to identify the main molecular pathways altered in BC cell lines after hypoxic exposure for 24 and 48 h. This analysis indicated that the genes showing a significant variation in expression levels were included in the following three pathways: cell cycle regulation (35 genes), HIF-1 α network (15 genes), and AURKA signaling (12 genes). The DEGs involved in these pathways were grouped in heat maps (Fig. 2). A considerable number of genes involved in proliferation and cell cycle control, through the G1/S and G2/M transitions, exhibited a significantly deregulated expression in all three BC cell lines (Fig. 2a). Among the DEGs in hypoxia, required for G1/S transition, *CDC6*, *CCND1*, *CCNE2*, and *CDK2* were tendentially down-regulated, whereas *CDKN1A* (p21^{Cip1}) was up-regulated. Instead, *CDC25A* was down-regulated in MCF-7 and SKBr3 cells and up-regulated in MDA-MB-231 cells. Moreover, we found that in MCF-7 and SKBr3 cells, hypoxia induces down-regulation of several genes required for G2/M transition, such as *CCNA2*, *CCNB1*, *CCNB2*, *CDC2* (*CDK1*), and *CDC25C*. The same results were shown by MDA-MB-231 cells under hypoxic conditions for 24 h (Supplementary Table S2).

In addition, significant increases in expression levels were also observed in key genes involved in HIF-1 α network (Fig. 2b), such as *VEGF-A*, *SLC2A1*, *JUN*, *FOS*, and

other genes encoding for glycolytic enzymes (*PGK1*, *PFKFB3*, *HK2*, *ALDOA*) (Supplementary Table S3). Finally, microarray analysis showed a significant variation in expression levels of some genes that regulate the mitosis included in AURKA signaling pathway (Fig. 2c). This analysis revealed that hypoxia induces a noteworthy down-regulation of *AURKA* expression. Other genes involved in the same pathway, such as *AURKB*, *JUB* (ajuba), and *TPX2*, were tendentially down-regulated in all three BC cell lines under hypoxic conditions (Supplementary Table S4).

AURKA down-regulation in BC cells under hypoxia

In particular, our analyses were focused mainly on effects of hypoxia on AURKA expression and molecular mechanisms that regulate this. As previously described, in all three examined BC cell lines, in response to hypoxia, a down-regulation of *AURKA* expression compared to normoxia (fold changes: -2.19 and -3.55 in MCF-7; -2.56 and -1.51 in MDA-MB-231; and -1.84 and -3.17 in SKBr3, at 24 and 48 h, respectively) and a concomitant decrease in expression levels of *JUB* (fold changes: -1.90 and -2.60 in MCF-7; -3.43 and -1.78 in MDA-MB-231; and -1.14 and -1.65 in SKBr3, at 24 and 48 h, respectively) were observed. Both *TPX2* and *AURKB* also showed a parallel down-regulation of their expression in hypoxia, except that in MDA-MB-231 cells exposed to hypoxia for 48 h (fold change: $+1.22$ and $+1.20$, respectively). Furthermore, microarray analysis revealed, following hypoxic exposure, significant changes in expression levels of AURKA-related genes that regulate the G2/M transition and mitosis, such as *CCNB1*, *CCNB2*, *CDC2*, *CDC25B*, and *TACC1*. All these genes exhibited a negative fold change (M), except *CCNB2* ($M = +1.73$) and *CDC2* ($M = +1.19$) in MDA-MB-231 cells, after hypoxia for 48 h (Table 1). In order to validate microarray analysis data, we evaluated the *AURKA* mRNA expression levels in the same BC cell lines under hypoxic conditions, using qRT-PCR and RT-PCR (Fig. 3a, b). The qRT-PCR analysis confirmed that hypoxia induces a *AURKA* down-regulation, with an increased reduction of mRNA expression levels after 48 h of exposure in MCF-7 and SKBr3 cells (0.15-fold and 0.2-fold, respectively), and with an initial decrease after 24 h (0.25-fold) followed by a partial expression increase after 48 h (0.45-fold) in MDA-MB-231 cells (Fig. 3a). Similar results were obtained by means of RT-PCR analysis (Fig. 3b). Interestingly, in parallel to *AURKA* mRNA down-regulation, we observed, through Western blot (WB) analysis, a reduction of AURKA protein levels in MCF-7 and SKBr3 cells after 24 and 48 h of hypoxia, compared to the control condition (normoxia). Differently, as demonstrated by microarray analysis, MDA-MB-231 cells showed a reduction in AURKA protein levels after 24 h, with a partial increase in

