**Intermittent hypoxia confers pro-metastatic gene expression selectively
through NF- κ B in inflammatory breast cancer cells**

Katrin Gutsche^{a,b,c}, Elisa Randi^{a,b}, Volker Blank^d, Daniel Fink^c, Roland H. Wenger^{a,b},
Cornelia Leo^{e*}, Carsten C. Scholz^{a*}

^aInstitute of Physiology, University of Zurich, 8057 Zurich, Switzerland, ^bZurich Center
for Integrative Human Physiology (ZIHP), University of Zurich, 8057 Zurich,
Switzerland, ^cDepartment of Gynecology, University Hospital of Zurich, 8091 Zurich,
Switzerland, ^dLady Davis Institute for Medical Research, Department of Medicine &
Department of Physiology, McGill University, Montreal, Quebec, Canada, H3T 1E2,
^eDepartment Women and Children, Cantonal Hospital Baden, 5404 Baden,
Switzerland

*These authors contributed equally to this manuscript.

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Corresponding authors:

Dr. Carsten Scholz
Institute of Physiology
University of Zurich
Winterthurerstr. 190
8057 Zurich
Switzerland
E-mail: carsten.scholz@uzh.ch
Tel: +41 44 63 55075

PD Dr. Cornelia Leo
Department Women and Children
Cantonal Hospital Baden
Im Ergel 1
5404 Baden
Switzerland
cornelia.leo@ksb.ch
+41 56 486 35 14

ABSTRACT

Inflammatory breast cancer (IBC) is the most aggressive form of breast cancer. Treatment options are limited and the mechanisms underlying its aggressiveness are poorly understood. Intermittent hypoxia (IH) causes oxidative stress and is emerging as important regulator of tumor metastasis. Vessels in IBC tumors have been shown to be immature, which is a primary cause of IH. We therefore investigated the relevance of IH for the modulation of gene expression in IBC cells in order to assess IH as potential regulator of IBC aggressiveness. Gene array analysis of IBC cells following chronic IH (45-60 days) demonstrated increased expression of pro-metastatic genes of the extracellular matrix, such as tenascin-C (TNC; an essential factor of the metastatic niche) and matrix metalloproteinase 9 (MMP9), and of pro-inflammatory processes, such as cyclooxygenase-2 (COX-2). Investigating the oxidative stress-dependent regulation of TNC, we found a gradual sensitivity on mRNA and protein levels. Oxidative stress activated NF-E2-related factor 2 (Nrf2), c-Jun N-terminal kinase (JNK), c-Jun and nuclear factor κ B (NF- κ B), but TNC upregulation was only dependent on NF- κ B activation. Pharmacological inhibition of inhibitor of NF- κ B α (I κ B α) phosphorylation as well as overexpression of I κ B α prevented TNC, MMP9 and COX-2 induction, whereas the pro-inflammatory cytokine interleukin-1 β (IL-1 β) increased their expression levels. Analysis of the gene array data showed NF- κ B binding sites for 64% of all upregulated genes, linking NF- κ B with IH-dependent regulation of pro-metastatic gene expression in IBC cells. Our results provide a first link between intermittent hypoxia and pro-metastatic gene expression in IBC cells, revealing a putative novel mechanism for the high metastatic potential of IBC.

Keywords: Intermittent hypoxia, oxidative stress, reactive oxygen species, ROS, NF- κ B, tenascin-C, inflammatory breast cancer

Abbreviations: ADAM19: a disintegrin and metalloproteinase 19; AP-1: activator protein-1; ARE: antioxidant response element; BSP: bisulfite sequencing PCR; CA9: carbonic anhydrase IX; CIH: chronic intermittent hypoxia; COX-2: cyclooxygenase-2; DCF: 2',7'-dichlorofluorescein; DMF: dimethyl fumarate; ECM: extracellular matrix; ER: estrogen receptor; HIF: hypoxia-inducible factor; HO-1: heme oxygenase 1; IBC: inflammatory breast cancer; IH: intermittent hypoxia; I κ B α : inhibitor of NF- κ B α ; IL1A : interleukin-1 α ; IL-1 β : interleukin-1 β ; JNK: c-Jun N-terminal kinase; Keap1: kelch-like ECH-associated protein 1; MAPK: mitogen-activated protein kinase; MMP2: matrix metalloproteinase 2; MMP9: matrix metalloproteinase 9; NAC: N-acetyl-L-cysteine; NF- κ B: nuclear factor κ B; Nrf2: NF-E2-related factor 2; Nrf3: NF-E2-related factor 3; ROS: reactive oxygen species; SFN: sulforaphane; SHC4: Src homology 2 domain containing family member 4; SMC1: Structural Maintenance of Chromosomes-1; tBHQ: tert-butylhydroquinone; TFBS: transcription factor binding site; TNC: tenascin-C; TREM1: triggering receptor expressed on myeloid cells; XRCC2: X-ray repair complementing defective repair in Chinese hamster cells 2

INTRODUCTION

Inflammatory breast cancer (IBC) is the most aggressive and most lethal form of primary breast cancer. While IBC is estimated to account for up to 5% of all breast cancer cases, it leads to approx. 8-10% of all breast cancer-related deaths [1-3]. IBC presents with a diffuse tumor formation and is highly metastatic with lymph node metastases being found in almost all and distant metastases in 30% of patients, respectively, at the time of diagnosis [2]. Treatment of IBC patients has proven to be challenging due to its rapid progression and aggressive nature [2,4]. The mechanisms underlying the aggressiveness of IBC are poorly understood, impeding the development of targeted therapy [2,5]. In addition, the investigation of molecular signaling pathways leading to the development of the aggressive phenotype of IBC is difficult, because of a limited availability of tumor material and with only two primary tumor-derived cell lines existing [5,6].