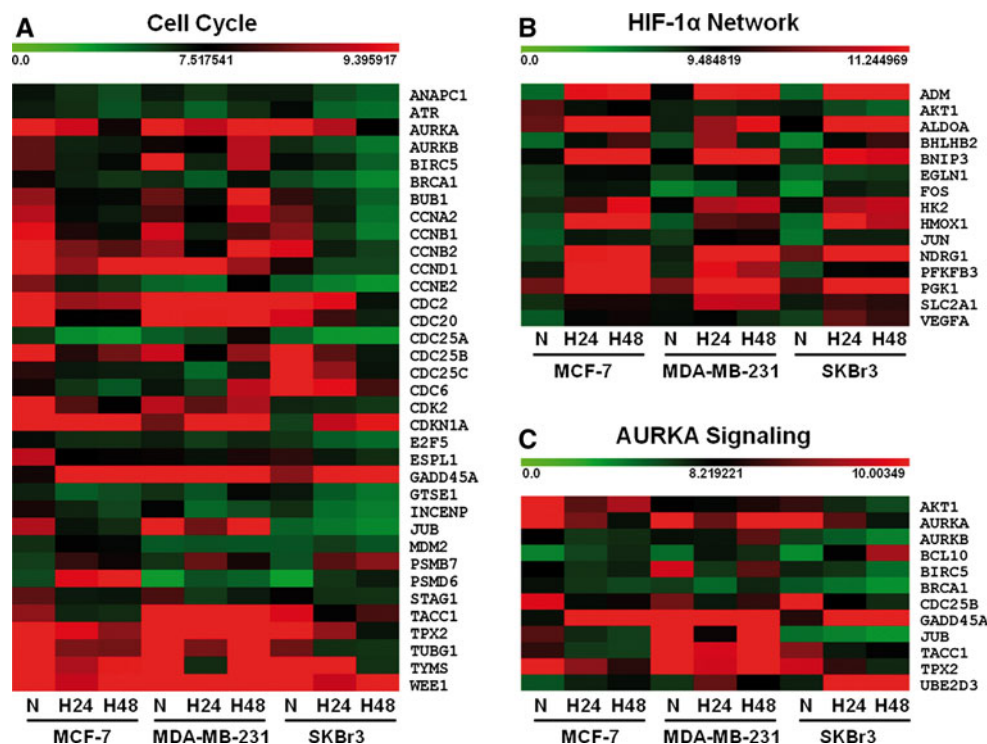


Fig. 2 Heat maps of common DEGs involved in main molecular pathways deregulated in hypoxia. Clustering of common DEGs involved in **a** cell cycle regulation pathway, **b** HIF-1 α network, and **c** AURKA signaling pathway. The heat maps were generated from microarray data reflecting gene expression values in MCF-7, MDA-MB-231, and SKBr3 cells exposed to hypoxia (3 % O₂) for 24 and 48 h in comparison to control cells cultured under normoxic conditions ($M > |1|$ and $P < 0.01$). Each row represents the expression levels for a single gene tested for different experimental

conditions. Each column shows the expression levels for the genes tested for a single experimental condition. The absolute expression value (log scale) of each gene is derived from the mean of two biological replicates. The color scale bar on the top represents signal intensity variations ranging from green (poorly expressed genes) to red (highly expressed genes). Black boxes indicate intermediate expression values. N normoxia; H24 hypoxia for 24 h; H48 hypoxia for 48 h

the expression after 48 h of exposure to hypoxia. Consistent with our findings, together with a reduction of the AURKA protein expression, we detected a decrease in expression levels of phospho-aurora A (Thr288) and an increase in HIF-1 α and VEGF-A protein levels in BC cells under hypoxia. However, HIF-1 α showed a lower expression level in MDA-MB-231 cells exposed to hypoxia for 48 h (Fig. 3c).

Hypoxia down-regulates AURKA expression in a HIF-1 α -dependent manner

Since HIF-1 α is a key transcription factor during hypoxia, we investigated its role in down-regulation of AURKA expression in BC cells exposed to hypoxia. To determine whether AURKA down-regulation was due to the expression of HIF-1 α , we evaluated AURKA expression variations in HIF-1 α -silenced cells. Therefore, HIF-1 α -dependent effects of hypoxia were reversed by silencing HIF-1 α mRNA expression with a specific siRNA pool targeting HIF-1 α (siHIF-1 α). A non-targeting control pool was used as a siRNA negative control (data not shown). Using qRT-PCR

analysis, specific knockdown of HIF-1 α was confirmed at the mRNA level and a significant increase in AURKA mRNA expression levels was observed in all three analyzed BC cell lines, following HIF-1 α silencing (Fig. 4a). In MCF-7 and SKBr3 cells, knockdown of HIF-1 α induced a greater increase of AURKA expression after 48 h of hypoxia (from 0.15-fold to 0.75-fold and from 0.2-fold to 0.7-fold, respectively) in comparison to that found after 24 h (from 0.45-fold to 0.65-fold and from 0.3-fold to 0.6-fold, respectively). In MDA-MB-231 cells, a lower increase was observed after 48 h of hypoxia (from 0.6-fold to 0.7-fold) with respect to that detected after 24 h (from 0.3-fold to 0.6-fold) (Fig. 4a). Similar results, after HIF-1 α silencing, were obtained by means of RT-PCR analysis (Fig. 4b). Specific knockdown of HIF-1 α was also evaluated at the protein level. WB analysis showed a good correlation with data previously obtained. In BC cells, silencing of HIF-1 α by RNA interference induced a significant increase in AURKA and phospho-aurora A (p-AURKA) protein levels after 24 and 48 h of hypoxia. Greater differences in protein expression were detected in hypoxic condition at 48 h, except that