Tumors frequently outgrow their blood oxygen supply, leading to insufficient oxygen levels (hypoxia) and subsequent induction of angiogenesis [7]. The induced tumor vessels are often not properly developed, leading to fluctuations of blood and hence oxygen supply [8]. Oxygen levels in solid tumors are therefore dynamic, with repeated cycles of hypoxia and reoxygenation, called chronic intermittent hypoxia (CIH) or cycling hypoxia [8]. CIH cycles can occur in short (minutes to hours) and long (hours to days) intervals [8,9]. High frequency cycles can be caused by differences in red blood cell flux through perturbations of the microvessel diameter, while slow frequency cycles can occur through restructuring of tumor microvessels, which has been observed in tumors to occur on a daily basis [8]. CIH, although not directly assessed yet in IBC, likely also occurs in IBC tumors, because analysis of IBC tumor tissue showed hypoxia-dependent gene expression (carbonic anhydrase IX (CA9) induction) in 50% of the tumors and overall a higher microvessel density

than in other breast cancer forms [4,10,11]. Moreover, approx. 90% of IBC tumor blood vessels are not fully developed [12]. Of note, CIH leads to enhanced tumor microvessel density, which could present a link to the increased microvessel density in IBC tumors [13]. In addition, CIH has been shown to be a stronger inducer of cancer metastases than continuous hypoxia [14,15]. However, the role of CIH in IBC progression and aggressiveness is unclear.

The median oxygen partial pressure of a tumor is 30-38 mmHg (corresponding to 4-5% oxygen content in the gas phase), but fluctuating local oxygen gradients lead to a varying oxygenation between different tumor areas and cells, and the actual oxygen partial pressure experienced by each tumor cell depends on its proximity to vessels, local blood oxygenation, and on the oxygen consumption of surrounding cells, including tumor stroma and infiltrating immune cells [16,17]. Because it is not possible to accurately recreate such a complex situation *in vitro*, two clearly distinct normoxic and hypoxic oxygen levels were used for the investigations of the effect of CIH on IBC cells (normoxia: 20.9% O₂, hypoxia: 0.2% O₂).

CIH is a major source of oxidative stress due to reactive oxygen species (ROS) generation [8,18,19]. Oxidative stress can affect gene expression through regulation of DNA methylation and transcription factor activity [18,20,21]. While the mechanism of oxidative stress-dependent regulation of DNA methylation is less clear, several transcription factors have been reported to be activated by IH/oxidative stress, including NF-E2-related factor 2 (Nrf2), hypoxia-inducible factor (HIF), activator protein-1 (AP-1) and nuclear factor κ B (NF- κ B) [18,21,22]. Nrf2 is the main regulator of the cellular antioxidant response [23]. HIF is the master regulator of the transcriptional response to hypoxia but its activation by oxidative stress is still highly debated [8,24-27]. ROS have been reported to stabilize HIF-1 α , but HIF-1 α was not affected by ROS in other studies [8,18,19,24,25,27-29]. AP-1 has been linked to the

regulation of a wide range of cellular processes, including proliferation, migration and inflammation and has been reported to play an important role in tumor cell proliferation in the context of oxidative stress [30,31]. NF- κ B is the master regulator of the cellular transcriptional response to inflammation [32,33]. In cancer cells, NF- κ B activity can increase the expression of genes that promote proliferation, invasiveness, metastasis and apoptosis [34,35].

The extracellular matrix (ECM) plays an important role in the development and progression of breast cancer [36]. For example, the matrix metalloproteinases 2 and 9 (MMP2, MMP9) and a disintegrin and metalloproteinase 19 (ADAM19) are remodeling enzymes of the ECM and play a major part in the development of tumor metastasis and invasion [36,37]. Tenascin-C (TNC), a large hexameric ECM glycoprotein, is highly expressed during development, almost absent during postnatal life, but re-expressed in case of injury and in the onset of tumorigenesis [38]. Increased expression of TNC has been related to poor prognosis, local and distant recurrence in breast carcinomas and metastasis formation [36,39]. Of note, TNC has been shown to be an essential factor of the metastatic niche [36].

In the current study, we investigated the impact of IH and the resulting oxidative stress on transcriptional regulation in IBC cells. We provide evidence that CIH and oxidative stress are important regulators of pro-metastatic gene expression specifically in IBC cells. Investigation of the underlying mechanism of upregulated TNC gene expression showed that it is dependent on NF- κ B activation but not on DNA methylation, Nrf2, AP-1 and HIF. Furthermore, 64% of all CIH-upregulated pro-metastatic genes can be regulated by NF- κ B. These data provide a mechanistic link between intermittent hypoxia and pro-metastatic gene expression in IBC cells, contributing novel insights into the regulation of IBC aggressiveness.

MATERIAL AND METHODS

Reagents

Tert-butylhydroquinone (30 μ M) (tBHQ; Sigma-Aldrich, St Louis, MO, USA), dimethyl fumarate (40 μ M) (DMF; Sigma-Aldrich), BAY11-7082 (12.5 μ M, 25 μ M) (Selleck, Houston, TX, USA), H₂O₂ (100 μ M, 500 μ M) (Sigma-Aldrich) and 2',7'-dichlorodihydrofluorescein diacetate (10 μ M) (H₂DCFDA; Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA) were dissolved in ethanol. L-Sulforaphane (10 μ M) (SFN; Sigma-Aldrich) and SP600125 (10 μ M, 20 μ M) (Selleck) were dissolved in DMSO and N-acetyl-L-cysteine (3 mM, 20 mM) (NAC; Sigma-Aldrich) was dissolved in H₂O. IL-1 β (2 ng/ml) (R&D Systems, Minneapolis, MN, USA) was dissolved in 0.1% BSA in PBS.

Cell culture and treatments

The human triple-negative IBC breast cancer cell line SUM149PT was kindly provided by Rachael Natrajan (London, UK) and was used for all subsequent cell culture experiments if not otherwise indicated. SUM149PT cells were cultured in equal parts of high-glucose DMEM (Sigma-Aldrich) and Ham's F-12 Nutrient Mixture (Gibco, Thermo Fischer Scientific, Waltham, MA, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1 μ g/ml hydrocortisone (Sigma-Aldrich), 5 μ g/ml human insulin (Sigma-Aldrich), 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich). Reagents were diluted to the required concentration in the appropriate medium without FBS and supplements. Cells were seeded at 70-80% confluency. BAY11-7082, SP600125 and N-acetyl-L-cysteine were added to the cells 1 h before addition of tBHQ. Normoxic experiments were performed in a humidified atmosphere containing 20.9% O₂ (131.8 mmHg) and 5% CO₂ (35.7 mmHg) in a cell

culture incubator (Binder, Tuttlingen, Germany). Hypoxic experiments were carried out in a humidified atmosphere containing 0.2% O₂ (1.26 mmHg) and 5% CO₂ in a gas-controlled glove box (Invivo2 400, Baker Ruskinn, Bridgend, UK) as previously described [40].