Table 1 Effects of hypoxia on expression of genes related to AURKA

ID Affymetrix	Gene symbol	MCF-7		MDA-MB-231		SKBr3	
		M _{H24}	M _{H48}	M _{H24}	M _{H48}	M _{H24}	M _{H48}
208079_s_at	<i>AURKA</i>	-2.19	-3.55	-2.56	-1.51	-1.84	-3.17
209464_at	<i>AURKB</i>	-1.76	-1.46	-1.14	+1.20	-1.06	-2.24
228729_at	<i>CCNB1</i>	-1.46	-2.15	-2.47	-1.15	-2.65	-4.65
202705_at	<i>CCNB2</i>	-1.91	-2.16	-1.38	+1.73	-2.51	-3.49
203213_at	<i>CDC2</i>	-2.00	-1.88	-1.12	+1.19	-1.52	-3.74
201853_s_at	<i>CDC25B</i>	-1.97	-1.42	-1.87	-1.31	-1.93	-3.27
225806_at	<i>JUB</i>	-1.90	-2.60	-3.43	-1.78	-1.14	-1.65
200911_s_at	<i>TACCI</i>	-2.18	-2.36	-2.19	-1.13	-1.73	-1.07
210052_s_at	<i>TPX2</i>	-1.73	-2.39	-1.22	+1.22	-1.07	-2.73

M_{H24} represents the fold change of hypoxia versus normoxia (control) at 24 h. M_{H48} represents the fold change of hypoxia versus normoxia at 48 h. All showed fold change (*M*) values are in logarithm scale. $M > |1|$ and $P < 0.01$

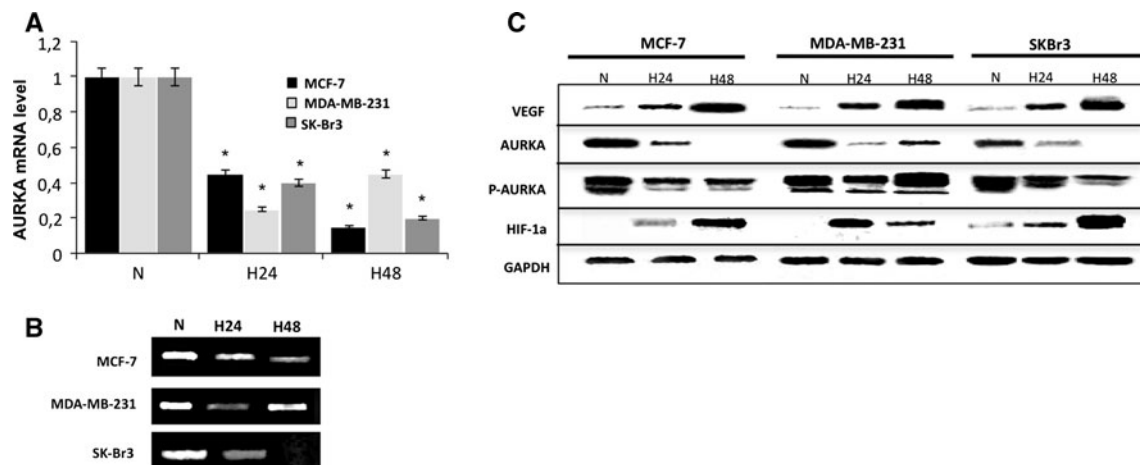


Fig. 3 Effects of hypoxia on AURKA expression in breast cancer cell lines. **a** Validation of microarray data by quantitative real-time PCR analysis. Changes in AURKA mRNA expression levels were determined in MCF-7, MDA-MB-231, and SKBr3 cells exposed to hypoxia (3 % O₂) for 24 and 48 h with respect to control condition (normoxia, 16 % O₂). Relative transcript levels were determined using the $2^{-\Delta\Delta C_t}$ method and normalized to β -actin mRNA (endogenous control). Normoxic condition values are taken as 1 and hypoxic condition values represent fold decrease relative to control condition. Data are presented as fold changes \pm SDs. Significant difference, hypoxia for 24 h (H24) or for 48 h (H48) versus normoxia (N),

$*P < 0.05$. **b** Quantification of AURKA mRNA expression using RT-PCR analysis. **c** Effects of hypoxia on VEGF, AURKA, p-AURKA, and HIF-1 α protein expression levels in MCF-7, MDA-MB-231, and SKBr3 cells. Protein expression was examined by Western Blot analysis with the indicated antibodies, by developing with the enhanced chemiluminescence reagent (ECL). p-AURKA represents phospho-aurora A (Thr288). The GAPDH house-keeping protein was used as loading control. The experiments were performed at least three different times and the results were always similar. N normoxia; H24 hypoxia for 24 h; H48 hypoxia for 48 h

in MDA-MB-231 cells. Furthermore, in all three BC cell lines, WB analysis showed a substantial hypoxia-mediated up-regulation of VEGF-A in the absence of siHIF-1 α and a considerable reduction in protein expression levels in cells transfected with siHIF-1 α (Fig. 4c).