mRNA analysis and data deposition

The RNeasy mini kit (Qiagen, Hilden, Germany) was used for total RNA extraction for the Affymetrix gene array analysis according to manufacturer's protocol. Affymetrix Human Gene 2.1 ST strip arrays were used to analyze transcript levels of one biological replicate of untreated cells and of cells cultured for 45 days in CIH. The data were deposited in Gene Expression Omnibus (GEO;

<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through the ID GSE81416

(reviewers access:

[http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ihgnacgezfkxli&acc=GSE8141](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ihgnacgezfkxli&acc=GSE81416)

6). For validation of the Affymetrix gene array results, three biological replicates of untreated cells and cells that were cultured for 45-60 days in normoxia or CIH conditions were analyzed by RT-qPCR as previously described [41]. The relative mRNA expression was calculated using the $\Delta\Delta C_t$ formula. U6 snRNA served as control gene and values were normalized to the average values of normoxic controls. For all further experiments, total cellular RNA was extracted as previously described [42]. Primer sequences were listed in Supplementary Table S3. mRNA copy numbers were calculated by comparison with serial dilutions of a calibrated standard, calculated relative to U6 snRNA levels and normalized to the starting time point if not otherwise indicated.

Bioinformatic analysis of Affymetrix gene expression array

For the analysis of the Affymetrix gene array results, protein coding genes, as listed in the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database, with ≥ 4 -fold increased transcript levels were used for bioinformatic analyses with MetaCore (<https://portal.genego.com/>) from Thomson Reuters. Enrichment analysis according to standard settings of the software was performed to rank the most relevant cellular processes. p values were calculated as $-\log_{10}$. To analyze literature-based transcriptional regulations, the “build network” option with the algorithm for “transcription regulation” was applied. In order to link single genes to the matrisome, analysis was performed via the matrisome project for each upregulated gene (<http://matrisomeproject.mit.edu/proteins/>) [43]. For detection of conserved transcription factor binding sites (TFBS), the web-based software oPOSSUM version 3 (<http://opossum.cisreg.ca/oPOSSUM3/>) was used [44-46]. The matrices of TFBS profiles included in oPOSSUM-3 were obtained from the 2010 release of the JASPAR database (<http://jaspar.genereg.net>) [47]. For detection of TFBS in the *TNC* promoter, the UCSC genome browser (<http://genome.ucsc.edu/>) was searched with the genome assembly released in February 2009 [48]. Further, the Swissregulon (<http://swissregulon.unibas.ch/fcgi/sr>) and the JASPAR databases were searched [49].

Protein detection and quantification

Proteins were extracted from cells using 0.4 M NaCl, 1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium-orthovanadate and protease inhibitor cocktail (P2714, Sigma-Aldrich). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and detected with antibodies against HIF-1 α (BD Transduction Laboratories, Allschwil, Switzerland); β -actin (Sigma-Aldrich); SMC1 (Abcam,

Cambridge, UK); tenascin-C (H-300, Santa Cruz Biotechnology, Dallas, TX, USA); Nrf2 (H-300, Santa Cruz Biotechnology); α -tubulin (Sigma-Aldrich); c-Jun, phospho-c-Jun(Ser63), phospho-c-Jun(Ser73), I κ B α , phospho-I κ B α , COX-2, MMP9 (all Cell Signaling Technologies, Danvers, MA, USA); and Nrf3 [50]. Detection of primary antibodies and recording of chemiluminescence were carried out as previously described [40]. Quantification of immunoblotting signals was performed using ImageJ 1.48v (<http://imagej.nih.gov/ij>) or as previously described [40]. Values were normalized to the respective loading control and to the average values of normoxic controls if not otherwise indicated.

Detection of secreted TNC by enzyme-linked immunosorbent assay (ELISA)

SUM149PT cell treatments were performed in equal volumes of medium and medium was collected following 24 h of tBHQ treatment and frozen at -20°C until analysis. Secreted tenascin-C was detected with the human Tenascin-C Large (FNIII-C) ELISA Kit (IBL, Japan) as per manufacturer's description.

siRNAs and plasmids

For siRNA experiments, cells were seeded to reach 60% confluency on the day of transfection. Nrf2 siRNA (ON-TARGET plus Human NFE2L2 siRNA, SMART pool), Nrf3 siRNA (ON-TARGET plus Human NFE2L3 siRNA, SMART pool), c-Jun siRNA (ON-TARGET plus Human JUN siRNA, SMART pool) and non-target siRNA (ON-TARGET plus Non-targeting Pool) were purchased from Dharmacon (GE Healthcare, Lafayette, CO, USA) and transfected using Lipofectamine 2000 (Invitrogen) and Opti-MEM (GIBCO) according to manufacturers' protocols. For plasmid transfections, cells were seeded to reach 70% confluency on the day of transfection. NC16 pcDNA3.1 FLAG Nrf2 was a gift from Randall Moon (Addgene plasmid # 36971) [51], pCMV4-3

HA/I κ B α was a gift from Warner Greene (Addgene plasmid # 21985) [52] and pcDNA3.1-hNFE2L3 has previously been published [50]. Treatments were performed after 24 h of transfection.

DCF assay

SUM149PT cells were seeded in 96-well plates and incubated with 10 μ M H₂DCFDA for 30 min in the dark. Subsequently, specific treatments were performed and fluorescence was measured using a 96-well fluorescence photometer (Infinite 200Pro, Tecan, Männedorf, Switzerland). For combined treatments with tBHQ and NAC, cells were pretreated for 1 h with NAC. Results were calculated as increase in fluorescence per well $((F_{tx} - F_{t0}) / F_{t0} \times 100)$, where F_{tx} = fluorescence at a certain time point and F_{t0} = fluorescence at 0 min [53]. For hypoxia-reoxygenation experiments, 96-well plates were incubated for 24 h in 0.2% oxygen. Cells were incubated with H₂DCFDA inside the hypoxia workstation and fluorescence measurements were performed immediately thereafter. For measurements during hypoxic incubation, plates were sealed at measurement time points with oxygen impenetrable foils (Brand GMBH+ CO KG, Wertheim, Germany) within the hypoxia workstation and measurements were performed as described.

Statistical analyses

If not otherwise indicated, results are presented as mean values $\pm$ standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed using Student's t-test and one-way ANOVA or two-way ANOVA where appropriate. P-values <0.05 were considered statistically significant.