At the same time, in order to further assess the HIF-1 potential role in regulation of hypoxia-mediated AURKA expression, BC cells were treated with DMOG, a cell-permeable inhibitor of both proline and asparaginyl

hydroxylases able to induce HIF-1 α stabilization in normoxia. Thus, treatment of MCF-7, MDA-MB-231, and SKBr3 cells with a PHD inhibitor (for 24 and 48 h) caused the activation of the HIF-1 pathway in normoxia. In fact, WB analysis provided results comparable to those previously described, leading to an increase in HIF-1 α expression levels and concomitant reduction of AURKA expression in the presence of DMOG. Interestingly, in MDA-MB-231 cells, a lower HIF-1 α induction was observed after 48 h of

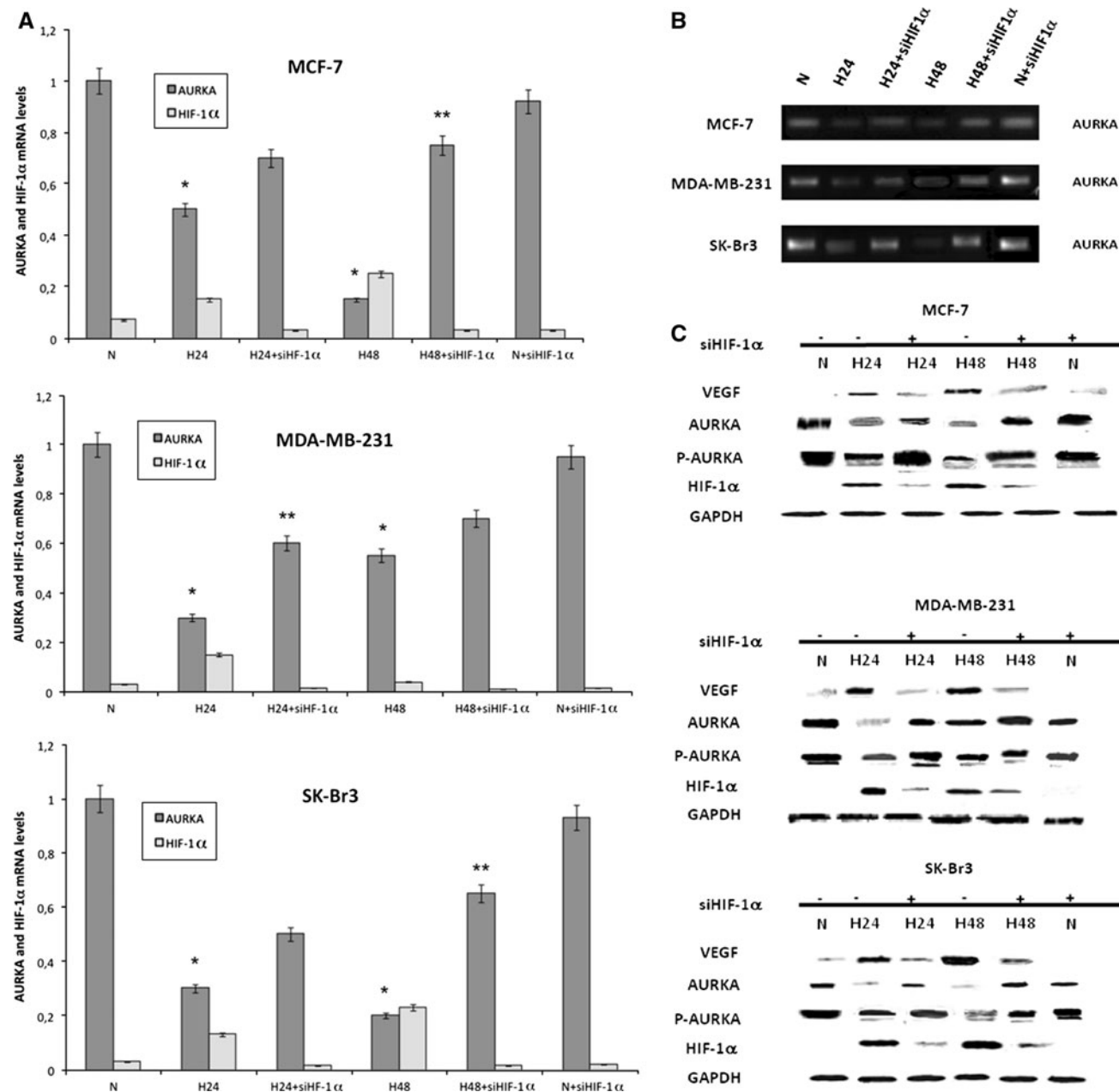


Fig. 4 Effects of HIF-1 α silencing on AURKA expression in breast cancer cells under hypoxic conditions. MCF-7, MDA-MB-231, and SKBr3 cells were transfected with a siRNA pool targeting HIF-1 α (siHIF-1 α) and subsequently exposed to hypoxia (3 % O₂) for 24 and 48 h. For all experiments, a non-targeting control pool was used as a siRNA negative control (data not shown). **a** Specific knockdown of HIF-1 α was confirmed at the mRNA level using qRT-PCR analysis. Variations in AURKA and HIF-1 α mRNA expression levels with respect to control condition (normoxia, 16 % O₂) were evaluated in breast cancer cells with and without siHIF-1 α . Relative transcript levels were determined using the $2^{-\Delta\Delta C_t}$ method and normalized to β -actin mRNA (endogenous control). Data are presented as fold changes \pm SDs. AURKA

mRNA expression values are in linear scale, while those of HIF-1 α are in logarithm scale. Significant differences: hypoxia for 24 h (H24) or for 48 h (H48) versus normoxia (N), * $P < 0.05$; H24+ siHIF-1 α versus H24, or H48+ siHIF-1 α versus H48, ** $P < 0.05$. **b** Quantification of AURKA mRNA expression after HIF-1 α silencing using RT-PCR analysis. **c** Effects of HIF-1 α silencing on VEGF, AURKA, p-AURKA, and HIF-1 α protein expression levels in breast cancer cells transfected (+) or not (-) with siHIF-1 α . Specific knockdown of HIF-1 α was assessed at the protein level by Western Blot analysis. p-AURKA represents phospho-aurora A (Thr288). The GAPDH protein was used as a loading control. The experiments were performed at least three different times and the results were always similar

incubation with DMOG with respect to treatment for 24 h, and a partial recovery of AURKA expression was detected (Fig. 5).