RESULTS

Chronic intermittent hypoxia increases pro-metastatic gene expression in inflammatory breast cancer cells.

In order to investigate the impact of CIH on IBC, the primary tumor-derived IBC cell line SUM149PT was cultured for 45 days in CIH conditions (15 cycles of hypoxia/reoxygenation; Fig. 1A) and gene expression was assessed by whole genome microarray hybridization. Using a four-fold difference relative to control as threshold for relevant gene regulation, 37 genes were identified to be upregulated and 51 genes to be downregulated in response to CIH. Analysis of associations of the upregulated genes with cellular processes via MetaCore (<https://portal.genego.com/>) showed that these genes are primarily involved in the regulation of the ECM (Fig. 1B). 13 out of the 37 upregulated genes were linked to the “matrisome”, which is composed of ECM and ECM-associated proteins (Supplementary Table S1) [43]. A second relevant process associated with CIH-upregulated gene expression was inflammation (Fig. 1B).

The CIH experiment was repeated to validate the expression levels of 10 out of the 37 upregulated genes. TNC, MMP2, MMP9 and ADAM19 were chosen because these proteins are important components and remodeling enzymes of the ECM, and since they have previously been shown to play a major role in tumor invasion and metastasis [36,37]. Triggering receptor expressed on myeloid cells (TREM1), interleukin-1 α (IL1A) and cyclooxygenase-2 (COX-2; gene name: *PTGS2*, prostaglandin G/H synthase 2) have been linked before to inflammatory processes, and COX-2 has been associated with a more aggressive breast cancer phenotype [2]. NF-E2-related factor 3 (Nrf3, gene name: *NFE2L3*) is a homolog of the transcription factor Nrf2 and might be part of the oxidative stress response [54]. Src homology 2 domain containing family member 4 (SHC4) plays a critical role in

migration of metastatic melanomas and potentially in the response to oxidative stress-induced DNA damage; and a SNP in X-ray repair complementing defective repair in Chinese hamster cells 2 (XRCC2), which is involved in homologous recombination DNA repair, was linked to spontaneous breast cancer development [55-57]. We analyzed the expression pattern of these genes in response to hypoxia, reoxygenation (both part of cycle 1 of the CIH experiment; Fig. 1A) and CIH (15-20 cycles; Fig. 1A). All genes except MMP9 demonstrated a significant upregulation following CIH (Fig. 2A). But also MMP9 showed an upregulation in response to CIH in all three independent experiments, only with a higher degree of variability. To analyze the expression patterns further, we directly compared the observed induction factors of the different treatments to each other and identified three differentially regulated groups (Fig. 2B). Group I showed only upregulated gene expression in response to CIH, while group II genes were already increased in response to a single reoxygenation stimulus (Fig. 2B), and group III was upregulated by all three treatments (Fig. 2B). These findings indicate that some of these genes are only sensitive to a higher oxidative stress such as CIH (group I), while others are already upregulated by a single reoxygenation event (group II). In group III, we most likely observed overlapping regulation between hypoxia and the oxidative stress response. Of note, no increase in prototypical HIF target genes (e.g. CA9 or glycolytic enzymes) was observed in the gene array following CIH, indicating that HIF-dependent gene expression was not present in our CIH conditions at the time of analysis. In order to determine if CIH also led to increased protein levels, we determined TNC protein expression following the three different treatments. TNC protein expression followed the same pattern as its mRNA, with no upregulation in response to hypoxia or a single reoxygenation event but with a significant increase following 15 and 20 cycles of CIH (Fig. 2C, D).

Overall, these results demonstrate that CIH increases pro-metastatic gene expression in IBC cells. In addition, we identified three distinct groups of regulated genes, indicating a differential sensitivity to oxidative stress and hypoxia among the investigated genes. The observed changes on mRNA levels translated into protein expression, indicating functional relevance.

Tenascin-C expression demonstrates a gradual sensitivity to increasing oxidative stress levels specifically in IBC cells

We next investigated the underlying mechanism of the CIH-dependent increase in pro-metastatic gene expression. We focused on TNC expression because its mRNA and protein levels were specifically upregulated in response to CIH but not to hypoxia, demonstrating that its regulation depends on repeated reoxygenation events (Fig. 2). Furthermore, TNC has been linked to breast cancer development, it plays a major role in tumor progression and metastasis, and it has been shown to be an essential factor of the metastatic niche [36]. Because CIH is a well-established oxidative stress inducer and likely leads to a more severe oxidative stress than a single reoxygenation event [8,18,58], we aimed to investigate the effect of increasing oxidative stress conditions on TNC-dependent gene expression. First, we compared the potential of different stimuli, a single hypoxia/reoxygenation phase, hydrogen peroxide (H_2O_2) and tBHQ, to induce oxidative stress in SUM149PT cells using the DCF assay (Fig. 3A-C). Within cells, tert-butylhydroquinone (tBHQ) autooxidizes via an intermediate step as semiquinone to tert-butylquinone in an oxygen-dependent manner, forming two oxygen radicals per each tBHQ molecule [59].

Hypoxia/reoxygenation led to a short but significant increase in DCF fluorescence at early time points (0.5 h) (Fig. 3A), while 100 μM H_2O_2 led to a prolonged, plateauing increase in fluorescence over 4 h (Fig. 3B). 30 μM tBHQ induced a steady increase

in fluorescence over 4 h and the observed signal was up to 4-fold higher than with single hypoxia/reoxygenation or H₂O₂ (Fig. 3A-C). Hypoxia did not increase DCF fluorescence, indicating that the reoxygenation phase following hypoxic incubation is necessary for a detectable increase in ROS levels (Supplementary Fig. 1A & B). This established the following order of ROS induction by these treatments: tBHQ (30 μ M) > H₂O₂ (100 μ M) > single reoxygenation.

DCF assay analysis of SUM149PT cells following the CIH experiment showed that there was no further increased fluorescence upon hypoxia/reoxygenation in comparison to cells that had not been cultivated in CIH (Supplementary Fig. S1C). This indicates that long-term cultivation of SUM149PT cells in CIH does not change the intrinsic cellular ROS production in response to reoxygenation.

Next, we investigated the impact of the different ROS sources on TNC mRNA and protein expression. Hypoxia/reoxygenation led to a significant but short and transient increase in TNC mRNA levels at 2 and 4 h (Fig. 3D), while H₂O₂ significantly induced TNC mRNA at 4-8 h (Fig. 3E) and tBHQ at 9 h and 24 h (Fig. 3F), with 5 to 9-fold higher TNC mRNA levels following tBHQ treatment compared to the other treatments (Fig. 3D-F). On the protein level, hypoxia/reoxygenation neither induced TNC (Fig. 3G) nor activated the positive control for oxidative stress, the redox-sensitive transcription factor Nrf2 (data not shown). CA9 mRNA levels and HIF-1 α protein levels were used as positive controls for hypoxia (Fig. 3G, Supplementary Fig. S1D). In turn, H₂O₂ increased TNC protein levels significantly after 6 h (Fig. 3H). Nrf2 protein was stabilized and its target gene heme oxygenase 1 (HO-1) was elevated in response to H₂O₂ treatment (Fig. 3H, Supplementary Fig. S1E). tBHQ treatment led to a strongly increased protein expression after 9 h and 24 h (Fig. 3I). Nrf2 protein levels and HO-1 mRNA were also elevated by tBHQ (Fig. 3I,

Supplementary Fig. S1F). Of note, the observed differences of activated Nrf2 protein levels between the treatments are in agreement with the differences of ROS measured by the DCF assay (tBHQ > H₂O₂ > single reoxygenation), further supporting a differential ROS induction between the treatments (Fig. 3A-C, G-I). tBHQ treatment also led to an increased secretion of TNC protein (Fig. 3J), further indicating the functional relevance of the observed TNC regulation.

In order to test if the observed ROS-dependent regulation of TNC also occurs in triple-negative non-IBC breast cancer cells, we investigated the effect of tBHQ treatment on TNC expression in the MDA-MB-231 and MDA-MB-468 cell lines. tBHQ treatment led to a significant increase in Nrf2 protein and HO-1 mRNA levels in MDA-MB-231 cells, indicating effective ROS induction, but did not increase TNC mRNA or protein levels (Supplementary Fig. S2A-C). In MDA-MB-468 cells, tBHQ treatment also led to increased Nrf2 protein levels but did not upregulate TNC protein (Supplementary Fig. S2D). These data indicate that the observed ROS-dependent regulation of TNC is specific to IBC cells.

Overall, TNC expression shows a gradual sensitivity to ROS levels specifically in inflammatory breast cancer cells, indicating that CIH increases TNC expression through increasing oxidative stress.

Antioxidant treatment prevents oxidative stress-mediated TNC induction

We next sought to determine if the observed TNC induction was directly dependent on ROS. SUM149PT cells were pre-treated with the antioxidant NAC for 1 h followed by the addition of tBHQ. NAC prevented tBHQ-mediated ROS production (Fig. 4A) as well as tBHQ-mediated upregulation of TNC protein levels (Fig. 4B). These results demonstrate that regulation of TNC expression is directly linked to oxidative stress and can be prevented by antioxidant treatment.

Oxidative stress-dependent TNC induction is independent of Nrf2 and Nrf3

DNA methylation is frequently involved in chronic changes of gene expression and can be regulated by oxidative stress [20]. Therefore, we first investigated a potential impact of CIH on DNA methylation as a molecular mechanism for the regulation of TNC expression. Although CpG-rich regions were identified in the *TNC* promoter and in the first untranslated exon of *TNC*, no difference in methylation following CIH was observed in the *TNC* promoter by methylation-specific PCR (MSP) (Supplementary Fig. S3) nor in the *TNC* promoter or the first untranslated exon of *TNC* by bisulfite sequencing PCR (BSP) (data not shown).

A main transcription factor activated by oxidative stress is Nrf2 [23]. Nrf2 protein was stabilized and Nrf2-dependent target genes were increased in SUM149PT cells following H₂O₂ and tBHQ treatment (Fig. 3H, I, Supplementary Fig. S1E, F). In addition, in response to CIH we observed elevated Nrf3 mRNA levels (Fig. 2A). Nrf3 belongs to the same Cap'n'Collar transcription factor family as Nrf2 and has also been linked to the cellular antioxidant response [54]. Furthermore, the *TNC* promoter can be regulated by BACH1, a transcriptional suppressor binding antioxidant response elements (AREs) (Fig. 5A) [60]. Following oxidative stress, BACH1 does not suppress ARE-dependent transcription anymore [60], allowing e.g. Nrf2 or Nrf3 to bind instead (Fig. 5A). Therefore, we transiently knocked down Nrf2 and/or Nrf3 in SUM149PT cells, followed by tBHQ treatment. No significant difference in tBHQ-dependent TNC induction was detected with single or combined Nrf2 and Nrf3 knockdown (Fig. 5B), while HO-1 induction, a known direct Nrf2 target gene, was significantly down-regulated (Fig. 5C) [23]. Interestingly, knockdown of Nrf3, which had previously been reported to be capable of both inducing and repressing gene transcription [61], increased HO-1 expression, indicating that Nrf3 is

a repressor of ROS-induced HO-1 expression (Fig. 5C). Single and combined knockdown or overexpression of Nrf2 and Nrf3 showed no major effect on basal TNC gene expression (Supplementary Fig. S4). In addition, the effect of two chemical Nrf2 activators on TNC expression was tested, SFN and DMF. SFN and DMF mediate stabilization and nuclear translocation of Nrf2 by modulating the inhibitory protein of Nrf2, kelch-like ECH-associated protein 1 (Keap1) [62,63]. SUM149PT cells were treated with 10 μ M SFN and samples were collected for up to 24 h. No change in TNC mRNA expression was detected, while the induction of HO-1 6 h after treatment demonstrated efficient Nrf2 activation (Fig. 5D). Treatment with 40 μ M DMF caused only a slight elevation in TNC mRNA levels after 24 h, but showed a significant HO-1 induction from 3 h to 9 h (Fig. 5E). Increased TNC mRNA levels through DMF stimulation correlated with increased ROS levels while SFN caused no significant ROS production and no elevated TNC mRNA levels (Supplementary Fig. S5). Of note, the increase in ROS following DMF treatment was much lower than the ROS levels measured after H₂O₂ treatment and negligible compared to the ROS production caused by tBHQ (Fig. 3B, C, Supplementary Fig. S5B).

Overall, these results demonstrate that oxidative stress-dependent TNC induction is independent of Nrf2 and Nrf3.