According to these results, we suggest that HIF-1 could act directly or indirectly as a possible negative regulator of AURKA expression in BC cell lines cultured in hypoxic conditions.

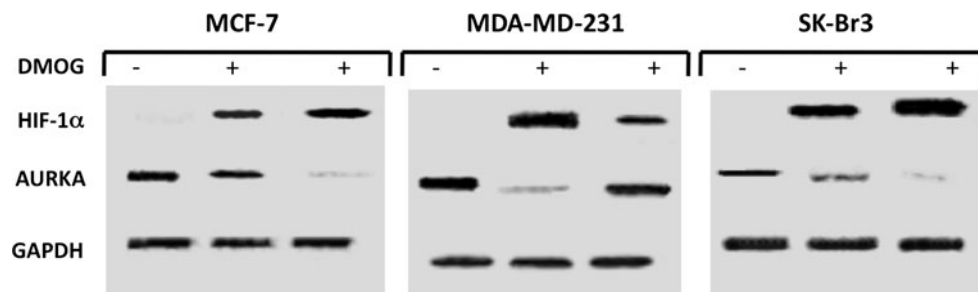


Fig. 5 Effects of HIF-1 α stabilization on AURKA expression. MCF-7, MDA-MB-231, and SKBr3 cells were incubated in the absence (–) or the presence (+) of 1 mM DMOG for 24 h (H24) and 48 h (H48) in normoxic conditions. Cells were lysed and protein extracts were

analyzed by Western Blot analysis, in order to assess HIF-1 α stabilization and AURKA expression levels. GAPDH protein was used as a loading control

Involvement of HIF-1 α in transcriptional regulation of *AURKA*

Since previously obtained results suggest that HIF-1 α could mediate *AURKA* down-regulation in BC cells under hypoxia, we aimed to obtain new insights into the regulation of *AURKA* expression. Therefore, we investigated the role of HIF-1 as hypothetical transcriptional repressor of the *AURKA* promoter. HIF-1 regulates the transcription of its target genes usually via binding to HREs. Sequence analysis identified three putative HREs sites into *AURKA* proximal promoter (HRE-1: –336/–332; HRE-2: –323/–319; and HRE-3: –240/–236) [14]. Thus, a ChIP assay was performed using an antibody against HIF-1 α in BC cells exposed to hypoxia, in order to evaluate the effects of hypoxia on HIF-1 α binding to the HREs into the proximal region of *AURKA* promoter. Then, quantitative PCR analysis, performed with specific primers for the *AURKA* promoter fragment containing the three HREs, revealed that HIF-1 α binds to the *AURKA* promoter. This analysis showed that HIF-1 α does not bind *AURKA* promoter in MCF-7, MDA-MB-231, and SKBr3 cells under normoxia. However, this binding was enhanced after hypoxic exposure for 24 and 48 h in MCF-7 and SKBr3 cells and maximal binding was observed after 48 h of hypoxia. Differently, increased binding was detected in MDA-MB-231 cells after 24 h of hypoxia followed by an evident reduction after 48 h (Fig. 6). The reduced HIF-1 α binding to *AURKA* promoter region, observed after 48 h of hypoxia in MDA-MB-231 cells, supports our hypothesis because, in response to a decreased binding, we found an increase in mRNA and protein levels of AURKA and p-AURKA. These results nicely correlate with those previously obtained from gene and protein expression analyses.

Also, in order to further investigate whether hypoxia was involved in regulation of *AURKA* promoter activity, we performed a luciferase reporter assay. For this purpose, an *AURKA* promoter fragment containing three putative HREs was cloned into pGL3 vector at the sites between