Oxidative stress-dependent TNC induction is independent of JNK and c-Jun activity.

Another transcription factor activated by oxidative stress and IH is AP-1, which is regulated by mitogen-activated protein kinase (MAPK) signaling [18,21,30]. AP-1 is a dimeric protein that can be composed of several different subunits of which the major protein subfamilies are Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) [30]. The *TNC* promoter contains ChIP-based binding sites for AP-1 subunits such as

c-Jun (Fig. 5A). tBHQ increased over a time course of 24 h c-Jun phosphorylation on serine 63 and 73, which enhances c-Jun transcriptional activity (Fig. 6A) [30]. In addition, total c-Jun protein levels were increased (Fig. 6A). c-Jun has been reported to be phosphorylated on serine 63 and 73 by the MAPK c-Jun N-terminal kinase (JNK) [30]. Therefore, we next investigated if tBHQ-dependent c-Jun phosphorylation could be prevented by the JNK inhibitor SP600125. One hour pre-treatment with SP600125 blocked tBHQ-dependent c-Jun phosphorylation (Fig. 6B), indicating that tBHQ activates the MAPK signaling cascade at the level of or upstream of JNK. However, the JNK inhibitor did not prevent tBHQ-mediated TNC induction (Fig. 6C). Furthermore, siRNA-mediated knockdown of c-Jun did also not significantly affect tBHQ-dependent TNC induction (Fig. 6D). These results show that oxidative stress activates MAPK signaling and c-Jun in IBC cells but the oxidative stress-dependent increase of TNC is independent of this mechanism.

Oxidative stress-dependent TNC induction is mediated by NF- κ B.

A further transcription factor activated by oxidative stress is NF- κ B, which also has a putative binding site in the *TNC* promoter (Fig. 5A). Hence, we next tested if tBHQ can activate NF- κ B in IBC cells and treated SUM149PT cells with tBHQ over a 24 h time course. This led to a time-dependent decrease of inhibitor of NF- κ B α (I κ B α) protein levels (Fig. 7A), demonstrating activation of the NF- κ B signaling pathway. Pre-treatment with BAY11-7082, which prevents phosphorylation and subsequent degradation of I κ B α [64], blocked the tBHQ-dependent decrease of I κ B α (Fig. 7B), indicating that tBHQ-mediated oxidative stress activates the NF- κ B signaling pathway at the level of or upstream of I κ B α phosphorylation. Inhibitor treatment 1 h prior to the tBHQ stimulus abrogated the increase in TNC expression (Fig. 7C),

indicating that NF- κ B regulates oxidative stress-mediated TNC induction. We expanded our investigations further to MMP9 and COX-2, which had shown increased expression in response to CIH (Fig. 2A, B) and which were upregulated in response to tBHQ treatment (Supplementary Fig. S6A). Pre-treatment with BAY11-7082 abrogated the tBHQ-dependent increase of MMP9 and attenuated tBHQ-dependent COX-2 upregulation (Fig. 7C). Overexpression of I κ B α also prevented the oxidative stress-dependent induction of TNC, MMP9 and partly of COX-2, overall confirming their regulation by the NF- κ B signaling pathway (Fig. 7D). H₂O₂, which induced TNC mRNA and protein expression (Fig. 3E, H), also increased NF- κ B activity in an NF- κ B dependent luciferase reporter gene assay in SUM149PT cells, indicating that the observed ROS-dependent NF- κ B activity in IBC cells is a general consequence of oxidative stress and not specific to the tBHQ stimulus (Supplementary Fig. S6B).

Treatment with the major pro-inflammatory cytokine IL-1 β , one of the best-characterized NF- κ B activating stimuli [64,65], showed a decrease of I κ B α levels at 30 min and a strong increase in TNC and COX-2 protein levels from 3-24 h and of MMP9 from 9-24 h (Fig. 7E). Analysis of breast cancer transcriptomic datasets revealed a significant, positive correlation between TNC and nuclear factor κ B p105 subunit levels (Supplementary Fig. S7). Together with the previous results, this demonstrates that oxidative stress-induced TNC, MMP9 and COX-2 expression is regulated by NF- κ B. Interestingly, when we investigated our CIH gene array results for transcription factor binding sites and known regulation by transcription factors using oPOSSUM and MetaCore (<http://opossum.cisreg.ca/oPOSSUM3/> and <https://portal.genego.com/>), 64% of all upregulated genes showed a possible regulation by NF- κ B (Fig. 7F, Supplementary Table S2). Analysis for binding sites for

AP-1 and Nrf2 revealed a putative regulation of 33% by Nrf2 and of 83% by AP-1 with 30.6% of these genes showing overlapping regulation by Nrf2, AP-1 and NF- κ B (Supplementary Fig. S8).

Overall, our results demonstrate that the oxidative stress-dependent increase of several pro-metastatic genes is specifically regulated by NF- κ B and that this transcription factor might also play an important role in the upregulation of further pro-metastatic genes in IBC cells in response to CIH.

DISCUSSION

Inflammatory breast cancer is the primary breast cancer form with the highest aggressiveness [2,3,66]. The underlying mechanisms are still unclear but are urgently needed to be deciphered in order to develop novel treatment options [5]. While a common feature of tumors, CIH is only beginning to be appreciated as an important regulator of cancer progression and metastases. CIH occurs when cancer cells outgrow the area of sufficient oxygen delivery and subsequent hypoxia-induced tumor angiogenesis leads to vessels with abnormal structures [8]. Such changes have also been demonstrated for IBC tumors [7,12]. CIH is a well-established source for oxidative stress, which can severely impact on gene expression, leading to increased cancer aggressiveness [19]. However, the relevance of CIH for IBC progression and metastases formation is unclear. In this study, we provide insights into the regulation of pro-metastatic gene expression through IH and oxidative stress in IBC cells, which appears to be mediated mainly by NF- κ B (Fig. 8).

We identified that CIH leads to increased expression of ECM proteins in IBC cells, such as TNC, MMP2, MMP9 and ADAM19, and of proteins involved in inflammatory processes, such as COX-2. TNC, MMP2, MMP9 and COX-2 have previously been shown to highly promote breast cancer metastasis, and to be significantly stronger expressed in IBC tumors than in non-IBC breast cancer tumors [2,36,66,67]. Tumor inflammation has also been associated with cancer progression and metastasis formation and COX-2 was identified as a major component of the IBC molecular signature and suggested for targeted therapy for IBC [2,68]. Overall, these data demonstrate that CIH leads to the upregulation of pro-metastatic gene expression in IBC cells, which likely contributes to the aggressive phenotype of IBC.

The transcriptional regulation of the TNC gene is complex and several different stimuli and transcription factors are involved [69]. TNC has previously been

shown to be regulated by AP-1, NF- κ B and Nrf2 [69-71], and although we observed a ROS-dependent activation of Nrf2, JNK and c-Jun, these factors did not regulate TNC in IBC cells. In our gene array, 33% of CIH-upregulated genes were identified to carry a putative Nrf2 binding sequence, but we did not observe any prototypical Nrf2 target gene, such as HO-1 or other genes of the cellular antioxidant defense system [23]. Therefore, it seems unlikely that Nrf2 plays a major role in the here observed gene expression and no clear link between Nrf2 activity and IBC has been reported so far.

The effect of oxidative stress on the HIF pathway is controversially discussed in the literature [8,18,19,24,25,27-29]. Here, in the CIH experiment, no HIF-1 α stabilization and no prototypical HIF-dependent gene expression pattern was observed. Following a stimulus such as hypoxia, HIF-1 α reaches in most cells its highest protein levels after 6-8 h and declines subsequently due to negative feedback loops, even during a still continuing HIF-1 α stabilizing stimulus [72-75]. Hence, it is not surprising that no HIF-1 α protein levels and no HIF-dependent gene expression were detected in the CIH experiments, analyzing the samples 48 h following the end of the hypoxia period. These results indicate that the observed gene expression was not due to the initial hypoxic stimulus but rather due to the generated oxidative stress, and that this regulation is independent of HIF-1 α .

The relevance of MAPK signaling and AP-1 activation in IBC is unclear, but overexpression of AP-1 subunits such as c-Jun and MAPK hyperactivation have been suggested [76,77]. While ROS-dependent TNC induction was independent of c-Jun, 83% of the CIH-upregulated genes could potentially be regulated by AP-1. Therefore, a contribution of AP-1 to CIH-dependent gene regulation in IBC cells cannot be excluded but would need further investigations to determine its relevance.

We also observed oxidative stress-dependent activation of the NF- κ B signaling cascade at the level of or upstream of I κ B α and TNC, MMP9 and COX-2 were regulated by activation of the NF- κ B pathway. TNC has previously been suggested as a putative NF- κ B target gene in IBC [66,78], but although TNC is regulated by this transcription factor in other cell types, to our knowledge an NF- κ B-dependent TNC regulation had not been shown in IBC cells before. Of note, chronic exposure of SUM149PT cells to IH did not change the ROS production in response to reoxygenation. This indicates that the observed gene regulation following CIH was either due to an increasing sensitivity of the IBC cells to ROS over the CIH time course, or that repeated ROS exposures were necessary to sufficiently activate NF- κ B for the regulation of gene expression.

The term “inflammatory breast cancer” could suggest an inflammation-driven cancer, but it originated from the clinical appearance of patients [4,79]. The role of inflammation in IBC aggressiveness is only beginning to be unraveled. Several studies indicated NF- κ B to be constitutively activated in IBC and to be a major contributor to IBC-specific gene expression and tumorigenesis [2,66,77,80]. As mechanisms for increased NF- κ B activity, infection with human cytomegalovirus (HCMV), loss of estrogen receptor (ER) and differential cytokine expression has been suggested [2]. In our study, we found that oxidative stress activates NF- κ B in IBC cells and that 64% of all CIH-upregulated genes can potentially be regulated or have been shown to be regulated by NF- κ B, such as COX-2 [81]. TNC, MMP9 and COX-2 gene induction by oxidative stress were directly linked to NF- κ B activation and increased TNC expression was associated with increased p105 expression in breast cancer tissue. Overall, these results demonstrate the importance of NF- κ B in pro-metastatic gene regulation in IBC cells in response to intermittent hypoxia.

Interestingly, TNC can activate toll-like receptor 4, which in turn activates NF- κ B, leading to a regulatory loop [78,82]. Furthermore, IH and inflammation can form a vicious circle *in vivo*, amplifying each other [83]. We therefore hypothesize that IH and oxidative stress play an important role in the constitutive activation of NF- κ B in IBC, which is facilitated through increased TNC expression.

In summary, CIH is a possible novel modulator of IBC aggressiveness, increasing pro-metastatic gene expression through activation of NF- κ B and maybe other transcription factors. Both CIH and NF- κ B should be further investigated as potential targets for therapy of inflammatory breast cancer. The impact of CIH should be considered in future studies of IBC tumor material, as CIH affects gene and protein expression and could lead to differences in expression levels between different biopsy sites. Taking into consideration the crosstalk between the tumor microenvironment and the cancer cells, such as between intermittent hypoxia and IBC cells, might allow us to develop novel approaches for IBC treatment.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Fig. 1: Genes regulated by chronic intermittent hypoxia (CIH) in inflammatory breast cancer cells.

(A) Experimental scheme of the CIH exposure regime, indicating the duration of exposure to hypoxia and normoxia, and the time points of sample collection. **(B)** Enrichment analysis of upregulated genes using MetaCore software to rank the most relevant cellular processes.

Fig. 2: Gene array validation reveals three distinct groups of genes regulated by CIH.

(A) Validation of gene array results for selected candidate genes in SUM149PT cells by RT-qPCR. **(B)** Heatmap showing the mRNA induction factors depicted in (A) following hypoxia, reoxygenation or CIH. **(C)** Immunoblot analysis of TNC protein levels in response to hypoxia, reoxygenation or CIH. **(D)** Quantification of TNC protein levels following hypoxia, reoxygenation or CIH. Hyp = hypoxia, Reox = reoxygenation; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t-test).

Fig. 3: Regulation of *TNC* gene expression by oxidative stress.