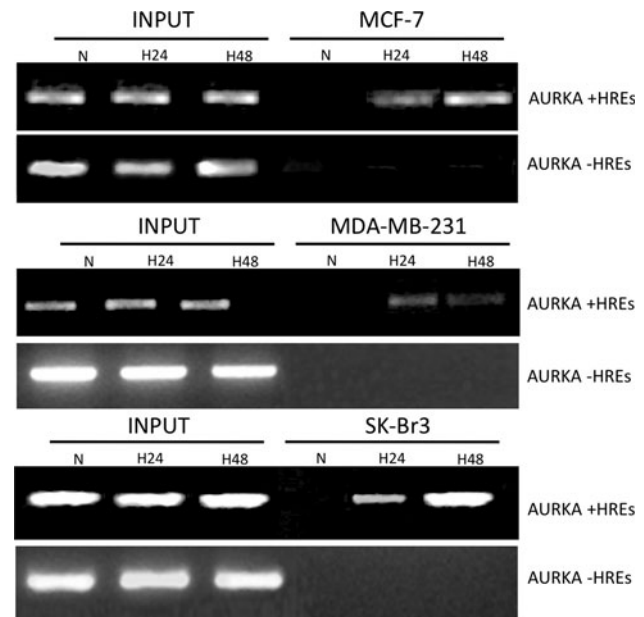


Fig. 6 Effects of hypoxia on HIF-1 α binding to proximal region of *AURKA* promoter. The binding of HIF-1 α to the proximal region (–336/–236) of *AURKA* promoter was tested by ChIP assay (as described in “Methods” section) using an antibody against HIF-1 α in MCF-7, MDA-MB-231, and SKBr3 breast cancer cells cultured in normoxic (16 % O₂) and hypoxic (3 % O₂, for 24 and 48 h) conditions. Interaction between HIF-1 α and *AURKA* promoter region containing HRE-1, HRE-2, and HRE-3 (*AURKA* + HREs) was analyzed by q-PCR. An *AURKA* promoter region containing no HREs was used as a control (*AURKA*–HREs). *N* normoxia; *H24* hypoxia for 24 h; *H48* hypoxia for 48 h

KpnI and XhoI giving the pGL3-*AURKA*-Luc plasmidic construct. BC cells were transfected with this construct and luciferase activity was measured after incubation under normoxia and hypoxia. We found that luciferase activity was significantly decreased in MCF-7, MDA-MB-231, and SKBr3 cells transfected with pGL3-*AURKA*-Luc and incubated in hypoxia (for 24 and 48 h) compared to cells transfected with empty vector (pGL3-basic-Luc), suggesting a hypoxia-mediated reduction of *AURKA* promoter activity (Fig. 7a).

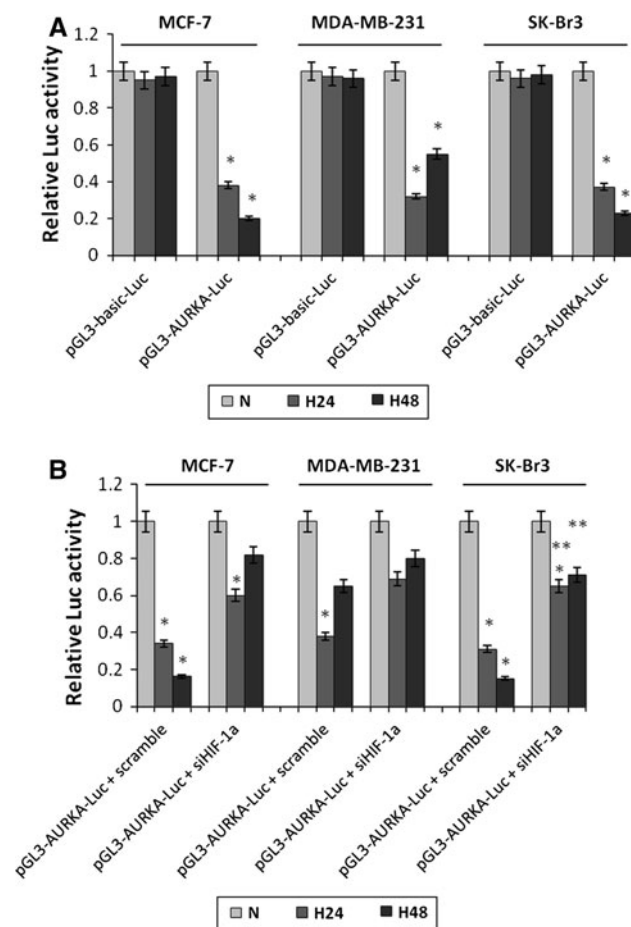


Fig. 7 Regulation of *AURKA* promoter activity under hypoxia. Transcriptional activity of *AURKA* promoter under hypoxia was evaluated by luciferase assay using the following plasmidic vectors: pGL3-*AURKA*-Luc, pGL3-basic-Luc (negative control), and pRL-TK. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Luciferase activity of cell lysates was determined by the Dual-Luciferase[®] Reporter Assay System (Promega). Data represent mean \pm SD from triplicate sets. Normalized luciferase activity in the respective controls was set to 1. **a** Breast cancer cells were co-transfected with the pGL3-*AURKA*-Luc firefly luciferase vector and pRL-TK *Renilla* luciferase vector or with the pGL3-basic-Luc empty vector and, after 24 h, incubated under normoxia or hypoxia (for 24 and 48 h). Significant difference, hypoxia for 24 h (H24) or for 48 h (H48) versus normoxia (N), * $P < 0.05$. **b** Cells were co-transfected with the pGL3-*AURKA*-Luc construct and a specific siRNA pool targeting HIF-1 α (siHIF-1 α) as well as pRL-TK vector and, after 24 h, incubated under normoxia or hypoxia (for 24 and 48 h). A non-targeting control pool (scramble) was co-transfected as a siRNA negative control. *Significant difference, H24 or H48 versus N in each experimental condition and **significant difference, H24 pGL3-*AURKA*-Luc + siHIF-1 α versus H24 pGL3-*AURKA*-Luc + scramble and H48 pGL3-*AURKA*-Luc + siHIF-1 α versus H48 pGL3-*AURKA*-Luc + scramble, $P < 0.05$