DCF assays to quantify the generation of intracellular ROS levels by **(A)** reoxygenation (following 24 h 0.2% O₂), **(B)** 100 μ M H₂O₂ or **(C)** 30 μ M tBHQ over 4 h. RT-qPCR analysis of TNC mRNA induction in response to **(D)** reoxygenation (following 24 h 0.2% O₂), **(E)** 100 μ M H₂O₂ or **(F)** 30 μ M tBHQ over the indicated time course. TNC immunoblot analysis and quantification of relative protein levels in response to **(G)** reoxygenation (following 24 h 0.2% O₂), **(H)** 100 μ M H₂O₂ or **(I)** 30 μ M tBHQ over the indicated time course. **(J)** Determination of secreted TNC protein levels in response to 30 μ M tBHQ over 24 hours by ELISA. Cell culture supernatants were used from samples of the experiment shown in Fig.4B. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (one-way ANOVA

followed by Tukey test for (D, G); Student's t-test (J); two-way ANOVA followed by Bonferroni posttest for all other statistical analyses).

Fig. 4: Effects of antioxidants on tBHQ-mediated TNC induction. (A) DCF analysis of tBHQ treatment (30 μ M) alone or following 1 h pre-treatment with 3 mM NAC. **(B)** Analysis and quantification of relative TNC protein levels in SUM149PT cells in response to 30 μ M tBHQ alone or following 1 h pre-treatment with 3 mM or 20 mM NAC. Quantified values were normalized to β -actin loading control and to the starting time point. n.s., not significant; **, $p < 0.01$; ***, $p < 0.001$ (Two-way ANOVA followed by Bonferroni posttest was applied for comparison of tBHQ treatment alone with the combinatorial treatment of tBHQ and NAC in (A); one-way ANOVA followed by Tukey test in (B)).

Fig. 5: Role of Nrf2 and Nrf3 in oxidative stress-dependent TNC regulation. (A) UCSC-integrated ENCODE data proximal to the transcriptional start site of the *TNC* gene. The transcription factor ChIP-sequencing track shows regions of transcription factor binding derived from ChIP-seq experiments performed by the ENCODE project. The intensity of the grey shading is proportional to the signal strength observed in any cell line contributing to the respective cluster. Enrichment levels of histone marks (H3K4Me1, H3K4Me3, H3K27Ac) were determined by ChIP-seq and indicate regions of active enhancers (H3K4Me1, H3K27Ac) or active promoters (H3K4Me3). DNase hypersensitive areas reflect regulatory regions. Further relevant TFBS found in JASPAR and from published results were added in rectangles [71]. A circle shows relevant, additional TFBS found in the Swissregulon database. JUN, c-Jun; ARE, antioxidant response element, targeted by Nrf2 and Nrf3. **(B)** Nrf2 or Nrf3

alone or Nrf2/Nrf3 in combination were knocked down for 48 h followed by treatment with 30 μ M tBHQ or solvent control (ethanol) for the indicated time points. TNC and (C) HO-1 mRNA levels were determined by RT-qPCR and normalized to 0 h siCtrl. (D) Cells were treated with 10 μ M SFN or solvent control (DMSO) and TNC and HO-1 mRNA levels were determined by RT-qPCR. (E) TNC and HO-1 mRNA levels were determined by RT-qPCR following treatment with 40 μ M DMF or solvent control (DMSO). n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (two-way ANOVA followed by Bonferroni posttest).

Fig. 6: Role of JNK and c-Jun activity in oxidative stress-dependent TNC

regulation. (A) Immunoblot analysis of total c-Jun and phosphorylated c-Jun protein after treatment with 30 μ M tBHQ or solvent control (ethanol) for the indicated time points. (B) SUM149PT cells were pre-treated for 1 h with different concentrations of the JNK inhibitor SP600125 (SP6) followed by a 9 h treatment with 30 μ M tBHQ or solvent control (ethanol). Total c-Jun and phosphorylated c-Jun protein was detected by immunoblotting. (C) Immunoblot analysis and quantification of relative TNC protein levels in response to 30 μ M tBHQ with or without 1 h pre-treatment with different concentrations of the JNK inhibitor SP600125 for the indicated time points. (D) Immunoblot analysis and quantification of relative of TNC protein levels after knockdown of c-Jun for 48 h and followed by treatment with 30 μ M tBHQ or solvent control (ethanol) for the indicated time points. n.s., not significant; **, $p < 0.01$; ***, $p < 0.001$ (one-way ANOVA followed by Tukey test).

Fig. 7: Role of NF- κ B in oxidative stress-dependent TNC regulation. (A)

Immunoblot analysis of I κ B α protein levels following treatment with 30 μ M tBHQ or

solvent control (ethanol) for the indicated time points. **(B)** I κ B α protein levels were analysed by immunoblotting following 1 h pre-treatment with different concentrations of the I κ B kinase (IKK) inhibitor BAY 11-7082 (BAY) and subsequent addition of 30 μ M tBHQ or solvent control (ethanol) for 9 h. **(C)** SUM149PT cells were pretreated for 1 h with 12.5 μ M BAY 11-7082 followed by 30 μ M tBHQ or solvent control (ethanol) for indicated time periods. TNC, MMP9 and COX-2 protein levels were analysed by immunoblotting and TNC levels were quantified. **(D)** Following transient overexpression of I κ B α for 24 h, cells were treated with 30 μ M tBHQ or solvent control (ethanol) for the indicated time periods and TNC, MMP9 and COX-2 protein levels were analyzed by immunoblotting. For the shown MMP9 immunoblot a previously cut membrane was used in an otherwise identical single experiment. **(E)** SUM149PT cells were treated with 2 ng/ml IL-1 β or solvent control (0.1% BSA in PBS) for the indicated time course and I κ B α , TNC, MMP9 and COX-2 protein levels were analyzed by immunoblotting. **(F)** Analysis of NF- κ B binding sites in all CIH-upregulated genes, observed in the gene array shown in Supplementary Table S1. The graph depicts the combined results of TFBS identification using the web-based software oPOSSUM and MetaCore. oPOSSUM and MetaCore identified TFBS profiles based on JASPAR and published results, respectively. n.s., not significant; ***, $p < 0.001$ (one-way ANOVA followed by Tukey test).

Fig. 8: Proposed model of the molecular mechanisms by which intermittent hypoxia mediates pro-metastatic gene expression via activation of NF- κ B in inflammatory breast cancer cells. Intermittent hypoxia upregulates pro-metastatic genes in inflammatory breast cancer cells through activation of the transcription factor NF- κ B.

Figure 1

Figure 2

Figure 3**A** **B** **C** **D** **E** **F** **G** **H** **I** **J**

Figure 4

Figure 5**A** **B** **C** **D** **E**

Figure 6

A

B

C

D

Figure 7**A** **B** **C** **D** **E** **F**

Figure 8