Furthermore, to evaluate the possible HIF-1 role in regulation of *AURKA* promoter activity, a specific siRNA pool targeting HIF-1 α (siHIF-1 α) was co-transfected with pGL3-*AURKA*-Luc plasmid in BC cells and a siRNA scramble was used as a negative control. After co-transfection, cells

were incubated under normoxia and hypoxia, and luciferase activity was determined. We observed that cells transfected with siHIF-1 α showed an increase of luciferase activity compared to cells transfected with negative control (scramble), indicating a higher induction of *AURKA* promoter activity after HIF-1 α knockdown. In particular, HIF-1 α -silenced MCF-7 and SKBr3 cells showed a higher increase of *AURKA* promoter activity after 48 h of hypoxia (Fig. 7b). Therefore, these data suggest that HIF-1 α could be involved in the negative regulation of *AURKA* expression, indicating a new possible transcriptional role of HIF-1 in BC cell lines.

Discussion

Nowadays, although oncologists have several available options (chemotherapy, hormone therapy, and biologic agents such as anti-angiogenic and anti-HER2 drugs), BC is still responsible for a significant percentage of cancer deaths in women [43, 44].

Hypoxia is a typical feature of the microenvironment of several solid tumors and is associated with poor prognosis in several cancer types, including BC [45]. Since tumor hypoxia is known to be an important factor for the expression of many genes involved in tumorigenesis and cell cycle regulation [46], we performed a microarray-based gene expression analysis in order to determine different expression profiles in MCF-7, MDA-MB-231, and SKBr3 BC cells in response to hypoxia for 24 and 48 h. This study allowed us to identify a set of 270 DEGs in hypoxia common to three BC cell lines. Our attention was focused mainly on the genes showing a significant variation in expression levels involved in proliferation, cell cycle progression and regulation, mitosis, and response to hypoxia. Three main molecular pathways were found altered in BC cell lines after hypoxia for 24 and 48 h: cell cycle regulation, HIF-1 α network, and *AURKA* signaling. Among the DEGs involved in proliferation and cell cycle control, *CDKN1A* (p21^{Cip1}), that can elicit the G1/S checkpoint, was up-regulated, while genes that further determine entry from G1 to S-phase, including *CDC6*, *CCND1*, *CCNE2*, and *CDK2*, were tendentially decreased in expression. Furthermore, *CDC25A* was down-regulated in MCF-7 and SKBr3 cells and up-regulated in MDA-MB-231 cells. Concomitant with decreased G1 to S-phase progression, a reduced expression of genes that regulate the passage of cells through G2/M, such as *CCNA2*, *CCNB1*, *CCNB2*, *CDC2* (*CDK1*), and *CDC25C*, was also demonstrated in MCF-7 and SKBr3 cells exposed to hypoxia for 24 and 48 h, and in MDA-MB-231 cells under hypoxia for 24 h. In addition, among the DEGs involved in HIF-1 α network, *VEGF-A*, *SLC2A1*, *JUN*, *FOS*, and other genes

encoding for glycolytic enzymes (*PGK1*, *PFKFB3*, *HK2*, *ALDOA*) were up-regulated in all three analyzed BC cell lines. Finally, genes that regulate the mitosis included in *AURKA* signaling pathway, such as *AURKA*, *AURKB*, *JUB* (ajuba), and *TPX2*, showed a significant down-regulation in expression levels. These changes could be responsible for genomic instability in BC. For the first time, our study showed that hypoxia induces a noteworthy down-regulation of *AURKA* expression in BC cell lines. The Aurora family kinases, especially aurora-A, play a particularly important role in the cell cycle and their deregulated expression is involved in many types of human malignancies [47, 48]. Several studies showed that aurora-A overexpression correlates with tumorigenesis, metastasis, and chemoresistance, confirming its pro-survival function in cancer cells [49–51]. Although in the literature there is evidence describing the activation of *AURKA* in different tumors, the mechanism of transcriptional up-regulation of *AURKA* in human BC is not yet elucidated [15]. Thus, the understanding of the regulatory pathways that mediate a reduced transcription of *AURKA* could be important, in order to identify new possible mechanisms counteracting not only BC progression but also the development of other tumors associated with *AURKA* overexpression. Therefore, our work focused mainly on effects of hypoxia on *AURKA* expression in BC cells and molecular mechanisms that regulate this. MCF-7, MDA-MB-231, and SKBr3 cells, in response to hypoxia for 24 and 48 h, showed a down-regulation of *AURKA* expression compared to normoxic condition, with a partial increase of expression after 48 h only in MDA-MB-231 cells. Moreover, microarray analysis revealed a parallel decrease in expression levels of *AURKB* and two *AURKA* co-activators, *JUB* and *TPX2*. However, according to the recovery of *AURKA* expression, *AURKB* and *TPX2* exhibited positive fold change in MDA-MB-231 cells exposed to hypoxia for 48 h, and *JUB* showed a partial increase of expression with respect to hypoxic condition at 24 h. Further analyses at protein level confirmed that hypoxia induces a decreased expression of *AURKA*, with an increased reduction of expression levels after 48 h of exposure to hypoxia in MCF-7 and SKBr3 cells, and with an initial decrease after 24 h followed by a partial recovery of expression after 48 h in MDA-MB-231 cells. Together with the reduction of the *AURKA* protein expression, an increase in HIF-1 α and VEGF-A protein levels was observed. However, HIF-1 α showed a lower expression level in MDA-MB-231 cells exposed to hypoxia for 48 h. From these data, HIF-1 α expression seems to be inversely related to *AURKA* expression.

Since HIF-1 α plays a pivotal role in the homeostatic mechanisms involved in the cellular response to hypoxic stress, we investigated its role in *AURKA* down-regulation

in BC cells exposed to hypoxia. To determine whether *AURKA* down-regulation was caused by increased expression of HIF-1 α in hypoxia, variations in *AURKA* expression were evaluated, both at mRNA and protein level, in BC cells transfected with a specific siRNA pool targeting HIF-1 α (siHIF-1 α). Thus, HIF-1 α -dependent effects of hypoxic exposure were reversed by siRNA-mediated silencing of HIF-1 α . Our data suggest that HIF-1 α could be involved in the regulation of *AURKA* expression in hypoxia because expression of siRNA against HIF-1 α reduced the down-regulation of *AURKA* in BC cell lines. In MCF-7 and SKBr3 cells, specific knockdown of HIF-1 α induced a greater difference of *AURKA* expression in hypoxia at 48 h in comparison to that detected at 24 h. In MDA-MB-231 cells, a lower difference was observed at 48 h with respect to that found at 24 h. At the same time, cells were treated with DMOG that induces an increase in HIF-1 α expression levels in normoxia, by inhibiting the PHDs. A lower *AURKA* expression was found in correspondence to higher HIF-1 α expression levels. These findings suggest that hypoxia could down-regulate *AURKA* expression in a HIF-1 α -dependent manner in BC cell lines. Since the molecular mechanisms through which *AURKA* is regulated in response to hypoxic condition are unknown in BC, the overall goal of this investigation was to obtain new insights on the regulation of *AURKA* expression in BC cells cultured under normoxia and hypoxia, by evaluating the possible HIF-1 role in transcriptional control of *AURKA* expression. HIF-1 regulates the transcription of its target genes usually via binding to HREs. Three putative HREs sites were identified into *AURKA* proximal promoter. In hepatocellular carcinoma, the stabilization of HIF-1 α not only induces the expression of genes involved in angiogenesis, glucidic metabolism, and survival but also causes *AURKA* up-regulation via its hypoxia-dependent binding to the HREs [14]. Moreover, in 2010, Xu et al. [52] reported that *VHL* inactivation stabilizes HIF-1 α inducing *AURKA* up-regulation in clear cell renal cell carcinoma (CCRCC) cell lines. Unlike the previous findings, we hypothesize a new possible mechanism where HIF-1 rather than inducing transcriptional activation could promote the *AURKA* down-regulation via its binding to HREs into proximal region of *AURKA* promoter in BC cells under hypoxia. Therefore, we investigated the potential role of HIF-1 as hypothetical transcriptional repressor of the *AURKA* promoter in the three BC cell lines. Our study showed that HIF-1 α does not bind to the *AURKA* promoter region containing three HREs in BC cells under normoxia, but its binding is enhanced after hypoxic exposure for 24 and 48 h in MCF-7 and SKBr3 cells, and a maximal binding is observed after 48 h of hypoxia. Differently, MDA-MB-231 cells showed an increased binding after 24 h of hypoxia followed by an evident reduction after 48 h. Our analyses indicated that

MDA-MB-231 cells show a different behavior concerning the *AURKA* expression after 48 h of hypoxia with respect to MCF-7 and SKBr3 cells. This could be attributed to a different expression level of HIF-1 α that binds to a lesser extent the *AURKA* promoter. Therefore, in support of our hypothesis, the increased HIF-1 α binding into the proximal region of *AURKA* promoter is related to a decrease in gene and protein expression levels of *AURKA*. Furthermore, to investigate whether HIF-1 regulates *AURKA* expression in hypoxia, luciferase assays were performed, showing a partial recovery of *AURKA* promoter activity after HIF-1 α knockdown. Therefore, these results suggest a new possible transcriptional role of HIF-1 that could determine a negative control of *AURKA* expression in BC cell lines under hypoxia. If HIF-1 acts directly as a possible transcriptional repressor of *AURKA* inhibiting the binding of RNA Polymerase II to its own site, or indirectly, preventing the binding of a transcriptional activator is yet unknown. This will be the subject of our future research as well as for a better understanding of the molecular bases of this down-regulation, by evaluating whether HIF-1 alone is directly involved in transcriptional control of *AURKA* in hypoxia or if other co-repressor proteins are implicated.

For the first time, the present study shows that hypoxia directly links HIF-1 with *AURKA* expression in BC, highlighting a possible pathophysiological role of this new pathway in this tumor and confirming HIF-1 as an important player linking an environmental signal to the *AURKA* promoter. Since *AURKA* is a key regulator of the chromosomal segregation process in mammalian cells and its down-regulation overrides the estrogen-mediated growth and chemoresistance in BC cells [53], these results could provide innovative approaches for the development of novel possible therapeutic strategies against BC.

Conflict of interest The authors declare no conflict of interest.

Ethical standards The authors declare that the experiments comply with the current laws.

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